LINDA RIKKERT.

**LET'S
GET
VESICLES**

**TUMOR-DERIVED
EXTRACELLULAR VESICLES IN
LIQUID BIOPSIES**

LET'S GET VESICLEs

*Tumor-derived extracellular
vesicles in liquid biopsies*

Linda Rikkert

Aan mijn ouders

The work described in this thesis was carried out in the groups *Medical Cell BioPhysics* at the *University of Twente*, Enschede, The Netherlands and the *Laboratory of Experimental Clinical Chemistry* at the *Amsterdam UMC, University of Amsterdam*, Amsterdam, The Netherlands and was financially supported by the *Netherlands Organisation for Scientific Research – Domain Applied and Engineering Sciences* (NWO-TTW), under the research program *Perspectief CANCER-ID* 14198.

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LET'S GET VESICLES
TUMOR-DERIVED EXTRACELLULAR VESICLES IN LIQUID BIOPSIES

DISSERTATION

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Introduction to this thesis.

Thesis motivation

Unreliable diagnostics results in performing unnecessary biopsies leading to overtreatment of prostate cancer patients (1). To improve clinical-decision making there is a need for a robust and low-invasive assay. Liquid biopsies, body fluids containing potential biomarkers, are therefore of great interest to obtain clinically relevant information.

One of these potential biomarkers in liquid biopsies are extracellular vesicles (EVs). EVs play a role in intercellular communication and EVs are present in body fluids such as blood and urine. In cancer patients, a minor fraction of EVs originates from tumor cells. It is already known that circulating tumor cells (CTCs) in blood from cancer patients have prognostic value (2). However, assessment of the CTC concentration is limited by their low concentration in blood. The *expected* tumor derived-EV (tdEV) concentration is higher compared to the CTC concentration, and measuring tdEVs may therefore increase the accuracy of screening (2, 3). If these tdEVs are indeed present in a higher concentration, detection of tdEVs directly in clinical samples, that is without the use of enrichment techniques, might be possible. However, EV detection is hampered by the presence of non-EV particles that are similar in size and density, like platelets and lipoproteins, and the high concentration of soluble protein (4, 5) and EVs from other origin, see Figure 1. Moreover, detection of EVs is complicated by their small size (> 30 nm), heterogeneity, and refractive index (6, 7).

To clinically use tdEVs as biomarkers, one needs to (i) distinguish EVs from non-EV particles and EVs from other origin, (ii) identify the cellular origin and functional characteristics, and/or (iii) examine the genetic content. Thus, optimized and novel technology platforms for the detection and characterization of unique characteristics of tdEVs, like antigen exposure, morphology, and size, need to be developed and validated.

Figure I.1. TEM image of blood plasma showing the presence of extracellular vesicles, but also the presence of non-EV particles like lipoproteins. The scale bar represents 200 nm.

Thesis contents

Chapter 1 provides an overview of the methods used in the Cancer-ID program to identify and characterize EVs. These methods analyze the optical, biochemical and mechanical properties of EVs. For each method the relevance for the EV field and new insights gained during the course of the Cancer-ID program are described. The goal of the Cancer-ID program is to evaluate which of these methods can be used to detect tdEVs in clinical EV samples.

Centrifugation is often used as a first step to isolate or concentrate biomarkers from whole blood. In *Chapter 2* a model is presented which can be used to determine the recovery of biomarkers by different centrifugation protocols. This model predicts that biomarkers are often co-isolated with other biomarkers, e.g. the co-isolation of platelets with EVs. This means study results cannot be assigned to a single biomarker.

Based on the same model, we developed a simplified and fast centrifugation protocol that is described in *Chapter 3*. This protocol can replace the more laborious, time-consuming double centrifugation protocol that is commonly applied in the EV field to obtain platelet-free plasma. Platelet removal by the new protocol is as effective as the old protocol, and results in a higher plasma yield.

Rate zonal centrifugation (RZC) can be used to separate co-isolated biomarkers based on size. Platelets and EVs end up in the same fractions after centrifugation and/or after size exclusion chromatography. RZC was successful for the separation of platelets and platelet-derived EVs. RZC, described in *Chapter 4*, can therefore be a step forward to enhance the purity of biomarkers for downstream analysis.

The next step in tdEV characterization is standardization of the EV detection techniques. In *Chapter 5* transmission electron microscopy (TEM) was evaluated, because TEM is often applied as a validation tool to ensure the quality and purity of an EV-containing sample. To improve the comparability and reproducibility of TEM images, we found that TEM images need to be taken at predefined locations. In a “head-to-head” comparison between images taken at predefined locations and by the current procedure of “operator selection”, it was clear that the latter does not provide an objective description of the EV sample.

In a small pilot study, flow cytometry (FCM) and surface plasmon resonance imaging (SPRi) were evaluated to determine whether prostate cancer patients could be discriminated from healthy controls based on the detection of tdEVs in plasma and/or urine. *Chapter 6* shows detection of tdEVs directly in clinical samples from prostate cancer patients was not possible using FCM and SPRi.

In the Discussion and Outlook the overall conclusion and findings of this thesis are discussed, as well as the future perspective of tdEVs for cancer diagnostics and to guide therapy for cancer patients. The complexity of EV samples remains a challenge for EV detection methods. tdEV enrichment is needed to decrease the background of non-EV particles and EVs from other cellular origin present in the blood.

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Chapter 1.

Cancer-ID: towards identification of cancer by tumor-derived extracellular vesicles in blood

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Abstract

Extracellular vesicles (EVs) have great potential as biomarkers since their composition and concentration in biofluids are disease state-dependent and their cargo can contain disease related information. Large tumor-derived EVs (tdEVs, $> 1 \mu\text{m}$) in blood from cancer patients are associated with poor outcome and changes in their number can be used to monitor therapy effectiveness. Whereas small tumor-derived EVs ($< 1 \mu\text{m}$) are likely to outnumber their larger counterparts, thereby offering better statistical significance, identification and quantification of small tdEVs is more challenging. In the blood of cancer patients, a subpopulation of EVs originate from tumor cells, but these EVs are outnumbered by non-EV particles and EVs from other origin. In the Dutch Cancer-ID program, we developed and evaluated detection and characterization techniques to distinguish EVs from non-EV particles and other EVs. Despite low signal amplitudes, we identified characteristics of these small tdEVs that may enable the enumeration of small tdEVs and extract relevant information. The insights obtained from Cancer-ID can help to explore the full potential of tdEVs in the clinic.

Introduction

Extracellular vesicles (EVs) are cell-derived particles with a phospholipid membrane. Because the membrane composition and content of EVs reflect the origin and state of the parental cells, EVs have become promising disease biomarkers (1-4). Participants from eight universities and 21 companies, who collaborate in the Dutch NWO Perspectief program Cancer-ID, aim to develop and evaluate technology to detect tumor-derived EVs (tdEVs) in blood as biomarker for cancer.

Throughout the project, two main challenges involved in the detection of EVs in blood became apparent. First, EV detection is hampered because EVs are outnumbered by the presence of non-EV particles in blood, like soluble proteins and lipoprotein particles at the low end of the EV size and density range, and platelets at the high end of the EV size and density range (5-7). Moreover, the concentration of larger lipoproteins, such as chylomicrons, depends on food intake, thereby emphasizing the need to discriminate EVs from other such particles. To illustrate this challenge, we know that 1 mL of human blood of metastatic castration resistant prostate cancer patients contains about 10 large ($> 1 \mu\text{m}$) tdEVs and we extrapolated this to 10^4 tdEVs in total. Furthermore, the blood contains up to 10^{16} lipoproteins, up to 10^9 platelets, and up to 10^{11} other EVs (5, 6, 8-10), see Figure 1.1.. The second challenge is the heterogeneity of EVs in many aspects, including morphology (11), size (11-13), membrane composition (13-18), and refractive index (19, 20), which complicates EV isolation, detection, and enumeration.

In sum, utilization of tdEVs as cancer biomarker requires (i) the discrimination of EVs from non-EV particles, (ii) identification of their cellular origin, and/or (iii) analysis of the EV molecular content. The insight that an EV-based cancer biomarker requires the ability to detect, identify and enumerate tdEVs amongst other particles plasma is an essential Cancer-ID outcome, because it defines the state-of-the-art. Therefore, we will use this definition to evaluate the ten techniques that were developed or improved throughout the project. The project includes techniques that (i) detect single particles attached to a surface, such as atomic force microscopy (AFM), electrochemical (EC) detection, scanning electron microscopy (SEM), and transmission electron microscopy (TEM), (ii) detect an ensemble of EVs attached to a surface, such as surface plasmon resonance imaging (SPRi), (iii) detect single EVs in suspension, such as flow cytometry

(FCM), or (iv) can measure either single or multiple EVs attached to a substrate or in a suspension, such as Raman microspectroscopy. The other evaluated technologies are integrated photonics lab-on-chip devices for Raman Spectroscopy, hybrid AFM-SEM-Raman, and immunomagnetic EpCAM (epithelial cell adhesion molecule) enrichment followed by fluorescence microscopic (FM) detection. The evaluated techniques including key characteristics are listed in Table 1.1.

Figure 1.1. Concentration, size and density of plasma particles. 3D representation of concentration, size and density of extracellular vesicles (dark green circle), platelets (blue) and the high-density lipoproteins (HDL, grey circle), low-density lipoproteins (LDL, grey triangle), very low-density lipoproteins (VLDL, grey star) and chylomicrons (CM, grey square) during fasting in blood. The average and standard deviation (lines) of the three parameters are indicated in the figure. Values are derived from literature (5, 6, 10, 21). The frequency of the large tumor derived extracellular vesicles (ltdEVs, light green circle) determined in the Cancer-ID program and the small tdEVs (stdEVs, light green square) estimated using the frequency of ltdEVs.

To compare all techniques, EVs derived from prostate cancer cell lines and EVs derived from platelet and red blood cell concentrates were distributed among the participants and measured. Based on the aforementioned requirements, we aimed to qualify the ability of a technique to (i) detect or image EVs, (ii) identify tdEVs, which involves differentiation of tdEVs from EVs and non-EV particles, and (iii) relate the measured signal or count to the concentration of tdEVs in plasma.

Table 1.1. Overview of the techniques used in Cancer-ID.

		Section §	Information obtained	Detection limit (nm)	Throughput ^a (×50 tdEV/hr)
Surface					
Single	TEM (11)	II.1	Morphology, Size	30	2×10^{-9}
	SEM (13)	II.2	Topography, Size	50	3×10^{-3}
	AFM (13)	II.3	Morphology, Bending modulus	30	2×10^{-4}
	Raman (13)	II.5	Chemical composition	80-320	9×10^{-4}
	Electrochemistry (15)	II.6	Concentration, Antigen expression	-	2
Bulk	SPRi (22)	II.7	Antigen expression	-	30^b
Suspension					
Single	NTA (23)	I	Particle size distribution	30	4×10^{-10}
	Raman (14)	II.4	Chemical composition	80-320	9×10^{-8}
	FCM (19)	II.8	Antigen expression, Refractive index	200	5×10^{-6}
	FM (18)	II.9	Antigen expression	1000	9×10^{-5}

“The column “Throughput” illustrates the specificity and sample handling capacity of the techniques used in this project by estimating the inverse value of the time needed (hr⁻¹) to detect the 50 tdEVs expected to be present in 1 µL of plasma. It demonstrates the drawbacks of conventional techniques (e.g. it would take 21 years to find all tdEVs in 1 µL of plasma using flow cytometry) and clarifies the need for in-situ enrichment and sensitive detection for diagnostic applications. Considering that the total area of all particles (lipoproteins and EVs, see Figure 1.1.) distributed over a densely packed monolayer is ~400cm², the following assumptions were made:

TEM) 2.2×2.2µm² imaging area, imaged in 1 min, with a capturing efficiency of 21%.

SEM) 50µm × 7mm capturing area, 10% capturing efficiency, 10% detected fraction (due to sensitivity limitations), 5 min per 10×10µm² image.

AFM) 50µm × 7mm capturing area, 10% capturing efficiency, 45 min per 25×25µm² image. Raman on surface) 50µm × 7mm capturing area, 10% capturing efficiency, 1% detected fraction (due to sensitivity limitations), 17 min per 30×30µm² image.

Electrochemistry) Processing any sample takes ~30 minutes regardless of the number of tdEVs.

SPRi) Processing time is 2 minutes.^b

NTA) This technique can process 100 particles in 15 minutes, i.e. 1010 measurements have to be performed to find all tdEVs in 1 µL of plasma.

Raman in suspension) 1012 measurements of 38 ms.

Flow cytometry) The sample must be diluted 109 times to ensure 1 detection event corresponds to 1 particle; 3 µL can be processed in 1 minute; 50% of particles fall below the detection limit.

Fluorescent microscopy) An area of 3×3µm² can be imaged in 1s; 99% of particles fall below the detection limit.

^bPlease note that 200 µL of sample is needed before the 10⁴ EVs/µL limit of detection is reached.

I. Preparation of EV samples

Two prostate cancer cell lines (PC3 and LNCaP) purchased from the American Type Culture Collection (ATCC, Manassas, VA) were used to obtain prostate cancer-derived EVs. The cell lines were cultured at 37 °C and 5% CO₂ in RPMI-1640 with L-glutamine (Lonza, Basel, Switzerland) supplemented with 10% v/v fetal bovine serum (FBS), and 1% v/v penicillin and streptomycin (Lonza). Medium was refreshed every second day. The initial cell density was 10,000 cells/cm² as recommended by the ATCC. The cells were washed three times with phosphate buffered saline (PBS; Sigma, Saint Louis, MO) when they reached 80–90% confluence. Next, FBS-free RPMI medium supplemented with 0.1% v/v penicillin and streptomycin was added to the cells. After 48 h of cell culture, the cell supernatant was collected and centrifuged for 30 minutes at 1,000 g. The supernatant was collected and aliquots were snap-frozen in liquid nitrogen and stored at –80 °C.

Red blood cell concentrate (150 mL) obtained from Sanquin (Amsterdam, The Netherlands) was diluted in a 1:1 ratio with filtered PBS (154 mM NaCl, 1.24 mM Na₂HPO₄·2H₂O, 0.2 mM NaH₂PO₄·2H₂O, pH 7.4; 0.22 µm filter (Merck Chemicals BV, Darmstadt, Germany)) and centrifuged three times for 20 minutes at 1,560 g. Platelet concentrate (100 mL) obtained from Sanquin was diluted in a 1:1 ratio with filtered PBS. Next, 40 mL acid of citrate dextrose (ACD; 0.85 M trisodiumcitrate, 0.11 M D-glucose, and 0.071 M citric acid) was added and the suspension was centrifuged for 20 minutes at 800 g. Thereafter, the supernatant was centrifuged three times (20 minutes at 1,560 g) to ensure removal of platelets. The supernatant was collected and aliquots of 50 µL were snap-frozen in liquid nitrogen and stored at –80 °C.

The particle size distributions of the EV samples were obtained using nanoparticle tracking analysis (NTA NS500; Nanosight, Amesbury, UK), equipped with an electron multiplying charge-coupled device (EMCCD) camera and a 405 nm diode laser (Figure 1.2.A.). Silica beads (105 nm; Microspheres-Nanospheres, Cold Spring, NY) were used to focus the microscope objective. Samples were diluted 10 to 2,000 times in filtered PBS to ensure the number of particles in the field of view was below 200 per image. Of each sample, 10 videos of 30 s were captured with the camera shutter set at 33.31 ms and the camera gain set at 400. All samples were analyzed with the

instrument software (NTA 2.3.0.15) using a threshold of 10, which was based on the exponential decay constant of the summed intensity histogram of all frames in each movie (MATLAB, v.7.9.0.529; Mathworks, Natrick, MA).

Figure 1.2.B. shows the measured particle size distributions of the EV samples. We estimate the smallest detectable EV for NTA to be 70-90 nm (22).

Figure 1.2. Particle size distributions of extracellular vesicle (EV) samples measured using nanoparticle tracking analysis (NTA). A) Schematic representation of the NTA setup. A laser beam illuminates the particles in suspension. The light scattered by particles undergoing random motion (white arrow) is collected by a microscope objective and detected by an EMCCD camera. The random motion of the particles under Brownian motion can be related to their size. B) NTA analysis results of the PC3 EV (green), LNCaP EV (blue), red blood cell EV (red), and the platelet (black) EV samples, respectively. The bin width is 10 nm. The mean particle size and concentration in the PC3 EV sample are 172 ± 4 nm and $1E8$ particles/mL, respectively. The mean particle size and concentration in the LNCaP EV sample are 167 ± 4 nm and $1E8$ particles/mL, respectively. The mean particle size and concentration in the red blood cell EV sample are 148 ± 4 nm, and the concentration is $1E8$ particles/mL, respectively. The mean particle size and concentration in the platelet EV sample are 89 ± 5 nm and $4E7$ particles/mL, respectively. Because the uncertainty in the determined concentration with NTA is unknown, the determined concentration should be interpreted as an order of magnitude estimate (22). Images adapted from (23, 24).

II.1 Transmission electron microscopy (TEM)

Cancer-ID specific method and operating principle

TEM is widely available, it has become the standard technique to confirm the presence of EVs in samples (25). TEM transmits electrons through sufficiently thin (< 100-200 nm for biological materials) samples to make images with possibly sub-nm resolution

(26). Particles from the sample are adhered to a carbon coated formvar grid. Because EVs compete with other negatively charged particles for space on the grid, removal of soluble proteins and/or salts, for example by size exclusion chromatography (SEC) (27) and/or concentration, is required prior to incubation with EV samples. In addition, because TEM is performed in vacuum, EV samples are fixed with paraformaldehyde. After fixation and adhesion, the grid is placed on a droplet of contrast agent (uranyl acetate). A filter paper is used to remove the excess of contrast agent and the grid is dried at room temperature (28).

Next, the grid is exposed to an electron beam and images are constructed based on the detected transmitted electrons (Figure 1.3.A.). The contrast agent scatters electrons efficiently and stains the background more efficiently than the EVs. Consequently, EVs appear as bright particles on top of a dark background.

EV definition

Water, the main cargo of an EV, is evaporated upon TEM. Therefore, EVs often appear as ‘cup-shaped’ (29-31) or ‘saucer/doughnut-shaped’ particles (32-34) (Figure 1.3.B.). Because water is not the major component of other particles, other particles maintain their original structure during TEM. For example, lipoproteins appear spherical and protein aggregates have an irregular shape. Therefore, we define EVs as cup-shaped particles larger than 30 nm (11).

Value added by Cancer-ID

We show that TEM images taken by operator selection, the current standard within the EV field, can be used to demonstrate the presence of EVs in a sample. However, the examination of the morphology of EVs by TEM shows an operator bias in their identification (11), which may lead to “cherry picking” and emphasizes the importance of an automated and objective assessment of EV identification. Two important steps to improve the comparability and reproducibility of TEM for monitoring the quality of EV samples, are (1) to take images at predefined locations, and (2) provision of both close-up and wide-field images, as adopted by MISEV2018 (35).

Figure 1.3. Transmission electron microscopy (TEM) of extracellular vesicle (EV) samples. A) Schematic representation of TEM imaging for EV samples. The sample on a grid is exposed to an electron beam and images are constructed based on the detected transmitted electrons. The uranyl acetate (UA), scatters electrons efficiently, which results in negative contrast. EVs and lipoproteins (LP) have a low electron density and are seen as bright particles in a dark background. B) TEM images of the EV samples from PC3 and LNCaP, and of red blood cells and platelets after size exclusion chromatography. The scale bar corresponds to 500 nm.

Relevance for cancer diagnostics

Although with appropriate sample preparation TEM can image EVs down to 30 nm, the contrast of TEM images is often insufficient to distinguish EVs from similar sized non-EV particles (Figure 1.3.B.). Moreover, to identify tdEVs, immuno-gold labeling is necessary. However, the main limitation of TEM for tdEV detection is the low throughput, see Table 1.1. Therefore, TEM is not a relevant technique for detection of tdEV in plasma samples.

II.2 Scanning Electron microscopy (SEM)

Cancer-ID specific method and operating principle

EV samples are fixed in paraformaldehyde, followed by gradual dehydration from 70% to 100% ethanol in water with a 10% concentration increment step every 5-10 minutes. Subsequently, chemical drying of the sample can be achieved using 1:1 hexamethyldisilazane (HMDS) in ethanol for 3-5 minutes, followed by 100% HMDS for 3-5 minutes more. EVs are dehydrated and dried to maintain their morphological and surface features with minimal deformation in the vacuum chamber of the SEM (36, 37). EV samples are coated with gold to increase the image contrast and avoid surface charging. Furthermore, the sample must be placed on a conductive substrate during imaging. The entire procedure is conducted at room temperature.

In SEM imaging, a focused beam of electrons scans the surface of a sample interacting with all atoms in the sample (Figure 1.4.A.). Detection of the secondary electrons, originating from the outer layers of the sample, enables to visualize the topography of a sample. The amount of backscattered electrons, originating from the deeper layers of the sample, is associated with the atomic number of the atoms in the sample.

EV definition

Since the LNCaP EV sample is derived from cell culture, we don't expect particles like lipoproteins to be present in this sample. Figure 1.4.B. shows round particles (white arrows) in lower and higher magnification, which we define as EVs.

Value added by Cancer-ID

We show that cells and EVs captured on functionalized substrates and in solution can be imaged by SEM.

Relevance for cancer diagnostics

SEM can be used to visualize the topography of tdEVs, as small as 50 nm but is unable to discriminate EVs from nonEV particles with a similar morphology. In order to confirm the nature of the particles, immunogold labeling or correlative techniques are required such as AFM, Raman, or fluorescence imaging. Furthermore, similar to TEM, the main limitation of SEM is the low throughput. Therefore, SEM is not a relevant technique for detection of tdEV in plasma samples.

Figure 1.4. Scanning electron microscopy (SEM) of extracellular vesicle (EV) samples. A) Schematic representation of a SEM setup. SED: secondary electron detector, BED: backscatter electron detector. The sample is illuminated by the electron beam. Electrons interact with the sample at different depths, resulting in emitted electrons from the surface (secondary electrons) and from deeper layers (backscattered electrons). B) SEM image of LNCaP EVs indicated by arrows. The large object in the left lower corner is part of a LNCaP cell floating in the cell supernatant and was imaged to show that the contrast of EVs is similar to cells. The scale bar represents 2 μm . Higher magnification allows imaging of smaller particles, possibly EVs, with lower contrast. The scale bar represents 500 nm.

II.3 Atomic force microscopy (AFM)

Cancer-ID specific method and operating principle

EVs are added onto a poly-L-lysine coated coverslip (38–41). Next, the well is filled with filtered PBS (0.2 μm filter; VWR International, Radnor, PA) and placed on the AFM. During AFM imaging, a cantilever with a nm-sized tip probes the sample surface (Figure 1.5.A.) (42). Deflection of the cantilever is measured with a laser and photodiode. AFM images are acquired in PeakForce Tapping® mode using minimal imaging force providing information about the topography of the samples surface. Mechanical properties can be obtained by applying a defined force perpendicular to the surface (indentation), providing force-indentation curves, as presented in Figure 1.5.B.

Figure 1.5. Atomic force microscopy (AFM) of extracellular vesicle (EV) samples. A) Schematic representation of the AFM setup. In AFM, a cantilever interacts with the sample and the reflected laser beam is detected by a photodiode. The experiments are performed in liquid (not depicted). B) Example of force-indentation curves (distance z) of the extend and retract response on an EV. AFM images of responses of LNCaP EVs (C) and platelet EVs (D) to an applied force before (first row) and after indentation (second row). Both the LNCaP and the platelet EVs can change shape upon indentation. The different responses are illustrated by the cross sections (bottom row), taken at the indicated spots in the corresponding AFM images above (red: before indentation; black: after indentation). Scale bars represent 50 nm.

EV definition

With AFM, we characterize an EV as a particle of at least 25 nm in height with a spherical shape. Aggregates typically have a non-spherical shape, and therefore can be excluded. The nanoindentation response is used to identify single EVs (39-41). A typical indentation curve is characterized by a (close-to) linear initial increase of force followed by a softening and finally bilayer pinching close to the substrate (Figure 1.5.B., red curve).

Value added by Cancer-ID

Unique characteristics, like deformability, of tdEVs compared to EVs of other origin still need to be explored. Examples of AFM measurements of LNCaP EVs and platelet EVs are shown in Figure 1.5.C. and D. Importantly, it should be noted that AFM imaging per se is not distinguishing between EVs and lipoproteins. Therefore, a good purification protocol is necessary (combining gradient-based and size-based isolation methods) in order to assure only EVs are present.

