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Biophotonics for diagnostic detection of extracellular vesicles

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ABSTRACT

Extracellular Vesicles (EVs) are versatile carriers for biomarkers involved in the pathogenesis of multiple human disorders. Despite the increasing scientific and commercial interest in EV application in diagnostics, traditional biomolecular techniques usually require consistent sample amount, rely on operator-dependent and time- consuming procedures and cannot cope with the nano-size range of EVs, limiting both sensitivity and reproducibility of results. The application of biophotonics, i.e. light-based methods, for the diagnostic detection of EVs has brought to the development of innovative platforms with excellent sensitivity. In this review, we propose an overview of the most promising and emerging technologies used in the field of EV-related biomarker discovery. When tested on clinical samples, the reported biophotonic approaches in most cases have managed to discriminate between nanovesicles and contaminants, achieved much higher resolution compared to traditional procedures, and reached moderate to excellent diagnostic accuracy, thus demonstrating great potentialities for their clinical translation.

Contents

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1. Introduction

Extracellular vesicles (EVs) are a heterogeneous group of particles, molecularly and physically defined, delimited by plasma membrane and constitutively released by cells of diverse tissue origin. Despite past controversies about nomenclature, in the last years the scientific community of the International Society for Extracellular Vesicles (ISEV) found consensus defining EVs the group of particles made up of exosomes, microvesicles, ectosomes and apoptotic bodies. To overcome the past confusion due to the inappropriate use of terminology and to help the convergence to a common language and comparable experimental settings and results, a paper [1] and its update [2] were published defining the main features of vesicles, a common terminology and the minimal requirements for experimental settings and characterization. Since their initial discovery, the consideration and interest about EVs have evolved: starting from the misleading opinion of scavenger particles for the discard of excess and unnecessary membrane and receptors, to the awareness of a role in the transfer of molecules and, later on, in the current high consideration in the physiological function of most body organs and in the pathogenesis of multiple disorders [3]. The above mentioned evolution finds its apparent demonstration in the number of research articles published in the last decade and the extensive reviews on the topic [4], the creation of its own scientific journal (Journal of Extracellular Vesicles) and the global market interest for EV-based diagnostics and therapeutics that is expected to progressively grow in the next years [5].

EVs are versatile carriers for proteins, lipids, and oligonucleotides and the increasing knowledge about their content has taken advantage of a wide range of techniques bringing to a continuously expanding list of molecules that is far from being easily characterized and summarized. Multiple attempts were made to schematically report the main constituents of EVs, building useful platforms like Evpedia, ExoCarta, Vesiclepedia, ExoRbase and EVmiRNA [6-11] that still cannot comprehensively describe the melting pot of molecules that characterizes EVs. The emerging role of EVs as signaling platforms for the intercellular communication among organs, their ability to initiate immune system cascade and mediate or interfere in physiological pathways have brought researchers to realize that conventional and standard procedures failed to approach the EV complexity because of technical limitations related to the nanodimensions of vesicles and because of their multifaceted nature. Actually, since the envision of EV complex nature, it became apparent that one of the main limitations would be related to the standardization of their isolation procedure and to the application of "cell-calibrated" techniques to natural nanoparticles. This brought to the release of position papers, editorials, guidelines and road maps [1,2,12-16] for EV research and to the creation of a digital platform, i.e. EV-Track, to guide researchers in reporting their experimental settings in order to help authors and readers in orienteering and avoiding misleading results [17,18].

In this review, we propose an overview of the most promising and emerging methods and technologies used in the field of EV-related biomarker discovery from a biophotonic-point of view. Traditional biomolecular techniques, including ELISA, Real Time-Polymerase Chain Reaction (RT-PCR), mass spectroscopy, often require consistent sample amount, rely on operator expertise for manual procedure steps, can be time- consuming and provide only partial information about the biochemical features of EV structure, thus imposing limitations to EV applied research. Moreover, the size range of EVs causes the interference of a plethora of common contaminants such as immune complexes, salt precipitates, and fluorescent antibody aggregates that can introduce artifacts in those techniques that do not guarantee sufficient sensitivity, compromising the reproducibility of results. Biophotonics is the application of light-based methods borrowed by physics and material sciences to the biological field. In particular, techniques dealing with light scattering and fluorescent phenomena that allow the modification and quantification of photons emitted directly or indirectly by the samples, can be considered part of the biophotonic approach. Biophotonic platforms, like vibrational spectroscopies and biosensors, offer valuable alternatives as they can usually reach the resolution limits required in EV research (see below), they often rely on functionalized substrates that can selectively immobilize and analyze EVs with no interference of contaminants and they often guarantee a higher multiplexing ability than the above cited traditional techniques. In addition, the herein considered methodologies have proved translation ability to the clinical scenario in the diagnostic framework [19,20], thus possibly accelerating the discovery of new EV-based biomarkers and the bench-to-bedside transition. The potential of EV-based assays in diagnostics are undoubtedly noteworthy, but the integration of innovative technologies, in the framework of a multidisciplinary strategy adopting orthogonal techniques, seems to be a crucial step to unscramble the vesicle-enclosed information about the prognosis of a disease and the predictive outcome of a therapy or of a rehabilitation process.

2. Diagnostic applications of EVs

EVs are natural carriers of various bioactive molecules with emerging significance in pathogenesis of multiple human disorders, including cancer [21-23], neurological disorders [24-27], inflammatory diseases[28], liver pathologies [29], cardiovascular disorders [30] and infectious diseases [31-33]. EV content in biofluids is influenced by the whole body homeostasis, being related to inflammatory or immune activated state, responses to exogenous stresses or nutritional states, physical exercise, disease conditions and regeneration processes. In case of an ongoing pathology, the circulating EV population may be influenced by the severity, the duration, and the organs involved in the disease, changing both its cargo and its outer composition [34,35]. For these reasons, they represent a target of crucial importance for those working on biomarkers, being expected to fulfill the criteria of early prediction, high prediction probability, and feasibility of measurement required for biomarkers. What's more, EVs are easily accessible, transporting tissue and pathology specific molecules in biofluids, like blood, urine and saliva, which can be periodically collected with minimal invasiveness. In particular, for diagnostic purposes, they were isolated from plasma, serum, cerebrospinal fluid, saliva and urine, making them potential diagnostic markers for the majority of complex human diseases [36-42]. Blood EVs were demonstrated to collect vesicles from multiple organs, including the non-accessible ones, i.e. Central Nervous System (CNS) [27,43,44], giving a snapshot of the physiological and pathological processes occurring in the brain. The source-related features of EVs make them identifiable and isolatable (at least theoretically) based on the typical intrinsic and well-defined properties, such as cell-lineage markers. Besides, EV signature critically depends on the stimulation and micro-environment of the donor cells and its release and changes in physicochemical properties were proved to be initial and rapid responders in the evolution of a disease. EVs act as vectors of different types of biological messages, i.e. mRNAs, microRNAs, proteins, phospholipids, bioactive lipids, and saccharides that are normally confined to cells or tissues. This is what makes them a complex mosaic of molecules, all of them possible biomarker candidates, but their kaleidoscopic nature is also what makes them friends and foes of researchers: most diagnostic

techniques validated for single molecules or cells require optimization and refinement to distinguish reliable EV data from artifacts. Their role in the development and evolution of human pathologies is continuously enriched with new discoveries, but from a diagnostic point of view, their overwhelming relevance as biomarker carriers is facing several obstacles: first of all, the standardization of methods, starting from the collection and isolation of biological samples, as well as the reproducibility of protocols, the technical sensitivity and the translation ability of the techniques [45]. The optimization of isolation protocols and the standardization of the characterization methodologies are the pre-requisites for the validation of EV-associated biomarkers and their alignment among different research laboratories is crucial to make results comparable, data discussion feasible, and goal achievement faster [12,46]. The ISEV scientific community has repeatedly stressed the need for transparency in reporting EV based results in order to improve their reliability once extracted from complex liquid biopsies [2,14,17]. It has to be noted that in the EV context, compared to conventional techniques and biomarkers, the importance of pre-analytical variables is increased because of the difficulty of many techniques to work in their optimal sensitivity range, with many traditional instruments remaining limited in their capacity to discriminate EVs from instrument noise, and thus making artifacts exert major impact on all subsequent information [47]. There is growing awareness for the need of experimental standards to monitor the efficacy of EV isolation and to improve the reliability and reproducibility of EV measurements in order to make them clinically relevant [48,49], still the field may require an assortment of standards, depending on the measurement-platform and the molecule (i.e. protein, lipid, oligonucleotide) that is considered as biomarker. Unfortunately, for the translation of EV research to clinics, one of the most prominent issues is the biospecimen itself, but many researchers have to rely on biospecimens from established bio-banks, in which the methods for collection, handling and storage were not intended for EV research. Few studies attend to the quality and consistency of human biospecimens that are the EV sources. The lack of rigor in terms of sample collection and handling is among the causes of general delay/failure in biomarker discover in the EV field. Beside the details of sample collection, storage, transportation and handling prior to arrival at the laboratory for downstream EV analysis, also information about donor characteristics like age, sex, diet, and co-morbidity are crucial to discover robust biomarkers. Such influencing parameters have not been deepened, yet, but can determine remarkable heterogeneity of samples and introduce unpredictable variables in the analysis. This wide knowledge gap about the variables affecting EVs makes critical specimen quality assessments essential prior to embarking on EV investigations.

Moreover, for the successful identification of EV biomarkers the downstream technological application is crucial. The intrinsic multidisciplinary nature of the EV field has brought researchers with different technological background to study vesicles, but this has also highlighted how differently the same issue can be investigated: from the single-EV to the bulk approach, from the selection of a single molecular marker to the identification of a panel of molecules that constitute a fingerprint for pathology-specific vesicles. Consequently, several questions arose: which is the best approach to use EVs in diagnostic? Which is the best specimen and isolation procedure? Can we standardize a protocol to be used as Standard Operating Procedure (SOP) for different types of analyses? Is the purest EV preparation procedure translatable to clinics? Such questions remain difficult to be answered. Of course biophotonic-based methods cannot solve all current difficulties in EV-based diagnostics, still they offer a new perspective in EV analysis and clinical application, proposing cutting-edge, technologically advanced solutions that can overcome some of the problems related to partial purification and limited yield of EVs, as it will be described below.

3. Biophotonic-based methods in EV research

Biophotonic-based methods take advantage of the physical properties of light to investigate the physico-chemical features of a biological sample. Among them, vibrational spectroscopies have already found wide application in the diagnostic field as they provide molecular information that can be rapidly acquired without the need for specialized stains or dyes, potentially simplifying analyses for point of care assessments while not requiring extensive specialized expertise once defined the analytical protocol [19,50-52]. Optical methods have the potential to accurately obtain clinically relevant properties of particles, they include a wide range of techniques that rely on the interaction of a laser light with the biological sample and detect the scattering of photons or the fluorescence effect, with emerging application in the EV field [53]. Optical methods like Dynamic Light Scattering (DLS) and simple Nanoparticle Tracking Analysis (NTA) have been widely used for the characterization of EVs for their ability to determine the relative size distribution of vesicles. As the quality of NTA data strongly depends on software settings and requires skilled operators, its use as quality control method appeared to be difficult. Asymmetrical-flow field-flow fractionation (AF4) coupled to a multi-angle light-scattering detector (MALS) is a method with the potential to overcome this issue [54]. Although the quantification of circulating vesicles has been evaluated by some authors with possible implication in disease diagnosis [31,55,56], DLS and NTA cannot distinguish EVs from similar-sized lipoprotein particles or small platelets, as no biochemical information is obtained, thus limiting its application in diagnostics. For this reason, in the present review, we will focus on those platforms that have shown the most promising results in the EV-based diagnostic by allowing a biochemical characterization of the sample for the discovery of a disease-related biomarker. The biophotonic platforms considered in the present review are summarized in Fig. 1.

