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Advances in Exosome Quantification Techniques

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Abstract

Accumulated evidence has indicated that exosomes play a vital role in many biological processes such as intercellular communication, antigen presentation, and waste management. Since the fingerprints of exosomes resemble their parental cells, they are widely recognized as next generation biomarkers for disease diagnosis, prognosis, and therapy. Being endogenous in nature, exosomes also present themselves as an exquisite vehicle for the delivery of therapeutic cargoes because of their immunological inertness and exceptional ability to elicit potent cellular responses. Therefore, the quantification of exosomes is crucial in facilitating exosome research and application. In this article, we summarize the progress in exosome quantification techniques with representative examples to illustrate their working principles with some discussion on challenges and prospects. We hope that this article will offer an overview of exosome quantification techniques and open up new avenues towards the development innovative concepts and instruments for exosome research and applications.

1. Introduction

Exosomes are membranous vesicles of endocytic origin excreted from cells containing multivesicular bodies when the membranes of these multivesicular bodies fuse with plasma membranes [1-3]. Exosomes have diameters ranging from 30 to 150 nm and densities between 1.13 and 1.19 g/mL [4,5]. The membrane of exosomes is made up of lipids and proteins, while proteins and nucleic acids such as DNA, messenger RNAs, microRNAs, and other non-coding RNAs are found in their lumens [6]. There are several different types of proteins present on exosomal membranes. These proteins are important in exosome isolation and quantification as they serve as recognition sites for specific protein binding. Surface proteins on exosomal membranes, for example tetraspanins, act as antigens for antibody binding. This specific interaction between antigen and antibody is then exploited in immunoaffinitive approaches for extraction and/or quantification of exosomes. To date, it has been confirmed that exosomes can be produced by most cell types in human body [7]. They can be found in most body fluids if not all, such as blood, urine, breast milk, and semen [8-13]. When examined under

an electron microscope, as illustrated in Figure 1 [14], exosomes have a characteristic cup-shaped morphology appearing as flattened spheres.

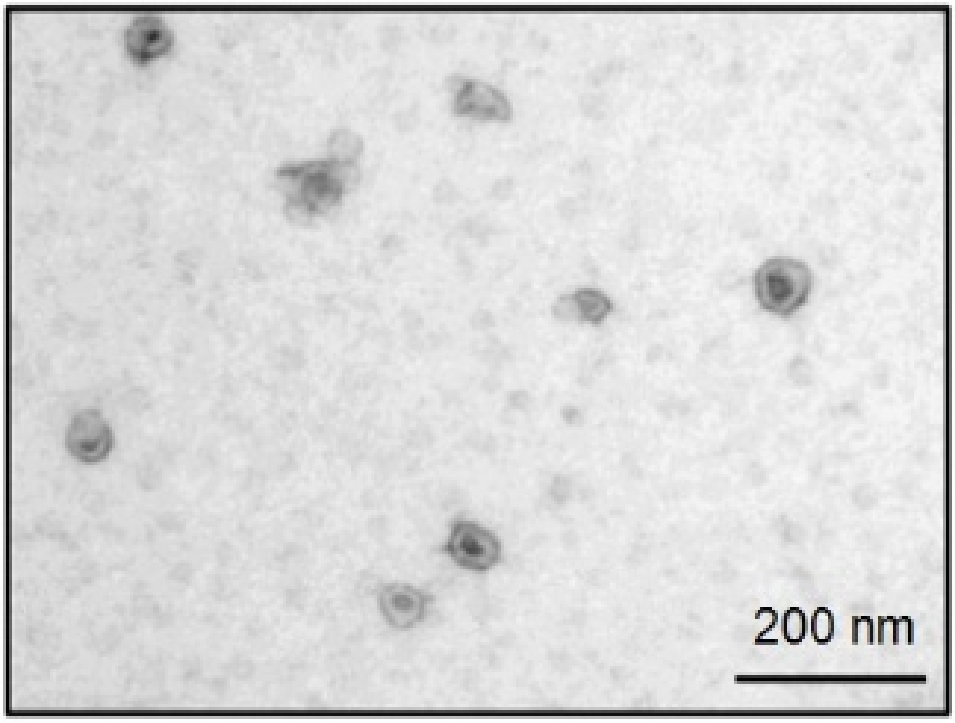

Figure 1. A scanning electron microscopic image of exosomes.
(Reproduced with permission from ref. 14.)

