Invited lecture/Review

Diagnostic Detection of Extracellular Vesicles Using Raman Spectroscopy

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Abstract:
This article addresses concisely the possibility of using Raman spectroscopy in detecting extracellular vesicles (EVs) for disease diagnosis. The article gives an overview about EVs, including their definition, roles in normal cells, relation to disease initiation and progression and the chances and challenges of targeting EVs for disease diagnosis. Furthermore, it gives a brief background about Raman spectroscopy, its relevant techniques and to what extent it can be used for single vesicle analysis (SVA). Lastly, it presents some recent trials in using Raman spectroscopy for diagnostic detection of EVs and discusses briefly the potentiality of applying Raman spectroscopy for diagnostic detection of EVs in clinics.

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1. Introduction

1.1. What is the extracellular vesicle?

Extracellular vesicles (EVs) are a heterogeneous group of particles, molecularly and physically delimited by plasma membrane, composed mainly of a phospholipid bilayer, and constitutively released by cells of diverse tissue origin through active secretion. (Lötvall, et al., 2014).

EVs have important roles in inter-cellular transferring of biomolecules such as proteins and nucleic acids, in addition to their physiological functions in many body organs and their involvement into the pathogenesis of many disorders (Yáñez-Mó, et al., 2015). In addition, it was found that the content of EVs in bio-fluids, such as saliva, blood, milk, seminal fluid and urine, is influenced by the body conditions in terms of nutrition, physical exercise, and health status. More particularly, in case of pathological conditions, the content of circulating EVs may be influenced by the diseased organ, severity and duration of the disease (Revenfeld, et al., 2014).

It was evident that various EVs types (including exosomes, microvesicles, and apoptotic bodies) contain subpopulations with unique biological functions that are highly involved in a broad range of biological processes (Willms et al., 2018; Kalluri et al., 2020).

This makes EVs a wealthy source of potential biomarkers for disease prognosis, vaccine production and drug delivery systemsms (Wiklander et al., 2019; Van Der Pol et al., 2010; Soung et al., 2017; Puente-Massagueret al., 2020). Also, EVs have an important property that it can be collected from non-accessible organs such as the brain (Shaimardanova et al., 2020). Thus, EVs-related studies have gained much attention during the past decades; nevertheless many of these studies dealt with bulk EVs and were based mainly on ensemble-averaging assays, such as proteomics for protein analysis and thin-layer chromatography for lipid analysis. These assays, despite being useful in many cases, masked the high heterogeneity between EVs in terms of their structure and composition. (Willms et al., 2018; Pick et al., 2018; Tkach et al., 2018).

For instance, ensemble measurements may be misinterpreted as a result of failing to figure out heterogeneities of molecular states of individual proteins or nucleic acids (Mathiasen et al., 2014).

In this regard, latest advances in single-vesicle analysis (SVA) have enabled deeper investigation of heterogeneity within EVs subpopulations and characterizing them on nanoscale (Pick et al., 2018).

This, undoubtedly, reveals valuable information about EVs and their potential use in clinical applications (Bordanaba-Florit et al., 2021). Wherein, SVA can reveal molecular states that govern the function and transport of the EV; also, it provides us with direct information regarding the heterogeneous composition of individual EVs, and offers statistically valid data that are often lost in ensemble-based experiments (Pick, et al., 2018; Chiang, et al., 2019).

Besides the high heterogeneity, another important motive to adopt SVA for disease diagnosis rather than bulk EVs-based methods is, in some cases, the low abundance of disease-related EVs (such as tumor-derived EVs) compared with similar-sized lipoproteins and EVs from other non-diseased organs that are usually present with much higher abundance in biofluids (Enciso-Martinez et al., 2020).

Based on the above, many researchers have developed about 20 techniques for SVA (Pick, et al., 2018; Chiang, et al., 2019). Among these techniques, Raman spectroscopy, which depends mainly on inelastic scatterings from biomolecules bearded on or contained in a single EV or a few EVs, is considered a potentially promising one.