3.1. Raman spectroscopy

Raman Spectroscopy (RS) is a vibrational investigative methodology, able to provide qualitative and quantitative information regarding the chemical components of the analyzed target. In RS, a monochromatic laser light, with specific emitting wavelength, is used to irradiate the sample. After the electromagnetic interaction with the chemical compounds that constitute the sample, the resultant light is mainly composed of elastically reflected light with the same frequency of the incident source (Rayleigh scattering). A minimum part of the incident light is inelastically scattered, slightly modifying its intrinsic energy due to the interactions with the molecules contained in the sample [57]. The energies of these photons correspond to the chemical bonds present in the target and thus provide information regarding the presence, abundance, concentration, modifications, environment, bi-dimensional and tridimensional structures [57]. The collected Raman signal consists of wavenumbers, usually expressed as Raman Shifts (cm^{-1}), on the abscissa and scattering intensities on the ordinates, resulting in a spectrum composed of peaks and bands at specific positions. The huge amount of available Raman databases allows the interpretation of the spectrum in a precise way, extracting information about almost all the biological tissues and fluids [58]. One example is provided by the codification of signals collected from proteins, with characteristic bands at specific positions, representing the orientation and the conformation of the secondary and tertiary structures in protein (Amide I, II and III) [59]. Similarly, large datasets

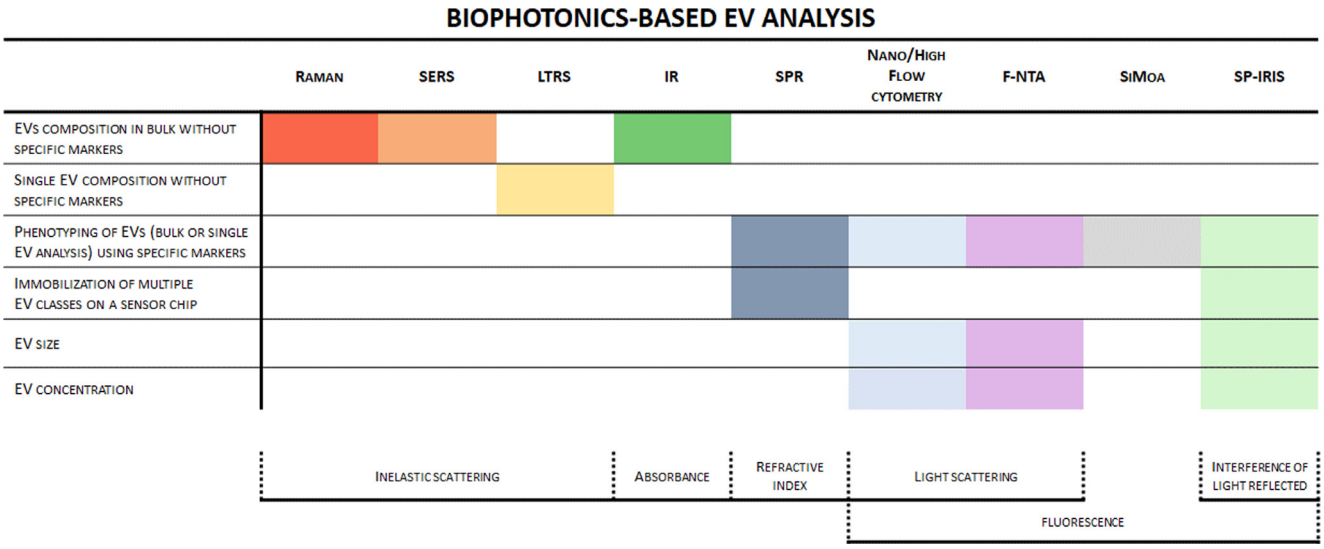

Fig. 1. Overview of the biophotonic platforms considered in the present review. Colored boxes highlight the main advantages related to EV research in diagnostics.

are available for the interpretation of the signals provided by lipids intercalated in the biological membranes [60]. RS ensures high chemical specificity, fast analytical procedure due to the minimal to no sample preparation, harmlessness for sample and high indication for the analysis of biological tissues, being inert to the aqueous background [61]. Due to the described advantages, RS has been successfully applied in the diagnostic field and proposed as potential device for the liquid biopsy of neurodegenerative pathologies, various cancer phenotypes and microbiological infections [51,62,63]. The precision and the comparability of the obtained Raman spectrum of a specific biological compound strictly depend on the calibration performed on the instrument. Usually, to ensure the correct positioning of the bands corresponding to the biological compounds, the calibration is performed using the silicon reference band at 520.7 cm^{-1} and ensuring in this way the precise repeatability of the analysis. In the same way, regarding the clinical and diagnostic applications, EVs from different subjects must be collected (healthy controls, pathological patients and subjects affected by similar pathologies), highlighting the spectral differences between the selected experimental groups and the consecutive attribution of such differences. In the last five years, the number of research articles proposing the RS as EVs investigative tool is drastically incremented, defining at the same time the optimal parameters for the analysis and the capability to interpret the obtained results. By means of RS, the biological composition of EVs can be characterized through the identification of the specific signature of the target components. Nowadays, this technique is mainly used combined with complex computational algorithms, in order to precisely assess the differences between two populations of EVs, identifying the biological families responsible for such differences (e.g. lipids, protein, nucleic acids, hormones) [64]. The first spectrum obtained from a synthetic vesicle was reported in 2003 from Kiselev *et al*, investigating the effects of the sucrose gradient at different concentration for EVs isolation procedures [65]. Exploiting the Raman effect, combined with other characterization methods, the first EVs specific spectral regions and bands were defined and attributed, observing the effects of external factors on the chemical orientation and modification of the structural lipids. Few years later, the production of informative Raman spectra from formalin-fixed vesicles and lipid nanostructures derived from human fibroblasts was carried out by Krafft *et al*, collecting a detailed spectrum of the samples and identifying with high precision the composition of the EVs and lipid structures [66]. Despite the long sample preparation procedure and the presence of a strong Raman inducer such as formalin, the work demonstrated the capability of RS not only to identify precisely the different nanometric structures, but also to qualitatively and semi-quantitatively determine the amount of the single lipids intercalated in the double layer (i.e. phosphatidylcholine and phosphatidylserine). The proposed approach, involved the preparation of human fibroblast cultures for the in vitro inclusion and consecutive Raman mapping for the identification of the spectral clusters of interest, resulting in a long sample preparation procedure and Raman signal collection due to the large maps performed.

The spectral region between 2600 and 3200 cm^{-1} was analyzed in detail to obtain structural information regarding the vibrational modes of methyl and ethyl groups present in the hydrophobic tail of membrane lipids. The acquisition of more information regarding the analytical procedure and the signal interpretation, allowed the analysis of EVs produced from eukaryotic unicellular organism not only to verify the effective lipid structure, but also to characterize the cargo related to each EV for drug delivery purposes [67]. In this work, the comparative proteomic and lipidomic analysis was developed concurrently with RS, laying the foundations for the Raman signature characterization. Starting from this work, RS was discussed together with the standard methods for vesicles characterization, proposing a potential role for the biochemical composition assessment, purity assessment and identification of components implied in their functional role [2,53,64,68-70]. The first label-free analysis of biological material directly derived from human samples, concerned the analysis of EVs collected from a human-derived erythrocyte concentrate [71]. The result of the study was the collection of a specific EV Raman fingerprint, attributing to selected spectral regions the signal provided by proteins and lipids, consecutively identifying the qualitative components of the nanostructure. Over the time, the RS analysis was deepen allowing the attribution of bands and peaks to specific lipids, proteins and nucleic acids used to characterize the EVs collected from different sources using the complex signal provided by the RS. The last example reported, regards the work of Gualerzi *et al,* that provides an example of the full exploitation of coherent RS potential, combining the signal collected with machine learning

algorithms for the decryption of the complex features contained in the Raman spectra of different in vitro sources [64]. In particular, they demonstrated that the fast and label-free coherent Raman approach, combined with multivariate analysis, is able to discriminate the EVs collected from human dermal fibroblasts, bone marrow derived and adipose tissue derive mesenchymal stem cells with accuracy of 94%, attributing the different discriminant regions to as many biological structures. In the great part of the described works, RS demonstrated the capability to rapidly and accurately characterize the bulk EVs, providing detailed information on the average composition of external and inner molecules. Despite the great potential, the main limitations concerning the application of RS regard the intrinsically weak Raman scattering and the probe volume of the analysis. To overcome these issues, RS was combined with other techniques highly enhancing the signal intensities and details provided by the analysis and allowing the identification at the single-EV level. The main Raman-based approaches are the Surface Enhanced Raman Spectroscopy (SERS) and the Laser Tweezers Raman Spectroscopy (LTRS).