Relevance for cancer diagnostics

Because of the nanometer position sensitivity and sub-piconewton force sensitivity, AFM can be used to determine the topography, morphology, and mechanical characteristics of single EVs, and differences between EVs of different origins can be investigated (38-41). Since with AFM only one particle can be observed at a time, AFM is not a suitable technique for tdEV identification and enumeration in plasma samples.

II.4 Raman microspectroscopy in suspension**Cancer-ID specific method and operating principle**

EV samples are diluted in PBS to a concentration of approximately 10^9 particles/mL (as measured by NTA) and placed on a well glass slide, covered with a glass cover slip, and sealed with glue. Next, the glass slide is placed under the microscope objective (Figure 1.6.A.). A Raman optical tweezer is used to (i) trap single particles diffusing near the high intensity part of the focus (Figure 1.6.A.), and (ii) detect both Rayleigh and Raman scattered photons synchronously. The trapping of a single particle is detected by Rayleigh scattering and the corresponding Raman spectrum discloses the chemical composition (14).

EV definition

The Raman spectra of submicrometer particles in biofluids have distinct spectral features depending on the nature of the particle or the source of EVs.

Value added by Cancer-ID

The procedure to trap, release and acquire sequentially the spectrum of single EVs in the focal volume is automated (14). Furthermore, EVs can be distinguished from lipoproteins and EVs from different sources, like PC3 EVs, LNCaP EVs, and red blood cell EVs. EVs show distinctive peaks at 1004 cm^{-1} and 1607 cm^{-1} (phenylalanine) (23), and a larger protein contribution at $2811\text{-}3023\text{ cm}^{-1}$ than lipoproteins (Figures 1.6.B. and C.). The Raman spectrum of red blood cell EVs is different from PC3 EVs and LNCaP EVs around $1200\text{-}1385\text{ cm}^{-1}$ and $1510\text{-}1631\text{ cm}^{-1}$. Further classification of EVs and lipoproteins was achieved by multivariate analysis and convolutional neural networks analysis (23, 43)

Relevance for cancer diagnostics

Differences in chemical composition are shown between EVs and lipoproteins, and tdEVs compared to red blood cell EVs. However, a limitation of Raman is the throughput. As an example, a typical acquisition time per EV is 1 second (14). It has become clear that enrichment of tdEVs is needed and a combination with another technique may be required to provide assurance that indeed tdEVs are being investigated.

Nevertheless, spontaneous Raman spectroscopy provides information on the chemical composition of single or multiple EVs in solution or on a surface in a non-invasive and label-free manner (14, 23, 44-48).

Figure 1.6. Raman spectroscopy of extracellular vesicle (EV) samples. A) Particles in suspension are loaded in a well glass slide that is mounted under a microscope objective. Incident light illuminates the sample and both Raman and Rayleigh light is backscattered, collected by the lens, and detected by a spectrograph. Raman spectra corresponding to single (B) and multiple (C) PC3 EVs (blue), LNCaP EVs (green), red blood cell EVs (red), and lipoproteins in plasma (black). Figure A is adapted from (14).

II.5 Integrated photonics lab-on-chip devices for Raman Spectroscopy

Cancer-ID specific method and operating principle

Two lab-on-chip devices were developed by Cancer-ID. From a technological perspective, Cancer-ID exploits the possibility of lab-on-chip devices to localize light in ways that are impossible with traditional optics. For example, compared to optical trapping using a microscope objective (section II.4), we expect that combining multiple beams will result in higher field gradients and therefore trapping of smaller single EVs. To proof the principle, device type 1 contains multiple waveguides which emit multiple beams of light towards the center of a well as shown in Figure 1.7.A.. The beams combine coherently to form multiple regions of high light intensity, each serving as an optical trap sufficiently strong to trap single submicrometer particles near the well center. The same concentrated light induces a Raman spectrum from the

trapped particle for label-free identification. To increase the throughput, the well may be replaced by a flow cell in future versions.

To increase throughput compared to optical trapping using a microscope objective (section II.4), device type 2 combines an enrichment step with the simultaneous detection of Rayleigh and Raman scattered light from multiple EVs. EVs in suspension bind to antibodies at the surface of a spiral waveguide, which is placed at the bottom of a microfluidic channel as shown in Figure 1.7.B.. A laser field propagates inside the waveguide and produces an evanescent field that probes the attached EVs simultaneously. The EVs will scatter some of this light with characteristic Raman shifts. A significant portion of this light re-enters the waveguide and can be collected from the entrance through the same objective that launched the excitation light.

EV definition

An EV is identified based on the acquired Raman spectrum of the trapped particle. The obtained spectra may be cross-referenced with EV spectra already acquired with standard spontaneous Raman tweezers (section II.4). Furthermore, using device type 2, EVs are bound to the surface of a spiral waveguide by a specific antibody.

Value added by Cancer-ID

Both devices are still under development, so the throughput and detection limit remain to be determined. In device type 1, integration of the light beam with a microfluidic channel opens new possibilities of controlled particle delivery to the trap and particle sorting with pressure driven flow which may allow the detection of smaller EVs. In device type 2 specific capture of tdEVs from plasma is possible by the use of antibodies coated on the surface of a spiral waveguide using the chemistry used in II.6 & II.7.

Relevance for cancer diagnostics

Based on the differences in chemical composition tdEVs can be distinguished from non-EV particles like lipoproteins, and EVs from other origin. Furthermore, enrichment can be achieved by the use of antibodies bound to the surface of a waveguide. Raman spectroscopy of EVs provides information on the chemical composition of single or

multiple EVs in a non-invasive and label-free manner and may be simplified using integrated photonics lab-on-a-chip devices.

Figure 1.7. Integrated photonics based lab-on-a-chip Raman spectroscopy. A) Device type 1: camera image of a device with 16 waveguides for trapping and 4 waveguides for detection. The device is actuated with light from an input fiber that is embedded in a fiber array unit (FAU) at the lower right-hand side. The various structures light up as a result of light scattering, causing some saturation of the camera. The solid red lines indicate the chip edges. 1: FAU. 2: Excitation-waveguide circuitry. 3: Micro fluidic bath with the central trapping region. 4: Detection-waveguide circuitry. 5: Light from the trap that is coupled out by the detection waveguides. Here, the detection waveguides collect light as a result of direct illumination and scattering. B) Device type 2: Spiral waveguide with the Raman pump light travelling inside the waveguide. The Raman signal is (partially) scattered back into the waveguide and collected at the front entrance.

II.6 AFM-SEM-Raman

Cancer-ID specific method and operating principle

The surface of stainless-steel substrates is modified with a carboxydecyl phosphonic acid monolayer to covalently link anti-EpCAM antibodies to the substrate (Figure 1.8.) (49). EVs are incubated in poly(dimethylsiloxane) (PDMS) microchannels. The microchannels are washed to remove non-specifically bound material. Next, EVs are incubated with paraformaldehyde in PBS for 15 minutes. The PDMS is removed by immersion in de-ionized water, 70% ethanol in water, and finally 100% ethanol for 5 minutes each step. Incubation of tdEVs was followed by washing and overnight drying. Alignment markers are embedded on the stainless-steel substrate by injecting patterned microfluidic channels with cyanoacrylate glue. The micro-scale alignment markers facilitate retracing individual EVs in the sample stages of the AFM, SEM and

Raman microspectroscopy. SEM is used here to select regions of interest and confirm that the surface is successfully functionalized based on the attachment of EVs (13).

Figure 1.8. Atomic force microscopy (AFM), scanning electron microscopy (SEM), and Raman spectroscopy of extracellular vesicle (EV) sample. Schematic representation of the system: antibody-functionalized stainless-steel substrate examined with SEM, AFM and Raman for correlated multi-modal analysis of individual EVs. Image adapted from (13).

EV definition

EVs are identified by SEM and a Raman spectrum with lipid-protein peaks ($2811\text{--}3023\text{ cm}^{-1}$) characteristic for EVs. The functionalization of the substrate ensures that the EVs are of epithelial cell origin permitting the determination of the mechanical characteristics, like deformability, of the tdEVs by AFM.

Value added by Cancer-ID

The use of only one technique is often insufficient to identify and characterize EVs, as discussed in the previous sections (35). For example, both EVs and lipoproteins appear to be spherical by SEM. By combining SEM with AFM and Raman, we measure characteristics like size, chemical composition, and deformability to add certainty to the identification of tdEVs (13).

Relevance for cancer diagnostics

Using a combination of AFM, SEM and Raman and the capture of tdEVs to a functionalized surface helps to distinguish EVs from non-EV particles and adds certainty to the origin of the EV.

In principle this platform does not require distinguishing tdEVs from other species since enrichment is done by the functionalized surface. Since SEM measurements are faster than AFM or Raman, SEM was used for initial confirmation of tdEV presence on a chip; after enrichment 1000 tdEVs (of > 100 nm) can be imaged in 1h. Since AFM detects the more abundant much smaller particles (> 30 nm) as compared to SEM (> 100 nm), the fact that AFM is slower in terms of imaged μm^2 per unit time, is offset by a greater number of observed tdEV per imaged μm^2 , such that 1,000 tdEVs can be imaged in 2h. For Raman, detection of 1,000 tdEVs would require about 100 measurements of 17 minutes each followed by several days of data processing.

II.7 Electrochemistry

Cancer-ID specific method and operating principle

Interdigitated nano-electrodes (nIDEs) are surface-modified with poly(ethylene glycol) diglycidyl ether to form an amine-reactive anti-fouling layer (Figure 1.9.A.) (52). Anti-EpCAM antibodies are covalently linked to this layer and the remainder of the surface blocked with bovine serum albumin (BSA). EV samples are introduced onto the device to allow binding to the electrodes. After incubation, a biotinylated reporter anti-EpCAM is introduced. The biotin moiety conjugates to streptavidin coupled to alkaline phosphatase (ALP). ALP, only present on EpCAM-positive particles, converts an electrochemically inert molecule (para-aminophenyl phosphate) into a redox-active species (para-aminophenol), to yield a first amplification phase. Next, the para-aminophenol undergoes redox cycling, providing a second amplification phase.

EV definition

An increase in the redox current upon binding of particles to the nIDEs defines the presence of EVs. EVs from different species can be distinguished from each other by employing targeted antibodies, yielding a very high selectivity. For example, the signal

from platelet EVs did not vary from the background signal, whereas the introduction of LNCaP EVs markedly increased the signal (Figure 1.9.B.) (15).

Value added by Cancer-ID

This new and sensitive technique was developed by Cancer-ID in collaboration with researchers from the NanoElectronics group at University of Twente, the Netherlands. Several examples of sensitive integrated systems for (td)EV detection exist (51-53). A unique feature of the technique discussed here is the ability to detect a low concentration of EVs with a low antigen expression. The linear response covers a broad range of concentrations, which largely overlaps with concentrations of tdEVs in patient blood.

Relevance for cancer diagnostics

Using electrochemistry, tdEVs can be discriminated from non-EV particles and EVs from other origin based on the expression of EpCAM. A dilution series of LNCaP EVs in PBS showed a linear response ranging from 5×10^3 - 10^9 tdEVs/mL (Figure 1.9.C.) (15), which overlaps with the expected tdEV concentration in plasma (10), showing this technique is promising to identify, count, and characterize tdEVs in the range of clinical samples. Evaluation of the technique with plasma patient samples and association of the read-out with clinical outcome remain to be tested.

The functionalized device is incubated with tdEV-containing samples and subsequently with reporter antibodies and redox mediator. In the experiments performed in the paper these incubations were done over excessively long periods (2.5 h in total) to maximize the efficiency but once optimized, can probably be performed in several minutes to 1h. The cyclic voltammetry measurements were performed in 20 minutes, regardless of the concentration of tdEVs ($5 \times 10^3 - 10^9$ per mL of sample).

Figure 1.9. Electrochemical detection of extracellular vesicle (EV) samples. A) Scheme showing selective capture and in-situ labeling of EVs followed by enzymatic amplification of redox species and redox cycling. B) Cyclic Voltammograms recorded for a very wide range of EV concentrations. C) Recorded current at 0.4 V for varying concentrations showing linear response over 6 orders of magnitude. Image adapted from (15).

II.8 Surface Plasmon Resonance imaging (SPRi)

Cancer-ID specific method and operating principle

The surface of a SPRi sensor is coated with a conductive gold layer and a 3D hydrogel-like layer to reduce non-specific binding of non-EV particles to the surface (Figure 1.10.A.). Antibodies are printed on 48 spots on the sensor (Figure 1.10.B.), including isotype controls and a control (PBS) to correct for dissociation and non-specific binding (16). Next, the surface is washed and deactivated by incubation with 2-amino ethanol followed by BSA. After an EV sample is exposed to the sensor, EVs bind to the antibody-coated sensor spot, which increases the refractive index near the sensor surface. This increase in refractive index is measured in time using the angle scanning principle of the IBIS MX96 instrument (IBIS Technologies, Enschede, The Netherlands) and corresponds to the number of particles captured on the spot (Figure 1.10.C.).

EV definition

With SPRi, EVs are identified based on their antigen exposure. EVs bind to antibodies printed on the sensor, e.g. anti-CD9, anti-CD63, anti-epidermal growth factor receptor (anti-EGFR), anti-EpCAM, anti-olfactory receptor 51E2 (anti-OR51E2), transient receptor potential cation channel subfamily M member 8 (TRPM8) and lactadherin, see Figure 1.10.D.. SPRi detects a difference in response on the antibody spots between EV samples derived from different cell lines.

Value added by Cancer-ID

Characterization of EVs by SPRi, using the IBIS MX96, revealed the ability to detect cell surface antigens present at relatively low antigen densities compared to cells, as their presence could not be detected by flow cytometry (16).

Relevance for cancer diagnostics

SPRi can be used to distinguish tdEVs from non-EV particles and EVs derived from other cells based on the antigen expression. The IBIS MX96 is able to detect antigens present at a low density on EVs compared to cells (16). However, the required EV

concentration to perform these measurements is high (2×10^8 EVs/mL), and not within the range of the expected tdEV frequency.

Figure 1.10. Surface plasmon resonance imaging (SPRi) analysis of extracellular vesicles (EVs).

A) Schematic of a SPRi set-up. A SPRi signal is generated when the sensor surface is illuminated at various angles with light and surface plasmons are excited (54). The resonance angle, specific angle (beam 2) where maximum plasmon excitation and minimal internal reflection occurs (55), depends on the refractive index contrast near the interface in the evanescent field. B) The 48 spots on the SPRi sensor surface can be coated with different antibodies. In this example only 9 antibody coated spots on the SPRi sensor surface are shown. C) An EV sample is exposed to the SPRi sensor and measured for 60 minutes. The attachment of an ensemble of EVs to a specific antibody spot causes a change in the refractive index and generates a SPRi signal over time. D) The SPRi signals after incubation with four prostate cancer-derived EV samples are shown. The two CD63-clones show the same results for all samples. All samples are slightly positive for CD63, EGFR, and CD9. A higher positivity is seen for EpCAM and Lactadherin. The SPRi signals for the 22RV1-EV sample are higher compared to the other samples.

II.9 Flow cytometry (FCM)

Cancer-ID specific method and operating principle

EV samples are diluted in PBS (21-031-CV; Corning, Corning, NY) to prevent swarm detection (56) and stained with fluorescently-labelled antibodies. Antibody aggregates are removed by centrifugation prior to use. The “antibody supernatant” is added to

the EV sample followed by a two-hour incubation step, which is stopped by diluting the incubated sample with PBS.

In a flow cytometer, the sample is hydrodynamically focused with sheath fluid to intersect a laser beam (Figure 1.11.A.). Scattered light and fluorescence from the particle are collected by a forward scatter detector, a side scatter detector, and multiple fluorescence detectors (57) (Figure 1.11.B.). The measured scatter and fluorescent signals per particle can be represented and analyzed using scatter plots as shown in Figure 1.11.C..

EV definition

EV identification by FCM is commonly based on the expression of one or more antigens, which are detected using fluorescent immunostaining. Recently, we found that the refractive index of particles can be used as an additional parameter to distinguish EVs from lipoproteins (19). We therefore define an EV as a particle that expresses detectable levels of one or more antigens, and has a refractive index < 1.42 .

Value added by Cancer-ID

Within Cancer-ID, technology to determine the size and refractive index of submicrometer particles was partly developed, evaluated and used to find new applications. Based on refractive index, for example, EVs can be differentiated from lipoproteins without antibody labeling (37). Refractive index determination was used to show that generic EV dyes, which are commonly used to label EVs in FCM measurements, do not label all EVs and do label non-EV particles (17). The combination of antibody labeling and refractive index determination could be used to increase specificity of EV detection. Furthermore, the side scatter sensitivity of a conventional flow cytometer was improved 30-fold by systematically modifying the hardware and a method was developed to quantify the scatter sensitivity of a flow cytometer.

Relevance for cancer diagnostics

FCM measures light scattering and fluorescence from thousands of individual particles per second. Although detection of the smallest single EVs is possible (58), only the most sensitive commercial flow cytometers are able to detect EVs with a diameter < 200 nm (59). Based on the combination of an antibody and the refractive index, it is possible

to discriminate tdEVs from lipoproteins and EVs from other origin. However, plasma samples are typically pre-diluted 10-100 times before measurements to prevent swarm detection. This dilution means the detection of the few tdEVs that might be present in the plasma sample is impossible.

Nevertheless, FCM provides information on the concentration, cellular origin and biochemical composition, size and refractive index of single EVs (12, 20, 60).

Figure 1.11. Flow cytometry of extracellular vesicle (EV) samples. A) In flow cytometry, a single particle suspension is hydrodynamically focused with sheath fluid (arrows) to intersect a laser. Light coming from the particle is collected by a forward scatter detector (FSC), a side scatter detector (SSC), and multiple fluorescence detectors (FL1, FL2, etc.). B) Fluorescence (green dashed line) is isotropic and can be used to determine antigen expression and cellular origin. Scatter (blue solid line) has an angular distribution that depends on the size and refractive index of the particle (here 200 nm polystyrene). Knowledge of the flow cytometer collection angles and Mie theory allows derivation of particle size and refractive index from the measured scatter signals (12, 20). C) Scatter plots of side scatter versus fluorescence for the PC3 EV sample stained with CD63-PE (left), the LNCaP EV sample stained with EpCAM-Alexa Fluor 647 (center), and the platelet EV sample stained with CD61-FITC (right). In PC3 EV sample 14.1% was found to be positive for the EV marker CD63, in the LNCaP EV sample 7.8% was found to be positive for cell surface epithelial marker EpCAM, and in the platelet EV sample 5.4% of the particles was found to be positive for CD61. BB: blocker bar, FL: fluorescence.

II.10 Immunomagnetic EpCAM enrichment followed by fluorescence microscopic (FM) detection

Cancer-ID specific method and operating principle

Blood is collected in CellSave blood collection tubes (Menarini, Huntingdon Valley, PA). After centrifugation of 7.5 mL of blood for 10 minutes at 800 g, the sample is placed in the CellTracks Autoprep (Menarini, Huntingdon Valley, PA). The Autoprep aspirates and discards the plasma whereas the blood cell fraction is incubated with anti-EpCAM ferrofluid (Figure 1.12.A., step 1). The particles (cells and EVs) bound to the ferrofluid are separated from the rest of the blood by the application of magnetic forces (step 2). Following the immunomagnetic isolation, EpCAM-enriched particles are stained with the nuclear dye DAPI and fluorophore-conjugated antibodies recognizing the epithelial specific cytokeratins 8, 18 and 19 (CK-PE) and the leukocyte specific marker CD45 (CD45-APC) (step 3). The stained sample is loaded in a cartridge and placed between two magnets configured in such a way that all stained EpCAM+ enriched particles homogeneously align on the glass slide on the surface of the cartridge (step 4). The cartridge is scanned using the CellTracks Analyzer II (Menarini, Huntingdon Valley, PA), a fluorescence microscope equipped with a 10x 0.45 NA objective (step 4). The images are analyzed using the open-source ACCEPT software to identify circulating tumor cells (CTCs), tdEVs, leukocytes and leukocyte EVs (step 5).

EV definition

tdEVs are defined as EpCAM+, CK+, DAPI-, CD45- particles. A gate for their automated enumeration from the CellSearch image data sets has previously been reported (18).

Value added by Cancer-ID

The application of a tdEV gate resulted in 0 events in a blood sample of a healthy individual and in 3,772 events in a blood sample of the same healthy donor after spiking with LNCaP EVs (Figures 1.12.B. and 12.C.).

Re-analysis of digitally stored image data sets of retrospective clinical studies using EpCAM-enrichment and FM already suggest that large tdEVs ($> 1 \mu\text{m}$), co-isolated with CTCs, are negatively associated with the overall survival of metastatic prostate, colorectal, breast and non-small cell lung cancer patients in a similar way as CTCs

(Figure 1.12) (61) and could contribute in monitoring the disease and assessing therapeutic efficacy. However, the existing technique was developed for the detection of CTCs and eliminates the detection of smaller tdEVs or tdEVs with low antigen density even if they have been isolated by the anti-EpCAM ferrofluid.

Relevance for cancer diagnostics

The CellSearch system can be used to enrich tdEVs based on their EpCAM expression, as EpCAM is not expected to be present on EVs in blood of healthy individuals (62, 63). However, tdEVs isolated by the CellSearch system are limited to the relative larger EVs ($> 1 \mu\text{m}$), as the plasma obtained after centrifugation at 800 *g* is discarded.

III. Cancer-ID insights

Cancer-ID delivered new techniques and new insights to explore tdEV detection. Taken the complexity of blood into consideration, the necessity of enriching biological samples for tdEVs becomes obvious. EVs secreted from prostate cancer cell lines and EVs derived from red blood cells and platelets, resembling the expected background of EVs in plasma, were used to explore the utility of different techniques. The size distribution of the EV samples was characterized by NTA, the EV size and/or morphology by TEM, SEM and AFM, the biochemical composition by Raman spectroscopy, and their antigen expression profile of EVs using SPRI, FM and FCM. The techniques were able to detect or image EVs present in EV samples from cultured tumor cells. However, discrimination between EVs and non-EV particles becomes difficult in complex samples like plasma, because non-EV particles outnumber EVs (Figure 1.1.). Furthermore, most techniques cannot identify the cellular origin of single EVs and relate the measured signal or count to the concentration of tdEVs in plasma. The results of all individual techniques pointed out that a combination of more than one parameter or technique increases the certainty that tdEVs are being investigated and immune affinity enrichment or detection is needed to cover the large size and density range of EVs.

EV isolation protocols have not been standardized within the EV field (64, 65). Size-based isolation techniques, such as size exclusion chromatography can purify samples from contaminating lipoproteins and soluble protein of a size below 70 nm (27).

Figure 1.12. EpCAM immunomagnetic enrichment and fluorescence microscopic (FM) detection of extracellular vesicle (EV) samples. A) Principle of the CellSearch system. ACCEPT analysis of two CellSearch cartridges corresponding to EpCAM enriched blood sample of a healthy donor without (B) and with LNCaP EVs spiked (C). CD45 is depicted in red, CK in green and DAPI in blue. The objects falling in the applied tdEV gate are depicted as blue dots in the scatter plots of CD45 Mean Intensity versus CK Mean Intensity. The other particles are shown as grey dots. Thumbnail examples of 4 objects are shown. The CD45+, CK+ particles are attached to the leukocytes, as illustrated. Scale bars indicate 6.4 μ m. Panel D and E show Kaplan Meier plots of overall survival of 956 metastatic colorectal, prostate, breast and non-small cell lung cancer patients. Patients were grouped based on their circulating tumor cells (CTC) (Panel D) or tumor-derived EV (tdEV) counts (Panel E) demonstrating the equivalent prognostic power of CTCs and tdEVs.

Furthermore, centrifugation is often used to isolate biomarkers from whole blood. In the Cancer-ID program, we developed a model to predict the behavior of particles (cells and EVs) in solution during centrifugation, and showed the co-isolation of for example platelets and large EVs after centrifugation (65). Furthermore, although the application of rate zonal centrifugation improved the separation of platelets from EVs, the aforementioned isolation techniques result in purification of EVs rather than enrichment of tdEVs.