1.2. Background about Raman Spectroscopy

Molecules can exist in different vibrational states; simply, this is the basic idea behind Raman spectroscopy. In infrared (IR) spectroscopy, when a molecule absorbs the IR radiation with the exact frequency, it will be excited from the ground state (v_0) to the first excited state (v_1), but in Raman spectroscopy, something different will happen. When we shine the material under study with a laser source, it can excite molecules into a virtual state. The

virtual state is not a real energy level of the molecule. After a very short time interval, the molecule can scatter that light back and return into its original ground state. In this case, the scattered frequency ν_r will equal the frequency of the incident laser beam ν_L (this is called Rayleigh scattering, see **Figure 1**). The Rayleigh scattering involves no energy loss, thus it is called elastic scattering (Larkin, 2017).

The other scenario can happen while the molecule is in the virtual state. The molecule can absorb a part of the laser energy, and scatter the remaining with lower frequency. In this case, the molecule will not return to its original ground state, but to the first excited state, for example. The scattered light will have lower energy and smaller frequency than the incident laser light. This is called the Stokes scattering (see Figure 1). The frequency of Stokes scattering can be expressed by the following equation (Tkach et al., 2018)

$$\nu_s=\nu_L-\nu_{01} \quad , \tag{1}$$

where ν_s is the frequency of the scattered light (Stokes), ν_L is the frequency of the incident laser source, and ν_{01} is the frequency of transition between the ground and the first excited state. It is noteworthy that ν_L is known from the laser source; also, the spectrometer itself measures ν_s; so by solving the previous equation we can find ν_{01} which is characteristic for this molecule (Larkin et al., 2017).

It is also possible for the molecule to start from an excited state, for example the first excited state. So, the laser source will excite it to a higher virtual state than that in the first case, and from that higher virtual state, the molecule can scatter all the light back and return to its ground state. It is noteworthy that the scattered photon in this case will have higher energy than the laser light, because the molecule gave up some energy. This effect is called anti-Stokes scattering (see **Figure 1**). The frequency of anti-Stokes scattering can be expressed by the following equation

$$\nu_{as} = \nu_L + \nu_{01} \quad , \tag{2}$$

where ν_{as} is the frequency of the scattered light (anti-Stokes), ν_L is the frequency of the incident laser source, and ν_{01} is the frequency of transition between the ground and the first excited state (Larkin et al., 2017).

As in the Stokes scattering, we can find ν_{01}, which corresponds to the vibrational excitation characteristic of the molecule, and this is the same information we look for in IR spectroscopy.

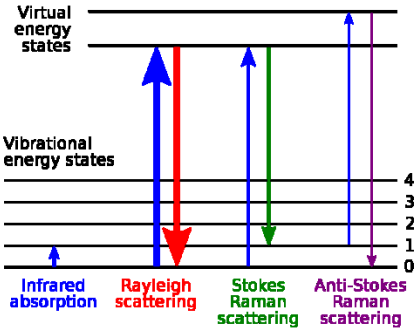

Figure 1. Schematic showing IR-absorption, Rayleigh scattering, Stokes and anti-Stokes scattering (Larkin et al., 2017)

Both Stokes and anti-Stokes lines are called Raman scattering. Raman spectroscopy yields complementary information to IR spectroscopy, as it allows the characterization of IR-inactive molecules (Larkin et al., 2017). Raman scattering is much less probable than Rayleigh scattering; and the relative probability of Stokes and anti-Stokes events is governed mainly by Boltzmann distribution. The energy of the inelastically scattered photons correspond to the chemical bonds present in the target and thus provides information regarding the presence, abundance, concentration, modifications environment of the molecules and their bi-dimensional and tri-dimensional structures (Rodriguez et al., 2006).