When exosomes are circulated in body fluids, they serve as shuttles for RNAs [2]. This allows functional information to be conveyed to neighboring cells or distant cells, thus promoting intercellular communication without cell-to-cell contact [15]. Reliant on their parental cells, exosomes carry a variety of specific nucleic acids and proteins to perform various functions. Exosomes secreted by immune cells, for instance, play an important role in antigen presentation; hence they are involved in cell signaling and exhibit anti-inflammatory properties [2]. On the other hand, exosomes secreted from tumor cells carry nucleic acids which are responsible for angiogenesis, cell proliferation, and cell survival [16].

A molecular biological approach to study the functions of exosomes is to investigate their multiple roles in transforming normal cells into cancer cells in the process of cancer formation, known as tumorigenesis. Exosomes take on a surprising number of roles, which are schematically depicted in Figure 2 [17]. Using tumor-derived exosomes as an example, the translation of the genetic information that they carry into cellular activity first turns healthy cells into a tumor microenvironment that triggers and enhances tumor cell proliferation. Next, due to their stability in body fluids, especially in blood, exosomes are also able to travel to distant cells to promote angiogenesis. Moreover, the immune system in the body is also affected by exosomes which block tumor recognition and impair the antitumor response system by inducing apoptosis of T-cells. In addition, it was observed that exosomes are

capable of recruiting bone marrow-derived cells to tumor and pre-tumor tissues for further cancer development [17]. This also explains why at most of the time the level of exosomes in cancer cells is much higher than that found in healthy cells.