By the use of affinity-based techniques using antibodies directed to antigens expressed on tumor cells but not on blood cells we demonstrated the enrichment of large ($> 1 \mu\text{m}$) EpCAM+ tdEVs from blood from metastatic cancer patients (18). EVs from different origin were eliminated in the enriched sample. Efforts for the immunomagnetic enrichment of smaller ($< 1 \mu\text{m}$) tdEVs from plasma samples based on EpCAM are ongoing. The frequency of small tdEVs shown in Figure 1.1. is based on an extrapolation from the frequency of the large tdEVs and this surely will need to be validated. Moreover, whether the small tdEVs have a similar relation with clinical outcome will need to be established. tdEVs likely encompass different subclasses for example those responsible for communication with the environment and those involved in the process of apoptosis of cancer cells and as such relation with clinical outcome or its cargo being informative on the optimal treatment will likely be different between these subclasses. Here only the EpCAM antigen was used to capture tdEVs, the use of different or a mixture of antibodies recognizing different cancer-specific antigens, such as VAR2CSA (66) and HsP70 (67, 68) could increase the capture efficacy and may identify different subclasses of tdEVs. Identification of tdEVs among the EpCAM enriched particles was obtained through identification of the presence of intravesical cytokeratins. The use of different components of the tdEV cargo might be important. Exploration of this cargo with label free technologies such as Raman and SPRI identified some alternative avenues that can be explored. The onset of retrieving data from the molecular content of EVs has also been explored in the Cancer-ID program. A challenge is retrieving sufficient RNA to represent the mRNA and long noncoding RNA transcriptome. As a first step, various EV RNA isolation kits were tested, and of the isolation kits tested, the Norgen total RNA isolation protocol resulted in the highest amount of RNA as determined by RT-qPCR of housekeeping and prostate-associated transcripts. Although this Norgen protocol will also extract

non-EV RNA from urine, RNA yield and coverage by RNAseq are considered of higher priority than purity for our EV-based biomarker efforts.

State-of-the-art integrated systems developed in the Cancer ID Perspectief program come close to reliably detecting tdEVs at clinically relevant concentrations at high throughput. Small tdEVs ($< 1 \mu\text{m}$) can be isolated using functionalized anti-EpCAM substrates and can be detected electrochemically in a label-free manner (15). Next, sorting of tdEV populations (as defined by fluorescence, by SPRI, electrochemically, or by Raman spectroscopy) can be used to perform downstream molecular analysis and reveal their genetic content which could play a critical role in identifying the best therapeutic strategy for cancer patients.

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Chapter 2.

Centrifugation affects the purity of liquid biopsy-based tumor biomarkers

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Abstract

Biomarkers in the blood of cancer patients include circulating tumor cells (CTCs), tumor-educated platelets (TEPs), tumor-derived extracellular vesicles (tdEVs), EV associated miRNA (EV-miRNA), and circulating cell-free DNA (ccfDNA). Because the size and density of biomarkers differ, blood is centrifuged to isolate or concentrate the biomarker of interest. Here, we applied a model to estimate the effect of centrifugation on the purity of a biomarker according to published protocols. The model is based on the Stokes equation and was validated using polystyrene beads in buffer and plasma. Next, the model was applied to predict the biomarker behavior during centrifugation. The result was expressed as the recovery of CTCs, TEPs, tdEVs in three size ranges (1–8, 0.2–1, and 0.05–0.2 μm), EV-miRNA, and ccfDNA. Bead recovery was predicted with errors $< 18\%$. Most notable cofounders are the 22% contamination of 1–8 μm tdEVs for TEPs and the 8–82% contamination of $< 1 \mu\text{m}$ tdEVs for ccfDNA. A Stokes model can predict biomarker behavior in blood. None of the evaluated protocols produces a pure biomarker. Thus, care should be taken in the interpretation of obtained results, as, for example, results from TEPs may originate from co-isolated large tdEVs and ccfDNA may originate from DNA enclosed in $< 1 \mu\text{m}$ tdEVs.

Introduction

Potential biomarkers in the blood of cancer patients include circulating tumor cells (CTCs) (1–3), tumor-educated platelets (TEPs) (4), tumor-derived extracellular vesicles (tdEVs) (5), EV-miRNAs (6), and circulating cell-free DNA (ccfDNA) (7,8). Typically, the first step to isolate a biomarker involves (differential) centrifugation, which isolates particles based on size and density. After centrifugation and optionally further processing, the biomarker is measured based on antigen exposure (CTCs and tdEVs) or on the composition of RNA (TEPs and EV-miRNAs) or DNA (ccfDNA). Fractions containing the biomarker of interest may be impure and contain substantial quantities of “contaminants”, for example, other biomarkers. These contaminants may affect the obtained signal and potentially lead to the misinterpretation of results. Therefore, we applied a model based on the Stokes equation to assess the purity of biomarkers after centrifugation according to protocols used by other groups to study their biomarkers of interest (1–8).

Materials and Methods

Stokes model

Figure 2.1. shows the principle of centrifugation. Before centrifugation, particles are uniformly distributed in a medium. Upon centrifugation, particles denser than the medium will travel toward the bottom of the tube. After centrifugation, the top fraction is collected, defined here as the “supernatant.” We define the “pellet” as the fraction remaining in the tube. The dashed line in Figure 2.1. is the interface between the supernatant and the pellet. The recovery of particles in the pellet is the fraction of particles in the pellet after centrifugation. This recovery depends on the starting point of the particle in the medium, as well as the densities and diameters of the particles. Consequently, the pellet will contain a high concentration of large and high density particles compared to the supernatant. For a swing-out rotor, the distance traveled by a particle can be modeled by the Stokes equation. In the Stokes equation, the speed of the particle results from the balance between the buoyant force and the drag force on a particle in a medium. In a centrifuge, the gravitational acceleration g depends on the distance from the axis of rotation.

Figure 2.1. Centrifugation to isolate biomarker of interest from whole blood. The position of a particle after centrifugation can be described with the Stokes equation (equation 2). Centrifugation of particles that are uniformly distributed throughout the sample causes large, high-density particles to move down to the pellet (below the dashed line), while small, low-density particles stay in the supernatant (above the dashed line).

$$(1) \quad g = R\omega^2$$

where R is the distance to the axis of rotation and ω is the angular velocity of the centrifuge. After time T , the distance R of a particle starting at R_0 is given by the following equation:

$$(2) \quad R_T = R_0 e^{\frac{d^2(\rho_P - \rho_M)}{18 S \eta} \omega^2 T}$$

where d and ρ_P are the diameter and the volumetric mass density of the particle and ρ_M and η are the volumetric mass density and viscosity of the medium. To allow nonspherical shapes such as platelets, the shape factor S is introduced ($S = 1$ for spheres). S accounts for the additional drag these nonspherical particles experience (9). Note that for nonspheres, d is the diameter of a sphere with equivalent volume. Model assumptions are (i) negligible wall effects, (ii) instantaneous formation of

the packed cell fraction during centrifugation of whole blood, and (iii) aspiration sufficiently gentle not to disturb the pellet (see (10,11) for a complete description of the assumptions in the Stokes model). The model was programmed in MATLAB (v2017a; MathWorks, Natick, MA) and applied to the evaluated centrifugation protocols.

Stokes model sample properties

Table 2.1. shows the assumed sample properties, which were derived from the literature for a temperature of 20 °C (see Supplemental Information Table S1 for additional background). If available, volumetric mass densities were taken from references that applied density media with neutral osmotic effects. Platelets have a log-normal volume distribution (12) and a shape factor S of 2.0 (9,13). Platelets can have a volumetric mass density of 1.05–1.09 g/mL, where the lower density is associated with platelets that have secreted their α -granule content but with an unaffected platelet volume distribution (14,15). Most EVs have a diameter of < 200 nm, which means that the volumetric mass density is substantially affected by the membrane density. A model describing EVs as cytoplasm enclosed by a cell-like membrane (16) leads to a size-dependent volumetric mass density. For EVs 100, 200, and 1,000 nm, the density is 1.099, 1.081, and 1.064 g/ml, respectively, which is in fair agreement with literature estimates between 1.08 and 1.11 g/ml (17–21). Finally, the mass density of ccfDNA is only available in cesium chloride media and the length of ccfDNA is unknown. Assuming a length of $< 1,000$ base pairs, the ccfDNA would be smaller than 50 nm.

Model validation

To validate the model, polystyrene beads were diluted in phosphate-buffered saline (PBS) or blood plasma. The concentration of beads was measured by flow cytometry before and after centrifugation at 300 g for 20 minutes or 2,700 g for 22 minutes using a Rotina 380R centrifuge (Hettich, Tuttlingen, Germany) or 15,800 g for 60 minutes using a SW 41 Ti rotor and Optima L-80 XP ultracentrifuge (Beckman Coulter, Fullerton, CA), all at 20 °C and with deceleration set to the minimum possible value. Polystyrene beads were 400, 799, 994, and 3,005 nm in diameter (Thermo Fisher Scientific, Waltham, MA) with a density of 1.05 g/mL. PBS (154 mM NaCl, 1.24 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.2 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$; pH 7.4) was 0.22 μm filtered (Merck, Darmstadt, Germany). Blood from anonymous healthy donors was obtained after written informed consent in accordance

with the Dutch regulations and approved by the medical–ethical assessment committee of the Academic Medical Center, University of Amsterdam. Whole blood was drawn using a 21G needle in EDTA vacutainers (BD Biosciences, San Jose, CA) and processed within 15 minutes after collection. Plasma was obtained from whole blood by double centrifugation at 2,500 g for 15 minutes at 20 °C using a Rotina 380R centrifuge. The supernatant was pooled between centrifugation steps and pooled before mixing with the polystyrene beads. The concentration of beads before and after centrifugation was measured on side scatter of a flow cytometer (FACSCalibur; BD, Franklin Lakes, NJ, 15 mW 488 nm laser, flow rate ~60 μ L/min calibrated by weight (22), SSC 400 V, gain 0, threshold 0), and data were analyzed using FlowJo (v10; FlowJo LLC, Ashland, OR).

Table 2.1. Stokes model sample properties.

Type and property	Value used in model	References
CTCs		
Density (g/mL)	1.053	(24, 25)
Diameter (μ m)	Uniform. 8-20	(26)
Platelets		
Density (g/mL)	Norm., mean 1.069, SD 0.0053	(14, 15, 27)
Diameter (μ m)	Lognorm., median 2.4, SD 0.6	(12, 28)
Shape factor	2.0	(9, 13)
EVs		
Density (g/mL)	Core 1.060, 8 nm shell 1.155	(17-21)
Diameter (μ m)	Uniform. 0.05-1	
ccfDNA		
Density (g/mL)	1.7	(16, 29)
Diameter (μ m)	≤ 0.05	(16)
Polystyrene beads		
Density (g/mL)	1.05	MFG
Diameter (μ m)		MFG
PBS		
Density (g/mL)	1.004	(30)
Viscosity (mPa.s)	1.193	(30)
Plasma		
Density (g/mL)	1.0253	(31, 32)
Viscosity (mPa.s)	1.75	(33-35)

Values at 20 °C. Lognorm.: Lognormal distribution, MFG: manufacturer specifications, Norm.: Normal distribution, SD: standard deviation, Uniform.: Uniform distribution.

Predicted performance of centrifugation protocols

We modeled the protocols as described in the literature and shown in Table 2.2. The dimensions of the low-speed centrifugation tube were derived from the 57.462 Sarstedt centrifugation tube and for ultracentrifugation from the 344059 Beckman Coulter centrifugation tube. For the model, we assume that centrifugation $< 10,000$ g is performed using a Rotina 380R centrifuge and centrifugation $> 10,000$ g using a SW41 Ti rotor and Optima L-80 XP ultracentrifuge. For most centrifuges, the set g-force is the force at the bottom of the tube. The force elsewhere is given by equation 1. This means that a 10 cm high sample in a Rotina centrifuge set to 2,500 g, has a g-force of 900 g at the top of the sample. The same centrifugation protocol on a rotor with a larger diameter will increase the g-force at the top of the sample and thus result in an increased recovery in the pellet. However, we did not model multiple rotor diameters, because most rotors suitable for large tubes have diameters comparable to the Rotina 380R. Results are expressed as the % recovery of CTCs, TEPs, tdEVs in three size ranges from large (1–8 μm , “apoptotic bodies”, “oncosomes”, or “tumor microparticles”), intermediate (0.2–1 μm , “microparticles”), to small (0.05–0.2 μm , “exosomes”) tdEVs, EV-miRNAs, and ccfDNA.

Table 2.2. Modeled centrifugation protocols.

Protocol	Blood volume and vacutainer	Protocol summary	References
CTCs / tdEVs	10 mL CellSearch	WB 7.5 mL:6.5 mL PBS 800g, 10 min $\rightarrow$ (10) Pel	(1-3, 5)
TEPs	6 mL EDTA	WB 120g, 20 min $\rightarrow$ (3) Sup 360g, 20 min $\rightarrow$ (10) Pel, wash 2x	(4)
EV-miRNA	6 mL EDTA	WB 900g, 7 min $\rightarrow$ (5) Sup 2,500g, 10 min $\rightarrow$ (5) Sup 500g, 10 min $\rightarrow$ (5) Sup	(6)
ccfDNA (Speicher)	10 mL PAXgene ccfDNA	WB 200g, 10 min, 1,600g, 10 min $\rightarrow$ (3) Sup 1,600g, 10 min $\rightarrow$ (3) Sup	(8)
ccfDNA (Thermo)	10 mL PAXgene ccfDNA	WB 1,600g, 10 min $\rightarrow$ (5) Sup 16,000g, 10 min $\rightarrow$ (10) Sup	(7)

ccfDNA: circulating cell-free DNA, CTCs: circulating tumor cells, EV-miRNAs: extracellular vesicle-associated miRNAs, min: minutes, TEPs: tumor-educated platelets, tdEVs: tumor-derived extracellular vesicles, WB: whole blood, $\rightarrow$ (xx) Pel: collect pellet xx mm above bottom of tube or buffy coat (for WB), $\rightarrow$ (xx) Sup: collect supernatant xx mm above bottom of tube or buffy coat (for WB).

Results

Model validation

Figure 2.2. shows the modeled and measured bead recovery in PBS (top) and plasma (bottom). As expected, the recovery of beads in the pellet increases with increased particle diameter, g-force, and centrifugation time. In addition, because plasma is more viscous than PBS, the recovery of beads in the pellet is lower in plasma compared to PBS. Modeled and measured recovery are in fair agreement (all errors < 8%) for both PBS and plasma at 300 g and 2,700 g, as well as for PBS at 15,800 g. The model overestimates the particle speed at 15,800 g for plasma, with a maximum error of 20%. Thus, the model is a reasonable approximation to predict the behavior of spherical particles when applied to the centrifugation protocols as shown in Table 2.2.

Figure 2.2. Centrifugation of beads diluted in PBS or plasma. The modeled bead recovery in pellet (blue dashed line) and supernatant (black, solid line) compared to measured bead recovery in pellet (open blue diamonds) and supernatant (closed black circles) for three centrifugation conditions.

Predicted performance of centrifugation protocols

Table 2.3 shows the predicted recovery of number of particles based on the centrifugation protocols as shown in Table 2.2. The volume of the supernatant plus the volume of the pellet equals the starting volume. The volume reduction can be calculated by dividing the starting volume by the volume of supernatant or pellet. To obtain the particle concentration after centrifugation, the predicted recovery in the number of particles needs to be multiplied with the volume reduction, which is shown in the last column of Table 2.3.

The CTC protocol recovers 100% of CTCs but co-isolates substantial fractions of TEPs, large tdEVs, and ccfDNA. In subsequent epithelial cell adhesion molecule (EpCAM)-based magnetic enrichment of CTCs, the TEPs and ccfDNA will be removed, but tdEVs exposing EpCAM will be co-isolated. The latter is confirmed by a previous study, in which the CTC fraction was shown to contain large EpCAM⁺ EVs (5).

The protocol to isolate TEPs predicts a yield of 71% together with 22% large tdEVs and < 3% CTCs, smaller tdEVs, and ccfDNA. Because the subsequent processing includes detection of all mRNA present in the sample, our model indicates that additional evidence is needed to prove that the obtained RNA profiles indeed originate from platelets.

EV-miRNA samples contain intermediate tdEVs (40%), small tdEVs (57%), ccfDNA (57%), and TEPs and large tdEVs (< 1%). By subsequent size exclusion chromatography (23), ccfDNA is removed, resulting in a relatively pure tdEV sample with high yield. The model predicts that the concentration of tdEVs is unaffected by the final centrifugation step of the protocol, which is confirmed by the authors upon inquiry (6).

For ccfDNA, two centrifugation protocols were evaluated. The Speicher protocol (8) predicts the 82% recovery of ccfDNA, together with large tdEVs (2%), intermediate tdEVs (63%), small tdEVs (82%), and negligible CTCs and TEPs. The Dawson protocol (7) yields 48% of ccfDNA, but with lower contamination of intermediate tdEVs (6%), small tdEVs (49%), and negligible CTCs, TEPs, and large tdEVs. Thus, both protocols do not yield pure ccfDNA. Because the volumetric mass density difference between DNA and plasma is at least ninefold higher than the density difference for EVs, it is difficult

to separate DNA from small EVs. Consequently, a 35 nm DNA “particle” travels at the same speed as a 100 nm EV.

Table 2.3. Predicted recovery (%) of centrifugation protocols.

Protocol	Change in number (% of original)					Vol. red.	
	CTCs	TEPs	Large tdEVs	Intermediate tdEVs	Small tdEVs	ccfDNA	(fold)
CTCs / tdEVs	100.0	39.4	81.1	16.7	13.3	13.3	2.9
TEPs	0.0	71.1	21.7	3.2	2.2	2.2	3.0
EV-miRNA	0.0	0.01	0.7	39.7	57.0	56.9	1.7
ccfDNA (Speicher)	0.0	0.05	2.4	63.4	82.0	82.0	1.2
ccfDNA (Thermo)	0.0	1.10^{-6}	0.0	8.1	50.7	50.2	1.7

ccfDNA, circulating cell-free DNA; CTCs, circulating tumor cells; EV-miRNAs, extracellular vesicle-associated miRNAs; TEPs, tumor-educated platelets; tdEVs, tumor-derived extracellular vesicles; Vol. red., volume reduction. Bold font highlights the target component.

Discussion

Centrifugation can be described with a model based on the Stokes equation. The model illustrates that centrifugation is effective at recovering particles that can be pelleted but that additional methods are needed to obtain a pure fraction. Particles with similar sedimentation rates are always co-isolated during centrifugation. This is especially relevant for TEP and ccfDNA protocols, because the co-isolated tdEVs may have substantial impact on the outcome. This model offers a tool to (re)design centrifugation protocols and at a minimum to establish whether available candidate protocols differ sufficiently from each other to warrant comparison. The model includes a shape factor to account for the nonspherical shape of platelets. This shape factor was set to 2.0 based on literature but was not experimentally validated.

For technologies evaluating the presence of CTCs, such as the CellSearch system, whole blood is centrifuged at 800 *g* for 10 min. For this application, the model predicts a 100% effective isolation of CTCs, in addition to a recovery of 81% of the large tdEVs and 39% of TEPs. However, because of the use of imaging to identify each particle, tdEVs and TEPs can be distinguished from CTCs, and thus, the co-isolation does not affect the determined CTC concentration. Nevertheless, a purer CTC sample may be

obtained by adjusting the centrifugation protocol. To reduce the recovery of TEPs or intermediate size tdEVs, the g-force or centrifugation time should be reduced. Because of their size, ccfDNA or small tdEVs' recovery in the pellet is mainly reduced by a reduction of the collection height.

The model predicts that a single biomarker cannot be purified by centrifugation alone. Substantial fractions of co-isolated other biomarkers remain present in all evaluated protocols, which may affect the obtained results and interpretation thereof.

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Supplemental Information

Sample properties

Platelets have a log-normal volume distribution (1), and a discoid shape with an aspect ratio of 4-10 (2). For the model, we assumed platelets to be an oblate spheroid with aspect ratio 7, leading to an S for platelets of 2.0 assuming the platelet moves at sufficiently low speeds such that all orientations are equally feasible (3). If this assumption is false, and the platelet is oriented in the energetically most favorable orientation, S would need to be 1.5 (3). Platelets can have a volumetric mass density of 1.05-1.09 g/mL, where the lower density is associated with platelets that have secreted their α -granule content but with a unaffected platelet volume distribution (4, 5). Typical mass densities of EVs determined in Optiprep are found between 1.08 and 1.11. This seems very high compared to typical volumetric mass densities for cells of 1.05-1.08 (6). However, because of the highly skewed particle size distribution this likely pertains to EVs smaller than 200 nm, which have a relatively large membrane content. Modelling an EV as a small cell, i.e. a 8 nm thick membrane consisting of 50% protein (volumetric mass density 1.30), 50% phospholipids (volumetric mass density 1.01) and filled with cytoplasm (volumetric mass density 1.06) (6), would mean that 100 and 200 nm EV have volumetric mass densities of 1.099, and 1.081 respectively. These densities are in good agreement with literature values, while a 1000 nm EV would have a density of 1.064, which is within the range of densities of a cell. Lastly, the mass density of DNA is only available in cesium chloride media, and the length of ccfDNA is unknown. Assuming a length shorter than 1000 basepairs, the ccfDNA would be smaller than 50 nm.

Table S1. Literature values for components in model.

	Property at 20°C, 1 bar	Values in article	Population/sample type	References
CTCs				
Density (g/mL)	1.05 +/- 0.002		lung carcinoma cell line	(7)
	1.056 +/- 0.004		cervical carcinoma cell line	(8)
Diameter (µm)	median 10.7	(measured in range 8-20)	276 castration resistant PCa	(9)
	median 11.0	(measured in range 8-20)	464 colorectal cancer patients	(9)
	median 13.1	(measured in range 8-20)	177 breast cancer patients	(9)
Platelets				
Density (g/mL)	1.05-1.09 @ 4°C	freq mean 1071 SD 6	17 normal donors	(4)
	1.04-1.08 @ 23°C		30 normal donors	(10)
	1.05-1.08 @ RT	freq mean 1067 SD 4.5	15 normal donors	(5)
Diameter (µm)	From spherical approximation of impedance based volume determinations			
	2.5 +/- 0.6 (mean +/- SD)	range 1-21, mean 8.2, SD 6.1 fL	55 normal donors	(11)
	2.3 +/- 0.6	lognormal mean 6.6 SD 4.3 fL	50 normal donors	(1)
Shape factor	"Platelets are discoids 0.5 um thick and 2-5 um diameter" -> aspect ratio 4-10			
	1.55, 2.03, 2.48	aspect ratio 4, 7, 10		(2) (3)
EVs				
Density (g/mL)	Excluded last round culture with exosome containing media, included only iso-osmotic gradient media			
	1.08-1.10		color. carcinoma cell line derived EVs	(12)
	1.09-1.11	sample in 0.25M sucrose before DGC	color. carcinoma cell line derived EVs	(13)
	1.094-1.143		breast cancer cell line derived EVs	(14)

Table S1. (Continued)

	Property at 20°C, 1 bar	Values in article	Population/sample type	References
cfDNA	1.093	some sucrose from previous steps	colorectal carcinoma ascites	(15)
	1.087	some sucrose from previous steps	melanoma in mouse model	(16)
Density (g/mL)	1.7		in CsCl gradient	(17)
Size (nm)	2 by 304 (1k base pairs)			(6)
PBS				
Density (g/mL)	1.004			(18)
Viscosity (mPa . s)	1.193			(18)
Plasma				
Density (g/mL)	1.026 (CI 1.024-1.027)	@ 4, 25, 37°C	25 normal donors	(19)
	1.0245 (CI 1.0215-1.0262)		32 normal donors	(20)
	1.0253 (CI 1.0217 - 1.0273)		122 pathological donors	(20)
Viscosity (mPa . s)	Converted to 20°C through 2.2% increase per °C (Arrhenius)			(21)
	1.6 - 1.9	1.1 - 1.3 @ 37°C	'normal range'	(22)
	1.70	1.52 @ 25°C	57 normal, no influence of sex, age	(23)
	1.80 (1.75/1.91)	1.24 (25p-75p 1.20-1.31) @ 37°C	Unspecified	(24)

25p-75p: 25-75 percentile, cfDNA: circulating cell-free DNA, CI: 95% confidence interval, Color.: Colorectal, CTCs: circulating tumor cells, DGC: density gradient centrifugation, EVs: extracellular vesicles, PBS: phosphate-buffered saline, PCa: prostate cancer, RT: room temperature, SD: standard deviation.