2. The basic principle of Raman spectroscopy-based SVA

Basically, an optical tweezer is an instrument that uses a highly focused laser beam to hold and move microscopic and submicroscopic particles such as nanoparticles (NPs), bacterial cells, blood cells and molecules such as DNA for a period sufficient to data acquisition for a single structure (Redding et al., 2015). In this context, laser tweezer Raman spectroscopy (LTRS), also known as Raman tweezer microspectroscopy (RTM), can trap an individual (or very few) vesicle in an aqueous medium, then excite them for Raman scattering which results in providing a characteristic vibrational fingerprint of surficial and internal proteins, lipids, nucleic acids and carotenoids of the single EV of interest. LTRS is considered the method of choice for globally studying the chemical and structural composition of the individually-trapped vesicles and monitoring variations in vesicle components with time (Tatischeff et al., 2012). The main advantage of this technique is the signal linearity, which allows both quantitative and qualitative characterization of a single vesicle. In addition, it requires no label and can provide a highly informative structural data (Tatischeff et al., 2012). On the other hand, the main disadvantage of LTRS is its low scattering efficiency that results in low Raman signal which highly extends the time required for data collection (Smith et al., 2015). However, the low strength of Raman signal can be compensated without losing information about single EV through using surface enhanced Raman spectroscopy (SERS).
In this context, SERS is a technique for enhancing Raman scattering from nanostructures. The scattering enhancement may be with a factor of up to 10^{11} that enables detection of a single molecules, thus characterization of a single EV, and eliminate the effect of high heterogeneity of EVs populations. (Blackie et al., 2009). In SERS, EVs are exposed to signal-enhancing substrates and/or nanoparticles to obtain an enhanced biomolecular signature (Stremersch et al., 2016). The mechanism of this enhancement is still under debate; however, there are two main theories that have interpreted this enhancement: the electromagnetic theory and the chemical theory. The electromagnetic theory proposes the excitation of localized surface plasmons, while the chemical theory proposes formation of charge-transfer complexes. (Blackie et al., 2009; Barbiellini, 2017).
Although label-free SERS can resolve the main drawback of LTRS, it brings another limitation, wherein this enhancement is highly dependent on the distance between the biomolecule and the substrate/nanoparticle, to the extent that it vanishes when this distance exceeds just a few nanometers (Cialla et al., 2014). So, this technique is mainly suitable for surfacial characterization of EVs (Bordanaba-Florit et al., 2021). Another drawback is that not all Raman modes are enhanced to the same extent, wherein those corresponding to molecular vibrations that are at right angles to the SERS surface are preferably enhanced (Cialla et al., 2014). This results in distortion of Raman spectrum and a relative difficulty in its interpretation (Bordanaba-Florit et al., 2021).

3. How Raman spectroscopy works for SVA?

3.1. Raman Microspectroscopy in Suspension

Raman microspectroscopy uses a Raman spectrometer integrated with the base of an upright optical microscope, such that a single laser beam is used for both trapping and excitation of the suspended EVs contained in a coverable glass-slide well (see **Figure 2A**). Raman scattering is collected using the objective lens that is corrected for the cover. Raman scattering is then detected using a CCD camera. Moving the objective along the *z*-axis allows focusing the laser focal spot inside the EVs suspension. The vesicular Brownian motion in the suspension is sufficient to bring them in a close proximity to the optical trap (i.e. the laser focal spot), where the net trapping force is enough to direct the EV to the focal spot (see **Figure 2A**). As long as the laser shutter is open, cumulative trapping of more vesicles occurs, until saturation of the spot volume. Once the first vesicle is trapped, it is defined through its Rayleigh spectrum, and the corresponding Raman spectrum is synchronously detected, which reveals the chemical composition of this EV. Extended monitoring time enables us to discriminate between the absence of any EVs (baseline), first individually trapped EV (1st step), and the successive trapping events. Regularly automated

clearance of laser spot, by closing the shutter, enables detection of many trapping events of single EVs (Enciso-Martinez et al., 2020a; Enciso-Martinez et al., 2020b). Using Raman spectroscopy for assessment of EVs was reviewed in (Lee et al., 2021).