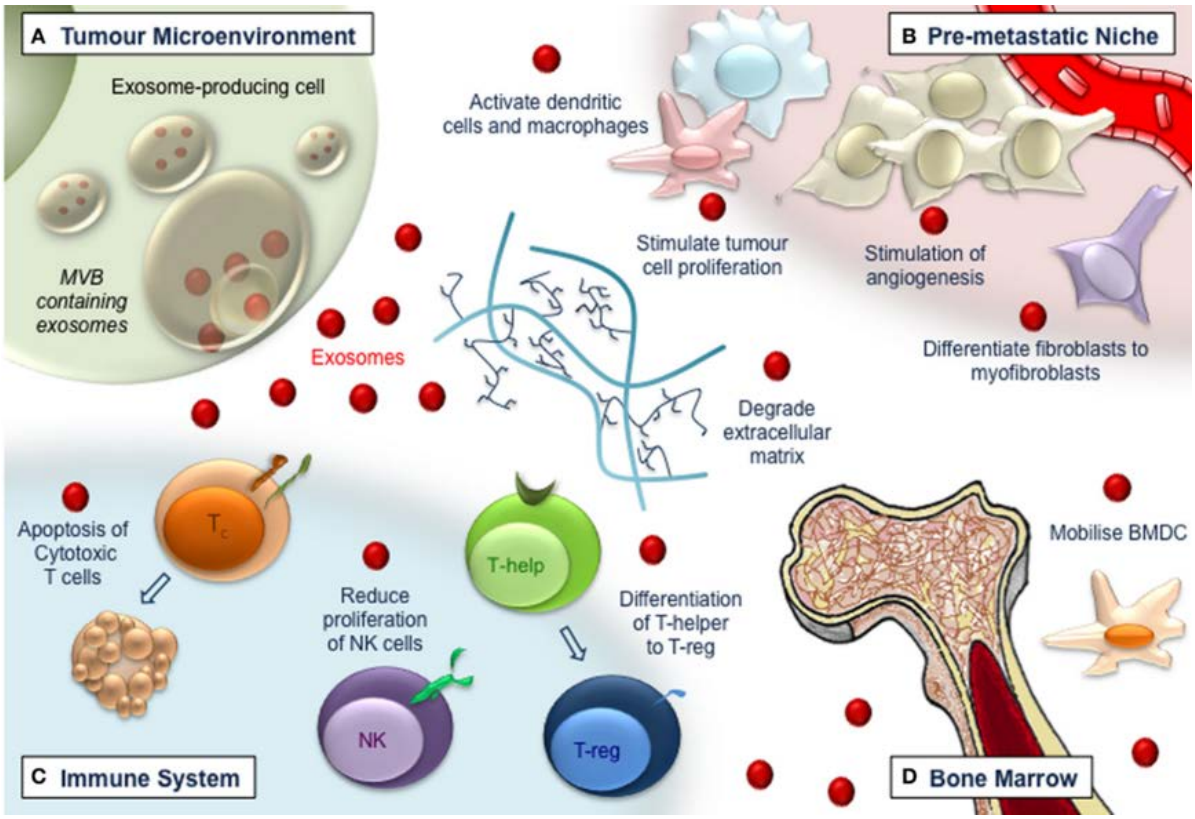

Figure 2. Schematic illustration of Exosomes (red spheres) performing multiple functions in cancer formation – tumorigenesis (Reproduced with permission from ref. 17.)

Exosomes have received much attention in the last decade because of their potential applications in molecular biology, biomedicine, and beyond. The acellular (non-living) mode of communication in exosomes, transport molecules involved in various cellular activities, and easy recovery from body fluids has made them stand out for diagnostic and therapeutic purposes [18]. It is worth mentioning that exosomes reflect RNA and protein contents in cells from which they are originated. Therefore, they are potentially useful for diagnosis and prognosis, as well as real-time monitoring of therapy by measuring the level of exosomes in body fluids [19]. As such, conventional needle biopsy which is invasive and painful can be alleviated if exosomes can be successfully used as biomarkers for diagnostic purposes. In the case of cancer, as compared to circulating tumor cells which are released only in small quantities, exosomes are secreted in large quantities into the circulation system [20]. The increased excretion of exosomes by cancer cells and their accumulation within the body is a key indicator in the malignant transformation process. This is well supported by the investigation conducted by Douglas and Taylor [21]. Due to their high abundance and good stability in body fluids, especially in blood, exosomes are being evaluated as a powerful and reliable source of circulating biomarkers for cancer [20] and are successfully used as biomarkers for several diseases, such as breast cancer, ovarian cancer, acute

kidney injury, Sjogren's syndrome, etc [22]. In more recent studies, exosomes are also being tested as potential biomarkers for other diseases, for instance, central-nervous system disorders like Alzheimer's disease [23]. Also, attempts have been made to transform exosomes into a promising therapeutic vaccine against cancer via antigen presentation and T-cell activation [24,25].

As a direct consequence of intensive research efforts, exosomes have now been widely considered as the next generation biomarkers, possibly serving as a liquid biopsy for many diseases and medical conditions since they largely reflect both the genetic and phenotypic status of their parental cells. Furthermore, exosomes provide an invaluable platform for clinical management of diseases because they bear the resemblance of their parental cells. For example, some potential applications include utilizing exosomes to assess the progress of pathology of many diseases and medical conditions like cancer and neurodegeneration, among others. As the first step towards a better understanding of exosomes and their association with various diseases and cancer in particular, exosomes have to be accurately and reliably quantified. However, their ultrasmall size poses significant challenges to the accuracy and reliability of their identification and quantification. Since exosomes are too small to be directly examined under an optical microscope or a flow cytometer [26], special considerations are required during the characterization and quantification of exosomes. For example, when attempting to detect exosomes by using a flow cytometer, swarms of exosomes are usually taken by the flow cytometer as single exosomes instead of true single exosome detection. In other words, multiple exosomes are simultaneously illuminated by the laser beam and counted as a single event signal, thus resulting in the underestimation of the concentration of exosomes [27]. Moreover, the development of exosome quantification techniques is further limited by their low refractive index and broad size distribution [28]. In the early days of exosome research, electron microscopes were frequently employed in the identification and morphological examination of exosomes. Even though electron microscopes still prove useful in direct visualization of exosomes and are engaged in laboratory practice, their high cost, bulky size, and stringent requirements of sample preparation have urged researchers to develop novel strategies and instruments/devices, hoping to circumvent the limitations of electron microscopy. In the following sections, different types of exosome quantification techniques and their applications will be discussed with typical examples to illustrate their working principles and possible advantages they could bring to exosome quantification along with some discussion on challenges and perspectives. It is anticipated that this article will serve as an entry point for those who are interested in developing more innovative concepts and strategies to more accurately and reliably quantify exosomes for uses at point-of-care.