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Chapter 3.

Platelet removal by single step centrifugation

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Abstract

The study of extracellular vesicles (EVs) in plasma requires removal of cells including platelets. At present, a two-step centrifugation protocol is recommended and commonly used. However, this protocol is laborious, time-consuming, and the yield of platelet-free plasma is low. Therefore, we developed an easy, fast and clinically applicable centrifugation protocol to produce essentially platelet-free plasma with a high yield for EV research. We compared the two-step centrifugation protocol to a single-step protocol at 5,000 g for 20 minutes. The removal of platelets was computationally predicted and experimentally validated. Flow cytometry was used to detect residual platelets and platelet-derived (CD61+) EVs. The single-step protocol at 5,000 g (i) is less laborious and approximately ten minutes faster, (ii) removes platelets as effective as the two-step centrifugation protocol, and (iii) has a ~10% higher plasma yield, whereas (iv) the recovery of platelet-derived EVs is comparable. For future research on plasma EVs we recommend the newly developed, easy and fast single-step protocol for preparation of platelet-free plasma for research on plasma biomarkers including EVs.

Introduction

Extracellular vesicles (EVs) are cell-derived nanoparticles that are abundantly present in body fluids like plasma. Plasma EVs have an outstanding biomarker potential, but studying such EVs requires removal of platelets during blood collection and handling (1). In the most applied protocol, blood is centrifuged at 2,500 g for 15 minutes to obtain platelet-poor plasma. Subsequently, the platelet-poor plasma is transferred to a new centrifugation tube and centrifuged at 2,500 g for 15 minutes to obtain essentially platelet-free plasma as shown in Figure 3.1. (2). This protocol was developed to optimize platelet removal, thereby improving the comparison of EV measurement results, and therefore this protocol was included in recent guidelines (3, 4). However, this protocol has several drawbacks. The protocol is laborious and time consuming, and the protocol is therefore difficult to implement in a clinical setting. A simpler protocol that is less operator dependent is likely to improve the quality of EV samples collected in clinical studies.

Previously, we developed a model to study how centrifugation protocols influence the purity of cancer biomarkers in blood (5). These biomarkers included circulating tumor cells, (tumor-educated) platelets, tumor-derived EVs, EV-miRNAs, and circulating cell-free DNA. In the present study, we applied this model to develop and validate an easier and faster protocol to prepare essentially platelet-free plasma without the loss of EVs.

Materials and Methods

Stokes model

We computed the protocol using the previously described model (5). Briefly, the Stokes equation describes the distance a particle travels through a medium, which depends on the diameter of the particle, volumetric mass density of the particle and medium, viscosity of the medium, time of centrifugation, and gravitational acceleration of the rotor. Centrifugation of particles that are uniformly distributed throughout the sample causes large, high density particles like cells to move down to the pellet (Figure 2.1. below the dashed line), while small, low density particles, like EVs stay in the supernatant (above the dashed line). The variables required to compute the model output were applied as described previously (5). Importantly, platelets were modeled as

oblate spheroids with a density ranging from 1.05-10.9 g/mL, independent of platelet size. EVs were modeled as spheres, with a size dependent density ranging from 1.10 g/mL for 100 nm diameter down to 1.06 g/mL for 1,000 nm diameter.

Model validation

Blood was obtained from ten healthy donors with informed consent in accordance with the Declaration of Helsinki and the study protocol was waived by the medical ethics committee of the Amsterdam University Medical Center. Whole blood was drawn using a 21G needle. The first 3.5 mL was discarded. For each of the ten donors, eight 3.2% citrate blood collection tubes of 3.5 mL (Greiner Bio-one, Kremsmünster, Austria) were collected, mixed gently with the coagulant and processed within 15 minutes. Seven blood collection tubes were centrifuged at 2,500 *g* for 15 minutes using a Rotina 380R centrifuge (Hettich, Tuttlingen, Germany) at 20 °C without brake (see Figure 2.1.). Platelet poor plasma was collected with a plastic Pasteur pipette (VWR, Radnor, PA), leaving 1 cm of plasma above the buffy coat and taking care not to disturb the buffy coat to circumvent cell contamination. Next, the approximately 9 mL of platelet poor plasma was pooled.

For the original two-step protocol, 3.5 mL of platelet poor plasma was transferred to a conical base tube (10 mL; Sarstedt, Nümbrecht, Germany). The tube was centrifuged a second time at 2,500 *g* for 15 minutes at 20°C without brake. After centrifugation, platelet-free plasma was collected leaving 100 μ L pellet as described in the original description of the two-step protocol (6). For the conservative two-step protocol, another tube containing 3.5 mL of platelet poor plasma was centrifuged a second time at 2,500 *g* for 15 minutes at 20 °C without brake. After centrifugation, platelet-free plasma was collected 1 cm above the bottom of the tube. For the single step protocol, one blood collection tube was centrifuged at 5,000 *g* for 20 minutes using a Rotina 420R centrifuge (Hettich, Tuttlingen, Germany) at 20 °C without brake, and platelet-free plasma was collected leaving 1 cm of plasma above the buffy coat. The remaining tube was used to measure the platelet concentration in whole blood.

Figure 3.1. Centrifugation protocols to remove platelets from whole blood. A) Using a commonly applied protocol, platelet poor plasma (PPP) is obtained by centrifugation of whole blood at 2,500 g for 15 minutes which will deplete cells and part of the platelets. A second centrifugation step at 2,500 g for 15 minutes of the supernatant depletes most of the residual platelets (PFP, platelet-free plasma). B) Using the single step centrifugation protocol, PFP is obtained by centrifugation of whole blood at 5,000 g for 20 minutes.

Flow cytometry

To measure the concentration of platelet and platelet-derived EV concentration by flow cytometry, 20 μL of each fraction was incubated with 2.5 μL anti-CD61-APC (Y2/51, final concentration 50 $\mu\text{g}/\text{mL}$; Dako Denmark, Glostrup, Denmark), for two hours at room temperature in the dark. Before labeling, antibody aggregates were removed by centrifugation at 18,890 g for 5 minutes at 20 $^{\circ}\text{C}$. The labeling was stopped by adding 200 μL phosphate buffered saline (PBS; 21-031-CV; Corning, Corning, NY) to the samples. Samples were pre-diluted in PBS if necessary to event rates below 5,000/s to prevent swarm when triggering on side scatter (12).

We performed flow cytometry measurements on an A60-Micro (Apogee; Northwood, UK) at a flow rate of 3.0 $\mu\text{L}/\text{minute}$. Samples were measured for 1.5 minutes triggered

on 405-nm side scatter. The side scatter trigger threshold corresponds to a side scattering cross section of 10 nm^2 (Rosetta Calibration; Exometry, Amsterdam, The Netherlands). A distinct platelet population was identified by side scatter and APC fluorescence, and EVs were identified by CD61-APC signal exceeding background fluorescence in the unstained sample (sample without antibody). Concentrations in this manuscript are the number of detected particles multiplied by the total sample dilution divided by flow rate and measurement time. Data were analyzed using FlowJo (v10, FlowJo, Ashland, OR) and Prism 7.0 (GraphPad, La Jolla, CA). Statistical analysis was performed using Prism 7.0. We applied the student's t-test to see if a statistical difference in residual platelet concentration was found between the two-step centrifugation protocol and the single-step centrifugation protocol.

Results - Discussion

The Stokes model suggests that the efficacy of platelet removal by a single-step centrifugation step at 5,000 g for 20 minutes should be comparable to the two-step centrifugation protocol without affecting the concentration of EVs for blood collection tubes up to 6 mL.

Figure 3.2. shows the measured concentrations of residual platelets and platelet-derived EVs for all centrifugation protocols. Left is the original two-step centrifugation protocol, in which approximately 100 μL of plasma is left above the pellet (see Figure 1) as described previously (6). For this protocol disturbance of the pellet is expected because the plasma is collected near the (cell) pellet, which would increase the concentration of residual platelets in the supernatant. We observed indeed such an increase compared to the other centrifugation protocols. Relative to the platelet concentration in blood (data not shown), median 0.5% (range 0.3-23.6%) of platelets remain in the sample after the original two-step centrifugation protocol.

To minimize disturbance of the pellet, we also performed a 'conservative' two-step centrifugation protocol where we left about 1 cm of plasma above the pellet. This effectively reduces the variability of the residual platelet concentration compared to the original two-step centrifugation protocol. For the conservative two-step protocol, median 0.1% (range 0.1-0.2%) of the platelets remain in the sample by this protocol.

The yield of plasma with the conservative two-step centrifugation protocol is ~77% compared to the original two-step centrifugation protocol.

Figure 3.2. Concentrations of residual platelets and platelet-derived extracellular vesicles (EVs) after centrifugation. The two-step 2,500 g protocol, 100 µL results in an increased concentration of residual platelets (closed symbols) compared to the other centrifugation protocols. Leaving 1 cm of pellet instead of 100 µL results in a lower residual platelet concentration. A single centrifugation step at 2,500 g for 15 minutes is not as efficient as a double centrifugation step. Replacing the two-step centrifugation protocol by a single 5,000 g step results in a lower residual platelet concentration compared to the original two-step centrifugation protocol. The EV concentration (open symbols) is similar for the two-step centrifugation protocol and single 5,000 g step protocol, suggesting that no EVs are lost at a higher speed and longer centrifugation time.

A single centrifugation step for 15 minutes at 2,500 g is less effective in removing platelets compared to either of the two-step centrifugation protocols. Median 1.6% (0.7 – 2.0%) of the platelets remain in the sample by a single-step 2,500 g protocol.

If we replace the two-step centrifugation protocol by the model-based protocol, that is by increasing the centrifugation speed from 2,500 g to 5,000 g as well as the time from 15 minutes to 20 minutes, platelet removal is about as efficient as the two-step centrifugation when leaving 1 cm of plasma above the pellet. No significant

difference ($p > 0.05$) between the protocols was found, but the plasma yield is ~10% higher compared to the original two-step centrifugation protocol, which implies that more plasma is collected and can be stored for biobanking. Median 0.1% (0 – 0.5%) of the platelets remain by this protocol. The concentration of platelet-derived EVs is comparable, suggesting that these EVs are not lost in the single 5,000 g protocol. Also the concentration of CD45 (leukocyte)- and CD235a (erythrocyte)-EVs are comparable between protocols (data not shown). Furthermore, as this protocol includes only a single aspiration step performed well above the pellet, this protocol is less prone to variation between users.

The single 5,000 g centrifugation step is easier, faster, and sufficient in platelet removal from whole blood with a higher plasma yield compared to the original two-step centrifugation protocol. Therefore, we recommend the newly developed protocol for preparation of platelet-free plasma for research on plasma biomarkers including EVs. Alternatively, a modified two-step protocol, with removal of plasma to about 1 cm above the pellet instead of 100 μ L, seems a good alternative when plasma is not the limiting factor. One has to bear in mind that we only studied blood collected from healthy subjects, and to which extent our data can be extrapolated to patient studies, needs further investigation.

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Chapter 4.

Separation of platelets from platelet-derived vesicles by rate zonal centrifugation

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Abstract

Biomarkers are separated based on density and/or diameter. However, the centrifugation protocol affects the yield and purity of biomarkers, for example isolation of platelets results in co-isolation with extracellular vesicles (EVs). The objective of this study is to assess the ability of rate zonal centrifugation (RZC) to separate platelets from co-isolated EVs. Using a linear Optiprep gradient, RZC was able to separate a mixture of beads with different diameters but similar density. Next, RZC was applied to samples containing both platelets and platelet-derived EVs (n=3). After RZC, all fractions were collected and stained with anti-CD61-Alexa 488 to measure the concentrations of platelets and platelet-derived EVs by flow cytometry. We confirm that RZC separates polystyrene beads with diameters of 140 nm, 380 nm and 1,000 nm. Next, we show that the majority of platelets occurs in fractions 8-19, whereas the majority of platelet-derived EVs is detectable in fractions 1-7. Furthermore, each fraction contains a different diameter range of platelets which suggests that separation is indeed diameter-based. In conclusion, RZC is able to separate platelets from EVs.

Introduction

The isolation of a biomarker from a body fluid is essential to evaluate the importance of the biomarker. Centrifugation is frequently applied to isolate biomarkers from whole blood based on differences in density and/or diameter (1-5). The distance a particle travels through a solution during centrifugation can be approximated using the Stokes equation:

$$(1) \quad \Delta x = \frac{d^2(\rho_D - \rho_M) g t}{18 \eta}$$

and depends on the diameter (d) and volumetric mass density (ρ_p) of the particle relative to the volumetric mass density (ρ_M) and viscosity (η) of the gradient. The g refers to the gravitational acceleration and the t to the time. Using this equation, we demonstrated that centrifugation protocols used to isolate biomarkers from blood from cancer patients result in impure biomarkers, due to the co-isolation and thus contamination with other biomarkers, e.g. the isolation of tumor-educated platelets results in co-isolation with tumor-derived extracellular vesicles (EVs) and vice versa (6, 7).

A commonly applied centrifugation method is density gradient centrifugation, where a sample is loaded on top of a density gradient. During density gradient centrifugation, particles move down the gradient until the difference between particle density and medium density is zero. Therefore, the separation principle is based on the density of a particle. In contrast, with rate zonal centrifugation (RZC), the gradient has a lower density throughout the entire gradient compared to density gradient centrifugation to ensure that the distance a particle travels through the gradient solely depends on the particle diameter (equation 1). RZC has previously been applied to isolate viruses, DNA, lysosomes and other nanoparticles (8-10).

At present, there is great interest to enhance the purity of biomarkers to improve the interpretation of data. In this study, we evaluate whether RZC is able to separate platelets from EVs based on diameter.

Materials and Methods

RZC requires loading of a sample on top of a gradient. If a single step gradient is used, the transition between sample and gradient can cause particles in the sample to form clusters at the top of the gradient. These clusters are bigger in diameter and have a higher sedimentation speed compared to single particles (equation 1). These clusters end up in the pellet or mix with the surrounding media and spread throughout the tube. This process is called droplet sedimentation (11). Based on preliminary results, we know that using a linear gradient in which the density increases towards the bottom of the tube prevents droplet sedimentation. Furthermore, the linear gradient has a slightly higher viscosity than the sample to facilitate sample loading without mixing with the gradient.

Linear density gradient

A gradient mixer (supplier unknown) was applied to generate a linear density gradient of 4% at the top to 8% Optiprep at the bottom (60% (w/v) solution of iodixanol in water; Sigma-Aldrich, St. Louis, MO), see Figure 4.1.. Bagamery et al. describe that sample manipulation-related platelet activation can be minimized by using Optiprep (12, 13). Optiprep was diluted in 0.22 μm filtered (Merck Millipore, Burlington, MA) phosphate-buffered saline (PBS; 154 mM NaCl, 1.24 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.2 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, pH 7.4). The gradient mixer chambers were filled with 4% Optiprep ($\rho = 1.027 \text{ g/mL}$) and 8% Optiprep ($\rho = 1.048 \text{ g/mL}$) respectively. Depletion of medium from the high density chamber causes medium from the low density chamber to flow into the high density chamber, where a magnetic stirrer mixes the media. A peristaltic pump (Minipuls 3; Gilson, Middleton, WI) pumped medium into a stainless steel syringe needle (alloy 316, 18 gauge, 6 inch pipetting blunt 90° tip; Sigma-Aldrich, St. Louis, MO). The needle tip was held at the meniscus by an injection pump (Perfusor segura FT; B. Braun, Hessen, Germany). In this fashion, a continuous linear Optiprep gradient was generated in the gradient tube (10 mL; Sarstedt, Nümbrecht, Germany). The optical density of successive fractions collected from three gradients was measured using a microplate reader (340 nm line, Spectramax i3; Molecular Devices, San Jose, CA) to demonstrate the linearity of the gradient (Figure S1A). Gradients were stored at 4 °C and used within two days after equilibration to room temperature.

Bead mixture

140 and 380 nm green fluorescent polystyrene beads and 1,000 nm non-fluorescent beads (Thermo Fisher Scientific, Waltham, MA) were used to validate the RZC setup. Each bead concentration was 0.03% w/v in 0.22 μ m filtered PBS supplemented with 0.1% sodium dodecyl sulfate in distilled water (20% SDS stock; Bio-Rad Laboratories Inc, Hercules, Ca).

Platelets and platelet-derived extracellular vesicles

Blood was obtained from three healthy donors with informed consent in accordance with the Helsinki Declaration and approved by the medical-ethical assessment committee of the Academic Medical Center, University of Amsterdam. For each donor, whole blood was drawn using a 21G needle, and the first vacutainer was discarded. Next, two 4.0 mL EDTA vacutainers (BD Biosciences, San Jose, Ca) were collected, mixed by gentle inversion and processed within 15 minutes. To obtain “tumor-educated platelets” as described by Best et al (1), vacutainers were centrifuged at 120 g for 20 minutes at 20°C and without brake in a Rotina 380R centrifuge (Hettich, Tuttlingen, Germany) to prepare platelet-rich plasma. The platelet-rich plasma was transferred to a 10 mL conical base tube (Sarstedt), and centrifuged at 360 g for 20 minutes at 20 °C and without brake to concentrate the platelets. The platelets were washed twice with 1 mL 0.05 μ m filtered (Nuclepore; GE Healthcare, Chicago, IL) 0.8% EDTA-buffer (Titriplex III in PBS; Merck Millipore, Burlington, MA). Finally, 500 μ L of 0.8% EDTA-buffer was added to the pellet and the pellets were pooled. After each centrifugation step, supernatant above the pellet was removed leaving some supernatant so the pellet was not disturbed. Therefore, plasma was still present when the washed pellets were pooled.

The mixture of platelets and EVs (1 mL) was loaded on size exclusion chromatography (SEC) columns (10 mL qEVOoriginal; Izon Science, Christchurch, New Zealand) to replace the blood plasma with 0.05 μ m filtered acid citrate dextrose-buffer (five parts PBS to 1 part acid citrate dextrose; 0.85 mol/L trisodiumcitrate, 0.11 mol/L D-glucose and 0.071 mol/L citric acid, supplemented with 0.32% trisodiumcitrate in PBS, pH 7.4, see Figure S2). Fractions 8 and 9 contained all particles larger than 70 nm, both platelets and EVs. These fractions were pooled for each donor as starting material for RZC.

Figure 4.1. Gradient setup. A) A 4% Optiprep solution is loaded into the left compartment of the gradient mixer. The 8% Optiprep solution is loaded into the right compartment of the gradient mixer together with a magnetic stirring bar. The 4% Optiprep is pumped into the gradient mixer using a roller pump, mixed with the 8% Optiprep by stirring, and layered into a centrifuge tube using a needle mounted to an injection pump. The Optiprep concentration gradually decreases to form a linear gradient. B) After sample loading and centrifugation the gradient is placed back into the setup. A needle is mounted to an injection pump. The needle is lowered while extracting the fractions into Eppendorf tubes using a roller pump.

Centrifugation and fractionation

500 μ L of starting material was loaded on the 4-8% Optiprep gradient using a plastic Pasteur pipette (VWR, Radnor, PA), which was placed against the wall of the tube just above the gradient. For the bead mixture, RZC was performed at 2,772 g for 100 minutes at 20 $^{\circ}C$ and without brake. Using equation 1, we predicted which combination of centrifugal force and time was needed to make sure the platelets would end up in the last fractions and EVs could spread out in the first fractions. RZC of the platelet- and

EV-containing samples was performed at 1,000 g for 30 minutes at 20 °C and with acceleration and deceleration set to the minimum possible value. Centrifugation was performed at 20 °C, since platelets are more sensitive to activation at 4 °C (14).

Figure 4.1.B. shows the fractionation setup. A needle aspirating from the top was used for fractionation (15). The tip of the needle was held at the meniscus while extracting twenty 0.5 mL fractions into Eppendorf tubes (Greiner Bio-One, Kremsmünster, Austria) using a peristaltic pump. Each fraction was weighed on an analytical balance (Sartorius, Göttingen, Germany) to determine the collected volume per fraction. Figure S1B shows that 87.7% (50/57) of the collected fractions were within the mean collected volume $\pm$ 40 μ L ($\sim$ one droplet). The volume per fraction was used to calculate the platelet and platelet-derived EV yield, which was defined by the percentage of platelets found in all fractions compared to the starting material. The platelet yield ranged from 30% to 40%, whereas the platelet-derived EV yield ranged between 66 to 146%.

Flow cytometry

Flow cytometry measurements were performed on an A60-Micro (Apogee; Northwood, UK) at a flow rate of 3.0 μ L/minute. Samples were measured triggered on 405-nm side scatter. The side scatter trigger threshold corresponds to a side scattering cross section of 10 nm² (Rosetta Calibration; Exometry, Amsterdam, The Netherlands).

Bead samples were pre-diluted in 0.22 μ m filtered PBS if necessary to event rates below 5,000/s to prevent swarm when triggering on side scatter (16). Bead samples were measured for one minute. Side scatter resulted in distinct bead populations.

To obtain the platelet and platelet-derived EV concentration by flow cytometry, 20 μ L of each fraction was incubated with 2.5 μ L anti-CD61-Alexa 488 (Y2/51, final concentration 0.01 mg/mL; Bio-Rad Laboratories, Hercules, CA), aggregates in the antibody solution removed by centrifugation at 18,890 g for 5 minutes at 20 °C) for two hours at room temperature in the dark. The labeling was stopped by adding 200 μ L PBS (21-031-CV; Corning, Corning, NY) to the samples. The experiment with the platelet- and EV-containing samples was performed at a later date than the bead sample. In the meantime we switched from custom-made PBS to commercially available PBS. Samples were measured for two minutes triggered on 405-nm side scatter. We selected

one minute of data in which the flow rate was stable. A distinct platelet population was identified by side scatter and Alexa 488 fluorescence, and platelet-derived EVs were identified by a gate at the Alexa 488 fluorescence channel exceeding background fluorescence levels in the unstained sample (sample without antibody), corresponding to 397 MESF FITC.

Provided concentrations are the number of detected particles multiplied by total sample dilution divided by flow rate and measurement time. Data were analyzed using FlowJo (v10.4.2, FlowJo, Ashland, OR) and statistical analysis was performed in Prism 7.0 (GraphPad, La Jolla, CA). We used Rosetta Calibration v1.08 (Exometry) to calibrate side scattered light to units of nm^2 and to relate side scattered light to the diameter ($> 200 \text{ nm}$) of platelets and platelet-derived EVs (17). We modeled platelets as solid spheres with a refractive index (RI) of 1.40 (18) using steps of 10 nm^2 in diameter.

Results

Rate zonal centrifugation of beads

We applied a mixture of beads of various diameters to validate that RZC separates particles with the same density but a different diameter. After centrifugation, distinct bands are observed in the gradient (Figure 4.2.). The position of the 140 nm and 380 nm beads are indicated and arrows indicate the expected positions of the 140 nm and 380 nm beads after RZC based on equation 1. The two extra bands seen below the 380 nm beads are likely doublets and triplets of the 380 nm beads. The graph shows the percentage of beads from the whole bead population measured by flow cytometry, with the peak percentage of beads in the same fraction as the bands. The flow cytometry data shows substantial broadening of the peaks which is likely caused by the fraction collection method we used, because the fraction boundaries can coincide with the bead bands.

Rate zonal centrifugation of platelets and platelet-derived extracellular vesicles

The starting material contained both platelets and platelet-derived EVs and was loaded on top of the gradient before centrifugation, see Figures 4.3. and S3. Almost no platelets are observed in fractions 1-5, and the majority of platelets is observed in

fractions 8-19, with an average scatter signal on the Y-axis increasing when moving further down the gradient (Figure 4.3. and 4.4.A. and Figure S3). Figure 4.4.B. shows that the mean diameter of platelets increases while moving down the gradient, confirming that each fraction contains platelets with a different diameter range.

The majority of platelet-derived EVs (64%) is observed in fraction 1-7 (Figure 4.3. and 4.4.A.; a logarithmic scale was used for the Y-axis of Figure 4.4.A. as the platelet-derived EV concentrations of donors 2 and 3 are low compared to donor 1).

Figure 4.2. Rate zonal centrifugation of a bead mixture. A mixture of polystyrene beads with the same density but different diameter (140 nm, 380 nm and 1,000 nm) is loaded on top of a 4-8% Optiprep gradient. After centrifugation at 2,772 *g* for 100 minutes the bead populations are visible as distinct bands. This is confirmed by the peaks in the flow cytometry data, which also reveal the 1,000 nm beads, which are not visible by eye because they are not fluorescent. The arrows show the expected position of each bead population calculated based on the Stokes equation.

