Synchronized Rayleigh and Raman scattering for the characterization of single optically trapped extracellular vesicles

Agustin Enciso-Martinez, Ir. a, Edwin van der Pol, Dr. b, c, d, Aufried T.M. Lenferink, Ing. a, Leon W.M.M. Terstappen, Prof. Dr. a, Ton G. van Leeuwen, Prof. Dr. b, d, Cees Otto, Dr. a,⁎

a Department of Medical Cell BioPhysics, TechMed Centre, University of Twente, Enschede, The Netherlands
b Department of Biomedical Engineering and Physics, Amsterdam University Medical Centers, Location AMC, University of Amsterdam, The Netherlands
c Laboratory Experimental Clinical Chemistry, Amsterdam University Medical Centers, Location AMC, University of Amsterdam, The Netherlands
d Vesicle Observation Center, Amsterdam University Medical Centers, Location AMC, University of Amsterdam, The Netherlands

Revised 3 October 2019

Abstract

Extracellular Vesicles (EVs) can be used as biomarkers in diseases like cancer, as their lineage of origin and molecular composition depend on the presence of cancer cells. Recognition of tumor-derived EVs (tdEVs) from other particles and EVs in body fluids requires characterization of single EVs to exploit their biomarker potential. We present here a new method based on synchronized Rayleigh and Raman light scattering from a single laser beam, which optically traps single EVs. Rapidly measured sequences of the Rayleigh scattering amplitude show precisely when an individual EV is trapped and the synchronously acquired Raman spectrum labels every time interval with chemical information. Raman spectra of many single EVs can thus be acquired with great fidelity in an automated manner by blocking the laser beam at regular time intervals. This new method enables single EV characterization from fluids at the single particle level.

© 2020 . Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Key words: Extracellular vesicles; Exosomes; Raman spectroscopy; Single particle analysis; Nanomedicine; Nanoparticles

Recent studies have shown that small lipid-membrane-bound vesicles called Extracellular Vesicles (EVs) derived from cancer cells play a role in tumor cell proliferation, migration, invasion, and metastasis, and the peripheral blood load of relatively large tumor-derived EVs is strongly associated with poor clinical outcome. The EVs, which may be as small as 50 nm, are able to transport proteins, nucleic acids and membrane components to neighboring or distant cells, influencing cellular behaviors. Hence, EVs derived from diseased cells, such as cancer cells, could trigger oncogenic signals and promote tumor development. Tumor-derived extracellular vesicles (tdEVs) can be present in body fluids, such as blood. However, their identification and characterization are challenging due to their heterogeneity, ultra-small size, low refractive index, size overlap with physiological EVs and contaminants in body fluids, and the lack of knowledge on their chemical composition. Specially, their broad size range overlaps not only with non-tdEVs, but also with lipoproteins and protein aggregates that fall in the EV size range. Thus, the ensemble average inherent to bulk analysis methods requires a sensitivity which techniques like ELISA or western blot unlikely have. Single particle analysis may overcome this problem and pave the road towards identification of rare tdEV populations.

There are well established methods for single particle analysis, such as nanoparticle tracking analysis (NTA), resistive pulse sensing and flow cytometry that together can provide the size, number, concentration, electrophoretic mobility and refractive index of EVs. However, these methods fail in providing information on the chemical composition of EVs, which is essential to identify their origin. In recent years, Raman spectroscopy (RS) has been used to contribute chemical information of EVs and tdEVs. RS is a label-free chemical
characterization technique based on the inelastic scattering of light by molecules. RS combined with optical tweezers, provides Raman spectra of particles trapped in the focal spot. Pioneered by Ashkin, optical tweezers use a diffraction limited beam to stably trap a particle in three dimensions. Particles in Brownian motion that enter the trapping focal spot experience a net force that brings them to the axial center of the laser beam waist slightly displaced from the focal plane.

Previous studies demonstrate trapping of single solid particles in the size range of EVs, such as silica beads, polystyrene beads and viruses. The trapping of single particles has been complemented with characterization techniques such as fluorescence spectroscopy and Raman spectroscopy. Although EVs have also been studied with Raman, single EV Raman measurements have never been experimentally confirmed. Indirect evidence has been used in some studies to presume single EV or liposome trapping and chemical characterization. However, the unequivocal proof that a trapping event involves a single EV and the ability to trap EVs at sufficiently high rates has not yet been shown.