An important advantage of this technique ***is*** the possibility to distinguish EVs from lipoproteins that are in the same size range, and to discriminate between various EVs from different sources (Rikkert et al., 2020). For example, EVs reveal their characteristic peaks at 1004 and 1607 cm^{-1} (Phenylalanine) in addition to their larger protein contribution at 2811 – 3023 cm^{-1} than lipoproteins (black lines in **Figures 2B & 2C**) (Enciso-Martinez et al., 2020a). Enciso-Martinez, et al. (2020a) could differentiate between individual EVs that were derived from two different prostate cancer cell lines, as shown in **Figures 2B & 2C**, i.e. PC3-cell line (blue) and LNCaP cell-line (green) and those derived from red blood cells (red).

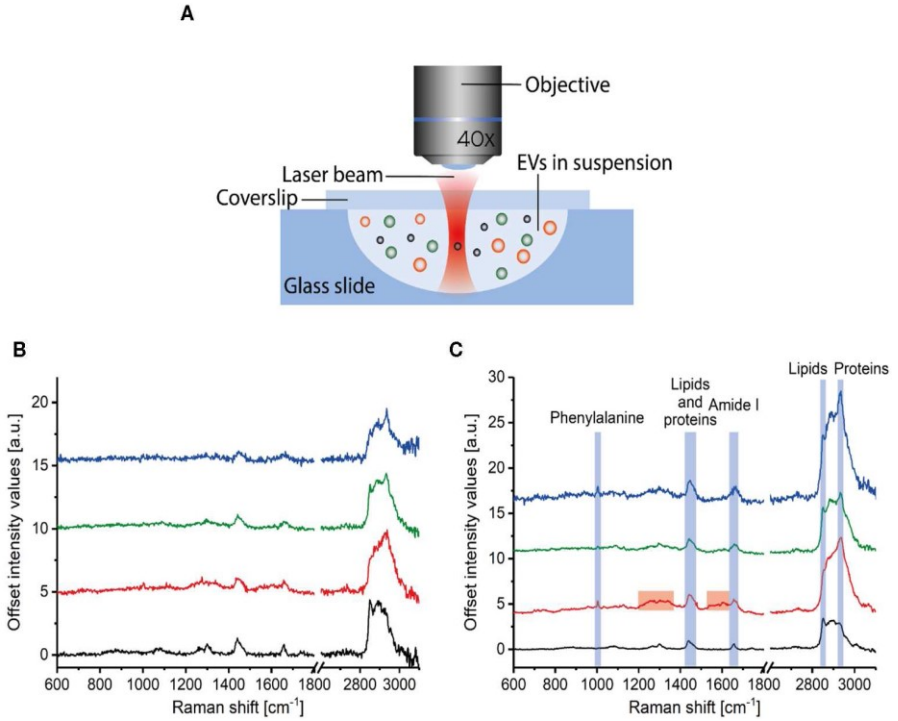

Figure 2. (A) EVs suspension loaded in the weel slide and mounted under the light microscope objective. Raman spectra correspond to single (B) and multiple (C) EVs derived from PC3-cell line (blue), LNCaP-cell line (green), red blood cells (red) and lipoproteins (black). (Enciso-Martinez et al., 2020a)

3.2. Lab-on-Chip device based Raman spectroscopy for SVA

Another interesting study adopted a lab-on-chip device for collecting Rayleigh and Raman spectra. This technique makes the advantage of the higher field gradient that results from coherently combining multiple laser beams that constructively interfere at certain spots on the chip (see **Figure 3**). Each spot serves as an optical trap for a single sub-micrometer EV; this enables trapping of smaller individual EVs for inducing Raman spectrum with the same trapping light (Rikkert et al., 2020).