3.1.1. Surface enhanced Raman spectroscopy

SERS is an inherently plasmonic method in which the coupling of photons to charge density oscillations provided by nanostructured metallic materials is exploited to enhance the Raman signal sensitivity up to 10^{15} orders of magnitude [72]. The absorption of the biological molecules to the nanostructure surfaces generates highly localized regions (~10 nm) of intense local field enhancement, called "hot spots", through the electromagnetic and chemical interactions [73]. The absorption, as well as the enhancement factor, strictly depend on the chemical composition, surface charge and on the shape of the nanomaterial. These parameters can be tuned achieving the enhancement of the Raman signal for the target structures, with the possibility to bind recognizer molecules (e.g. antibodies, aptamers, integrins) on the nanostructure surface to selectively enhance specific molecules inside complex biofluids in an "active targeting-like" process [62]. SERS is a powerful investigative methodology already applied to the clinical detection of potential biomarkers including nucleic acids, proteins, lipids and also for the identification of microorganism and eukaryotic cells [74]. There are mainly two ways to apply the SERS approach: use the metallic (mainly gold or silver) Nanoparticles (NPs) as probes mixed in the biofluid or use nanostructured metallic substrates as biosensors [75]. Both the approaches ensure a high and detailed content of structural information, with the possibility of molecules quantification and multiplexing analysis. Nowadays, the combination of EV isolation strategies with SERS applications, has enabled mainly the characterization of different vesicle populations through their unique molecular component (e.g. phospholipids, cholesterol, phosphatydilcolin, surface proteins) [76]. The first example is provided by the work of Stremersch *et al* using gold nanoparticles as SERS probes deposited directly on EVs after the isolation through concentration gradient [77]. The analytical parameters were finely tuned identifying the optimal ranges for the main variables involved in the SERS processes for biological fluids, including the EVs:NPs ratio, incubation time for the determination of the optimal hotspots and the attribution of the enhanced peaks and bands. As a result, they were able to identify EVs collected from cancerous or healthy cells with specificity and sensitivity ranging from 92 to 100% applying the partial least square analysis to the collected spectra [77]. An example of SERS substrate was presented by Park *et al*, depositing the EVs isolated from normal and cancer cells on a $CuSO^{4-}$ induced aggregated gold nanoparticles substrate [78]. In addition, in this case, the specificity and sensitivity levels were between 95 and 97%, but with the advantage to perform the analysis on a reusable biosensor that ensures a high SERS enhancement. A similar approach was adopted by Dong *et al*, modifying the SERS substrate and focusing on the importance of the different electrochemical material properties [79]. In this case, they used a gold-coated titanium oxide honeycomb-structure, able to capture EVs and to provide the SERS enhancement needed for vesicles detection, by means of the "slow light effect" of the structure. The beehive (or inverse opal structure) is able to absorb sound waves by means of multi-scattering processes. Moreover, titanium oxide materials are anisotropic with a high refractive indexes and scattering properties, allowing the entrapment of light, in this case, inside the pores. As a result, the slow light effect is able to enhance the SERS signal not only at the single-molecule level, but also for larger structures including EVs. The importance of the SERS inducer relies in its intrinsic physical and chemical properties, in fact also slight variations in materials composition and nanometric structure can lead to huge variations in the collected Raman spectrum. The aggregation of EVs induces signal variations with dependency on the position and material aggregation on the SERS substrates. To overcome this limitation, researchers applied plasmonic nanomaterials with well-ordered and repetitive nanostructures ensuring the uniformity of the signal all over the sensor surface. The provided homogeneous and repeatable signal could be used for the monitoring of the processes involved in EVs isolation and sample preparation. Lee *et al* created a thin silver layer coated on ordered nanobowls in the nanometric range, which provided a uniform distribution of hot spots for the reproducible EVs Raman spectrum all over the biosensor surface [76]. Using this substrate, they monitored the emission of new characteristic SERS peaks during the EVs drying process, allowing the monitoring of chemical modifications in lipid membrane and cargoes within the vesicles.

As mentioned before, the identification and quantification of specific EV subpopulations can be achieved also using NPs functionalized with target specific ligands [75]. The active targeting technique allows the identification of specific EV fractions, such as surface molecules, characterizing only the discriminant part of biological fluid mixtures. Lee *et al* performed a selective SERS process of EVs using a thiolated peptide ligand connected to the surface of silver NPs, identifying through the SERS signal the EVs belonging to $\alpha 3\beta 1$ integrin overexpression [80]. Several studies reported similar approaches in order to achieve the concomitant EVs subpopulations selection and analysis using SERS, exploiting the different electrochemical properties on EVs [75,81]. The last reported example involves the application of different NPs to select and analyze vesicles, resulting in a relatively fast (2 h) and sensitive methodology. Zhong *et al* presented a SERS detection method using magnetic NPs as capturing substrate and silver/gold core shell nanorods functionalized with specific antibodies as SERS tags [82]. Besides the optimal results, Zhong and colleagues were able to directly isolate and analyze EVs from culture medium without pre-enrichment of the vesicles.

In the last decades, several studies reported different combination of SERS enhancer with several combined approaches for simultaneous EV isolation and analysis [81]. All the described SERS approaches presented significant results in terms of EVs characterization and differentiation, highly incrementing the knowledge about plasmonic approaches for the fast and detailed vesicles identification. Despite all the reported advantages, also the SERS-based approaches possess intrinsic limitations, mainly regarding the modification of the signal on the base of the used nanostructure, not existing nowadays a standardization for SERS procedures, and about the characterization at the single-EV level.

3.1.2. Laser tweezers Raman spectroscopy

LTRS is a methodology able to trap in place, using the emitted light, a single particle (nm to μm) long enough to perform a single structure data acquisition. This approach was proposed to over-

come the limitations regarding the weak Raman signal respect to the intrinsic fluorescence of the biological materials and to the background signals provided by several SERS substrates. Since the Raman spectrum of a immobilized particle can be acquired in both solution and air, the LTRS can limit the signal provided by the background obtaining the characterization of the single molecule also in its physiological environment [83]. Moreover, also the temporal responses of the single particle to different environments can be monitored, investigating for example the effects of different sample preparations respect to the untreated material [84]. There are several trapping techniques, each of which present advantages and limitations. The most popular approaches regards the radiative pressure force, photophoretic force, holographic trapping and single shaped laser beam usually combined with SERS substrates or probes to increment the weak Raman signal [83]. For example, Dai *et al* obtained the characteristic signal of single-EV using the "Raman enabled nanoparticle trapping analysis" [85]. Combining optical forces and label-free Raman, they acquired the spontaneous Raman signal confirming the discriminant morphological-chemical profiles of EVs collected from cancer and healthy cells. Moreover, the single-EV analysis was able to discriminate in the cancer EV population the presence of two categories expressing different surface markers, confirming through multivariate analysis the reliability of the methodology. The laser tweezer approach has been adopted with slight variations for the identification of EVs isolated from different sources including protozoa, bacteria, eukaryotic cells in various pathological and physiological conditions, tumor cells, synthetic EVs and vesicles isolated from various human biofluids [81,86-90]. Among the encouraging results obtained, Kruglik *et al* were able to characterize the EVs through RS combined with laser tweezer, acting on the fine discrimination between the vesicles nucleic acids and carotenoids content [86]. This demonstration confirmed the potential of RS to sensitively identify also the nucleic acids transported by EVs, which possess an high potential as pathological biomarkers including mRNA, miRNA and siRNA [91,92]. The more sophisticated combination of RS in laser tweezer regime, with the application of SERS inducers achieved the full characterization of the single-EV collecting a highly detailed and sensitive signal. Kaufman *et al* developed a plasmonic nanoholes array in which flowing EVs were captured and trapped by means of the laser tweezer application [93]. The encouraging results of the work reported a label-free, fast and accurate discrimination of EVs isolated from mesenchymal stem cells trapped on the plasmonic platform. The main advantage of the LTRS regards the passage from the bulk EVs analysis to the single-EV signal, allowing the identification of the discriminant molecules present in the specific captured vesicle.

3.2. Fourier-transform Infrared spectroscopy

Fourier-transform Infrared (FT-IR) spectroscopy is a vibrational, non-destructive method which allows the simple and reproducible analysis of the tissue with only small amounts of material. It is based on the change in the dipole moment in a molecule, working on the principle of vibrating molecular bonds and the resulting absorption wavelengths, which depend on the involved atoms and strength of intermolecular interactions. Despite providing specular results as RS, FT-IR mainly deals with non-aqueous samples, because of the strong absorption bands of water. Moreover, compared to RS, FT-IR has higher sensitivity but it does not provide the possibility of confocal resolution, and its lateral resolution is 10–20 μm (compared to 1–2 μm of Raman spectroscopy). Nonetheless, it has been widely used for diagnostic purposes thanks to the possibility to obtain quantitative information from the biological sample and to the fast acquisition (few minutes). These properties make this technique a suitable tool for the point-of-care (POC) analysis. Under the economic point of view, commercially available instrumentation is usually less complex and expensive than the corresponding Raman spectrometers [19,94]. In the EV field, FT-IR was used as fast and simple characterization method of EV isolated from cultured cells [95]. The IR spectra were used to gain general information concerning the molecular constituents and the structures of EVs and apoptotic bodies after drying the sample. Results demonstrated that the typical IR spectra of apoptotic bodies resemble those of the parent cells, whereas EVs differ from the cell source due to changes in the spectral features of protein secondary structure. Furthermore, Mihály and colleagues introduced a new parameter, i.e. the spectroscopic protein to lipid ratio, to classify different pre-isolated EV subpopulations. IR spectroscopy was demonstrated to represent a reliable, fast and relatively cheap screening approach for EV isolation which requires only minimal amount of sample and no complex sample preparation procedures [95], and it was proved to be able to assess differences in the purity level of EV preparations and differences in EV subtypes (large vs small EVs) [96]. Although firstly proposed for research purposes to efficiently classification of EV subpopulations, in 2016 another research group deposited a patent that suggested FT-IR for diagnosing, prognosing and monitoring pathophysiological states, like cancer [97]. Results related to the analysis of EVs isolated by a combined protocol of ultrafiltration and size exclusion chromatography from the supernatant of cultured breast cancer cells were reported. Spectral data demonstrated that three different cell lines owed distinct biochemical features related to their cellular origin and possibly by the extension to cancer cells and to the tumor stage [97]. FT-IR was also demonstrated to have potentialities in the identification of the biochemical fingerprint of blood [98] and salivary EVs [99]. Compared to blood EV results that were characterized by significant contribution of protein components [98], maybe due to the selected isolation procedure, IR spectra of salivary EVs revealed differences in multiple biomolecules and in particular differences were observed in the peak at 1072 cm^{-1}, corresponding to absorbance bands of phosphate bonds within nucleic acids [99]. Such observation made authors conclude that the content of nucleic acids in cancer EV can be lower than that from healthy EV, although further investigation is needed.

Collectively, FT-IR can be considered as an alternative and/or a complement to other methods used for fingerprinting EV subpopulations, including other spectroscopic methods, such as RS, as it was demonstrated to be able to identify general features shared between several EV subtypes or specific to one sub-type of EVs. Still, the identification of the proper isolation method is crucial for the downstream application of IR data.

3.3. Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) is a sensitive analytical technique able to detect variations in the mass adsorbed on top of a thin gold layer in real time and without the need of any label. In particular, SPR is a phenomenon that occurs when polarized light, shone through a prism on a chip covered by a thin metal surface, is reflected by the metal film as a mirror. At the precise angle of incidence of the laser light that causes the minimum of the intensity of the reflected light, polarized photons are able to interact with free electrons of the metal layer, inducing the generation of a wave of plasmons across the surface and thereby reducing the reflected light intensity. The angle at which the reflected light intensity reaches the minimum (SPR angle) is dependent on the refractive index in the immediate vicinity of the metal surface. Therefore, when a biomolecule is adsorbed on the surface, its mass changes the local refractive index producing a shift of the SPR angle that is detected by the SPR signal. In this way, it is possible to detect

the presence of a molecule of interest and to track interactions between biological molecules without the use of any other marker. SPR is considered the gold standard for real time monitoring of molecular interactions characterized by an extraordinary versatility; indeed it could be used for a broad range of biomedical applications, even in clinical laboratories, and for the analysis of complex biological fluids [100]. The ability of SPR based sensors to detect low concentration of multiple biomarkers concomitantly and in a label-free manner makes it adaptable for the development of new diagnostic tools [20,101].