Here, we propose a new method to optically trap single EVs and simultaneously disclose their inherent chemical composition by means of RS. The method distinguishes individual particle trapping events by the detected Rayleigh scattered light while synchronously measured Raman spectra enable the chemical characterization of individual EVs. Rayleigh scattering and Raman scattering are, respectively, elastic and inelastic scattering of light by particles in a medium. Further, our method involves a purge of the optical trap at regular time intervals. Hence, long time series of Rayleigh–Raman scattering events can be acquired starting each time from an empty trap. In this way, many individual trapping events can be acquired in an automated manner. Our method can be directly applied to diluted EV samples and does not require any EV isolation steps. The Raman spectra of multiple individual EVs enable a chemical fingerprint of the sample. We introduce and validate the method with polystyrene (PS) and silica (Si) beads. Subsequently, we show that synchronous Rayleigh–Raman scattering enables the identification of single EVs on the basis of the Raman spectral signature, which is related to their molecular composition.

Methods

PC3-derived EVs

PC3 cells provided by the American Type Culture Collection (ATCC) were used as a model to produce prostate cancer-derived EVs. Cells were cultured at 37 °C and 5% CO2 in RPMI-1640 with L-glutamine medium (Lonza, Cat. No.: BE12-702F) supplemented with 10% (v/v) fetal bovine serum (FBS), 10 U/mL penicillin and 10 μg/mL streptomycin. The initial cell density was 10,000 cells/cm² as recommended by the ATCC. Medium was refreshed every second day. At 80 to 90% confluence, cells were washed three times with phosphate buffer solution (PBS) and cultured in FBS-free RPMI-1640 with L-glutamine medium (Lonza, Cat. No.: BE12-702F) supplemented with 1 U/mL penicillin and 1 μg/mL streptomycin. After 2-3 days of cell culture, cell supernatant was collected in a 15 mL tube (Cellstar® tubes, Greiner Bio-one) and centrifuged at 800 xg at room temperature for 10 min (Centrifuge 5804, Eppendorf). The pellet containing dead or apoptotic cells and large cell fragments was discarded. The collected supernatant containing PC3-derived EVs was stored in aliquots of 500 μL (Micro tube 0.5 mL, PP, SARSTEDT) at −80 °C. Samples were thawed in a water bath at 37 °C before use. EV size distribution was determined using NTA (NanoSight NS500). An EV concentration of 1.9×10⁸ particles/mL with a mean diameter (± standard deviation) of 157 (± 79) nm was found (Figure S1).

Polystyrene (Ps) and silica (Si) beads

To validate our technology, we used NIST traceable polystyrene (PS) beads (Nanosphere; Thermo Fisher, Waltham, MA, USA) with mean diameters of 100, 125, 147, 296 and 799 nm and plain silica (Si) beads (Silica Kisker, Steinfurt, Germany) with mean diameters of 182, 315, 405 and 548 nm. The concentration was determined by NTA (NS500, Malvern, UK). Diameters of the silica beads were obtained by TEM. The corresponding refractive indices of PS and Si beads are 1.586 and 1.464, respectively. The suspensions of beads were diluted in MilliQ to a concentration of 3×10⁸-3×10⁹ particles/mL to optimize the single particle trapping rate. PS and Si particle suspensions were vortexed for 3 s and re-suspended 3 times before experiments to prevent particle aggregation that may have occurred during storage. A suspension of 50 μL of each monodisperse sample of beads was then used for synchronous Rayleigh and Raman scattering measurements.

Sample preparation

PS beads, Si beads, and EVs derived from PC3 cells were used in a concentration of 3.6×10⁸-5×10¹⁰ particles/mL and diluted in MilliQ (beads) or PBS (EVs) to this concentration if necessary. A 50 μL suspension of each particle type was placed on a well glass slide (BMS Microscopes; 1.0-1.2 mm thick, Cat. no.: 12290). The sample was covered with a glass cover slip (VWR Ltd, thickness No. 1, diameter: 22 mm, Cat. no.: 631-0158) and sealed with glue (EVO-STIK, Impact) onto the well glass slide (Figure 1, A). The glass was then cured at room temperature for ~30 min. The closed well glass slide was placed on the microscope table under the objective of the Rayleigh–Raman spectrometer.