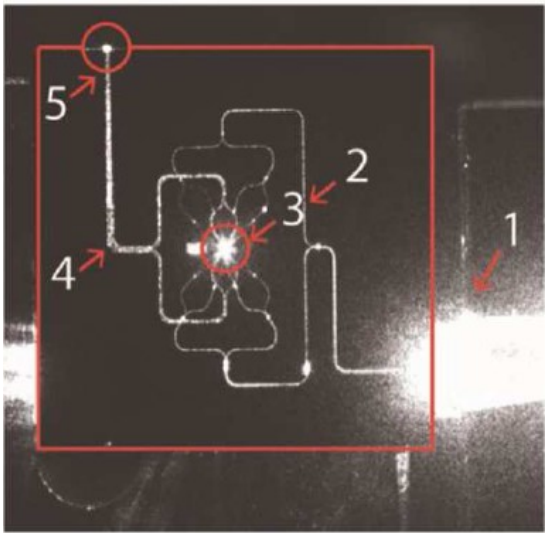

Figure 3. Lab-on-chip-based Raman spectroscopy. The device is operated with the light from an input fiber that is embedded in a fiber array unit (FAU) at the lower right-hand side. The solid red lines indicate the chip edges. 1, FAU; 2, excitation-waveguide circuitry; 3, microfluidic bath with the central 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 (Rikkert et al., 2020).

4. EVs-based diagnosis

The idea of using EVs in disease diagnosis is based on assessment of the disease-related EV (sub)population(s). EVs are isolated from their respective bio-fluids, getting their characteristic fingerprint using Raman spectroscopy, thus figuring out the presence, absence or progression of the disease of interest. In this context, it is noteworthy that the EVs-based diagnosis was already studied for some diseases; whereas among these, cancers diagnosis got the most attention.

4.1. Cancer

Among the first pioneering works, SERS was performed by Stremersch et al. in 2016 for discrimination between cancerous and normal cells. Gold NPs were deposited directly on the EVs and were used as a SERS probe. The researchers could optimize the main SERS parameters, including optimal EVs: NPs ratio and their incubation time for characterization of individual EVs and identifying it with specificity and sensitivity ranged from 92 to 100% (Stremersch et al., 2016).

In 2017, Park et al. deposited EVs collected from normal and cancerous cells on a precipitated gold NPs substrate for SERS experiment. The identification specificity and sensitivity ranged from 95 to 97%, but with the advantage of providing a reusable bio-sensing substrate (Park et al., 2017).

Another interesting approach integrated both immunoassay and Raman spectroscopy, wherein SERS analysis was also used successfully to identify pancreatic cancer-related EVs with amplifying the Raman signal through sandwiching the immunoassay with NPs that highly surplused the traditional ELISA (Li et al., 2018).

4.2. Skeletal Muscle Diseases

Some other pathologies, such as skeletal muscle diseases, have gained high interest in many preliminary studies that have addressed biogenesis and various roles of EVs in pathophysiology of skeletal muscle atrophy (Wang et al., 2022). Also, a recent proteomics-based study demonstrates that skeletal muscle (SKM)-interstitium EVs display unique protein and miRNA profiles that are distinct from plasma EVs. This study has defined some potential marker proteins for SkM-EVs including ATP2A1, β-enolase, calsequestrin