Moreover, in the last years, some studies have shown the success of using SPR-based platforms for the detection of EVs from biological samples according to the presence of specific membrane molecules, as the range in which SPR technique is able to induce signal variations (200 nm) fits the dimensions of EVs, that have a mass relevant enough to be able to generate a SPR signal. The SPR-based platforms for the EVs studies allow to analyze intact vesicles and to study the expression of molecules on their surfaces because EV membrane effectively increases the local molecular mass adsorbed on the chip with a kind of intrinsic SPR enhancing property. The first studies applying the SPR technology for the research in the field of EVs were aimed at determining the concentration of vesicles isolated from conditioned media from the human mast cell line [102,103] or at obtaining a label-free profiling of the main proteins of EVs, present in samples from breast cancer, multiple myeloma, ovarian and lung cancer [104-107]. In the latter case, Liu and coworkers developed an SPR-based biosensor for the lung cancer diagnosis able to detect epidermal growth factor receptor and programmed death-ligand 1 as EVs-associated biomarkers. In particular, they found higher levels of these markers in non-small cell lung cancer cells derived EVs than in normal cells; the analysis was also performed in human serum samples, showing higher detection sensitivity than ELISA assay [107].

Although most of the studies dealing with SPR characterization of EVs are related to cancer research, now the application field is expanding including coronary heart [108] and brain diseases [109]. This is due to the demonstrated potentiality of the technology to be effective in the detection and concomitant characterization of EVs, thus improving the already existing diagnostic platforms.

Different settings of SPR platforms have been tested in order to improve the sensitivity and specificity of the analysis, according to the specific biomedical application of each biosensor. In every case, a crucial point is the set-up of the surface chemistry for the creation of the array to be used for the detection of EVs of interest; accurate surface modification strategies have been developed in order to limit non-specific binding and screen the presence of EVs in a specific way. In these works, after EV isolation by ultracentrifugation or size exclusion chromatography, vesicles were captured on the gold coated surface of the chip by means of specific antibodies or aptamers [110] and protocols were adopted to reduce the unspecific binding on the sensor surface. In this way, the ability of SPR to detect relative low concentration of EVs from cell supernatant, from human plasma or urine was assessed [111,112].

Importantly, the improvement of the sensitivity of the proposed platforms has been recently achieved thanks to the combination of nanotechnology with the SPR-based biosensors. In this framework, a successful attempt to improve SPR sensitivity in EV detection is represented by the development of a homemade SPR chip, the nano-plasmonic sensor nPLEX [106], which took advantages of the optical properties of nanomaterials used for the amplification of SPR signal. Indeed, the proposed sensor relies on a nanostructured chip where nanoholes of<200 nm match EVs size range allowing improved sensitivity. The multiplex label-free feature of SPR sensor is guaranteed by specific antibodies bound on the surface that were demonstrated to be effective in detecting EVs in both cell supernatant and patients' ascite. Furthermore, the combination of nanostructures chip and gold nanoparticles (spherical and star shaped) proved to be an effective integration of nanotechnology approaches to increase the sensor sensitivity, a pivotal issue when handling with complex human fluids and heterogeneous vesicle populations.

Another example of SPR-based platform which uses nanomaterials to enhance its sensitivity is given by Fan *et al* [113], who developed a high-sensitive sensor to effectively distinguish EVs from non-small cell lung cancer cells with a limit of detection of 104 particles/μL thanks to functionalized gold nanoparticles. In particular, gold nanoparticles functionalized with anti-CD63 antibody were injected after the EV samples causing remarkable increase of SPR signal. Indeed, plasmonic nanoparticles are widely adopted to enhance the SPR signal, thanks to their mass, optical properties, such as higher refractive index, and also their easy preparation and good compatibility.

In this latter work, as in many other studies, researchers used the SPR imaging (SPRi) technique for the vesicle subtypes screening, developing a specific antibody microarray to purify multiple tissue-specific vesicles, according to the presence of validated protein markers and demonstrating the interaction also with the CCD differential images on defined areas on the chip [114]. Indeed, the SPRi method combines the multiplexing analysis with the imaging capability that enables users to visualize the entire working area in real time. So, it allows the phenotyping of vesicles, similarly to conventional microarrays that track multiple markers in parallel with limited amount of sample and reagents [115], but with the advantage of the "label-free" approach. Actually, the protein microarrays started to be considered for the detection and phenotyping of EVs since 2013, when the first EV-Array was developed. The pioneering work of Jørgensen and colleagues allowed the development of a highly sensitive array able to detect and phenotype EVs in a high-throughput manner estimating the concentration of EV from plasma of healthy subjects using a panel of antibodies against 21 different cellular surface antigens and cancer antigens. [115]. Indeed, the developed platform was an open system, easy to transform and develop further with other markers of interest, but it required a detection cocktail of biotinylated antibodies against the tetraspanins followed by fluorescence-labelled streptavidin that can be overcome by the SPRi biosensors. Moreover, the SPRi method allows to simultaneously quantify specific EVs subtypes, thus providing quantitative information thanks to the proportional relationship between SPR signal and the number of bound vesicles per area.

A SPRi-based biosensor was recently developed to simultaneously detect and characterize EVs with different neural origins present in plasma of human patients. Purified EVs were captured on the SPRi chip using specific antibodies to separate astrocyte-, neuron-, oligodendrocyte- and microglial-derived EVs and then characterized on the same chip with molecules targeting specific antigens of interest for the pathogenesis of Alzheimer's Disease (AD). It was possible to obtain a molecular profile of each specific subpopulation of EVs individuating the presence and the relative amount of translocator protein (TSPO), β-Amyloid and ganglioside M1 [43,109].

Another recent interesting work showed a proof-of-concept of a system based on Surface Plasmon Resonance Microscopy that integrates the SPR biosensor and advanced plasmonic microscopy for the multifunctional analysis of EVs. The system was applied for the analysis of EVs secreted from human lung cancer A549 cell lines, demonstrating its capability in providing information regarding not only the biochemical properties of EVs membrane proteins and binding affinity between antibody and EV, but also their physical properties, including size distribution and concentration [116].

Another new platform that was developed is based on the combination of SPR detection and surface plasmon-enhance epifluorescence for the mesenchymal stem cell-derived EVs efficient detection and monitoring, using magnetic nanoparticles as labels for the enhancement of the SPR sensor signals [117].

Therefore, considering the huge potentiality and the high tunability of the SPR technique, the application of such a methodology to complex biofluids without preliminary purification of vesicles needs to be further examined and improved in the field, thus moving the use of such biosensors closer to the clinical practice.

3.4. Flow cytometry

Flow cytometry (FC) is a key technology for both the qualitative and quantitative characterization of single cells or biological particles within a sample, providing a rapid multi-parametric analysis. The fundamental principle on which the FC is based is the light scattering and the fluorescence emission when a laser strikes the unlabeled or labeled moving particles in a hydro-dynamically focused fluid stream. Scattering and emission signals are measured for each individual particle that passes through the excitation source, generally a laser, thanks to an optical-electronic coupling system with lenses, filters, mirrors, beam splitters, and detectors. Light scattering is measured in two different directions: the forward direction (FSC or Forward Scatter) which can provide the relative size and at 90° (SSC or Side Scatter) which indicates the granularity or the internal complexity of the cell. Most flow cytometers employ a single laser, however, some systems can operate with two or more different lasers at the same time.

FC is a powerful technique that has applications in multiple disciplines such as molecular biology, immunology, virology and cancer [118,119], representing a convenient approach to detect and characterize EVs in order to provide information about the diameter, via scattered light, and information about the concentration and phenotype, via fluorescent labelling [120-123]. In fact it is estimated to be utilized in 90% of EVs researches [124] and most applications are based on fluorescence monitoring.

The fluorescence-based approach can involve the use of generic dyes to stain the phospholipid membrane or, principally, a fluorochrome-conjugated antibody to identify different vesicles subsets within heterogeneous pools [125,126]. Uniform and bright fluorescent labelling without the generation of label-associated artefacts is a prerequisite for fluorescence threshold-based analysis of individual nano-sized vesicles [127]. Coumans *et al* [128] compared several generic fluorescent markers and the results obtained showed that none of the tested markers stains all EVs (100% of sensitivity) and only EVs (100% specificity). In fact, fluorescent markers may also stain lipoproteins leading to a misidentification of EVs.

Most traditional and commercial flow cytometers were designed for the analysis of cells, that are significantly larger than EVs, and they have the lower detection threshold around 300–500 nm [129-131], therefore they are not suitable for EVs analysis. Others problems are the low refractive index as compared with that of cells or other nanoparticles [132] and a very high background signals generated from sample buffer which can drown out the small signals created by EVs [133]. With the aim to improve the resolution and the separation of the EVs from the electronic noise, cytometers with a better optical-electronic system have been developed [127,134,135]. These modern cytometers use well-characterized polystyrene or silica beads as a method of size calibration. An approximate EV detection limit of 100 nm was obtained by Brisson and colleagues by use of a Beckman Coulter Gallios [136] and by the Wauben group using a custom BD influx [126]. As reported by the Brisson group [136,137], fluorescence triggering allows the detection of a higher count of EVs in plasma compared to scattered-based triggering: Anx5-positive EVs detected were 50-fold higher. These data have been confirmed by a recent study on identification of mesenchymal stem cell-derived EVs using phycoerythrin as dye [138]. In addition, great progress has been made regarding the standardization of EVs measurement using FC, such as sample preparation, immuno-staining and particle size calibration protocols [46,130]. For example, prefiltration of antibodies to remove aggregates followed by detergent lysis were proved to be effective at excluding background fluorescence [139]. On the contrary, Stoner *et al* bypassed the wash step for the separation of unbound dye and dye aggregates from EVs by optimizing dye and staining conditions [140].

Improved sensitivity and resolution over standard flow cytometers (sd-FC) have been demonstrated by several studies: for example, with the design of a standardized protocol using fluorescent beads, Robert *et al* have obtained an increase of 8- to 20-fold in microparticles derived from platelets count compared with sd-FC [141]. Another example is reported by Morales-Kastresana *et al*, who identified a strategy that robustly labels individual EVs using a high resolution flow cytometric system demonstrating its relevant sensitivity and specificity for EV detection. Indeed, the assay is able to identify artifactual shifts in the fluorescence of the background noise due to unbound label [142].

Also, Stoner and co-workers developed a custom flow cytometer with improved sensitivity for resolution of EVs, changing laser power, signal integration times and electronics. The limit of detection of this system was estimated to be 70–80 nm with a fourfold increase in instrument sensitivity that should translate to a twofold reduction in the detection limit to < 40 nm [140].

Similarly to hs-FC, nano-flow cytometry is an emerging tool and allows the direct and single-particle measurement of EVs. This technology has reached the resolution below 100 nm thanks to narrow laser beam, a more sensitive detector, and instrument configuration. For this reason, it has the capacity to cover the entire range of EVs, enabling a multi-parameter and quantitative analysis of individual EVs [143]. By nano-flow cytometry, Choi and colleagues reported the profiling of EVs from three different cancer cell lines (lung, breast, and epidermoid cancers) with the aim to investigate the expression of TF/F3 and EGFR, known to participate in cancer progression [144]. Instead, Salmond *et al* have optimized the analysis using this emerging technology to determine the levels of mammaglobin-a positive EVs in breast cancer patient unpurifed plasma, using mammaglobin-a as a breast cancer marker [145].