Figure 4.3. Flow cytometry scatter plots of platelets and platelet-derived extracellular vesicles (EVs) in fractions of rate zonal centrifugation (RZC) from a representative donor. The first graph shows the gates set for platelets and platelet-derived EVs for the unstained starting material (control). The starting material, loaded on top of the gradient before centrifugation, contained both platelets and platelet-derived EVs. The platelet population is missing in the first fractions. However, moving further down the gradient shows the platelets are mainly present in fraction 8-19 and the scatter signal on the y-axis shifts while moving down through the gradient. The majority of platelet-derived EVs is present in fraction 1-7. In fractions 1 and 2 we observed two subpopulations of CD61+ EVs, of which the upper population is not observed in the other fractions. At present, we have no explanation for the presence of this subpopulation.

Discussion

The use of biomarkers is complicated by their differences in physical characteristics such as density and diameter. Moreover, during sample preparation frequently biomarkers are co-isolated. To overcome these limitations we introduce RZC as a technique to separate biomarkers. RZC is not an alternative method to isolate platelets and EVs from blood. The goal of this paper was to show as a “proof of principle” that particles with a likely similar density can be separated by RZC based on size.

RZC has never been applied to samples containing mixtures of platelets and EVs. We demonstrate that platelets and platelet-derived EVs can indeed be separated by RZC.

Figure 4.4. Platelet and platelet-derived extracellular vesicles (EV) concentration and diameter measured in rate zonal centrifugation fractions (RZC) for three donors. A) The highest concentration of platelets (closed symbols) are present in fraction 8-17. The highest concentration of platelet-derived EVs (open symbols) are present in fraction 1-7. B) The platelet and platelet-derived EV diameter in RZC fractions for donor 1-3. The platelet diameter range (> 1 μm) was obtained using Rosetta calibration. The platelet diameter range shifts to the right while moving through the gradient, which confirms that the separation of platelets from platelet-derived EVs (200 nm to 1 μm) by RZC is based on diameter.

Furthermore, the platelet diameter range differs slightly in each fraction suggesting that RZC may be used to isolate monodisperse platelet or EV populations. Calibration of detection instruments is currently performed using polystyrene and silica beads. However, these beads differ in RI compared to EVs (19). Therefore, monodisperse biological reference material of for example EVs is preferred to calibrate detection techniques because then corrections for differences in RI are not necessary.

Lipoproteins present in the starting material can independently migrate through the gradient, although the density for lipoproteins is too low and the diameter is too small to travel through the gradient in the configuration as used in the present study (20). Co-migration of lipoproteins with platelets and/or EVs is possible, and this would affect the density and size of platelets and/or EVs, and therefore the fraction in which a platelet or an EV accumulates. Nevertheless, these effects are mitigated by removal of the majority of lipoproteins by SEC prior to RZC (21).

Figure 4.2. shows a difference between the expected and measured results. The linear increasing density and viscosity throughout the gradient make it difficult to predict the precise location of the beads by using equation 1. Therefore, we also tested in practice before adjustments were definitive. RZC relies on the assumption that the density between particles in a solution is equal. In this study, we assumed that the densities of platelets and EVs are equal. However, the density of platelets is reported to range between 1.061-1.091 g/ml (22, 23) and the density of EVs between 1.02-1.19 g/mL (24-27). Therefore, an overlap in platelet diameter range is shown in the different fractions. Furthermore, we have to keep in mind that activated platelets may change in shape, density and diameter (28).

In conclusion, based on the results from the bead mixture we show that RZC can be used to separate particles with the same density but a different diameter. Furthermore, we applied RZC to a biological sample and show that RZC can partially separate platelets from EVs.

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Supplemental Information

Figure S1A) Linearity Optiprep gradient. The linear relation between the optical density measured at 340 nm wavelength of Optiprep concentrations (4%-8%) and each fraction measured with the Spectra-max i3 spectrophotometer. A gradient mixer produces a linear gradient for donor 1-3 ($R^2=0.99$, $R^2=0.94$, $R^2=0.96$ respectively).

B) Volume of rate zonal centrifugation fractions. The volume in each fraction is determined by weighing the Eppendorf tube with sample and subtracting the weight of an empty Eppendorf tube (average of 20). 87.7% (50/57) of the fractions is within the range of one drop (40 μ L) below and one drop above the average volume fractionated.

Figure S2A) Rate zonal centrifugation of 140 nm (blue) and 380 nm (orange) green fluorescent beads in pool plasma at 2,772 g for 100 minutes. When the beads are in plasma ($\rho = 1.025$ g/ml), beads spread throughout the gradient. Therefore, SEC is used to replace plasma by PBS.

B) Rate zonal centrifugation of 140 nm (blue) and 380 nm (orange) green fluorescent beads in pool plasma at 2772 g for 100 minutes after size exclusion chromatography. When the beads are in PBS, a clear band of beads is visible.

Figure S3. The platelet concentration was also measured by the FACSCalibur (BD, Franklin Lakes, NJ). 5 μ L of each fraction was incubated with 5 μ L of CD61-Alexa 488 (final concentration 0.01 mg/mL), and 35 μ L HEPES buffer (137 mM NaCl, 20 mM Hepes, 5.6 mM D-glucose, 0.1 % BSA, 3.3 mM NaH_2PO_4 , H_2O , 2.7 mM KCl, 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, pH 7.4). After 30 minutes incubation in the dark, the labeling was stopped by adding 2 mL of HEPES buffer to the samples. Samples were measured for 1 minute. The concentration of CD61+ particles in each sample was measured on the side scatter (15 mW 488 nm laser, flow rate calibrated by weight, SSC 400 V, gain 0, threshold 0). The flow cytometer was calibrated using BD Calibration beads (unlabeled, FITC, APC, PE, PerCP). An unstained sample was used as a control. Starting material which is loaded on top of the gradient contains CD61+ platelets. In fraction 1 the count of these platelets is low compared to fractions further down the gradient. The platelets are mainly present in fraction 8-17 and the scatter signal moves upwards. Fraction 13 was removed because no CD61+ population was visible.

Chapter 5A.

**Quality of extracellular vesicle images
by transmission electron microscopy is
operator and protocol dependent.**

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Abstract

Transmission electron microscopy (TEM) has nm resolution and can be used to distinguish single extracellular vesicles (EVs) from non-EV particles. TEM images of EVs are a result of operator selection. To which extent operator selection reflects the overall sample quality, and to which extent the images are comparable and reproducible, is unclear. In a first attempt to improve the comparability and reproducibility of TEM to visualize EVs, we compared images taken by operator selection to images taken at predefined locations from the same grids, using four EV TEM preparation protocols, a single EV-containing sample and a single TEM instrument. Operator selection leads to high quality images that are comparable between the protocols. In contrast, images taken at predefined locations reveal differences between the protocols, for example in number of EVs per image and background quality. From the evaluated protocols, for only one protocol the TEM images taken by operator selection are comparable to the TEM images taken at predefined locations. Taken together, images taken by operator selection can be used to demonstrate the presence of EVs in a sample, but seem less suitable to demonstrate the quality of a sample. Because images taken at predefined locations reflect the overall quality of the EV-containing sample rather than the presence of EVs alone, this is a first step to improve the comparability and reproducibility of TEM for monitoring the quality of EV-containing samples.

Introduction

Extracellular vesicles (EVs) are cell-derived particles with a phospholipid bilayer and diameter between 30 and 1,000 nm (1,2). Transmission electron microscopy (TEM) is the most widely used instrument to monitor the quality and purity of EV-containing samples, because TEM is able to discriminate single EVs from similar sized non-EV particles (3,4). Therefore, TEM is often applied to demonstrate that a sample (i) contains EVs, and (ii) is sufficiently pure, e.g. for therapeutic application or downstream analysis (5). Cryo-EM provides similar information and is less prone to artefacts (6,7), but cryo-EM is not widely available and more time consuming than TEM.

At present, TEM images are made by “operator selection”, meaning that an operator makes images at locations where EVs are present and well visible. To which extent this procedure provides a comparable and reproducible impression of the overall sample quality, however, is questionable. At present, there is a strong tendency to improve the comparability and reproducibility in (bio)medical science, including the new field of EV research (5,8,9). Therefore, as a first step, we compared images taken by operator selection to images taken at predefined locations from the same TEM grids. We used four commonly applied EV TEM preparation protocols, but minimized the contribution of other variables by using a single EV-containing sample and by imaging on a single TEM instrument. This procedure eliminates differences due to inter-sample variation, and enables a straightforward and “head-to-head” comparison between the images obtained by operator selection versus images taken at predefined locations.

Materials and Methods

Protocol selection

TEM preparation protocols for EV samples were selected based on a literature search in Web of Science. Articles in English published between January 2011 and November 2016 were included. From the 2,602 included articles, we selected authors that are authorities in the EV field based on co-authorship on the MISEV criteria (5), and co-authored at least eight articles which include TEM images of EV samples. From these research groups the most recent protocol was selected. The resulting nine protocols (10-18) are summarized in Table 5.1. These protocols contain three main elements: (i) fixation to preserve EV

morphology, (ii) mounting of EVs to a TEM grid, and (iii) negative staining to enhance the contrast between EVs and the background. From Table 5.1., it is clear that each protocol has many variables, and there is no variable similar between protocols. Particularly surprising is the absence or presence of fixation using glutaraldehyde (GA), osmium tetroxide (OsO_4), and paraformaldehyde (PFA). Therefore, we selected one protocol of every fixative to evaluate in this study, except OsO_4 due to safety regulations of our TEM facility. Furthermore, we also included one protocol which applied UV treatment of the TEM grid to enhance EV adhesion. These protocols are labelled A-D in Table 5.1. Authors provided additional protocol information upon our request. Even though glow discharging the grid is often performed before TEM to enhance adhesion, we did not apply glow discharge because none of the selected protocols mentioned this.

Table 5.1. Preparation protocols for transmission electron microscopy of extracellular vesicles.

Protocol	A		B		C		D		
Fixation									
Fixative	-	-	GA	GA	GA	GA	GA	OsO ₄	PFA
Concentration (%)	-	-	2.5 + 2 sucrose	2	1	1	2.5	0.5	0.1
Time (min)	-	-	-	-	-	-	10	-	≥1000
Mounting									
Grid material	Cu	Cu/Pd	Ni	Cu	Cu	-	-	-	Cu
Mesh	400	-	200	200	-	-	300	-	200
Film	-	Formvar	Formvar	Formvar	-	Formvar	Formvar	Formvar	Formvar
Coating	C	C	C	C	-	C	C/UV	-	C
Time (min)	2	-	20	5	-	-	10	10 - 30	7
Wash	Blot	-	-	2 x MilliQ	-	-	-	3 x H ₂ O	-
Negative staining 2x									
Substance	UA	UA + MC	NanoVan	UA	-	UA	UA	UA	UA
Concentration (%)	2	-	-	1.5	-	-	2	1% in 50% EtOH	1.75%
Time (min)	<2	-	-	0.2	-	-	10	15	7
Wash	-	-	-	-	-	-	-	3 x	-
Sequence	Mt, St	Mt, St	Mt, Fx, St	Fx, Mt, St	Fx, Mt, St	Fx, Mt, St	Mt, Fx, St	Fx, Mt, St	Fx, Mt, St
Reference	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)

- not described and/or not performed; Fx, fixation; GA, glutaraldehyde; MC, methylcellulose; min, minutes; Mt, mounting; PFA, paraformaldehyde; St, negative staining; UA, uranyl acetate

Extracellular vesicle sample

We applied the protocols to a single urinary EV sample, because urine has low concentrations of non-EV particles such as lipoproteins and protein aggregates (19). Urine was obtained from five overnight fasting healthy male individuals. This study was carried out in accordance with the Dutch law on research with human subjects. The study protocol was waived by the medical ethics committee of the Academic Medical Center, University of Amsterdam. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The urine was pooled and centrifuged to remove cells (8 x 50 mL pooled urine, 10 minutes at 180 g, 4 °C, followed by 20 minutes at 1,560 g, 4 °C) using a Rotina 46RS centrifuge (Hettich, Tuttlingen, Germany). Cell-free urine aliquots (1 mL) were frozen in liquid nitrogen and stored at -80 °C. Prior to analysis, twelve cell-free urine aliquots were thawed at 37 °C and removed from the water bath immediately after thawing. These aliquots were pooled and centrifuged (10 minutes at 1,560 g, 4 °C) to remove precipitated salts. Next, EVs were concentrated from the supernatant by ultracentrifugation (60 minutes at 154,000 g, 4 °C) using a TLA-55 rotor and Optima MAX-XP ultracentrifuge (Beckman Coulter, Fullerton, CA) and washed once in 0.05 µm filtered (Nucleopore, GE Healthcare) phosphate-buffered saline (PBS; 154 mM NaCl, 1.24 mM Na₂HPO₄·2H₂O, 0.2 mM NaH₂PO₄·2H₂O, pH 7.4; supplemented with 0.32% trisodium-citrate). The concentrated EV suspension was divided into four samples, one for each protocol (Figure 5.1.). Although ultracentrifugation may introduce artefacts in the sample, the same artefacts should be present in all protocols. TEM imaging is possible without the ultracentrifugation step, but requires more images to visualize the same number of EVs. The product of the sample preparation procedure has been characterized previously using TEM, flow cytometry, nanoparticle tracking analysis, and tunable resistive pulse sensing (18, 20). For reference, a particle size distribution obtained by nanoparticle tracking analysis is added to the Supplemental Information (Figure S1).

To verify that the selected final protocol can handle also other relevant types of sample, we also imaged a platelet free plasma sample (30 mL citrate blood from one overnight fasting donor, centrifuged twice for 15 minutes at 2,500 g, protein concentration reduced by size exclusion chromatography (21)) by applying this protocol. This sample has been characterized by TEM and tunable resistive pulse sensing previously (22).

TEM preparation protocols

The protocols (A-D in Table 5.1.) were performed at room temperature on the same day. All used liquids were filtered by 0.22 μm filters (Merck, Darmstadt, Germany). All TEM grids were obtained from Electron Microscopy Sciences (Hatfield, PA). We prepared three grids per protocol. Grids were air dried at the end of each protocol.

Protocol A (10): 10 μL of the EV sample was pipetted onto a 400 mesh copper grid with carbon-coated formvar film and incubated for 2 minutes. Excess liquid was removed by blotting. The grid was briefly placed on 10 μL of 2% uranyl acetate (w/v; Merck, Darmstadt, Germany), followed by blotting to remove excess liquid. This last step was repeated.

Protocol B (13): The EV sample was fixed 1:1 with 2% glutaraldehyde (v/v; Sigma Aldrich, Saint Louis, MO) for 30 minutes. 6 μL of fixed sample was pipetted onto a 200 mesh copper grid with carbon-coated formvar film, and incubated for 10 minutes. Excess liquid was removed by blotting. The grid was washed twice by brief contact with 100 μL MilliQ water, followed by blotting to remove excess liquid. Next, the grid was placed on 30 μL of 1.5% uranyl acetate (w/v) for 12 seconds.

Protocol C (16): A 300 mesh formvar/carbon film nickel grid was placed in a laminar flow cabinet to treat with UV light (TUV30W G30T8, Philips, Eindhoven, the Netherlands) for 15 minutes. Then, the grid was incubated on 15 μL of EV sample for 10 minutes. Next, the grid was fixed by placing it on 15 μL of 2.5% glutaraldehyde in PBS (v/v). After 10 minutes, the grid was placed on 15 μL 2% uranyl acetate (w/v) for 10 minutes.

Protocol D (18): The EV sample was fixed 1:1 with 0.2% paraformaldehyde (w/v) for at least 18 hours. A 200 mesh copper grid with carbon-coated formvar film was incubated onto 10 μL of fixed sample for 7 minutes. Next, the grid was placed on 10 μL of 1.75% uranyl acetate (w/v) for 7 minutes.

Figure 5.1. Experimental setup. A single urinary EV sample was thawed and centrifuged to remove precipitated salts. The supernatant was concentrated by a double ultracentrifugation step. Next, the pellet was divided over four Eppendorf tubes to perform the TEM preparation protocols (A-D). From each protocol three grids were imaged at one operator selected location and at five predefined locations per grid. This resulted in a total of eighteen images per protocol.

Transmission electron microscopy

Images were acquired using a single TEM instrument (Fei, Tecnai-12; Eindhoven, the Netherlands) at 80 kV. Illumination was performed with e-beam spot size 2 and filament 30. Images result from the average of two 700 ms acquisitions on a Veleta 2,048 x 2,048 side-mounted CCD camera and Imaging Solutions software (Olympus, Tokyo, Japan). The image intensity was 5,000-6,000. All grids were oriented with the “1” mark on a fixed position in the TEM specimen holder. To find a magnification that shows as many vesicles as possible on a single image with sufficient detail to distinguish EV morphological features, we evaluated images at magnifications of 150,000x, 98,000x, and 49,000x (Figure 5.2.). The higher magnification image appears to have a better background quality. However, this is due to selection of the image area, as shown in panels B and C. From the insets, it can be seen that a lower magnification

results in lower contrast and less sharp images compared to a higher magnification. These differences are small, however, and do not interfere with the identification of a cup-shaped particle. Thus, for the rest of the study, we evaluated images at 49,000x magnification ($2.2 \times 2.2 \mu\text{m}$ / image).

Figure 5.2 . Impact of magnification on image quality. The operator selected an image location containing a high number of EVs and relatively low background, and imaged this location at magnifications of 150,000x, 98,000x and 49,000x. The insets show the same 230×230 nm area at each magnification. The contrast and sharpness of the image decrease at lower magnification. However higher magnification reduces background if the background is no longer part of the image area.

We obtained six images per grid, for a total of eighteen images per protocol. In concordance with common practice in the EV field, the operator selected a high quality image per grid. This image contained a high number of EVs and relatively low background. To provide an ‘indication of the heterogeneity of the EV preparation studied’ (5), the common practice is to include an overview image showing the surroundings of the high quality image at lower magnification (Figure 5.3.). To get a general impression of the grid, we imaged at five predefined locations for every grid of each protocol.

TEM image evaluation

Because the urinary EV sample mainly contains EVs, we define EVs as particles with a diameter ≥ 30 nm with sufficient contrast for semi-automated segmentation (Figure S2) using the Quick selection tool of PHOTOSHOP v11.0.2. (Adobe Systems, San Jose). A custom Javascript was used to extract the number of EVs per image, and the surface area per EV. The EV diameter was calculated from the surface area using $d = \sqrt{(4A/\pi)}$,

which approximates the EV diameter in suspension (18). EVs on the edge of the image, or overlapping with annotations were excluded because the diameter of such EVs could not be determined. In TEM, a commonly applied morphological definition of EVs is “cup-shape” (23, 24, 25). Therefore, the same procedure was performed to select cup-shape EVs only (Figure S2).

Figure 5.3. Overview of images after operator selection. TEM images obtained from a single urinary EV sample by the four evaluated protocols. The white outlines indicate the image that we would normally show. The overview image is typically shown in Supplemental Information to “show the heterogeneity of the sample”. Panel letters correspond to protocol name, scale bar represents 1 μm and is the same for all images.

All the TEM images were rated from 1 (poor) to 5 (excellent) by four researchers based on image quality and the background quality. High image quality means the image

is suitable for EV research, high background quality means the absence of non-EV particles that interfere with EV recognition. The images were placed in random order to blind the researchers to the protocol. Contrast is defined as the difference between EVs and the background. Background is defined as visible proteins, salts, and large non-EV aggregates. Both a low contrast, as well as high background will interfere with the overall recognition of EVs.

Statistics

Statistical analysis was performed in Prism 7.0 (GraphPad, La Jolla, California, USA). The coefficient of variation (%CV) is defined as the standard deviation divided by the mean times 100%. For each image, the number of EVs, the % cup-shape, the image quality and the background quality was evaluated. Statistical analysis was performed to determine whether observed differences between grids or between protocols can be explained by chance alone. Within each protocol, an analysis of variance (ANOVA) was applied to compare the five images for each grid. The student's t-test was applied to compare the operator selected images to the images taken at predefined locations. Between protocols, the student's t-test was applied to compare the 15 images for each protocol. No significant differences ($p > 0.05$) are expected if the TEM image is not substantially affected by either the practice of operator selection or the TEM preparation protocol.

Results

To study the effects of four protocols on the obtained TEM images, we imaged a single EV sample using these four protocols. We evaluated each protocol by both the current practice of operator selection as well as by images taken at predefined locations. The comparison between protocols is based on the number of EVs, % cup-shape EVs, image quality and background quality.

Operator selection

The current practice is that an operator selects a high quality image, and includes an overview image of the surroundings. Figure 5.3. shows the overview image of a high quality image (white square) for each protocol. High quality TEM images can be

obtained with all protocols by operator selection, although some differences are visible between the protocols with regards to the number of EVs per image, % cup shape EVs, contrast, and background. Nevertheless, based on Figure 5.3., the quality of the obtained images seems to be only modestly affected by the four different protocols.

Qualitative evaluation

Figure 5.4. shows the TEM images obtained by both operator selection and at predefined locations for protocol A. The EVs are easily visible and recognizable, because the contrast is high and the background is low. TEM images taken by operator selection are comparable to the TEM images taken at the predefined locations. Therefore, operator selection produces images that are representative for protocol A.

The TEM images obtained with protocol B are shown in Figure 5.5. While the contrast and background are low overall, within a single grid extreme differences in contrast are present. Several images are almost devoid of EVs, and other images lack contrast, making it difficult to recognize EVs. Operator selection may be needed for a high quality EV image, because of the differences in contrast between images obtained at predefined locations.

The contrast in TEM images obtained with protocol C (Figure 5.6.) is high. EVs are visible in all images but there is moderate to high background in most images. In images with high background it is difficult to identify EVs. To obtain a high quality EV image operator selection is needed, but the resulting image does no longer represent the grid.

Figure 5.4. TEM images obtained with protocol A. TEM images obtained after operator selection and at predefined locations for three grids. Scale is the same for all images, bar represents 500 nm.

Figure 5.5. TEM images obtained with protocol B. TEM images obtained after operator selection and at predefined locations for three grids. Scale is the same for all images, bar represents 500 nm.

Figure 5.6. TEM images obtained with protocol C. TEM images obtained after operator selection and at predefined locations for three grids. Scale is the same for all images, bar represents 500 nm.

Figure 5.7. TEM images obtained with protocol D. TEM images obtained after operator selection and at predefined locations for three grids. Scale is the same for all images, bar represents 500 nm.

Figure 5.7. shows the TEM images obtained with protocol D (18). In most images, the contrast is high, but so is the background which contains visible salt deposits. Due to the background, identification of EVs is the most difficult in protocol D. A predefined location is likely to yield a poor image, and operator selection is tedious.

Quantitative evaluation

For all images, we quantified the number of EVs, the % cup-shape EVs, the image quality, and the background quality. The data are summarized in Figure 5.8. and in Table S1. Because we used a single EV-containing sample and made all images using a single TEM instrument, we can compare the data obtained with the four protocols (Table 5.2). The size distributions of EVs do not differ between protocols (Figure S1).

Operator selection leads to TEM images with a higher number of EVs, higher image quality and higher background quality compared to images taken at predefined locations. Furthermore, the TEM images are more similar between protocols (Figure 5.8. and Supplemental Information Table 1). Because there are three operator selected images per protocol, we did not perform statistical comparisons of operator selected images between protocols.

For predefined images within protocol A, the differences were not significant for image quality and background quality (both $p = 0.07$). For Protocol B, the differences were not significant for any of the parameters ($p = 0.58$ - 0.87). For protocol C differences were not significant for number of EVs per image ($p = 0.08$), % cup-shape ($p = 0.77$), and image quality ($p = 0.11$). For protocol D all the differences were significant ($p < 0.05$).

Within protocols, the number of EVs per image ranged for protocol A from 83 to 233, for protocol B 40 to 165, for protocol C 35 to 207, and for protocol D 91 to 273. Protocols A and D resulted in a 1.8-fold higher mean number of EVs with a lower %CV compared to protocols B and C. The number of EVs per image were comparable between protocols A and D ($p = 0.73$), and between protocols B and C ($p = 0.99$).

The number of cup-shape EVs per image ranged between 3 and 96. The % cup-shape was lowest for protocol A (14%) followed by B and D, 29% and 28%, respectively. Protocol C had the highest % cup-shape EVs, 46%.