Optical setup and measurements

The Rayleigh–Raman spectrometer is based on a home-built Raman spectrometer integrated with the base of an upright optical microscope (Olympus BX41). A single laser beam from a Coherent Innova 70C laser (λexc=647.089 nm) was used for optical trapping and illuminating beads and EVs. Rayleigh and Raman scattering was collected with a cover glass corrected dry objective (Olympus, 40x, NA: 0.95). Rayleigh and Raman scattering was separated in a homebuilt spectrometer and simultaneously detected with a single CCD camera. The spectrometer had an average dispersion of ~2.3 cm⁻¹ (0.11 nm) wavenumber per pixel over the CCD camera surface with 1600 pixels along the dispersive axis and 200 pixels perpendicular to this axis. The spectral resolution was ~3.0 cm⁻¹. The laser
power was measured underneath the objective and adjusted to 70 mW. By moving the objective along the z-axis, the laser focal spot is focused inside the solution, ~37 μm below the cover slip. The focal spot has a theoretical diameter of 370 nm and a Rayleigh range of 1160 nm based on full width half maximum. Rayleigh and Raman scattering spectra were typically acquired 256 times with an acquisition time of 38 ms over a period of ~9.7 s. The trapped particles were then released from the optical trap by blocking the laser beam with a shutter for ~1 s. This measurement cycle was repeated, typically, 100 times, which resulted in 100×256 = 25,600 Rayleigh–Raman spectra per measurement.

Backscattering calculations

Light scattering calculations based on Mie theory were used to relate the measured backscattering to the diameter and refractive index of PS and Si beads. The Rayleigh scattering intensity was compared with Mie scattering calculations of the intensity averaged over the collection angle of the microscope objective as a function of particle diameter. From these measurements it was theoretically predicted that with the present embodiment of the setup the Rayleigh light scattering signal was sufficiently intense to detect trapping of individual EVs, in spite of the small refractive index difference of EVs ($n=1.37$) from water ($n=1.33$). We use the Mie scripts of Mätzler in MATLAB (2018b, MathWorks, USA) to calculate the amplitude scattering matrix elements, which describe the relation between the incident and scattered field amplitudes of a sphere. Our model incorporates particle diameter and refractive index (RI), RI of the medium, the illumination wavelength, and the numerical aperture of the microscope.

Results

Brownian motion of EVs in suspension occasionally brings the EVs sufficiently close to the trap to experience a net trapping force directing towards the focal spot. Once an EV is trapped, an intense increase in the amplitude of the Rayleigh light scattering occurs, and this can be seen as a discrete step in a time trace (Figure 1, B). Such time trace results from the integration of the Rayleigh band over 50 pixels around 0 cm$^{-1}$ plotted over time. As long as the shutter to the laser remains open, successive trapping and accumulation of particles occur until the focal volume becomes saturated with particles. Hence, monitoring the time trace over prolonged time enables to distinguish between zero (baseline), single (first step) and multiple trapping events, which can be enumerated (Figure 1, B and C). Synchronous acquisition of Raman spectra with the Rayleigh scattering allows us to assign a Raman spectrum to individual particles trapped (Figure 1, D). Purging the laser focus from trapped particles at
regular time intervals enables the acquisition of many single EV trapping events in an automated manner over long periods of time, thus monitoring the composition of a suspension of EVs on the level of individual, single EVs with fidelity. Rayleigh and Raman scattering spectra were typically acquired 256 times with an acquisition time of 38 ms over a total period of ~9.7 s per cycle. After purging the laser focus a new cycle started.

To validate our technology, single PS and Si beads of various sizes (SI) were optically trapped and synchronous Raman acquisition was performed. To increase the event throughput of our Rayleigh–Raman setup we have automated the trapping and release of particles by acquiring both Rayleigh and Raman scattering signals over time while closing a laser shutter to purge the trap from particles. Subsequent opening of the shutter starts another cycle of trapping events from an empty trap. For example, Figure 2, A shows the intensity time traces of both, the Rayleigh and the Raman scattering signals from an empty trap. For example, Figure 2, A shows the intensity time traces of both, the Rayleigh and the Raman scattering signals of 100 nm polystyrene beads. Integrating the characteristic 1003 cm\(^{-1}\) polystyrene Raman peak and plotting the intensity versus time result in a step-wise time trace that correlates with the Rayleigh time trace. This correlation confirms that the trapped particles indeed are polystyrene beads.