These recent advances in FC present new opportunities to detect the full EVs diameter range and subtypes and understand intercellular communication. However, in part due to the limitations of instrument detection and sensitivity and the lack of robust methods, numerous challenges have emerged during the analysis of EVs with FC. In order to overcome these issues, a consensus framework for the minimum information that should be provided concerning EV-FC, called MIFlowCyt-EV, has been drafted by researchers from ISEV, ISAC and ISTH [49]. This framework includes the MISEV guidelines [2] and MIFlowCyt (Minimum Information for Studies of EVs) standard [146], thus obtaining a specific and detailed document on the use of FC for EVs analysis. The main objective is not to draw up a specific guideline, but a template for the reporting of EV-FC results that should be applied to all works regarding this topic.

3.5. Fluorescence based approaches

Fluorescence is the property of a material to absorb light of a particular wavelength and re-emit it at a different wavelength, usually longer. In the field of EV research, fluorescence based methods have been widely used to track and characterize vesicles

taking advantage of fluorophores, i.e. organic dye molecules conjugated to antibodies or specific proteins, including green fluorescent protein (GFP) labelled proteins or produced by the EV-releasing cell. Most cells and vesicles exhibit no intrinsic fluorescence thus they are labelled providing information on the biochemical composition and on the cellular origin of EVs. Differently from the above cited vibrational spectroscopy techniques, fluorescence based method rely on a pre-existing knowledge about the antigen expressed by the considered vesicle. Moreover, fluorescent multilabeling analysis has limitations due, for example, to the non-specific binding of the fluorophore-conjugated antibodies or to the formation of fluorescent aggregates that cannot be easily distinguished from EV clusters [53]. In the following sections, we will focus on those fluorescence-based approaches that have shown the most promising application in EV diagnostics.

3.5.1. Fluorescent nanoparticle tracking analysis

Among the fluorescence based methods, fluorescent Nanoparticle Tracking Analysis (F-NTA) is a nanoparticle-based method for the characterization of the immunophenotype of EVs. Thanks to nanoparticles conjugated with fluorescent antibodies, F-NTA allows the detection of size, concentration, biochemical composition and cellular origin of vesicles at high speed. Differently from NTA which visualizes vesicles by light scattering using a light microscope and relates the brownian motion to particle size, this technique measures fluorescence light emitted by fluorophores associated to particles thus overcoming a limitation of conventional NTA that can accurately measure vesicle size and concentration, but cannot phenotype them.

To perform F-NTA, a high sensitivity camera is incorporated to the optical system in order to detect EVs labeled with specific fluorescent antibodies and allowing their phenotype [147]. Fluorophores are excited and only fluorescently labeled vesicles are tracked, demonstrating the ability of a technique typically used for nanoparticle characterization to be improved and effectively help EV applied research.

Different studies have used quantum dots for application with F-NTA, but sometimes they have presented obstacles, such as the diminished activity of the antibodies conjugated with quantum dots and the lack of protocols for the separation between EV and quantum dots. An improvement of these platforms was developed in a recent work showing that indirect immuno-labelling using quantum dots within the NTA platform offers a robust method that enables immunophenotypic EV characterization with estimation of the EVs concentration and size distribution [148].

3.5.2. Single-Molecular array

Over the past decade, it has become necessary to develop methods for the recognition of very low protein concentrations in order to identify new diagnostic protein markers and monitor disease progression.

Single-Molecular array (SiMoA), also called digital ELISA, is an ultrasensitive immunoassay that allows the detection of proteins via ELISA assay using a completely automated and easy-to use instrument.

The main difference compared to conventional immunoassays concerns sensitivity: mainstream immunoassays are limited to 10^{-13} M, while SiMoA has improved the sensitivity of over 1200-fold thanks to the ability to trap single molecules in femtoliter-sized wells [149]. This method, developed in 2012 by Chang and co-workers, is based on the isolation and the counting of individual immunocomplexes on paramagnetic beads [150]. Briefly, microscopic paramagnetic beads coupled with specific antibody capture the target proteins; then they are labelled with an enzyme capable of generating fluorescence linked to a second antibody, exploiting a multi-step immunosandwich approach. These complexes are suspended in a solution containing a fluorogenic substrate to the enzyme and finally loaded in femtoliter wells. White light images of the arrays are used to identify the wells that contained beads, while the emitted fluorescence is used to distinguish the presence ("on" well) or not ("off" well) of enzymatic activity in each well containing a bead. SiMoA signal is expressed in Average Enzyme per Bead (AEB), which is determined by counting the number of wells containing both a bead and a fluorescent product relative to the total number of wells containing beads. If the fraction of "on" beads (f_{on}) is < 0.7, the AEB is determinate digitally using the Poisson distribution; if f_{on} is > 0.7 the AEB is determinate with an analog approach using the average intensity of wells containing a bead in an array [151]. This technology has been widely employed for the detection of several protein biomarkers [150,152-154], but it was recently applied also in a SiMoA prototype targeting EV markers, allowing for the profiling of EVs and colorectal-cancer EVs directly from cancer cell culture supernatants or plasma from cancer patients [155]. This study highlighted higher EV levels in colorectal cancer patients compared with healthy people and benign tumor patients, demonstrating good performance of both the tumor-EV assay (Epcam-CD63) and the universal EV assay (CD9- CD63) for tumor diagnosis. Instead, in another work, this technology was employed to assess the enrichment of EVs detecting levels of the immune checkpoint protein, programmed death ligand 1 (PD-L1), from EVs within plasma in patients with non-small cell lung carcinoma and triple negative breast cancer [156]. In conclusion, the improvement in sensitivity shows by SiMoA using ELISA reagents, compared to the conventional immunoassay, resulted in a revolutionary approach for the measurement of critical concentration of protein biomarkers with possible diagnostic benefits.

3.6. Single Particle Interferometric Reflectance Imaging Sensor

The Single Particle Interferometric Reflectance Imaging Sensor (SP-IRIS) technology, which is mostly applied for the detection of viruses in the blood [157], is now another emerging technique in the field of EV. Indeed, it allows the dynamic detection of molecular binding of single EVs from biological fluids on a solid surface, combining microfluidics with immunodetection and interferometric imaging. The EV sample is injected onto a chip with a microfluidic system that allows the sample to pass over successive silicon chips coated with molecules that can recognize surface EV proteins. The bound EVs can be visualized by interferometric imaging where the signal results from the interference between the scattered field from the vesicle and the reference field of the substrate. In the EV field of research, SP-IRIS on the ExoView™ platform was developed for the digital and label-free counting and sizing of captured nanovesicles (≥50 nm) based on an interferometric principle [158-161]. Moreover, the SP-IRIS platform can be used to quantify EVs depending on the expression of EV marker proteins, by labelling the bound EVs with fluorescent antibodies and detect them by fluorescence based on co-localization of EV surface markers [162]. An innovative application of this technique was recently applied by introducing the use of membrane-sensing peptides as ligands to capture small EVs. The label-free, single-particle counting and sizing analysis was combined with fluorescence co-localization immune staining with anti-CD9, anti-CD63 and anti-CD81 antibodies, demonstrating a binding capacity of peptides to select EV higher than anti-tetraspanins antibodies [163].

4. Translation to clinics

The application of biophotonic methodologies to unmet need in EV-based research have allowed to overcome significant

limitations in terms of sensitivity and specificity, still, the experimental settings and models used to verify the applicability of the proposed techniques need further optimization to fit a real clinical scenario. In particular, the search for rare events, i.e. the complex combination of strictly specific biomarkers on single vesicle in the blood flow [164], requires the pure-preparation of EVs through laborious and time consuming isolation protocols that should be standardized to better fit the amount of samples (both numbers and volumes) of a diagnostic laboratory. The selection of the appropriate isolation method for EVs is crucial, especially when dealing with blood samples, to avoid the interference of non-EV particles in the analysis (i.e. lipoproteins), introducing artifacts and non-specific variability in the results [165]. Such goal can be reached by biophotonic-based platforms that owe remarkable high throughput features [166,167], but we have to mention that the limited purity degree of EV preparation has been often overcome by the use of specific markers, i.e. antibodies, for the functionalization of substrates or nanoparticles in the application of biophotonic-based platforms. Moreover, what has progressively emerged is the lack of gold standard procedures for EV isolation, quantification, detection and characterization. Some methods are becoming "de facto" gold standard because of the extent of their application and use, for example NTA for vesicle quantification [165] and Western Blot for protein detection, but the identification of specific biomarkers on EVs has no reference method for the proper validation. For these reasons, we would like to sensitize authors dealing with EVs in diagnostics to the importance of a proof of concept with real clinical samples in their works and to correlate the experimental data obtained by the application of biophotonic-based methods with approved and well-known clinical parameters, i.e. neuro-imaging data, soluble factor quantification in blood or clinical scales. What's more, the literature review proposed here has highlighted that authors often strive for considerable technological improvement, for high throughput methods that require expensive instruments and laborious sample processing, with the risk to hinder the translation to clinics. Indeed, the time required for the sample preparation and the repeatability of the pre-analytical processing are as crucial as the sensitivity of their analytical platform, thus the optimal combination of isolation protocol and high throughput analytical method is necessary. Moreover, the cohort selection is a central issue: usually the reported works in the field engage great effort in the technological development, but lack of a subsequent clinical validation step and correlation with current clinical scales, especially for neurological disorders of the elderly with high incidence of comorbidities.

Despite the great challenge, the wide literature described in previous sections, confirmed the potential application of biophotonic-based methods for the diagnostic analysis of vesicles, especially when they rely on commercially available instruments with proved application on cell and tissue diagnostic assays. The translation from the analysis of in vitro collected EVs to the vesicles isolated directly from patients affected by different pathologies, allowed the constitution of various accurate and sensitive diagnostic tools for the detection and monitoring of cancer, neurodegenerative disorders and diabetes.

4.1. Neurological disorders

Neurological disorders are a wide range of diseases comprising neurodegenerative and cerebrovascular disorders that often lack a straightforward diagnostic process and specific biomarkers, but can require time-consuming and invasive procedures to reach a definitive diagnosis. The possibility to isolate EVs of neural origin from blood [27,43,44] opened the way to a new era in the diagnosis of such diseases, especially for the possibility to gain insights in the complex processes occurring in the brain that is favored by the "leakiness" of the blood brain barrier that often accompanies the brain pathologies, increasing the amount of neural EVs transferred to the periphery [168,169]. The variegated nature of vesicles' cargo that includes proteins, oligonucleotides and lipids, has been used to design multiple molecular assays focused, for example, on the emerging role of miRNAs [170-172]. Still, results are often controversial because of limited overlap between the findings and lack of reproducibility for diverse reasons, i.e. isolation methods and sensitivity of the assay among the others [173].The biophotonic approach has the potentiality to go beyond the idea of a unique biomarker and to identify a complex biomarker that can be treated as continuous variable in relationship with brain damage.