Each image was scored on a scale from one (poor) to five (excellent) by four researchers. Based on rating, the mean image quality was 3.0, 2.5, 2.8 and 2.4 for protocols A, B, C and D, respectively (Table S1). Between protocols there was a 1.3-fold difference in image quality score, and within protocols the variability in quality was comparable (%CV 24-29), except protocol B, which had a %CV of 47. Images from protocol A were rated highest for suitability for EV research (Figure 5.8.C). Differences in image quality were not significant between protocols A-B ($p = 0.06$), A-C ($p = 0.34$), B-C ($p = 0.23$), and B-D ($p = 0.68$).

The mean background quality was 3.4, 3.4, 2.8, and 2.2 for protocols A, B, C and D, respectively (Table S1). The relatively high scores for protocol A and B are probably due to less non-EV particles that interfere with EV identification (Figure 5.8.D). The background quality differed between protocols, except between protocols A and B ($p = 0.28$).

5A

Figure 5.8. Summary of image properties by protocol and grid. Total number of EVs per image (A), % cup-shape of total EVs per image (B), image quality (C), and background quality (D) are shown for the four TEM preparation protocols. Each symbol represents one image. Circles indicate images taken at predefined locations, x indicates images after operator selection. Horizontal bar shows the mean value of predefined image locations per grid.

Table 5.2. Statistical comparisons between grids of each protocol (ANOVA), between operator selected and predefined locations (student's t-test), and between protocols (student's t-test).

EVs / image					% cup-shape				
	A	B	C	D		A	B	C	D
A	*0.002	<0.001	0.001	0.73	A	*<0.001	0.005	<0.001	<0.001
B		*0.65	0.99	<0.001	B		*0.65	0.008	0.87
C			*0.08	<0.001	C			*0.77	<0.001
D				*0.005	D				*0.05
Operator selected	0.005	0.002	0.009	0.02	Operator selected	0.29	0.07	0.01	0.03

Image quality					Background quality				
	A	B	C	D		A	B	C	D
A	*0.07	0.06	0.34	0.002	A	*0.07	0.28	<0.001	<0.001
B		*0.58	0.23	0.68	B		*0.87	<0.001	<0.001
C			*0.11	0.02	C			*0.03	0.01
D				*0.02	D				*<0.001
Operator selected	0.07	0.15	0.10	0.03	Operator selected	0.13	0.92	0.59	0.03

*p-value shown, *Between grids of same protocol.*

Discussion

The ideal TEM EV preparation protocol results in TEM images that truly represent the EV sample. These images should enable the assessment of the presence of EVs as well as the quality and purity of the sample. Furthermore, providing such images for all EV-related publications should facilitate the comparability between results obtained in different studies (5).

To improve the reproducibility and reliability of TEM results, the influence of the preparation protocol should be minimized by developing a consensus protocol. Ideally, operator influence should be minimized or even eliminated. If this is not possible, the procedure of operator selection should be well defined, and only allow the operator to eliminate obvious flaws, but not to allow cherry picking the 'best' images.

The common practice within the EV field is to have an operator select a high quality image of EVs, and to show the heterogeneity of the sample by providing an overview

image. Operator selection results in high quality images but influences the results through observer bias. While the image contrast and background are clearly protocol dependent based on the images taken at predefined locations, the influence of the protocol is incorrectly marginalized by the operator selection. In our facility, the operator searches for a field with a high number of EVs and low background. The parameters of our previous standard protocol (protocol D) had been extensively optimized using operator selection. In hindsight, operator selection stood in the way of a proper optimization because protocol differences were effectively minimized by operator selection. In our view, predefined locations are the least subjective, and operator selection without any restrictions (e.g. time or area searched) the most subjective approach to image selection. Regardless of the method for image selection, it may be needed to discard an entire grid due to overall quality issues, for example due to ruptures in the formvar film, cleanliness, etc. Furthermore, the inclusion of an overview image proved ineffective to demonstrate that the images within the white squares in Figure 5.3. were not representative of the grids.

The selection of nine different protocols used in the EV field (Table 5.1.) shows that there are at least thirteen parameters within each protocol that may influence the TEM images. Because several parameters are interdependent there is no straightforward way to optimize a protocol. Based on predefined image locations, the comparison of four protocols applied in the EV field demonstrates that the protocol choice affects the outcome of the experiment through differences in number of EVs per image, contrast and background quality. Low contrast and/or high background interfere with recognizing EVs. Protocol A is straightforward, results in a high number of EVs with high contrast, and a low background, and can also be applied to identify EVs in plasma, see Figure S3. Before removal of proteins in plasma, the background overshadows all EVs, but after protein removal by size exclusion chromatography protocol A was suitable (21).

Based on our results, a standard TEM protocol is needed, and should include all properties listed in Table 5.1., and should include a clear guideline on how to select image locations. The selection of locations should be as objective as possible. A standardized protocol for sample preparation and imaging will enhance the comparability and reproducibility of TEM within the field of EV research.

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Supplemental Information

Supplemental Data 1. Literature search string.

Search performed in Web of Science on November 22 2016:

TOPIC: (exosome) *OR* **TOPIC:** (extracellular vesicle) *OR* **TOPIC:** (cell-derived vesicle) *OR* **TOPIC:** (cell-derived microparticle) *AND* **TOPIC:** (transmission electron microscopy) **Refined by:** **DOCUMENT TYPES:** (ARTICLE) **Timespan:** 2011-2016.

Indexes: SCI-EXPANDED, SSCI, A&HCI, ESCI.

Figure S1. EV diameter histograms by TEM and NTA. Panels A-D correspond to protocols A-D. Histograms were normalized to 100% area under the curve, and have bin width 25 nm. Solid lines show total EVs, dashed lines cup-shape EVs. The diameter histograms have substantial overlap, and for protocols A-D respectively the median EV diameter was 47, 46, 44, 46 nm. The maximum observed diameter was 392, 292, 219, and 267 nm for protocols A-D respectively. The observed differences in size may be random because the number of EVs > 150 nm were 54, 16, 7 and 66, out of a total of 1496-2619 EVs. The differences in diameter may be explained by differences in shrinkage or flattening of EVs, and possibly a difference in the captured population of EVs. No EVs larger than 500 nm were observed, and by definition we did not include any EVs smaller than 30 nm. Panel E shows the particle size distribution of the urinary EV sample (100x diluted in PBS) detected by nanoparticle tracking analysis (NS500, Nanosight, UK). Particle size distribution were derived from 30 measurements of 10 seconds at camera level 14.

Figure S2. EV selection and automatically determined contours for TEM preparation protocols A-D. All EVs recognized by the selection tool were manually selected. The images were also analyzed by selection of cup-shape EVs. The green outline shows the contour determined by an automated script, which is used to determine the diameter of each particle. Identified EVs which were smaller than 30 nm were excluded from the analysis (pink outline). Examples of selected cup-shape EVs and excluded non cup-shape particles are shown on the right for each protocol. Scale bar represents 500 nm for the overview images, the examples are 250 x 250 nm.

Figure S3. TEM images of plasma after size exclusion chromatography obtained with protocol A at predefined locations. EVs are visible on TEM image of plasma sample after size exclusion chromatography. Scale is the same for all images, bar represents 500 nm.

Table S1. Summary data obtained by four TEM preparation protocols.

Protocol	A	B	C	D
Total EVs / image	168 (30)	100 (37)	100 (52)	175 (32)
Cup-shape EVs / image	21 (41)	32 (80)	44 (54)	48 (30)
Percentage cup-shape	14 (58)	29 (61)	46 (30)	28 (22)
Image quality score	3.0 (29)	2.5 (47)	2.8 (27)	2.4 (24)
Background quality score	3.4 (29)	3.4 (33)	2.8 (34)	2.2 (50)

Mean value shown with CV in parenthesis. Image quality and background quality score can range from 1 (poor) to 5 (excellent).

Chapter 5B.

Letter to the editor

Alain R. Brisson

Dear Editor,

I read with interest the article by Rikkert and colleagues “Quality of extracellular vesicle images by transmission electron microscopy is operator and protocol dependent” (1). In this article, two approaches for imaging extracellular vesicles (EV) by negative staining electron microscopy (EM) are compared. One approach consists in recording images at locations selected by the operator, while in the second approach, images are recorded at predefined locations on an EM grid, that is, at random locations not selected by the operator. The Authors conclude that images recorded at predefined locations reflect the overall quality of EV samples, while the operator selection approach is less suitable to evaluate the sample quality and leads to results influenced by operator bias.

I wish to express my disagreement with this overall conclusion. I consider that, in the case of heterogeneous samples like EV, analyzed by negative staining EM, it is preferable to record images at operator selected positions, unless and until optimized preparation protocols are applied. In my opinion, the Authors make a confusion between the sample quality, defined in the article as the content and purity in EV, on the one hand, and the quality of the preparation protocol, on the other hand.

The aim of an EM study is to provide a faithful description of a sample; in the case of EV samples, this means describing the morphology and size distribution of EV, the presence or absence of non-vesicular particles and aggregates, and determining the relative amount of the various components. Imaging a sample by negative staining EM involves three consecutive steps, namely (i) adsorption of the material contained in a sample on a carbon support film, (ii) staining and air-drying, and (iii) EM imaging per se (2, 3). Step (3) is a quasi-ideal operation, in the sense that the recorded image is an exact representation, magnified, of the material present locally on the grid (at 2-nm resolution for negative staining). On the other hand, steps (1) and (2), which together constitute the preparation protocol, are complex and poorly controlled processes (2-4). Ideally, the material deposited on the carbon film should reflect faithfully the sample content, and the material and stain deposit should be homogeneously distributed over the entire EM grid. With such an ideal preparation protocol, equivalent results would be obtained by imaging a sample at predefined locations and at operator selected locations.

However, anyone with some experience in EM of negatively stained samples is aware that the actual situation is different. Most often, the amounts of adsorbed material and stain deposit vary across the grid. This reflects the variability in the local adhesion properties of the support film and is a sign of preparation protocols of poor quality. Results obtained by three out of the four protocols selected by Rikkert and colleagues correspond to this situation, as clearly illustrated in Figures 5.5.–7. of (1). In this case, images recorded at predefined locations are likely to be impacted, if not dominated, by flaws from the preparation protocol, and are thus expected to provide an incorrect representation of the sample itself. This is the reason why, in common practice, the operator scans the EM grid at low magnification and searches for areas which present a larger amount of adsorbed material and/or a more homogeneous distribution of material and stain. It is logical and legitimate that the operator selects these areas and not areas of lesser quality in terms of material spreading and staining. In doing so, the operator applies a conscious bias, based on his/her experience and knowledge from previously published literature.

In conclusion, current efforts towards the development of optimized preparation protocols for EV imaging by negative staining EM, as presented in Rikkert and colleagues' article, are highly valuable. This is a challenging task, given the heterogeneity and diversity of EV samples, e.g. from body fluids or cell conditioned media (5,6), and the large variety in carbon film adhesion properties (4).

Finally, in the context of this article, a major advantage of cryo-EM over negative staining deserves to be emphasized. Indeed, in cryo-EM, objects are (in general) not directly adsorbed on a support film, but are contained within frozen nanodroplets suspended over a perforated film (7,8). The absence of adsorption issue correlates with the highly homogeneous distribution of objects, which characterizes cryo-EM grids (9). Therefore, in the case of cryo-EM, it is foreseeable that imaging EV samples at predefined positions, say, in a fully automated manner or with minimal intervention from the operator, will become a routine approach.

Response to letter to the editor.

Linda G. Rikkert

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Dear Editor,

We appreciate the letter by renowned electron microscopist Alain Brisson in response to our article. As stated in his letter, “*The aim of an EM study is to provide a faithful description of a sample*”. In the case of electron microscopy, the faithful description of a sample is based on the obtained images. Sources of variation contributing to the obtained images are (i) sample heterogeneity (“*the morphology and size distribution of EVs, the presence or absence of non-vesicular particles and aggregates*”), (ii) the preparation protocol (“*complex and poorly controlled processes*”), and (iii) the operator.

When influences of sample heterogeneity and preparation protocol were controlled, operator-selected images showed less variation than images taken at predefined locations. This finding is the result of “confirmation bias”, which is defined as “*evaluating evidence that supports one’s preconceptions differently from evidence that challenges these convictions*” (9, 10). In the case of EM, the operator looks for image locations that confirm the expectation. While it is common in EM to assume that the operator influence on the study outcome is negligible, this assumption is at odds with the results of our study and the knowledge of confirmation bias. Therefore, in our view the “*faithful description of a sample*” is best reflected by images taken at predefined locations.

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Chapter 6.

Detection of extracellular vesicles in plasma and urine of prostate cancer patients by flow cytometry and surface plasmon resonance imaging

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Abstract

Large ($> 1 \mu\text{m}$) tumor-derived extracellular vesicles (tdEVs) enriched from the cell fraction of centrifuged whole blood are prognostic in metastatic castration-resistant prostate cancer (mCRPC) patients. However, the highest concentration of tdEVs is expected in the cell-free plasma fraction. In this pilot study, we determine whether mCRPC patients can be discriminated from healthy controls based on detection of tdEVs ($< 1\mu\text{m}$, EpCAM+) and/or other EV subtypes, in cell-free plasma and/or urine. The presence of EV subtypes in plasma and urine samples from mCRPC patients ($n=5$) and healthy controls ($n=5$) was determined by flow cytometry (FCM) and surface plasmon resonance imaging (SPRi) using an antibody panel and lactadherin. For FCM, the concentrations of marker positive (+) particles and EVs (refractive index < 1.42) were determined. Only the lactadherin+ particle and EV concentration in plasma measured by FCM differed significantly between patients and controls ($p = 0.017$). All other markers did not result in signals exceeding the background on both FCM and SPRi, or did not differ significantly between patients and controls. No difference was found between patients and controls based on the detection of tdEVs. For FCM, the measured sample volumes are too small to detect tdEVs. For SPRi, the concentration of tdEVs is probably too low to be detected. Thus, to detect tdEVs in cell-free plasma and/or urine, EV enrichment and/or concentration is required. Furthermore, we recommend testing other markers and/or a combination of markers to discriminate mCRPC patients from healthy controls.

Introduction

Circulating tumor cells (CTCs) and large ($> 1 \mu\text{m}$) tumor-derived extracellular vesicles (tdEVs) that are measured with the CellSearch are both associated with poor survival of metastatic castration-resistant prostate cancer (mCRPC) patients (1-4). Furthermore, changes in the concentrations of CTCs and tdEVs after initiation of therapy can be used to monitor treatment response. CellSearch uses EpCAM immunomagnetic enrichment in combination with immunofluorescence labeling to identify CTCs and large tdEVs from the cell fraction of centrifuged whole blood, and defines both by expression of cytokeratin and lack of the leukocyte marker CD45. Discrimination between CTCs and tdEVs is obtained by cell morphology features, and the presence of a nucleus in case of CTCs. For mCRPC patients, a median of ~ 7 CTCs and ~ 116 large tdEVs per 7.5 mL of blood was reported (i.e. ~ 0.009 CTCs and ~ 0.015 large tdEVs per mL) (1). As the concentration of EVs increases with decreasing size (5), a 100-1,000 fold higher concentration of small ($< 1 \mu\text{m}$) tdEVs is expected to be present in blood of mCRPC patients, resulting in an expected concentration of $\sim 1.5 - 15$ small tdEVs per μL of blood. The majority of these small tdEVs should be present in cell-free plasma of mCRPC patients (6), and may directly be measured in plasma without the need of enrichment techniques. In this study, we therefore performed a pilot study to determine whether mCRPC patients can be discriminated from healthy controls based on the presence of small tdEVs ($< 1 \mu\text{m}$, EpCAM+) and other EV subtypes, directly in cell-free plasma and/or urine. Since EVs $< 1 \mu\text{m}$ are below the detection limit of the CellSearch Analyzer, we used flow cytometry (FCM) and surface plasmon resonance imaging (SPRi) in this study.

Methods

Minimal detectable marker⁺ concentration for FCM

To determine the minimal detectable marker⁺ particle concentration for FCM, we performed a spiking experiment (see Supplemental Information for a description of the methods used). PC3 cell-derived EVs, identified by CD63-PE, were spiked into plasma at different volumetric dilutions. CD63 was chosen because CD63 had the highest expression on the PC3 EVs compared to other markers tested (data not shown). The minimal detectable marker⁺ particle concentration was defined as the

concentration at which the number of detected CD63+ particles exceeded the 95% confidence interval based on pure plasma.

Sample collection

Blood and urine were obtained from five mCRPC patients and five healthy controls. See Table 6.1. for donor characteristics. Samples were collected with written informed consent in accordance with the Helsinki Declaration and approved by the medical-ethical assessment committee of the Academic Medical Center, University of Amsterdam (NL64623.018.18). For each donor, whole blood was collected using a 21G needle, and the first vacutainer was discarded. Next, three 2.7 mL citrate vacutainers (BD Biosciences, San Jose, CA) were collected, mixed by gentle inversion and processed within 20 minutes. Vacutainers were centrifuged at 2,500 *g*, 20°C for 15 minutes without brake in a Rotina 380R centrifuge (Hettich, Tuttlingen, Germany). Plasma was collected until approximately 0.5 cm above the pellet using a 5 mL Pasteur pipet (VWR, Radnor, PA). The plasma was pooled and transferred to a conical base tube (10 mL; Sarstedt, Nümbrecht, Germany), and centrifuged at 2,500 *g*, 20 °C for 15 minutes. The supernatant was transferred to a conical base tube and divided over 75 µL cryovials (Sarstedt), followed by snap-freezing in liquid nitrogen and storage at -80 °C.

First catch-urine (no prostate massage) was collected in 125 mL routine urine collection cups free of any preservatives. Urine samples were placed on melting ice (0°C) and transported to the laboratory within one hour after collection. The urine collection cup was inverted five times to resuspend the cells. To remove the cells, two aliquots of 20 mL urine were centrifuged at 500 *g*, 4 °C for 20 minutes without a brake using a Rotina 46RS centrifuge (Hettich). The supernatant was pooled, vortexed for at least 10 s and aliquots of 1 mL were pipetted in cryovials (Sarstedt), followed by snap-freezing in liquid nitrogen and stored at -80 °C.

Table 6.1. Metastasized castration-resistant prostate cancer (mCRPC) patients and healthy controls characteristics.

Variable	mCRPC patients (n = 5)	Healthy controls (n = 5)
Age in years, mean (range)	80 (66-83)	28 (19-40)
Ethnicity	Caucasian	Caucasian
Mean (range) serum PSA level (ng/mL)*	6.0 (0.1-17.6)	NA
Mean (range) total Gleason score (biopsy tissue)	7.8 (6-9)	NA
Previous treatment for prostate cancer**	Yes	NA

mCRPC: metastasized castration-resistant prostate cancer; NA: not applicable; PSA: prostate specific antigen;

*last determined PSA level before inclusion **including surgery, hormone therapy, radiotherapy, cryotherapy, and/or chemotherapy.

Sample preparation

All samples were thawed in a 37 °C water bath. For the FCM measurements, the urine samples showed a high level of autofluorescence in the FITC channel, which disappeared after size exclusion chromatography (SEC). Therefore, for each urine sample, 150 µL of urine was loaded on SEC columns (qEVsingle/70 nm; Izon Science, Christchurch, New Zealand), followed by elution with phosphate buffered saline (PBS; 21-031-CV; Corning, Corning, NY). The first 1250 µL eluate was discarded, and the next 500 µL eluate, containing particles with a diameter larger than 70 nm, was used for the FCM measurements. In preliminary experiments, undiluted urine samples caused negative SPRI responses, which disappeared after 5-fold dilution in PBS. Therefore, all samples were pre-diluted 5-fold in PBS for SPRI.

Markers

A panel of fourteen markers was selected for measurements with FCM and SPRI on the plasma and urine samples. We included lactadherin, CD9, CD63 and CD81 which are associated with EVs (7, 8), CD45 expressed by leukocytes, CD61 expressed by platelets, CD146 expressed by endothelial cells and CD235a expressed by erythrocytes. In addition, we included EpCAM expressed on cells of epithelial cell origin (1), PSMA expressed on prostate cells (9) or markers reported to be overexpressed on cancer cells CD24, CD47, CD142, HER2, (10-13), and an isotype control. An overview of marker target proteins, clones, concentrations and suppliers is shown in Table 6.2..

In FCM, the PE-conjugated isotype control resulted in PE positive concentrations higher than the PE positive concentration found for any of the actual markers. The PE-conjugated isotype control was therefore considered unreliable. Instead, measurements of marker in PBS were used to obtain an estimation of the background.

Samples were measured simultaneously by FCM and SPRI, alternating between samples from patients and controls. PBS samples were measured after the patient and control samples.

Flow cytometry

Plasma samples were pre-diluted in PBS to event rates between 2,000 and 3,000/s to prevent swarm detection when triggering on side scatter (14). The pre-dilution varied per plasma sample, and ranged between 10-fold dilution and 100-fold dilution. The urine samples were diluted through the use of SEC and no additional dilution was necessary to prevent swarm detection. Antibody aggregates were removed by centrifugation at 18,890 g, 20°C for 5 minutes prior to use. 20 μ L of pre-diluted sample was incubated with 2.5 μ L marker (for concentrations see Table 6.2.) for two hours at room temperature in the dark. The staining was stopped by adding 200 μ L PBS to the samples. All samples were analyzed on an A60-Micro (Apogee; Northwood, UK) at a flow rate of 3.0 μ L/minute. Each sample was measured for four minutes or until 500,000 events were detected, triggering on 405-nm side scatter using a threshold corresponding to a side scattering cross section of 10 nm² (Rosetta Calibration; Exometry, Amsterdam, The Netherlands). Taking into account the sample dilution and measurement time, a total of approximately 0.008 to 0.08 μ L for plasma and 1 μ L SEC eluate of urine was analyzed.

Table 6.2. Markers used for flow cytometry (FCM) and surface plasmon resonance imaging (SPRi).

Marker	Target protein	Clone	Concentration (µg/mL)		Conjugate	Company
			FCM	SPRi		
CD9	Tetraspanin	M-L13	2.5	5	PE	BD
CD24	Sialoglycoprotein	SN3	Unknown	5	PE	TFS ¹ ; eB ²
CD45	Protein tyrosine phosphatase, receptor type C	HI30	50	5	FITC	Bl ¹ ; Sony ²
CD47	Integrin associated protein	CC2C6	8	5	PE	Bl
CD61	Integrine beta-3	VI-PL2	3	5	PE	TFS ¹ ; BD ²
CD63	Tetraspanin	H5C6	6.25	5	PE	BD ¹ ; CGS ²
CD81	Tetraspanin	JS-81	5	5	PE	BD
CD235a	Glycophorin A	JC159	100	5	PE	AT ³
CD142	Tissue factor	HTF-1	9	5	PE	BD ¹ ; eB ²
CD146	Melanoma cell adhesion molecule	P1H12	12.5	5	FITC	Sony ¹ ; eB ²
EpCAM	Epithelial cell adhesion molecule	VU1D9	6	5	PE	TFS ¹ ; custom ³
HER2	Erb-b2 receptor tyrosine kinase 2	24D2	25	5	PE	Bl
Lactadherin	Milk fat globule-EGF factor 8	NA	41.5	5	FITC	HT ³
PSMA	Glutamate carboxypeptidase II	REA408	11	5	PE	MB
IgG1	Immunoglobuline G	X40	50	5	FITC/PE	BD

¹FCM; ²SPRi; ³Same conjugated antibody used for FCM and SPRi. AT: Agilent Technologies, Santa Clara, CA. BD: BD Biosciences, San Jose, CA. Bl: Biolegend, San Diego, CA. CGS: Cell Guidance Systems, Cambridge, UK. custom: Own hybridoma cell line, Enschede, Netherlands. eB: eBioscience, San Diego, CA. FITC: fluorescein isothiocyanate. HT: Haematologic Technologies, Essex Junction, VT. MB: Miltenyi Biotec, Bergisch Gladbach, Germany. NA: not applicable. PE: phycoerythrin. Sony: Sony Biotechnology, San Jose, CA. TFS: Thermo Fisher Scientific, Waltham, MA.