Figure 2, B shows a magnification of a part of the time trace in Figure 2, A. In the absence of particles in the laser focus, both scattering signals show a constant baseline. Trapping events can easily be observed from stepwise increases in the amplitude of the scattering signals. These signals increase upon the arrival and trapping of the first, second, third and fourth bead (labeled as 1, 2, 3 and 4, respectively in Figure 2, B). The average Rayleigh signal per time interval (0, 1, 2, 3 and 4 in Figure 2, B) is shown in Figure 2, C. Synchronous Raman acquisition labels each time interval with a Raman spectrum. The average Raman spectra of polystyrene for the five time intervals in Figure 2, B are shown in Figure 2, D.

The discrete nature of the time traces can be better visualized by segmenting all the levels in the Rayleigh signal and plotting the median value of each level versus time, as shown in Figure 3, A. Here, the time trace corresponds to the trapping and release of polystyrene beads with a diameter of 125 nm. The median values of each level in the Rayleigh intensity time traces are represented with a triangle. Figure 3, B shows a typical boxplot with a low standard deviation of the Rayleigh scattering intensities corresponding to the discrete trapping of beads. Five levels of scattering can be identified, namely the baseline (no trapped beads), and the first, second, third and fourth step or trapping event. Figure 3, C (black squares and red circles, right axis) shows the experimentally obtained backscatter intensity of the PS (\(n=1.586\)) and Si (\(n=1.464\)) beads in water (\(n=1.3317\)) versus their diameter. The backscatter intensity values correspond to the amplitude of the Rayleigh scattering for the first trapping events (e.g. blue box in Figure 3, B). These values have been plotted versus diameter and theoretically described by Mie theory, taking into account the diameter and refractive index of the beads as well as the optical configuration of the setup. Mie theory predicts an angle-dependent oscillatory behavior of the scattering cross section (Figure 3, C, left axis) for the collected backscattered light.

After developing and validating a procedure to trap single beads and measuring their Rayleigh scattering and Raman spectrum, we proceeded to investigate suspensions of EVs derived from a prostate cancer cell line (PC3 EVs) by synchronized Rayleigh–Raman scattering. Their intensity time traces are shown in Figure 4, A. The associated Raman scattering time trace was obtained from the integration of the lipid-protein band in the
Raman spectrum between 2811 cm$^{-1}$ and 3023 cm$^{-1}$. The Rayleigh and Raman time traces show synchronous steps associated with trapping events and provide a tool to confirm the presence of a single EV in the trap. The mean Rayleigh intensity for PC3 EVs is $1.74 \times 10^5$ with an SD of $1.6 \times 10^5$ ($n=94$). Figure 4, B shows a magnified portion of the time trace in Figure 4, A with four time intervals labeled as 0, 1, 2, 3 and 4, corresponding to no trapping, first, second, third and fourth trapping events, respectively. The synchronously measured Raman spectra are extracted from the time trace and correspond to the Raman fingerprint of the particles trapped at the respective time intervals. Figure 4, C shows the Raman spectra of four different single PC3 EVs that correspond to the four time intervals presented in Figure 4, B.

To obtain a Raman fingerprint of PC3 EVs, we segmented the first particle trapping events from the Rayleigh time traces and thus obtained the time intervals where individual EVs generated a Raman spectrum. Figure 4, D shows the mean Raman spectra after baseline correction and noise removal for 94 PC3 EVs. Typical lipid and protein bands are shown at 1004, 1128, 1300, 1340, 1443, 1607, 1636-1690 cm$^{-1}$, as well as in the high frequency region between 2811 and 3023 cm$^{-1}$. Among other Raman peaks found in PC3 EVs are: 1247 (Amide III)$^{27}$ and 1663 (DNA and Amide I of proteins)$^{27}$, further confirming the trapping of EVs.

**Discussion**

Identification and characterization of a sub-population of EVs (e.g. tdEVs) require a method working at the single EV level. To achieve this, optical trapping and subsequent chemical characterization by Raman spectroscopy have been shown to be an attractive approach, because it is essentially a label-free approach that requires minimal sample preparation and enables chemical characterization of EVs in fluids. However, previous studies
have shown that Raman spectroscopy requires long integration
times of many seconds up to minutes,\cite{13-17} leading to the problem
of trapping multiple particles. One option is to decrease the
concentration of EVs and avoid any multiple trapping events, but
the disadvantage is that at such low concentrations the chance of
actually trapping an EV of interest, such as tdEVs, becomes low,
and the acquisition of a sufficient large number of Raman spectra
from single EVs may take many hours.