Trying to take advantage of the multifaceted nature of EVs and not to rely on a single molecule as biomarkers, the biophotonic approach was tested on clinical specimens of patients affected by neurodegenerative disorders. In particular, the Raman analysis was successfully applied on EVs collected from peripheral blood for the bulk characterization of the vesicle signature in Parkinson's Disease (PD) and Amyotrophic Lateral Sclerosis (ALS) onset. Regarding PD, Gualerzi *et al* focused the attention on the prion-like spreading of misfolded α-synuclein through the body using EVs as vehicle of diffusion [174] (Fig. 2A). Following this hypothesis, they analyzed EVs isolated from serum of PD patients comparing the results with the one obtained from healthy controls. The applied methodology regarded the standard vesicles purification through size exclusion chromatography and ultracentrifugation, using a Raman-grade substrate for the bulk characterization of the vesicles isolated from the experimental groups. Multivariate analysis was adopted in order to create an automatic classification model able to deeply investigate the complex signals collected after the analysis. Beside differences between the two EV populations related to lipid and protein secondary structures, one of the principal spectral variations was attributed to the carbohydrates belonging to the EV glycocalyx, confirming the fundamental role attributed to the surface saccharides modification in the pathological states. An important further step was performed correlating the Raman data processed through multivariate analysis, with the clinical scales used nowadays. As results, the reliability of the Raman-methodology was confirmed providing additional support to the diagnostic and prognostic potential, identifying a statistical correlation with the clinical scales related to the degree of PD progression and to the motor impairment. A similar approach, using a Raman-grade substrate for the EVs bulk analysis, was subsequently adopted by Morasso *et al* for the characterization of the plasma EVs in ALS [175]. Once more, EVs (in particular large EVs isolated by ultracentrifugation) from the pathological samples presented differences compared to the healthy controls in specific spectral regions related principally to the protein and lipid structures. The data were confirmed through multivariate analysis, characterizing the Raman signature of the EVs circulating during the ALS onset. Peripheral blood from PD patients was the source of EVs separated by immunoaffinity assay and analysed by flow cytometry to investigate a specific immune profiling of EVs. Actually, this approach allowed the identification of 37 EV surface markers, 17 differentially expressed between PD and atypical parkinsonism. Differently from Raman spectroscopy, this type of analysis relies on the identification of single molecules, but what emerges is the need for a combined marker to achieve the best performances. Indeed, the receiver operating characteristic (ROC) curve calculated using linear weighted combination of the 3 markers showed better diagnostic performance respect to single markers [176].

Despite the great number of nano-plasmonic biosensors that have been developed in the last years, only few of them have been tested with clinical samples, whereas most of them foresee a diagnostic application based on *in vitro* results. Among those, a SPR platform based on multiple protein markers was tested for AD

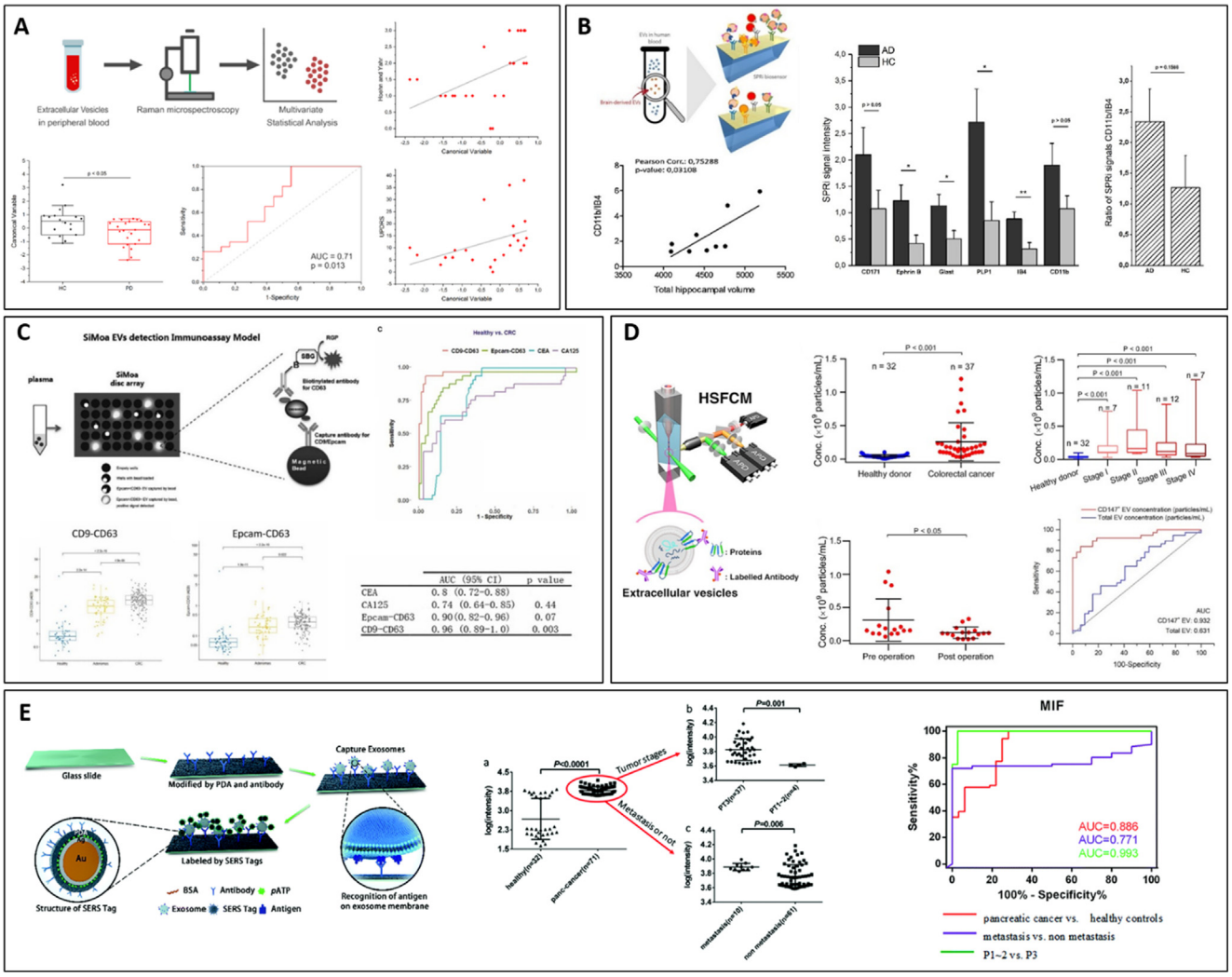

Fig. 2. Examples of works reporting the application of biophotonics-based techniques on the analysis of EVs in clinical samples. Selected works report reliable clinical conclusions, demonstrated by the calculation of the diagnostic potential (ROC curve values) and/or the correlation of experimental EV data with clinical parameters or scales. A: **Raman-based analysis of EVs.** The study was conducted on EVs isolated from serum of patients with Parkinson's disease (PD) and matched healthy controls (HC). The box plot represents the distribution of the canonical variable resulting from the classification model PCA-LDA showing that the means of HC and PD groups are significantly different; ROC analysis was reported as well as the correlation of Raman data with clinical scales. *Adapted with permission from* [174]. B: **SPRi-based analysis of EVs**. The study was conducted on EVs isolated from plasma of patients with Alzheimer's disease (AD) and matched HC. SPRi data demonstrate differences in the relative amount of brain-derived EVs between AD and HC subjects; the correlation between SPRi data (CD11b/IB4 ratio) and the total hippocampal volume obtained through MRI test was reported. *Adapted with permission from* [109]. C: **SiMoa technology for the analysis of EVs**. The study was conducted on EVs isolated from plasma of patients with colorectal cancer (CRC) by CD9-CD63 and Epcam-CD63 assays; ROC curves were obtained with CD9-CD63 SiMoa, Epcam-CD63 SiMoa and traditional serological markers (CEA and CA125) with best performance for the CD9-CD63 SiMoa. *Adapted with permission from* [155]. D: **Laboratory-built HS-FCM for the analysis of EVs.** Simultaneous detection of side scattering and two-color fluorescence of single EVs. The study was conducted on EVs isolated from plasma of patients with colorectal cancer (CRC) and HC. Differences were reported in CD147 + EVs concentrations in HC vs CRC patients, HC vs CRC patients at different cancer stages, and in CRC patients before (pre-operation) and after (7–10 days post-operation) cancer surgical removal were reported; ROC curves for CD147 + EVs and total EVs concentration *Adapted with permission from* [122]. *Copyright 2018 American Chemical Society.* E: **PDA chip and SERS tag for the analysis of EVs**. The study was conducted on EVs isolated from serum of patients with pancreatic cancer and HC. Differences were reported in the anti-migration inhibitory factor (MIF) in pancreatic cancer patients vs HC, P3-stage tumors vs P1–2-stage tumors, metastasis vs non-metastasis); ROC curves were calculated for MIF. *Adapted with permission from* [179] - *Published by The Royal Society of Chemistry.*

diagnosis [43,109]. The multiplexing ability of the SPRi-biosensor allowed the identification of EVs of neural origin from plasma, after size exclusion chromatography isolation by means of commercially available columns. SPRi data demonstrated significant variations in the relative amount and cargoes of specific brain-derived EV populations compared to healthy subjects. In particular, the activation phenotype of microglia EVs and the lipid composition of EVs were proposed as potential biomarkers of disease progression that could be correlated to the structural changes in the brain anatomy of AD patients [109] (Fig. 2B). Another interesting study was done by Muraoka *et al* who analyzed EVs isolated from the cerebrospinal fluid (CSF) of patients with cognitive and neuropsychiatric symptoms who are at risk for chronic traumatic encephalopathy caused by a history of repetitive mild traumatic brain injury. Muraoka *et al* performed a proteomic profiling of EVs, and in particular they used an updated version of SiMoA to quantify total tau and tau phosphorylated on threonine181 levels of CSF-derived EVs of patients and age- and sex- matched healthy subjects. Results showed a positive correlation between CSF t-tau, p-tau181 and EV t-tau, p-tau$_{181}$ levels in patients but not in the healthy subjects; these data together with other EV proteomic analysis have confirmed that CSF is a relevant biofluid for biomarker discovery with lots of EV-

Table 1
Summary of the main reported applications of biophotonic based methods for the diagnosis of neurological disorders. Only research articles dealing with clinical samples were considered.

Disease	EV source	EV isolation method	Analysis method	Cohort patients/Controls	Accuracy or diagnostic potential	Correlation to clinical scales	Ref.
Parkinson's disease (PD)	Peripheral blood (serum)	SEC (Izon qEv single) + ultracentrifugation (120,000 g 70 min 4 °C)	Raman spectroscopy	22 patients / 18 healthy controls	ROC AUC 0.71	UPDRS and Hoehn and Yahr	Gualerzi et al. [174]
	Peripheral blood (plasma)	10,000 g 15 min x2; 20,000 g 30 min at 4 °C	Flow cytometry	10 PD patients / 5 multisystem atrophy /5 atypical parkinsonism / 20 healthy controls	ROC AUC 0.908	MMSE and MoCa scores; disease duration; Hoehn and Yahr	Vacchi et al. [176]
Alzheimer's disease	Peripheral blood (plasma)	SEC (Izon qEV)	SPR imaging	10 patients / 10 healthy controls	–	Magnetic Resonance Imaging	Picciolini et al. [109]
Amyotrophic Lateral Sclerosis	Peripheral blood (plasma)	Differential ultracentrifugation	Raman spectroscopy	20 patients / 20 healthy controls	ROC AUC 0.64 (small EVs) ; ROC AUC 0.84 (large EVs)	–	Morasso et al. [175]
Chronic Traumatic Encephalopathy (CTE)	Cerebrospinal fluid	PS affinity method (MagCapture Exosome Isolation Kit)	SiMoA	15 patients at risk of CTE /16 healthy controls	–	–	Muraoka et al. [177]
Neurological disorder	Cerebrospinal fluid	No EV isolation step required	SP-IRIS	1 patient	–	–	Daaboul et al. [158]

associated proteins related to neurodegenerative diseases processes in early stages [177] (Fig. 2C). CSF was also the target for SP-IRIS approach that was tested, as proof of concept, to verify the ability of the method to identify EVs. Although no specific diagnostic need was envisioned, the significant improvement of the work was related to the possibility to detect EVs without pre-isolation processing [158].