2. Exosome Quantification Techniques

Prior to quantifying exosomes in biological samples like culture media and biological fluids, necessary isolation and concentration steps are often carried out first, because other than exosomes,

there are cell detritus and a few other types of membranous vesicles present.[3] The isolation of exosomes is generally accomplished by ultracentrifugation, ultrafiltration, precipitation, or immunoaffinity-based exosome isolation [29]. Unfortunately, most of these exosome isolation techniques are still under development with limited efficiency [30]. Nonetheless, the success of a reliable exosome quantification largely depends on the quality of the isolated/concentrated exosomes. Amongst all exosome isolation techniques developed, ultracentrifugation, occasionally in conjugation with density gradient centrifugation, is currently considered as the gold standard. It is, however, not without limitations. Since exosomes are extremely sensitive to most of the operating parameters of ultracentrifugation, it is practically impossible to optimize and standardize all of them to compile a set of standardized protocols, not to mention the large varieties of sample composition. In addition, the effect of a very long period of ultracentrifugation on the integrity of exosomes remains unclear. Experimental evidence has already indicated that substantial amounts of exosomal proteins and RNAs are lost after ultracentrifugation [31,32].' On the contrary, with the capability of isolating pathology-specific exosomes, immunoaffinitive approaches appear to be able to preserve the integrity of exosomes. However, their high cost, low efficiency, and low capacity greatly hinder their wide-spread applications [33]. For instance, in order to successfully isolate exosomes, expensive antibodies that target specific exosomes must be engaged. However, due to cell heterogeneity, some cells of the same type may not express that target. Consequently, the exosomes secreted by those cells will not be targeted by the antibody, thereby leading to a low isolation efficiency. Other factors such as antigen modulation and the blockage of antigenic epitopes also affect the isolation efficiency. The limited efficiency in purification made the measurement of the number and size distribution of exosomes more challenging. Despite increasing interest in exosomes, the progress of exosome research has been largely hampered due to a lack of standardization and poor consensus on various methods for the isolation, quantification, and characterization of exosomes [34,35].' Therefore, great care must be taken when selecting exosome isolation techniques.

In principle, exosome quantification relies either on their characteristic physical properties like size, mass, and density, or on membrane proteins presented on their surface. In the following section, we will primarily focus on various techniques developed based on these properties for the quantification of exosomes. They are immunoaffinity capture (IAC) which is well known for its highly specificity; asymmetrical-flow field flow fractionation (AF4) coupled with a multidetection system which is the newest technique among all; nanoparticle tracking analysis (NTA) which is the most popular technique capable of detecting the size range between 30 to 1000 nm [36]; dynamic light scattering (DLS) which is an user friendly and universal method; and lastly surface plasmon resonance (SPR) which is a real-time, fast, and specific quantification technique. Each technique has its own advantages and drawbacks. Therefore, it is very important to consider the background information of target exosomes like what types of antigens present and the expected results when evaluating the suitability of the technique to be

utilized for quantification. We will end this article with a comparison of the five groups of techniques discussed as well as the challenges and perspectives of exosome analysis.