Data analyses were performed in MATLAB R2018b (Mathworks, Natick, MA) and FlowJo (v10.6.1; FlowJo, Ashland, OR). First, for samples with a minimum count rate of 250 counts/s, time points of the data in which the count rate deviated > 25% from the median count rate were removed to account for fluctuations in the flow rate. Next, fluorescent gates were determined for every marker, resulting in the gates as mentioned in the supplemented MIFlowCyt. Positive (+) events are defined as events with a fluorescent signal exceeding the gate. Using this conventional gating strategy, all marker+ particles are taken into account, including non-specific binding of markers to lipoproteins or protein aggregates. Recently, we showed that marker+ EVs can be distinguished from other marker+ particles using the refractive index (RI)

(15). Therefore, we also determined the RI of all particles > 200 nm using Flow-SR as described previously (15). Briefly, using the ratio of side versus forward scatter, Mie theory, and a mathematical model of the optical configuration of the flow cytometer, the diameter of each particle can be determined. The RI can subsequently be derived from a lookup table of side scatter versus diameter. In the present study, marker+ particles include all particles above the size detection limit (Table 6.3.) and positive for a marker, whereas marker+ EVs include particles > 200 nm with an RI < 1.42 and positive for a marker.

Concentrations provided are the number of detected events corrected for the total sample dilution, flow rate and measurement time. Because for urine, the dilution caused by SEC was unknown, the marker+ concentration is expressed as marker+ particles/EVs per μL SEC eluate. For a detailed description of the flow cytometer configuration, operating conditions and data analysis please see the MIFlowCyt list.

Surface plasmon resonance imaging

SPRi measurements were performed using the MX96 SPRi device (IBIS technologies B.V., Enschede, the Netherlands) as described by Stravers et al (16). Markers (5 $\mu\text{g/mL}$) were printed in triplicate on a sensor surface (Easy2Spot type-G; Ssens B.V., Enschede, The Netherlands) using a Continuous Flow Micro-spotter (CFM) 2.0 (Wasatch microfluidics LLC, Salt Lake City, UT). Three spots were used as control spots, and active sites on the sensor surface were inactivated (16). In total four sensors were used, each analyzing six samples, i.e. five plasma or urine samples and one negative control (PBS).

Before starting the six sample runs, three regeneration runs were applied, consisting of i) a baseline phase of two minutes, ii) an association phase of 15 minutes, whereby regeneration buffer (0.1 M Glycine (Merck, Darmstad, Germany), pH 3.06 and 0.3% (v/v) Triton-x100 (Sigma-Aldrich, Darmstadt, Germany)) is flowed back and forth over the sensor surface, and iii) a dissociation phase of two minutes, whereby the regeneration buffer is replaced by PBS. Next, 300 μL of each pre-diluted sample was placed into the SPRi to allow measurement of each sample in duplicate. A single sample run consisted of i) a baseline phase of two minutes, ii) an association phase of 60 minutes, whereby the sample is flowed back and forth over the sensor surface and

iii) a dissociation phase of four minutes. After each sample run, the chip surface was regenerated to break the formed antigen-antibody bonds before the next sample run was started. Taking into account the sample dilution, 26 μ L of plasma and urine was analyzed per sample run.

File conversion and analysis was performed as described by Stravers et al (16). SPRi signals in PBS were set to zero to account for differences in the initial spot values. The first 100 s of the association phase was removed from the analysis to account for RI differences of buffers. The mean SPRi signals of each marker of the last 50 s of the association phase, corrected for the reference spot, was used as the maximum response. The difference between the maximum response and the response after 100 s of each spot was used to calculate the mean response in resonance units (RU). While three spots were printed for each marker, we included data from the two spots with the highest relative ligand density (RLL) because of printing artifacts in some spots. A data point represents the RI change during one hour per sample averaged over the two spots. To correct for degeneration of the sensor and for differences in the total particle concentration between samples, the response per sample was normalized by dividing the response for one marker by the sum of all marker responses for that sample. The mean of the duplicates was calculated to obtain a response per marker per donor.

Data analysis

Median and standard deviation (SD) of the data was calculated per marker. The median is preferred over the mean, because the median is less influenced by outliers and therefore more representative given the small number of samples. Statistical analysis was performed using a Student's t-test. All analyses were performed in MATLAB R2018b.

Results

Minimal detectable marker+ particle concentration for FCM

Figure 6.1.A. shows the side scatter versus CD63-PE fluorescence scatter plots for pure plasma, and for PC3 EVs spiked in plasma at a 1:1,000 and 1:1 volumetric dilution. Figure 6.1.B. shows that at a volumetric dilution of 1:1,000, the number of detected CD63+ particles exceeded the 95% confidence interval for pure plasma. Correction for

the particle concentration of the PC3 EV sample and the percentage of CD63+ particles in the sample, led to a minimal detectable concentration of 15 marker+ particles/ μ L. Since the A60-micro FCM used in this study has a higher scatter sensitivity and similar fluorescence sensitivity than the A50-micro used in the spiking experiment, we expect the minimal detectable concentration for the A60-micro to be similar or lower than 15 marker+ particles/ μ L.

Flow cytometry

Figure 6.2. shows the marker+ concentration measured by FCM in plasma (Figure 6.2.A-B.) and urine (Figure 6.2.C-D.) of patients and controls, and in PBS. Figure 6.2.A and C show the marker+ particle concentration obtained using conventional gating, that is including all marker+ particles. Figure 6.2.A. shows that for all markers studied, the marker+ particle concentrations exceeded the background measured in PBS ($p < 0.05$). However, only the lactadherin+ particle concentrations differed significantly between patients and controls ($p = 0.042$). The EpCAM+ and PSMA+ particle concentrations were similar in plasma samples from patients and controls, making it unlikely we detected tdEVs.

Figure 6.2.B. shows the RI of marker+ particles > 200 nm in plasma of patients and controls, and in PBS. When using the RI to identify true marker+ EVs ($RI < 1.42$) in the sample, marker+ EVs were found for all markers except for CD146, and EpCAM in controls, which did not exceed the background measured in PBS ($p > 0.05$). EpCAM and PSMA mainly stained particles with an $RI > 1.42$, which most likely are lipoproteins (15). Again, only lactadherin showed a significant difference in the marker+ EV concentration between patients and controls ($p = 0.017$).

Marker+ concentrations in urine (Figure 6.2.C.) were approximately 10-fold lower than marker+ concentrations in plasma. Marker+ particles were found for lactadherin, CD9, CD63, CD81, CD61, CD146, CD235a in patients, CD24, CD47 in patients, CD142, and HER2 in patients ($p < 0.05$). However, neither of these marker+ concentrations differed significantly between patients and controls. When using the RI to identify true marker+ EVs in the sample, concentrations exceeding the background in PBS

were found for all markers except for CD146, CD235a in controls, CD142 in controls, EpCAM in controls, and PSMA in patients ($p > 0.05$).

Figure 6.1. A) Side scatter versus CD63-PE fluorescence for pure plasma (left), plasma spiked with PC3 extracellular vesicles (EVs) at a 1:1000 volumetric dilution (centre) and 1:1 volumetric dilution (right). B) Detected number of CD63+ particles (black dots) at volumetric dilutions of PC3 EVs in plasma. CI: confidence interval.

However, again none of the markers exceeding the background showed a significant difference in the marker+ EV concentration between patients and controls.

Taken together, the lactadherin+ particle and EV concentration in plasma differed significantly between patients and controls as measured by FCM. All other markers tested on plasma and urine samples that showed concentrations exceeding the background, did not show a significant difference between patients and controls.

Figure 6.2. Marker+ concentration by flow cytometry per µL plasma (A-B) or urine (C-D) from metastatic castration-resistant prostate cancer patients (red, n=5), healthy controls (grey, n=5) and phosphate buffered saline (blue, n=2). Panels B and D show the refractive index (RI) of marker+ particles >200 nm. Data shown represent the median and standard deviation (whiskers) per group. Lact: lactadherin.

Surface plasmon resonance imaging

Figure 6.3.A. shows the normalized SPRi responses per marker as measured for plasma from patients and controls. Particles expressing lactadherin, CD63, CD81 in patients, CD61 in patients, CD146, CD24 in patients and CD47 in patients were found in plasma, since their responses exceeded the IgG response ($p < 0.05$). However, none of these marker responses differed significantly between plasma of patients and controls.

Figure 6.3.B. shows the normalized SPRi responses per marker as measured for urine from patients and controls. Particles expressing CD235a in controls, CD47 in controls and CD142 were found, since their responses exceeded the IgG response ($p < 0.05$). Similar as in plasma, none of these markers differed significantly between plasma of patients and controls.

Figure 6.3. Surface plasmon resonance imaging (SPRi) response for plasma (A) or urine (B) of metastatic castration-resistant prostate cancer patients (red, n=5), healthy controls (grey, n=5) and phosphate buffered saline (blue, n=2) for different markers. Data shown represent the median and standard deviation (whiskers) per group. Lact: lactadherin; RU: resonance units.

Discussion

In this study, we determined whether mCRPC patients could be discriminated from healthy controls based on the presence of EV subtypes in plasma and/or urine samples, using FCM and SPRi. FCM was chosen because FCM is frequently used to measure the concentration of EVs in clinical samples such as plasma or urine. However, common flow cytometers do not detect EVs of smaller sizes and low antigen densities. For example, the FCM used in this study has an EV size detection limit of 160 nm and an antigen detection limit of 70 PE molecules (Table 6.3.). In SPRi, multiple EVs captured on a surface coated with markers induce a change in the index of refraction, thereby generating the SPRi signal. All EVs, also the EVs which are too small to be detected by FCM, may contribute to the SPRi signal. However, since an ensemble of EVs is attached to the surface, evaluation of the concentration of EVs is not possible with SPRi (17).

Only for the lactadherin+ particle and EV concentration in plasma measured by FCM, a significant difference was found between patients and controls. Use of the RI to identify true marker+ EVs increased the specificity by exclusion of marker+ lipoproteins ($RI > 1.42$) (15). The cause of the significant difference in lactadherin+ particle and EV concentration may be due to the presence of prostate cancer, but the influence of the age (18) and treatment differences between the groups cannot

be excluded (Table 6.1.). All other markers did not result in signals exceeding the background in PBS, or showed no significant differences between patients and controls.

Various explanations for the absence of a significant difference in small tdEVs between patients and controls are conceivable. First, no detectable differences are present in EV subtypes $< 1 \mu\text{m}$ in the plasma and/or urine samples of the two groups. The small EVs may be bound to or taken up by leukocytes or endothelial cells, and thus will be absent in the cell-free plasma after centrifugation (19).

Secondly, extrapolation of the concentration of CTCs and large tdEVs found in a previous study (1), results in an expected small tdEV ($< 1 \mu\text{m}$, EpCAM+) concentration of 1.5-15/ μL in plasma (20). Based on our spiking experiment, we expect the minimal detectable concentration for the A60-micro to be 15 marker+ particles/ μL . However, since we measured plasma volumes $< 0.1 \mu\text{L}$, it was impossible to detect the few tdEVs that might be present in the plasma sample. For SPRe, a minimum concentration of 10^5 marker+ particles/ μL is needed to obtain a signal (Table 6.3.). Therefore, it is likely that the concentration of tdEVs is too low to be detected with SPRe.

A third explanation is that the actual small tdEV concentration is less than the expected small tdEV concentration based on the extrapolation of CTC and large tdEV concentrations (20). For example, the detected concentrations of small (here 160-1,000 nm) leukocyte EVs was 9.3×10^3 per μL plasma in controls. The concentration of large ($> 1 \mu\text{m}$) leukocyte EVs was found to be ~ 900 per μL blood in controls (21). Thus, the concentration of small leukocyte EVs was not, as mentioned in the introduction, 100-1,000 fold higher than the concentration of large leukocyte EVs, but only 10-fold higher.

Another explanation is that the antigen density on small EV subtypes may be below the antigen detection limit for FCM. For example, the detection limit for EpCAM-PE used in this study was 70 mean equivalent soluble fluorophore (MESF; Table 6.3. and supplemented MIFlowCyt). Since the dye to protein ratio of PE-conjugated antibodies is typically 1, this means that an EV should have a minimum of 70 EpCAM molecules for it to be detected as EpCAM+, assuming the staining efficiency is 100%. If we extrapolate the measured EpCAM density on prostate cancer cell lines LNCaP and

PC3, CTCs described by Rao et al (21) to EVs with a diameter of 300 nm, the expected number of EpCAM molecules is ~30, which is below the detection limit for EpCAM in this study.

Biggs et al (22), showed a difference in the detection of PSMA+ particles between controls and prostate cancer patients with a Gleason score higher than 8. However, the PSMA clone (3/E7) used by this and one other study (22, 23) differs from the one used in our study (REA408), and is not commercially available. Logozzi et al (24) detected a higher concentration of EVs expressing CD81 and PSA in blood from prostate cancer patients compared to controls. However, in that study the EVs were concentrated by ultracentrifugation before measurements. In our study, the goal was to detect EVs directly in plasma and urine and therefore we did not concentrate the EVs before measurements.

To investigate whether small tdEVs are present in the plasma and/or urine of patients, we recommend to perform an enrichment or concentration enhancement step to increase the concentration of small tdEVs. Furthermore, other clones/markers and/or a combination of markers may be needed, as used by Nanou et al (1). Moreover, for FCM, the development of brighter fluorophores and/or more sensitive fluorescence detectors is required to allow detection of the low number of antigens present on EVs. For SPRi, EV concentrations need to be increased to reach the minimal detectable concentration, and/or the SPRi response per EV needs to be increased. Furthermore, SPRi has not often been applied to plasma samples, therefore further optimization may be needed to prevent non-specific binding of for example (lipo)proteins.

In conclusion, only the lactadherin+ particle and EV concentration measured in plasma by FCM differed significantly between patients and controls. Based on this result, FCM may be more promising for application to clinical samples compared to SPRi, taken into account the before mentioned challenges. In the future, larger cohort studies are needed to confirm the presence of small tdEVs in blood plasma and urine, and to see whether such EVs can be used to discriminate between prostate cancer patients and controls.

Table 6.3. Characteristics of the methods used for the detection of extracellular vesicles (EVs).

	Flow cytometry	SPRi
Detection of	Single EVs	Ensemble EVs
EV size detection limit	≥ 160 nm*	All EVs
Particle detection	In suspension	On surface
Signal caused by	Light scattering, fluorescence	Refractive index change
Minimal # EVs/μL	1×10^3 **	2×10^5 (17)
Antigen detection limit	70 molecules (PE)	1
Preparation steps	Sample dilution, fluorescent marker staining	Sample dilution, printing markers on sensor
Preparation time	2.5 hours	2 hours
Measurement time / sample	1-4 minutes***	1 hour
Output	Antigen expression, concentration, size, refractive index	Antigen expression

As determined using Rosetta Calibration (Exometry, Amsterdam, The Netherlands) using a core-shell model with refractive index (RI) of 1.38 for the core, 1.48 for the shell and a shell thickness of 4 nm. ** Based on the 95% confidence interval for buffer only measurements ($n = 3$). * Increases with the number of markers to be analyzed.*

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Supplemental Information

Methods spiking experiment

PC3 EVs

The prostate cancer cell line PC3 was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and used to produce prostate cancer-derived EVs. The cell line was cultured at 37 °C and 5% CO₂ in RPMI-1640 with L-glutamine medium (Lonza, Basel, Switzerland) supplemented with 10% v/v fetal bovine serum (FBS), 10 units/mL penicillin, and 10 µg/mL streptomycin. Medium was refreshed every second day. The initial cell density was 10,000 cells/cm² as recommended by the ATCC. When cells reached 80–90% confluence, they were washed three times with PBS and FBS-free RPMI medium supplemented with 1 unit/mL penicillin and 1 µg/mL streptomycin was added to the cells. After 48 h of cell culture, cell supernatant was collected and centrifuged for 30 minutes at 1,000 g. The supernatant was pooled, and aliquots of 50 µL were frozen in liquid nitrogen and stored at –80°C.

Plasma

Blood was obtained from four healthy and overnight fasting male donors with written informed consent in accordance with the Declaration of Helsinki. The study protocol was waived by the medical ethics committee of the Amsterdam UMC. Whole blood was drawn using a 21G needle. The first 3.5 mL was discarded. In total 27 citrate vacutainers of 9 mL (BD, San Jose, Ca) were collected, mixed gently with the coagulant and processed within 15 minutes after collection. Plasma was obtained from whole blood by double centrifugation at 1,560 g for 20 minutes at 20°C. After centrifugation, supernatant was pooled, and aliquots of 50 µL were frozen in liquid nitrogen and stored at –80°C.

Spiking experiment

PC3 EV and plasma samples were thawed on melting ice. Plasma was diluted 10-fold in citrate PBS (154 mmol/L NaCl, 1.24 mmol/L Na₂HPO₄·2H₂O, 0.2 mmol/L NaH₂PO₄·2H₂O, pH 7.4; 50 nm filtered (Nuclepore, GE Healthcare), supplemented with 0.32% trisodium citrate). Volumetric dilutions of 1:2, 1:5, 1:10, 1:50, 1:100 and 1:1000 of PC3 EVs in the diluted plasma was performed. 20 µL of plasma spiked with PC3 EVs was stained with 4 µL of CD63-PE (7.5 µg/mL, clone CLBGran/12, Beckman Coulter,

Brea, CA), 4 μ L of CD61-FITC (25 μ g/mL, clone Y2/51, Dako, Glostrup, Denmark), 4 μ L of CD235a-FITC (50 μ g/mL, lot 83040, Dako, Glostrup, Denmark), 4 μ L of CD45-FITC (50 μ g/mL, clone HI30, Biolegend, San Diego, CA), and 4 μ L of citrate PBS. Samples were incubated for 60 minutes at room temperature in the dark. The staining was stopped by addition of 200 μ L citrate PBS.

All samples were analyzed for four minutes on an A50-Micro (Apogee, Northwood, UK) using side scatter triggering, at a flow rate of 4.5 μ L/minute. PE signals were compensated for signal spill over from the FITC channel, and a gate identifying CD63-PE positive signals was set at 49 arbitrary units. The number of measured CD63-PE+ particles per dilution were determined, and compared to the 95% confidence interval determined using the number of CD63-PE+ particles found in plasma, assuming Poisson statistics. The minimum required concentration was determined by calculating the concentration of CD63-PE+ PC3 EVs present in the sample at the minimal volumetric dilution, using the concentration of CD63-PE+ particles in the original PC3 EV sample.

Discussion & Outlook.

Discussion & Outlook

To improve the disease management of prostate cancer, there is need for a robust and low-invasive assay to increase the specificity of prostate cancer detection and provide information on what treatment should be administered when. Body fluids, like blood, contain potential biomarkers which behold clinically relevant information. Within the Cancer-ID program, universities and companies joined forces to detect and characterize one of these potential biomarkers, tumor-derived extracellular vesicles (tdEVs).

The presence of 5 or more circulating tumor cell (CTCs) in 7.5 mL of blood is associated with poor survival in metastatic prostate cancer patients. tdEVs have similar prognostic value but are present in a higher concentration compared to CTCs (1). If tdEVs can be detected with similar sensitivity and specificity as CTCs, tdEVs may improve the accuracy of the severity of the cancer. Furthermore, ideally screening can be simplified when tdEVs can be identified and quantified directly in a biofluid.

However, moving down in size from CTCs to tdEVs reveals new challenges. These challenges are that one needs to be able to (i) detect tdEVs, (ii) differentiate tdEVs from other EVs and non-EV particles like lipoproteins and platelets and (iii) relate the measured signal to the concentration of tdEVs in blood.

Ability to detect or image EVs

Multiple characteristics of EVs can be quantified, including morphology, size, deformability, refractive index, biochemical composition, and these characteristics can be applied to identify EV and differentiate between the EVs of different cell origin, eventually leading to the tdEV concentration (2). Evaluation of EV detection techniques shows that most techniques are able to detect or image EVs and provide characteristics of EVs (*Chapter 1*). However, discrimination between tdEVs and non-EV particles and EVs from other cells is a challenge for all EV detection techniques. In addition, most techniques are in an early stage of development, and not yet ready to be used for clinical samples.

Differentiation of tdEVs from non-EV particles

One of the main outcomes of this thesis is that detection of tdEVs is difficult due to the abundant presence of non-EV particles in blood. We expect concentrations of 10^{16} /mL of lipoproteins, up to 10^9 /mL of platelets, and up to 10^{11} /mL of EVs from non-tumor origin compared to 10^4 /mL of tdEVs in human blood of metastatic castration resistant prostate cancer patients (3-6). This means that we should expect the non-EV particles to outnumber tdEV by 1,000,000,000,000-fold, and EVs to outnumber tdEVs by 10,000,000-fold. Because of the influence of especially non-EV particles, in this thesis we improved pre-analytical procedures like the depletion of non-EV particles to enhance detection and characterization of tdEVs.

Centrifugation is most frequently applied to isolate biomarkers, like CTCs (7-9), tumor-educated platelets (10), tdEVs (11), EV-miRNAs (12), and circulating cell-free DNA (13, 14) from whole blood. However, centrifugation protocols have not been standardized within the EV field. To predict the behavior of blood-borne biomarkers during centrifugation and the purity of these biomarkers, we developed a model based on the Stokes equation (*Chapter 2*). Based on this model, we show that tdEVs, obtained by centrifugation, are co-isolated with other biomarkers, like tumor-educated platelets (15). This means caution is needed with the interpretation of results because these results may originate from another biomarker than the one under investigation. This developed model can be a new tool to help to (re)design centrifugation protocols to establish whether available candidate protocols differ sufficiently from each other to warrant comparison, to increase the purity of biomarkers, and to simplify the centrifugation protocol.

Based on the same Stokes model, we developed a new protocol to prepare platelet-free plasma (*Chapter 3*). This centrifugation protocol is as effective as the current standard protocol (16) to obtain platelet-free plasma from blood, but the protocol is easier, less time consuming and less laborious. Furthermore, with the new protocol a higher plasma yield is obtained with an EV concentration comparable to the old protocol. Since the new protocol includes one centrifugation cycle instead of two cycles, the potential for pipetting errors is minimized.

Because of the overwhelming concentrations of non-EV particles and EVs from other cell origin in blood, a purified tdEV sample is desirable for downstream analysis. Rate zonal centrifugation is a new technique introduced in this thesis to separate EVs from platelets. We show separation of platelets from EVs based on size by RZC is possible (*Chapter 4*). The lipoproteins and soluble proteins will not travel into the gradient, and thus will also be markedly reduced in the EV sample compared to the sample before RZC.

A validation tool is needed to ensure the purity and quality of an EV sample and to standardize EV detection techniques. One of the recommended techniques that is currently recommended by the EV community is transmission electron microscopy (TEM) (17). However, we show that the results obtained by TEM are hampered by the influence of the operator, who selects regions of interest for imaging (*Chapter 5*). This selection causes a subjective assessment of the purity and quality of EV samples. Therefore, TEM images based on operator selection can only be used to confirm the presence of EVs in a sample and not to ensure the purity and quality of an EV sample.

Based on our results, we recommend to obtain images at predefined locations to improve the comparability and reproducibility of TEM results. Images taken on predefined locations reflect the real quality and purity of an EV-containing sample rather than the images taken by operator selection, which merely demonstrate the presence of EVs (18). Thus, to further reduce the influence of the operator or protocol, a consensus protocol should be developed and automation of the assessment of EV samples should be explored.

The challenge is the concentration and size of tdEVs

One approach to differentiate tdEVs from EVs originating from other cells is by antigen expression, which reveals the cellular origin of EVs. This was applied in *Chapter 6*, where we used antigen-based methods, flow cytometry (FCM) and surface plasmon resonance imaging (SPRi). We evaluated whether tdEVs can be detected in plasma and/or urine from prostate cancer patients, and whether these tdEVs can be used to discriminate between prostate cancer patients and healthy controls. However, we were unable to detect tdEVs directly in the patient samples. Because in both FCM and

SPRi we measured plasma volumes $< 0.1 \mu\text{L}$, it was impossible to detect the few tdEVs present in the plasma sample.