4.2. Cancer

In the cancer field, we all strive for the search of new specific, easily accessible biomarkers that can be detected very early at the disease onset and thus save millions of lives all over the world. It is thus not surprising that huge literature has been published proposing innovative methodologies, including biophotonics, to detect EVs released by cancer cells in most biofluids, especially the accessible ones like blood, saliva and urine.

Indeed, among the biophotonic platforms, Raman-based approaches have been consolidated for the analysis of vesicles collected from patients affected by cancer, taking advantage and inspiration from the previously reported applications of RS in medicine and the commercially available instruments, already tested in the clinical setting. The principal aim for RS in EV diagnostics regards the characterization and identification of the pathological EVs biochemical features, which are compared with the physiological ones, for the creation of an automatic classification model able to firstly discriminate the signal collected from the different groups, and in some cases to stratify and monitor the pathological phenotypes and progression. When peripheral blood is considered as a source for cancer biomarkers, a promising Raman-platform has been developed and directly applied on plasma and serum of patients affected by prostate cancer and compared with the signals provided by healthy subjects and non-cancer patients (benign hypoplasia) [98]. The methodology regards ultracentrifugation steps for the isolation of the desired vesicles populations from the blood-derived samples, and consecutive drop deposition on Raman-grade substrates for the bulk Raman analysis focused on the biochemical modifications occurred in EVs collected from prostatic cancer. Despite the low number of samples collected, Krafft *et al* identified a significant reduction of alpha-helix proteins and enhancement of beta-sheet-rich proteins as specific signature of the prostate cancer circulating EVs [98]. In the same work, samples were processed and analyzed also by FT-IR obtaining comparable results, thus identifying differences in the protein components of serum and plasma EVs, especially in the secondary structures. Nonetheless only the Raman data and not the FT-IR spectra were used for the multivariate analysis and to test the ability of the spectroscopic analysis of blood EV to specifically recognize cancer patients from healthy subjects. Authors demonstrated that the Raman approach can detect variations in the EV content related to cancer and they speculate that the observed variations in circulating EVs could be possibly explained by systemic changes caused by progressive malignant disease [98].

Although PSA quantification is a routine diagnosis approach or prostate, novel reliable biomarkers are required for diagnosis improvement and reduction of over-diagnosis (high false positive results generated by benign prostatic hypertrophy, prostatitis or non-cancerous pathologies) and over-treatment. For this reason, multiple laboratories investigated the potential of EVs for prostate cancer diagnostic purposes. Plasma samples from patients were analyzed by FC, after accurate calibration of the instrument to set the lower limit of resolution of the size of the vesicles (110 nm). Double staining of vesicles with PSA and CD81, allowed the identification of a higher percentage of double positive vesicles in patients with prostate cancer compared to healthy controls and subjects with benign hypertrophy, although no accuracy of the

method was calculated [56]. A wider panel of antibodies was used to investigate EV content in plasma and urine of prostate cancer patients by FC and SPR. First of all, they report a 10-fold higher EV content in urine than plasma, but, looking at the antigenic profile, the same authors highlight the limitation of the considered FC method that cannot detect EVs smaller than 130 nm, but criticize also the SPRi platform as they encountered problems with the EV concentration. In this regard, authors avoided an EV isolation and concentration step that might have helped in the detection of EVs by SPRi, together with optimization of the SPRi platform as they suggest. The FC results found that lactadherin positive EV concentration in plasma was different between patients and controls, even though they cannot exclude influence of age and treatments for the observed results [111]. Similarly, FC was tested to verify its ability to diagnose colorectal cancer by EV detection on a much bigger cohort of patients (37 patients vs 32 controls), demonstrating the ability of the method to identify CD147-positive EVs isolated from plasma with excellent diagnostic potential as calculated by the ROC curve (AUC 0.932) [122] (Fig. 2D). The controversial FC results on clinical samples highlight one of the main limitations of the technique, i.e. the strict dependence on the type of instrument and on the preselection on the specific antigen.

This latter point can be overcome by the above cited spectroscopy methods (FT-IR and RS) that consider the whole spectrum

Table 2
Summary of the main reported applications of biophotonic based methods for the diagnosis of cancer. Only research articles dealing with clinical samples were considered.

Disease	EV source	EV isolation method	Analysis method	Cohort patients/controls	Accuracy or diagnostic potential	Correlation to clinical scales	Ref.
Prostate Cancer	Peripheral blood (plasma and serum)	Differential ultracentrifugation (12,000 g 1.5 h at 4 °C; 120,000 g 1.5 h at 4 °C)	Raman spectroscopy and FT-IR	4 prostate cancer / 4 benign prostatic hyperplasia / 1 bronchial carcinoma / 1 healthy control	–	–	Krafft et al. [98]
	Peripheral blood (plasma)	Differential ultracentrifugation (12,000 g 30 min; 110,000 g 1 h at 4 °C × 2)	Flow cytometry	15 prostate cancer / 15 benign prostatic hyperplasia / 15 healthy controls	–	PSA quantification assay	Logozzi et al. [56]
	Peripheral blood (plasma)	No EV isolation required only PBS dilution	Flow cytometry and SPR	5 prostate cancer patients / 5 healthy controls	–	–	Rikkert et al. [111]
	Urine	SEC (Izon, qEVsingle)	Flow cytometry and SPR	5 prostate cancer patients / 5 healthy controls	–	–	Rikkert et al. [111]
Colorectal cancer	Peripheral blood (plasma)	Differential ultracentrifugation (100,000 g 2 h at 4 °C × 2)	Flow cytometry	37 colorectal cancer patients /32 healthy controls	ROC AUC 0.932	Cancer stage	Tian et al. [122]
	Peripheral blood (plasma)	Differential ultracentrifugation (10,000 g 40 min; 100,000 g 2 h)	SiMoA	163 colorectal / 51 adenomas / 14 nasopharyngeal,, 20 breast / 20 renal cell cancer patients/ 5 lung cancer patients / 20 healthy controls	ROC AUC 0.96 for CD9-CD63; ROC AUC 0.90 for Epcam-CD63	–	Wei et al. [155]
Ovarian and endometrial cancer	Peripheral blood (serum)	Differential ultracentrifugation (10,000 g 30 min at 4 °C; 120,000 g 70 min at 4 °C × 2)	Raman spectroscopy	3 endometrial cancer/ 3 ovarian cancer / 2 healthycontrols	Accuracy 99.4% (PCA-hierarchical clustering)	–	Rojalin et al. [178]
Ovarian cancer	Ascites samples	Filtration (0.2 μm membrane filter)	SPR (nPLEX platform)	20 ovarian cancer patients/ 10 non-cancerous patients (cirrhosis)	ROC AUC 0.968 for EpCAM; ROC AUC 0.90 for CD24; ROC AUC 0.995 for EpCAM + CD24	–	Im et al. [106]
Pancreatic cancer	Peripheral blood (serum)	Ultracentrifugation (120,000 g 4 h x2)	SERS	71 pancreatic cancer / 32 healthy controls	ROC AUC 0.886 for MIF-SERS platform	Tumor Node Metastasis (TNM) classification stages	Li et al. [179]
Lung cancer	Peripheral blood (serum)	Total Exosome Isolation Reagent	SPR	5 lung cancer patients / 5 healthy controls	–	–	Liu et al. [107]
	Peripheral blood (plasma)	Differential ultracentrifugation (10,000 rpm for 45 min at 4 °C; 50,000 rpm 1 h at 4 °C × 2) + total RNA isolation (miRNeasy kit)	SERS for miRNA detection	3 lung cancer patients / 3 healthy controls	–	real-time PCR	Ma et al. [180]
Oral Cancer	Saliva	Differential ultracentrifugation (12,000 g 20 min, 120,000 g 70 min at 4 °C)	FT-IR	21 oral cancer patients / 13 healthy controls	Accuracy 88.89% (PCA–LDA with cross-validation)	–	Wong at al [181].
Multiple Myeloma	Peripheral blood (serum)	Serial centrifugation (16,000g 45 min; 100,000g 2 h) + discontinuous sucrose gradient + ultracentrifugation (100,000 g 16 h at 4 °C)	SPR	10 multiple myeloma/ 5 monoclonal gammopathy / 10 healthy individuals	–	–	Di Noto et al. [105]

and the overall complex biochemical composition of EVs, the so-called fingerprint, as a biomarker. Such methods found further improvement in the clinical application by the introduction of the computational approaches to decrypt the complex and numerous information provided. An elaborate example was presented by Rojalin *et al* in the discrimination of ovarian and endometrial cancer using the Raman analysis of serum EVs [178]. The SERS effect was induced using a nanoporous silicate scaffold functionalized with silver nanoparticles, achieving beside the enhancement of the Raman signal, also the filtration and immobilization of EVs from complex solutions. The further enzymatic treatment, allowed the collection of the Raman spectra from distinct EV sections analyzing the lipid, protein and extraluminal domain of glycocalyx corona. This last component was identified as the main responsible for the clinical significance, confirming the ability of the Raman-based approaches not only to distinguish the whole signature of the biological components, but also to attribute a specific loading to each component. Despite the reported remarkable internal heterogeneity within one EV isolate that might reflect chemically defined subpopulations of EVs or a platform intrinsic limitation, the control and cancerous samples were distinctly classified. SERS analysis was used also to detect cancerous EVs in the blood of patients with pancreatic [179] (Fig. 2E) and lung cancer [180]. While the first one set up a sandwich immunoassay with nanoparticles for signal amplification with excellent diagnostic ability compared to standard ELISA assays, the latter takes advantage of SERS to identify a specific miRNA, miRNA-21. Ma and colleagues started from the assumption that the limited concentration of cancerous EV-miRNA in blood, thus the SERS effect can facilitate the amplification of signal and improve sensitivity of the assay. Authors combined a stable SERS reporter element made up of metallic nanoparticles with a duplex-specific nuclease-assisted signal amplification for miRNA detection. After miRNA binding to the DNA probe, the nuclease cleaves the heteroduplex releasing the SERS reporter element to be detected. The amplification of the signal allowed to reach a detection limit as low as 5 fM that makes the assay feasible for the detection of miRNA from blood EVs. Actually, the methodology was successfully tested, using limited volumes of patients' samples, even if it still needs further optimization in order to obtain higher selectivity and sensitivity [180].