In this study, we have successfully investigated a new
approach of synchronized Rayleigh and Raman scattering that
enables to measure Raman spectra of single EVs from samples
with $1.9 \times 10^9$ EVs/mL at a reasonably high trapping rate. In this
approach, the Rayleigh scattering was used to detect the arrival
of each individual particle in the trap from a large increase in the
Rayleigh scattering signal. Synchronously, the Raman scattering
signal was obtained with the same acquisition time as the
Rayleigh scattering signal. Integration over the lipid-protein high
frequency band enabled to obtain Raman time traces that
coincide with the Rayleigh time traces. The time synchronicity is
essential to enable an unequivocal relation between single EV
trapping events visualized with Rayleigh scattering and the
characteristic Raman fingerprint of that EV.

In order to establish the method of synchronous Rayleigh–
Raman scattering, we first measured PS and Si beads in
suspension. PS and Si beads with a refractive index of 1.586 and
1.464, and a diameter down to 100 nm and 182 nm, respectively,
were easily trapped. Individual trapping events could be detected
(Figure 2) and the time-synchronized Raman scattering assigned
the familiar polystyrene spectrum to each PS particle. Mie theory
was used to predict the scattering cross section of single PS and Si
beads of different sizes. The prediction is in agreement with the
experimental backscatter intensity values obtained from optically
trapped single PS and Si beads.

Unlike PS and Si beads, EVs have a lower refractive index
\cite{25-31} making their refractive index contrast small
with respect to the aqueous solution with a refractive index of
1.3317. Such small refractive index contrast also results in a
decrease of the trapping force. However, we were able to trap and
identify single PC-3 EVs from a sample containing EVs with a
diameter of 157 (± 79) nm, according to NTA. In addition, the
low integration time (38 ms) and subsequent automated data
analysis allowed the identification of single EVs in the focal spot
and Raman spectra acquisition from approximately 400 single-
EVs per hour. The signal processing that we have implemented
detected the Rayleigh scattering signal as a way to monitor and
prove the trapping of single EVs. This enables the identification
of smaller particles as the Rayleigh signal is much stronger ($\times
\sim 10^3$ to $10^4$) than Raman scattering, whose weak scattering
process can miss the detection of very small particles. Our
method has the additional advantage of identifying single EVs
irrespective of their Raman spectra due to the fact that it does not

Figure 4. Rayleigh and Raman scattering signals of PC3 EVs. (A) Time trace of Rayleigh (blue) and Raman (orange) scattering signals. This dataset contains 100
cycles $\times 256 = 25,600$ spectra. (B) Magnified cycle from (A) indicating 4 time intervals with single trapped PC3 EVs (labeled as 1, 2, 3 and 4), whose
corresponding Raman spectra are shown in (C). (D) Mean Raman spectrum (thick line) and SD (shaded area) for 94 PC3 EVs.
rely on thresholding of a characteristic Raman peak or band integration. This becomes critical when trying to identify rare EV populations within a mixture of many other EV types. While the Rayleigh scattering signal allows the detection of single particles irrespective of their chemical composition, the Raman signal is used to identify the type of particles trapped. Other studies have relied on fluorescence signal to identify single EVs. Immuno-staining can disclose molecular-specific information, but it complicates sample preparation and workflow and requires specific markers to be used for rare EV populations. Our method is label-free and does not require additional steps to disclose the chemical information of an EV from its medium.

Although label-free Raman spectroscopy is not specific to molecules, the global information in Raman spectra enables to differentiate among different vesicles as previous studies of non-labeled EVs have demonstrated. Our results show that the spectral region between 2811 and 3023 cm⁻¹, assigned to proteins and lipids, is the highest Raman intensity band for EVs (around 3× higher than the highest peak in the fingerprint region for PC3 EVs). This region in the Raman spectrum between 2811 and 3023 cm⁻¹ enables an identification of different types of small single EVs and eventually tDEVs in plasma from cancer patients. The throughput of our technique can still be improved, e.g. by increasing the length of the time traces.

In conclusion, by synchronous acquisition of Rayleigh and Raman light scattering during optical trapping of sub-micrometer particles in suspension, we have been able to identify single trapping events at a high rate. This method could allow the identification of rare populations of EVs, such as tDEVs, where otherwise the ensemble average obscures the signal from single rare EVs. The synchronized detection of Rayleigh–Raman scattering and the ability to characterize single individual nanoparticles open a vast number of applications in different fields, e.g. identification of micro-plastics in contaminated water, virus detection, atmospheric science and catalysis, among others.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2019.102109.

References