The concentration of tdEVs relative to non-tdEVs and non-EV particles, results in long measurement times needed to obtain a representative sample of tdEVs, for example 1,000 tdEVs. Table D.1. illustrates the main challenge of tdEV enumeration. TEM, FCM and SPRi are able to detect EVs, discriminate between tdEVs and non-EV particles and EVs from other origin, and were applied in this thesis. The typical measured volume in one hour is shown. Note that we used the currently used protocols at Amsterdam UMC/University of Twente. Thus, the volume measured in one hour may be improved considerably with automation and other throughput improvements, but these gains will not come easily. We also took into account the pre-dilutions needed to perform these techniques. For example, before TEM imaging of plasma EVs, size exclusion chromatography is performed to remove soluble proteins, and size exclusion chromatography dilutes the sample (19). For FCM, plasma needs to be pre-diluted to prevent swarm detection (20). Plasma is also pre-diluted before detection of EVs by SPRi to reduce viscosity. To be clinically applicable, the time needed to detect 1,000 tdEVs should certainly be less than a day. This table demonstrates that enrichment or concentration will be needed before the detection of tdEVs in plasma is possible within reasonable time. Speeding up the measurement time only to obtain a result in one day requires a 70 times reduction in measurement time in the best case scenario of FCM.

Table D.1. Measured sample volumes after one hour for three EV detection techniques.

Technique	mL/h*	Pre-dilution	tdEVs/h**	Hours for 1,000 tdEVs
TEM	$1,2^{-6}$	2	0.006	172,176
FCM	$1,8^{-1}$	1,000	1.800	556
SPRi	$4,0^{-5}$	5	0.080	12,500

* TEM: one minute per $2.2 \times 2.2 \mu\text{m}^2$ image, assuming 100% capture from the sample, FCM: Apogee A60, SPRi: IBIS MX96 (21) ** Assuming concentration of 10,000/mL

Outlook

Compared to earlier studies, we have moved multiple steps forward. In this thesis we explored several ways to improve pre-analytical procedures. For example, platelets can

be co-isolated with EVs during centrifugation and we introduce a technique which can be used to separate platelets from EVs.

There are many uncertainties in our knowledge of the particles in plasma in the 100-5,000 nm size regime. To estimate the sensitivity needed to detect tdEVs, the size and concentration distribution of all 100-5,000 nm particles present in blood is needed. Additionally, the number of antigens per EV is poorly understood. Extrapolation from cell antigen concentration suggests that 100 nm EV will need to be distinguished based on a single antigen (22). A possible approach may be superresolution microscopy using single molecule sensitive detectors with samples embedded in an agarose matrix. This requires non-bleaching fluorescent dyes (no PE, FITC, APC), and thus will require optimization of staining protocols. To its advantage, no major dilutions are needed, and the measured volume can be accurately determined. Nevertheless, the sample volume that can be measured in one hour is $3,5 \times 10^{-3}$ mL, so also this technique will not become clinically applicable without enrichment.

The complexity of blood and the size and concentration of EVs leads to the measurement times shown in Table D.1.. From Table 1.1., the necessity of enriching biological samples for tdEVs is evident (*Chapter 1*). Enrichment can be performed based on size, density, or antigens. Size and density based methods may remove non-EV particles, but are probably ineffective against EVs other than tdEVs. Targeted depletion is probably difficult because of the wide variety of non-tdEVs. Antigen-based enrichment has been used in this thesis. For example epithelial cell adhesion molecule (EpCAM) based immunomagnetic enrichment has been applied for the isolation of large tdEVs ($> 1 \mu\text{m}$) from whole blood of metastatic cancer patients. These tdEVs were strongly associated with poor clinical outcome (1).

As mentioned previously, there may be very few antigens per EV in the sizes around 100 nm. Whether a cocktail of antibodies is needed (including for example Her2 and PSMA) to recover these smaller EVs, and whether the immunomagnetic enrichment will still work for this small EV size remains to be investigated.

The priority of future research should be on tdEV enrichment to decrease the background of non-EV particles and EVs from other cellular origin present in

blood. However, enrichment is not perfect, and will co-isolate contaminants. The use of a combination of different detectable parameters, like antigen expression, diameter, refractive index (23), Raman spectrum, elastic modulus at a rate of 10^6 EV/hour or faster likely will be needed. Alternatively, techniques that do not rely on the enumeration of EVs may be investigated, for example molecular biology on the (enriched) bulk sample. This will be essential for success of a biomarker based on a 'rare' EV subpopulation.

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Summary.

Summary

Prostate cancer diagnostics can be unreliable and/or invasive. Therefore, there is an urgent need for a robust and low-invasive assay to improve cancer diagnostics and to monitor therapy effectiveness over time. Measuring cells from the tumor that are circulating in the blood have prognostic value, but the low concentration of these circulating tumor cells (CTCs) limits the detection and their application as a cancer biomarker.

Compared to the low concentration of CTCs in blood from cancer patients, tumor-derived extracellular vesicles (tdEVs) are expected to be present in a higher concentration. Therefore, tdEVs may improve the accuracy for prostate cancer screening. However, tdEVs in blood are outnumbered by non-EV particles such as lipoproteins which overlap in size and density, platelets, and by EVs originating from other cell types. Furthermore, EVs are small in size and are heterogeneous in morphology, composition and refractive index. Therefore, moving down in size from CTCs to tdEVs reveals new challenges.

The aim of this thesis is to (i) evaluate detection techniques used to detect and characterize tdEVs, and (ii) investigate tdEVs as a biomarker for cancer.

Techniques used in the (td)EV field were evaluated and optimized, or new techniques were developed to distinguish tdEVs from non-EV particles and EVs from different cellular origin. Optical, biochemical and mechanical properties of EVs were obtained. *Chapter 1* describes the relevance of each technique for the EV field, development of new techniques, and new insights that were obtained that are relevant to implement measuring tdEVs in the clinic.

Standardization of pre-analytical procedures is an important step towards the detection of rare EVs, including tdEVs. One of these pre-analytical procedures is the isolation of biomarkers from biological samples. To assess the purity of biomarkers after centrifugation, a technique often used for the isolation or concentration of biomarkers from blood, we applied a model based on the Stokes equation in *Chapter 2*. We found that biomarkers are often co-isolated with other biomarkers, e.g. the co-

isolation of “tumor-educated platelets” with EVs. Therefore, a next step is to enhance the purity of the biomarker of interest and reduce the influence of other co-isolated biomarkers, to improve the interpretation of data.

We used the same model to develop an easy and fast centrifugation protocol to prepare essentially platelet-free plasma as a starting material for EV analysis. The one-step centrifugation protocol described in *Chapter 3* can replace the now commonly used but laborious, time-consuming double centrifugation protocol. Platelet removal by the new protocol is as effective as the old protocol, and results in a higher plasma yield whereas the EV recovery is comparable. For future research on plasma EVs, the new one-step protocol for preparation of essentially platelet-free plasma is recommended for research on EVs and other plasma biomarkers.

To enhance the purity of biomarkers, in *Chapter 4* we used rate zonal centrifugation (RZC) to separate platelets from EVs based on diameter. Platelets and platelet-derived EVs in platelet rich plasma were successfully separated by RZC and therefore RZC is a step forward towards enhanced purity of biomarkers for downstream analysis of for example the EV content.

A validation tool to objectively ensure the quality and purity of an EV-containing sample is lacking. In *Chapter 5* we evaluate transmission electron microscopy (TEM) as a validation tool. We show that operator selection, the current standard within the EV field, does not provide an objective description of the EV-containing sample and can only be used to confirm the presence of EVs in a sample, but not to confirm the quality and purity of a sample. A first step to improve the comparability and reproducibility of TEM for monitoring the quality and purity of EV-containing samples is to obtain images at predefined locations. These images reflect the overall quality of the EV-containing sample rather than the presence of EVs alone.

Because we expected tdEVs to be present in a higher concentration than CTCs, we hypothesized that the detection of tdEVs directly in clinical samples such as plasma or urine, without the use of enrichment techniques, would be possible. As a proof of principle, in *Chapter 6* we used flow cytometry (FCM) and surface plasmon resonance imaging (SPRi) for comparison to evaluate whether tdEVs can be detected in plasma

and/or urine from prostate cancer patients, and whether based on this analysis patients can be discriminated from healthy controls. We were unable to detect tdEVs in the patient samples. We speculate this is due to the measured sample volume is too small to detect tdEVs. The expected tdEV concentration is 1.5-15/ μ L in plasma. We measured plasma volumes $< 0.1 \mu$ L. Therefore, it was impossible to detect the few tdEVs present in the plasma sample.

The complexity of blood remains a challenge for tdEV detection. EV detection techniques need to be able to detect, identify and enumerate 10^4 tdEVs amongst 10^{16} other particles in 1 mL plasma. State-of-the-art integrated systems developed in the Cancer-ID Perspectief program come close to reliably detecting tdEVs at clinically relevant concentrations at high throughput. However, future research needs to focus on (i) tdEV enrichment and/or concentration prior to analysis to decrease the background of non-EV particles and EVs from other cellular origin present in the blood, and (ii) the use of a combination of parameters, like antigen expression combined with the refractive index, or (iii) a combination of techniques to increase the certainty that a tdEV is evaluated and to reveal multiple unique characteristics of tdEVs.

To summarize, this thesis provides insights into the (i) complexity of EV samples, (ii) the status of current state-of-the-art EV isolation and detection techniques, and (iii) how to overcome some of the limitations. These insights pave the road towards where the state-of-art techniques should be in order to realize the use of tdEVs for cancer diagnostics and to guide therapy of cancer patients.

Samenvatting.

Samenvatting

Het stellen van een diagnose voor prostaatkanker is niet altijd betrouwbaar en wordt vaak uitgevoerd op een invasieve manier. Daarom is behoefte aan een robuuste en minimaal-invasieve test om de diagnostiek van prostaatkanker te verbeteren en om de effectiviteit van therapie te volgen. Het meten van circulerende tumorcellen in het bloed van prostaatkankerpatiënten heeft een prognostische waarde voor het verloop van de ziekte, maar omdat deze cellen slechts in een laag aantal aanwezig zijn, is het meten van deze tumorcellen als “biomarker” voor de diagnose prostaatkanker gering.

De verwachting is dat van tumorcellen afkomstige extracellulaire blaasjes, tumorcelblaasjes, in een hogere concentratie aanwezig zullen zijn dan de circulerende tumorcellen. Het meten van de concentratie tumorcelblaasjes kan dus de nauwkeurigheid en dus ook de betrouwbaarheid voor prostaatkankerscreening verbeteren.

Bij het meten van tumorcelblaasjes in bloed doen zich drie problemen voor. In het algemeen zijn alle celblaasjes moeilijk te meten omdat ze klein zijn, onderling sterk verschillen in uiterlijk en samenstelling, en omdat ze weinig licht verstrooien. Daar komt bij dat de concentratie tumorcelblaasjes in het bloed laag is ten opzichte van de concentratie celblaasjes die afkomstig zijn van andere cellen, en zijn andere deeltjes aanwezig zoals lipoproteïnen en cellen zoals bloedplaatjes, die in grootte en dichtheid overlappen met celblaasjes.

Het doel van dit proefschrift is om meetmethoden te evalueren die worden gebruikt om tumorcelblaasjes te detecteren en te karakteriseren, en om te onderzoeken of tumorcelblaasjes kunnen worden gebruikt als biomarker voor prostaatkanker.

De meetmethoden die momenteel worden gebruikt om (tumor)celblaasjes te onderzoeken zijn geëvalueerd en geoptimaliseerd. Tevens zijn nieuwe technieken ontwikkeld om tumorcelblaasjes te kunnen onderscheiden van deeltjes die geen celblaasjes zijn. Door dit onderzoek is inzicht verkregen in de optische, biochemische en mechanische eigenschappen van celblaasjes. *Hoofdstuk 1* geeft een overzicht van de relevantie van elke meetmethode voor het onderzoeksveld naar celblaasjes, de nieuwe

meetmethoden die zijn ontwikkeld, en nieuwe inzichten die relevant kunnen zijn voor het implementeren van het meten van tumorcelblaasjes in de kliniek.

Het standaardiseren van pre-analytische methoden is een belangrijke stap om betrouwbaar zeldzame typen celblaasjes, zoals tumorcelblaasjes, beter te kunnen meten. Een veelvuldig gebruikte pre-analytische methode is centrifugeren. Centrifugeren wordt gebruikt om biomarkers uit lichaamsvloeistoffen te isoleren of te concentreren. Om de zuiverheid van biomarkers na centrifugeren te beoordelen, passen we in *Hoofdstuk 2* een model toe dat is gebaseerd op de Stokes-vergelijking. De resultaten van dit model tonen aan dat biomarkers die zijn geïsoleerd of geconcentreerd met behulp van centrifugeren vaak verontreinigd zijn met andere biomarkers. Een voorbeeld hiervan is het samen isoleren van tumoropgeleide bloedplaatjes en celblaasjes, twee bekende biomarkers voor kankeronderzoek, uit bloed. Dus, door de zuiverheid van biomarkers te verbeteren, bijvoorbeeld door aangepaste protocollen voor centrifugeren, komen betere en betrouwbaardere resultaten binnen handbereik.

Hetzelfde Stokes-model is gebruikt om een eenvoudig en snel protocol te ontwikkelen om bloedplaatjesvrij plasma te bereiden uit bloed als startmateriaal voor onderzoek aan celblaasjes. Het in *Hoofdstuk 3* beschreven protocol is een verbetering ten opzichte van het huidige protocol, dat omslachtig en tijdrovend is. Beide protocollen zijn even efficiënt in het verwijderen van bloedplaatjes, maar het nieuwe protocol heeft een betere plasma opbrengst zonder de concentraties celblaasjes negatief te beïnvloeden. Voor toekomstig onderzoek naar celblaasjes in bloed bevelen wij dan ook het nieuwe protocol aan om betrouwbaar en snel bloedplaatjesvrij plasma te maken.

Om de zuiverheid van biomarkers te verbeteren, hebben we in *Hoofdstuk 4* scheiding op basis van “zonale sedimentatie” toegepast om bloedplaatjes te scheiden van celblaasjes op basis van hun verschil in diameter. Door deze techniek toe te passen, kunnen biomarkers zoals celblaasjes worden geïsoleerd met minder verontreinigingen, waardoor de betrouwbaarheid van resultaten zal verbeteren, denk hierbij bijvoorbeeld aan het analyseren van de samenstelling van celblaasjes.

Momenteel is er geen manier om de kwaliteit en de zuiverheid objectief te beoordelen van een preparaat dat celblaasjes bevat. In *Hoofdstuk 5* is onderzocht of transmissie-

elektronenmicroscopie (TEM) geschikt is als mogelijke instrument om de kwaliteit en zuiverheid te onderzoeken. In dit hoofdstuk wordt aangetoond dat het selecteren van beelden door de gebruiker een ander beeld geeft ten opzichte van beelden gemaakt op vooraf bepaalde locaties. Door zelf te selecteren ontstaat geen objectief beeld van het preparaat en deze manier van selecteren kan alleen worden gebruikt om de aanwezigheid van celblaasjes aan te tonen. Omdat deze methode momenteel de huidige standaard is binnen het onderzoeksveld aan celblaasjes, is deze methode dus ongeschikt om de kwaliteit en zuiverheid van het preparaat te bepalen. Een eerste stap om de vergelijkbaarheid en reproduceerbaarheid van TEM beelden te verbeteren, is dan ook het nemen van beelden op vooraf bepaalde locaties. Deze beelden weerspiegelen de kwaliteit van het preparaat aantoonbaar beter dan beelden die zijn genomen door gebruikers.

Als tumorcelblaasjes daadwerkelijk aanwezig zijn in een hogere concentratie dan circulerende tumorcellen, dan kunnen tumorcelblaasjes mogelijk direct in lichaamsvloeistoffen zoals plasma of urine worden gedetecteerd zonder het gebruik van isolatie- en/of concentratiemethoden. Om de proef op de som te nemen, zijn flowcytometrie (FCM) en oppervlakte-plasmonresonantiebeeldvorming (SPRi) gebruikt om te onderzoeken of tumorcelblaasjes daadwerkelijk rechtstreeks kunnen worden gemeten in plasma en/of urine van prostaatkankerpatiënten, en of op basis van deze meting patiënten en gezonde controles kunnen worden onderscheiden. Echter, zowel met FCM als met SPRi werden geen tumorcelblaasjes gedetecteerd, waarschijnlijk omdat het daadwerkelijk gemeten volume plasma of urine onvoldoende is om tumorcelblaasjes te kunnen meten. De verwachte concentratie tumorcelblaasjes is ongeveer 1,5-15 per microliter plasma, maar het gemeten plasmavolume voor bijvoorbeeld flowcytometrie was minder dan 0,1 microliter.

De complexiteit van bloed is een uitdaging om tumorcelblaasjes te meten. De toegepaste meetmethoden moeten in staat zijn om 10^4 tumorcelblaasjes te identificeren en te tellen in aanwezigheid van 10^{16} andere deeltjes in 1 milliliter plasma. Moderne en nieuw ontwikkelde meetmethoden, zoals ontwikkeld in het Cancer-ID Perspectief-programma, komen wel in de buurt.

Toekomstig onderzoek zal zich moeten richten op het verrijken van tumorcelblaasjes vóór de meting om de achtergrond bestaande uit deeltjes die geen celblaasjes zijn en andere typen celblaasjes, te verminderen. Daarnaast verdient het aanbeveling om verschillende gemeten eigenschappen van individuele deeltjes, zoals antigeenexpressie en brekingsindex, te combineren. Tenslotte zal het gebruik van meerdere technieken de betrouwbaarheid vergroten, waardoor mogelijk ook nieuwe en unieke kenmerken van tumorcelblaasjes kunnen worden onthuld.

Dit proefschrift geeft inzicht in (i) de complexiteit van de samenstelling lichaamsvloeistoffen die celblaasjes bevatten, (ii) de toepasbaarheid en tekortkomingen van de huidige methoden die worden gebruikt om celblaasjes uit lichaamsvloeistoffen te isoleren en vervolgens te detecteren, en (iii) hoe een aantal van deze tekortkomingen kunnen worden overwonnen. Deze inzichten laten zien wat methoden moeten bereiken om het meten van tumorcelblaasjes daadwerkelijk zinvol te maken voor nieuwe kankerdiagnostiek.

List of publications.

List of publications

Journal Publications

Staining specificity of extracellular vesicles by flow cytometry using refractive index

Leonie de Rond, Sten F.W.M. Libregts, **Linda G. Rikkert**, Chi M. Hau, Edwin van der Pol, Rienk Nieuwland, Ton G. van Leeuwen, and Frank A.W. Coumans.
Journal of Extracellular Vesicles, 2019.

Response to letter to the editor

Linda G. Rikkert, Rienk Nieuwland, Leon W.M.M. Terstappen, and Frank A.W. Coumans.
Journal of Extracellular Vesicles, 2019.

Centrifugation affects the purity of liquid biopsy-based tumor biomarkers

Linda G. Rikkert, Edwin van der Pol, Ton. G. van Leeuwen, Rienk Nieuwland, and Frank A.W. Coumans.
Cytometry Part A, 2018.

Quality of extracellular vesicle images by transmission electron microscopy is operator and protocol dependent

Linda G. Rikkert, Rienk Nieuwland, Leon W.M.M. Terstappen, and Frank A.W. Coumans.
Journal of Extracellular Vesicles, 2018.

Label-free prostate cancer detection by characterization of extracellular vesicles using raman spectroscopy

Wooje Lee, Afroditi Nanou*, **Linda G. Rikkert***, Frank A.W. Coumans, Cees Otto, Leon W.M.M. Terstappen, and Herman L. Offerhaus
Analytical Chemistry, 2018,

The histophysiology and pathophysiology of the peritoneum

Juliëtte O.A.M. van Baal, Koen K. van de Vijver, Rienk Nieuwland, Ron C.J.F. van Noorden, Willemien J. van Driel, Auguste Sturk, Gemma G. Kenter, **Linda G. Rikkert**, and Christianne A.R. Lok.
Tissue and Cell, 2015.

*authors contributed equally

Conference contributions

Oral presentations

Extracellular vesicles as a liquid biopsy for cancer patients

5th Cancer-ID General Assembly Meeting, April 12 2019, Rotterdam Netherlands

Liquid biopsy based tumor biomarkers

4th Cancer-ID General Assembly Meeting, April 9 2018, Groningen Netherlands

Characterization of tumor-derived extracellular vesicles

3rd Cancer-ID General Assembly Meeting, March 27 2017, Amsterdam Netherlands

Strategies to identify rare extracellular vesicles in human body fluids (invited)

Gordon Research Conference Biologic Effects and Therapeutic Potential of Extracellular Vesicles, August 24 2016, Newry Maine

Cancer-ID data analysis

2nd Cancer-ID General Assembly Meeting, April 4 2016, Rotterdam Netherlands

Strategies to identify rare extracellular vesicles in human body fluids

ISEV, May 4-7 2016, Rotterdam Netherlands

Posters

Centrifugation affects the purity of liquid biopsy-based tumor biomarkers

Linda G. Rikkert, Edwin van der Pol, Ton G. van Leeuwen, Rienk Nieuwland, Frank A.W. Coumans

ISEV, April 24-28 2019, Kyoto Japan

Centrifugation affects the purity of liquid biopsy-based tumor biomarkers

Linda G. Rikkert, Edwin van der Pol, Ton G. van Leeuwen, Rienk Nieuwland, Frank A.W. Coumans

NL-SEV, November 3 2018, Amsterdam Netherlands

Strategies to identify rare extracellular vesicles in human body fluids

Linda G. Rikkert, Frank A.W. Coumans, Rienk Nieuwland, Leon W.M.M. Terstappen

ISEV2018, May 2-6 2018, Barcelona Spain.

Characterization of Extracellular Vesicles using Raman Spectroscopy for Label-free Cancer Detection

Wooje Lee, Afroditi Nanou, **Linda G. Rikkert**, Frank A.W. Coumans, Cees Otto, Leon W.M.M. Terstappen, Herman L. Offerhaus

ISEV2018, May 2-6 2018, Barcelona Spain

Characterization of extracellular vesicles by transmission electron microscopy: comparison of negative staining protocols

Linda G. Rikkert, Rienk Nieuwland, Leon W.M.M. Terstappen, Frank A.W. Coumans

NL-SEV, November 17 2017, Utrecht Netherlands

Characterization of extracellular vesicles by transmission electron microscopy **Linda G.**

Rikkert, Leon W.M.M. Terstappen, Rienk Nieuwland, Frank A.W. Coumans

DutchBiophysics, October 2-3 2017, Veldhoven Netherlands

Cancer-ID: Strategies to identify rare extracellular vesicles in human body fluids

Linda G. Rikkert, Frank A.W. Coumans, Leonie de Rond, Rienk Nieuwland, Leon W.M.M. Terstappen

Amsterdam Cancer Center Retreat, January 13-14 2017, Noordwijkerhout Netherlands

A model to study the role of extracellular vesicles in the interaction between ovarian tumor cells and the peritoneum

Linda G. Rikkert, Anita N. Böing, Frank A.W. Coumans, Koen K. van de Vijver, Jan Stap, Gemma G. Kenter, Rienk Nieuwland, Christianne A.R. Lok

ISEV, May 4-7 2016, Rotterdam Netherlands

Supervising

- M.Sc. Literature Study of L. Holland on 'miRNA content of extracellular vesicles: what goes wrong?', 2016.
- B.Sc. project of I. Jak on 'Concentration of extracellular vesicles by electrophoresis', 2017.
- B.Sc. project of R. Haring on "Stable and reproducible trans epithelial electrical resistance measurements", 2018.
- B.Sc. project of M. Engelaer on "Rate zonal centrifugation of extracellular vesicles", 2018.

Curriculum Vitae.

Curriculum Vitae

Linda Rikkert was born in Wierden on August 18, 1991. After finishing the Athenaeum at the O.S.G. Erasmus in 2009, she studied Life, Science and Technology at the University of Groningen. In 2013 she obtained a Bachelor of Science with a Major in Biomedical Sciences. Then she moved to Amsterdam, to study Oncology. In 2014, she did an internship at the Laboratory of Experimental Clinical Chemistry and Vesicle Observation Center of the Amsterdam UMC, University where she developed a 3D culture model to study metastasis of ovarian cancer. In 2015, she did an internship at the Sickkids hospital, Toronto, Canada, where she studied the development of mixed chimerism after radiation and blood transplantation in leukemia patients. In 2015 she obtained her Master of Science degree in Oncology at the VU University. In October 2015, she started her PhD as part of the Cancer-ID program (www.utwente.nl/tnw/cancer-id) at the Department of Medical Cell BioPhysics of the University of Twente, Enschede, and at the Laboratory of Experimental Clinical Chemistry and Vesicle Observation Center. She was involved in coordination of the program, improvement of the isolation and detection of tumor-derived extracellular vesicles in clinical samples.

Dankwoord.

"Ik ben mij er eentje"

- Herman Finkers -

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