Vibrational spectroscopy coupled to machine learning techniques were used also to build discrimination models for the diagnosis of oral cancer. In particular, EVs from whole saliva samples of oral cancer patients and healthy individuals were analyzed by FT-IR. Actually, saliva is an excellent biofluid for diagnostics, it includes multiple different molecules that undergo active and passive processes of transport from oral cavity cells, salivary glands and plasma to saliva, thus representing potential source biomarkers with the advantage of the possibility to collect sample with non-invasive procedures. Besides, the reduced amount of proteins in saliva compared to plasma or serum, makes saliva an easier substrate for FT-IR. The PCA–LDA discrimination model correctly classified the samples with accuracy of 95%, and the SVM showed a training accuracy of 89% after cross-validation accuracy of 89% [181].

Trying to limit sample preparation and to use commercially available instruments that were proved to be suitable for a clinical setting, SiMoA technology was used to detect EVs from plasma of colorectal cancer patients. Thanks to only two pairs of antibodies, CD9-CD63 and Epcam-CD63, authors analyzed a significant number of clinical samples looking for Epcam positive EVs released by colorectal cancer cells. The ROC curves were calculated to evaluate the diagnostic performance of the assay, demonstrating an excellent performance of the proposed platform, that significantly ameliorated the diagnostic potential of traditional biomarkers [155]. To the best of our knowledge, this is the only reported work with a significant cohort of clinical samples that can be considered reliable in its clinical conclusions.

Among the multiplexing biosensor developed in the last years, nPLEX system and its next-generation version were used to investigate the diagnostic application for ovarian cancer and pancreatic ductal adenocarcinoma [106]. Data obtained from the characteri-

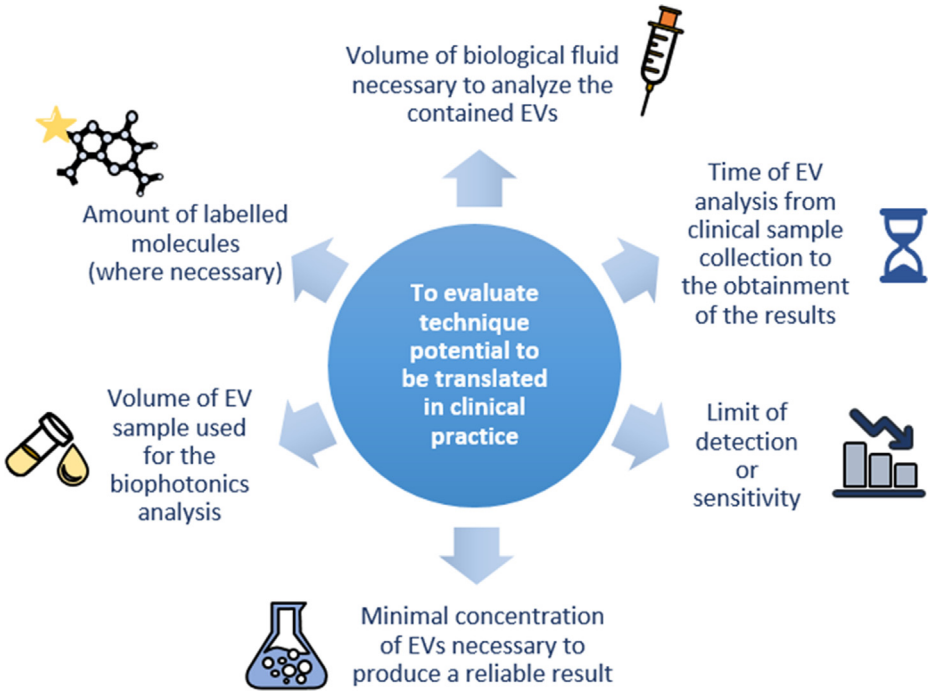

Fig. 3. Helpful technical information for the comparison between methodologies, allowing the evaluation of technique potential to be translated in clinical practice.

zation of EVs isolated from ascites of patients by identification of EpCAM and CD24 on EV surface, demonstrated the excellent accuracy of the method as determined by the ROC curves (accuracy of 93% for EpCAM and 87% for CD24). On the contrary, considering peripheral blood as accessible EV source, SPR biosensors were also used for lung cancer [107] and multiple myeloma [105]. In the latter case, the SPR biochip was functionalized with heparin, demonstrating higher binding of EVs from serum of multiple myeloma compared to those extracted from specimens of healthy individuals and patients with monoclonal gammopathy of undetermined significance [105]. Despite sustaining the potentiality of biosensing in the characterization of EVs and the possibility for a quick and reliable label-free sorting of multiple myeloma EVs, the proposed methodology was tested after laborious and time consuming EV isolation and would require a validation on EVs purified by means of a faster procedure thus confirming its potentialities in diagnostics.

4.3. Other diseases

Despite the broad interest and potential application, only Roman *et al* analyzed bulk EVs isolated from urine of patients affected by type 2 diabetes mellitus and from healthy subjects by RS with the aim to verify differences in biochemical structure of the two EV populations able to diagnose the pathology onset and to obtain information regarding the pathological mechanism [182]. The Raman analysis was successfully applied to identify different EVs biochemical features between the two groups, in particular the study highlighted an abundance in phenylalanine rich-protein in the pathological EVs and a significant difference in the protein secondary structures (Amide I) present in the vesicles. As result, Roman *et al* proposed a new diabetes mellitus biomarker able to provide significant information regarding the hyperglicemic state and renal complications.

5. Conclusions

Biophotonics is a growing field of research to tackle the gap between clinics and technological development. The strong interdisciplinary of biophotonics fosters a gold mine of ideas, discoveries and innovation in the EV biomedical field. Up to now, conventional methods were shown to be only partially effective in EV phenotyping and analysis with one of the major obstacles relying on the sensitivity and reproducibility of results. Contaminants, low EV concentration, limited discrimination between single and clustered vesicles and laborious protocols are only some of the limits imposed by current diagnostic methods. The biophotonic-based approaches described in the present review have in most cases managed to ameliorate the discrimination between nanovesicles and contaminants in the clinical sample by the enrichment of specific EV population or by increasing the performances of the proposed methodologies, in terms of higher resolution compared to traditional procedures or both, leading to moderate ore even excellent diagnostic accuracy. We believe that all the proposed and herein described biophotonic methods have great potentialities, especially those like RS, SPR and SiMoA that have already a long history of application on clinical samples for diagnostic purposes. Beside the outstanding sensitivity, platforms like RS and SPR have two major advantages: i) limited amount of sample required; ii) limited pre-processing procedure. As many of the authors highlight in their papers, the amount of vesicles needed for the analysis consists in few microliters (2–5 μl). Despite some authors proposed complex purification procedures, most of them (Table 1 and Table 2) refer to differential ultracentrifugation as preferential procedure. Such method is certainly time-consuming and brings to limited EV yield, but it is also known for its limited purification ability [68,183]. For these reasons, some commercially available and automatable methods, like size exclusion chromatography and tangential flow filtration, might be considered in the future as more reliable and reproducible procedures for EV isolation and purification, to replicate the reported results, possibly with better results.

Of course, biophotonics cannot find solutions to all of the limitation of current isolation methods, still we believe that the herein considered works demonstrate the possibility to identify a common guideline for specimen handling and EV isolation, a protocol that could be used as SOP for different types of analyses, without the need for an extremely pure EV preparation. Actually, most of the methods tested on clinical samples (Table 1 and Table 2) have demonstrated their remarkable performances on relatively simple, even though laborious and time consuming, isolation protocols (mainly differential ultracentrifugation), suggesting the possibility to propose a common SOP, translatable to clinics, that would also simplify the comparison of results. If effort would be made in defining an easy SOP (not laborious, not expensive, possibly automatable, with good yield) and authors would accept to adhere to it, their technological effort in developing high throughput and innovative methods would be rewarded by clearance on any EV isolation doubts and a more straightforward demonstration of applicability. Effort should be spent also in the transparent and detailed reporting of technological parameters, like for example detection limit, resolution, required sample volume, etc. We suggest here a check list for the accurate reporting of technological parameters and experimental requirements that would be crucial for the comparison of methods and the acceleration of the translation to clinics (Fig. 3).

Besides, it is worth noting that the Raman based platforms can rely on the availability of commercially available portable instruments that can be considered affordable for any diagnostic laboratory. Of course, not all the reviewed approaches can apply to portable instruments. For those methods where a more sophisticate instrument is required, we suggest careful considerations about the balance between high resolution, time-consuming procedure and cost-effectiveness. In the RS field, the bulk vs the single EV characterization is an example of this controversial concept: while the single EV approach guarantees higher resolution and specificity, a measurement takes considerable time (with the current state of the art, obtaining Raman spectra from 1000 vesicles would take hours [171]), whereas the bulk approach obtains information from clusters of vesicles, with limited acquisition time and possibly with portable cost-effective Raman instruments, thus with higher translatability. Similarly, the SPR based biosensors rely on expensive instruments for the analysis, but the proposed platforms can be scalable with a large number of sensing units integrated into a single chip, they are usually label-free (i.e. no need for secondary labeling) or, in some cases, inexpensive metallic nanoparticles can be used for signal enhancement reducing the amount of required reagents. Indeed, the huge potentiality of the SPR/SPRi technique for the EV studies could be expressed through the development of a SPR biochip and a kit with all the necessary reagents including the ones for the signal amplification, customized and available for a specific EV analysis. The production of these SPR-based arrays, created with an optimized and standardized protocol, could allow to bring the technology into a clinical setting. Moreover, the integration of microfluidic systems on SPR sensing platform could be a possible solution for the development of point-of-care (POC) devices, thanks to the advantages of automation, that allows improved reproducibility and precise control over each reaction step, small volumes, rapid processing and potentially enhanced sensing efficiency increasing the throughput of a single SPR chip with a proper design [184].

Still, we believe that the variability in the considered EV isolation methods, the controversies about the EV subtype of interest (small or large EVs) and the relative proportion of different EVs in a given sample [185], together with the "habit" for those working in a clinical setting to look for a specific pre-determined protein, genetic, or lipid biomarker is slowing the evolution and application of biophotonic platforms to diagnostics. For example, concerning the isolation method, the "standard" differential ultracentrifugation procedure has slight modification in every laboratory, limiting the comparison of data and sensitivity results from different platforms [45]. Most of the reported biophotonic platforms were tested on simplified experimental settings, mainly cell supernatants, and when their efficacy on complex biofluids was tested, just few samples were considered. In particular, the selected cohort of patients rarely allows for the calculation of the diagnostic potential of the proposed methodologies.

To make a step forward in the diagnostic application of biophotonic platforms, we underline the need for i) proper selection of specimens; ii) adequate selection of patients' cohort and correlation with current clinical scales; iii) easy and automatable EV isolation protocol that can fit the routine of a diagnostic laboratory; iv) reduced time of the analysis.

Moreover, the optical technologies can generate a large amount and many types of biomedical data. The availability of these biomedical big data can push the advance of machine learning in biology, medicine and pharmaceutical science.

The future of biophotonics application in EVs studies is bright and promising.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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