2, caveolin-3, and desmin. Furthermore, the same study shows that four micro RNAs (miRNAs) (including miRs-1, -206, -431, and -486), that are abundantly expressed in muscles, are significantly concentrated in the interstitium EVs. In particular, miR-1 and miR-206 in the interstitium EVs were 45- and 20-fold higher than those in plasma EVs, respectively. Thus the presence and/or relative abundance of these markers can be targeted for Raman fingerprinting-based diagnosis of skeletal muscle diseases in near future (Watanabe et al., 2022).
Positive correlation between muscle protein synthesis and degradation and muscle mass during disuse atrophy and regrowth, and the serum-EVs miR-203a-3p content was obtained by Van Pelt et al. (2020) indicating the potential of targeting serum-EVs miR-203a-3p as a biomarker for monitoring and diagnosing muscle mass and protein turnover-related diseases. In this regard, miRNAs transferable via EVs are considered key mediators for many skeletal muscle processes including their development, regeneration, functioning, and diseases. This makes miRNAs potential biomarkers for skeletal muscle disease diagnosis (Wang H and Wang B, 2016; Xu et al., 2022).
Raman spectroscopy was used in many studies for diagnosis of skeletal muscle diseases based on the whole cellular composition (Fosca et al., 2022). However, to the best of my knowledge, no studies were reported so far about using Raman spectroscopy for targeting SKM-EVs for diagnosis of SKM-disorders. The aforementioned preliminary studies, among many others, reveal some potential SKM-EV biomarkers that may be targeted for diagnostic detection using Raman fingerprinting in future.
It is worth mentioning that Raman spectroscopy was used successfully in many studies for direct in situ detection of miRNA, with detection limit reached in a recent study to 0.21 fM that is similar or even better than polymerase chain reaction (PCR), but simpler, faster, less invasive, non-destructive and less expensive (Fosca et al.,2022; Cao et al., 2017; Driskell et al., 2008). This, undoubtedly, opens doors for optimizing Raman spectroscopy in diagnostic detection of single EVs-related markers, including miRNas and proteins, in clinics.

Furthermore, it is worth mentioning that many papers have reported that EVs usually carry bioactive molecules that are significant and related to other human pathologies such as hepatopathologies (Balaphas et al., 2019), neuropathologies (Shaimardanova et al., 2020) and cardiovascular disorders (Osteikoetxea et al., 2016).

5. EVs-based diagnosis from laboratory to clinics

5.1. Single EV for disease diagnosis - Is it sufficiently mature?

Generally, using EVs for disease diagnosis faces many challenges. Basically, the identification and isolation of the disease-related vesicles from complex bio-fluids is the first and most important prerequisite. In this context, unfortunately, there are no standardized isolation and characterization protocols for different types of samples and diseases so far. This complicates the potential application of EVs in clinics (Bordanaba-Florit et al., 2021).
Another challenge arises, in my opinion, from the uncertainty that the compositional characteristics of each EV reflect the compositional properties of its parental cell. Since recent studies have shown that there are many subpopulations of EVs, with distinguished functions, originating from the same parental cell type (Bordanaba-Florit et al., 2021).
So, in my opinion, what is more critical for EVs-based diagnosis than just isolation and characterization of EVs is studying and determining the physiological or pathophysiological conditions under which the same parental cell type produces a certain (sub)population(s) of EVs. Thus, we can correlate a certain (sub)population(s) of EVs or even the relative abundance of (sub)populations to the initiation or progression of the disease.

5.2. Is Raman spectroscopy a reliable characterization technique for EVs?

In principle, Raman spectroscopy has many advantages for detecting structural characteristics of biomolecules, wherein it ensures high degree of chemical specificity, rapid analytical process because sample usually requires little or even no preparation, in addition to being harmless to biological samples, and above all it is inert to the aqueous background.

(Bordanaba-Florit et al., 2021). However, the experimental settings for applying this technique still need further optimization to be suitable for a real scenario in clinic; (Bordanaba-Florit et al., 2021) for instance, although the enhanced Raman signals provide high specificity and sensitivity, there are some limitations with regard to signal modifications under the effect of the used nanostructures. Thus, it still needs more standardization of procedures (Min et al., 2021).

So, there is a greater chance for Raman spectroscopy to be optimized for clinical applications through introduction of advanced computational approaches that can decrypt the complex information provided by Raman spectrum (Gualerzi et al., 2021).

Conflicts of Interest: The author declares no conflict of interest.

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