2.1 Immunoaffinity capture (IAC)

IAC is a technique that utilizes highly specific immunoaffinitive interactions between the characteristic surface proteins (antigens) present at exosomal membranes and labeled antibodies added externally [37]. An example of a commonly used target protein is tetraspanin [38]. Antibodies which are labeled with either signaling tags (detection antibody) or anchored on a solid support (capture antibody) are then exposed to a sample containing exosomes. Successful binding of the labeled antibodies to the antigen aids in readout by either showing a difference in color intensity or producing a fluorescence signal for quantification.

Enzyme-linked immunosorbent assay (ELISA) based IAC often leads to enhanced specific capture of exosomes with much increased efficiency in recovery of pure and intact exosomes from body fluids [34,35]. It allows isolation, quantification, and characterization of exosomes. The type of ELISA that discussed here is a sandwich ELISA which is the most commonly used variety of ELISA. In this assay, as shown in Figure 3, the exosomes to be analyzed are sandwiched between two antibodies: the capture antibody and detection antibody [39]. The four key steps in ELISA include coating, blocking, capturing, and readout. Coating of an immunoassay plate with a specific antibody and blocking of unbound sites on the plate by a totally unrelated protein allow exosome capturing; while the addition of enzyme-linked, usually horseradish peroxidase (HRP), secondary antibodies or antigens (e.g. anti-CD9, anti-CD63, and anti-CD81) for specific binding to the target antigen or antibody on the exosomes allows for signal amplification and detection. By exploiting enzymatic reactions, analytical signals are amplified after adding substrates of the enzymatic reactions. The readout is usually performed using a microtiter plate reader [34,40].

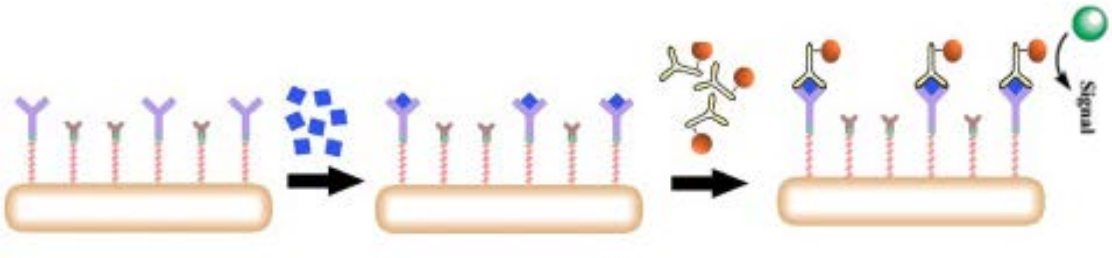

Figure 3. Schematic illustration of sandwich ELISA.

More recently, time-resolved fluorescence immunoassay (TR-FIA) against commonly expressed transmembrane proteins CD9 and CD63 was developed by Duijvesz et. al. to study exosomes as diagnostic biomarkers for prostate cancer [29]. CD9 and CD63 are transmembrane proteins that are seven to ten times abundant in exosomes and are often use as universal biomarkers for exosomes

[41,42]. Unlike ELISA, the first batch of antibodies against these proteins are biotinylated for capturing exosomes while the second batch is labeled with a europium complex for time-resolved fluorescence detection. As such, TR-FIA does not require an enzymatic reaction. In addition, as the analytical signal is fluorescence, a lower background signal and a longer fluorescence lifetime emitted by the europium complex achieved higher sensitivity and a wider dynamic range than those of conventional ELISA [43].

Because of their great potential in diagnostics, prognostics, and therapeutics, there has been a great demand for portable devices in order to bring exosome analysis to clinical practice at point-of-care. Researchers have devoted great efforts to search for such devices that are capable of delivering results in a timely manner with great affordability to replace research-type bulky and expensive instruments. In this regard, fully-integrated microfluidic and electrochemical devices have been developed and they are capable of meeting the above-mentioned criteria of routine clinical tests of exosomes at point-of-care. The attractive features of microfluidic and electrochemical devices are their inherently high portability and compatibility with microfabrication technology, simplicity in operation, and high selectivity and sensitivity when coupling with IAC and signal amplification strategies. A good example of this type of electrochemical devices is personal glucose meters currently employed in diabetic homecare.

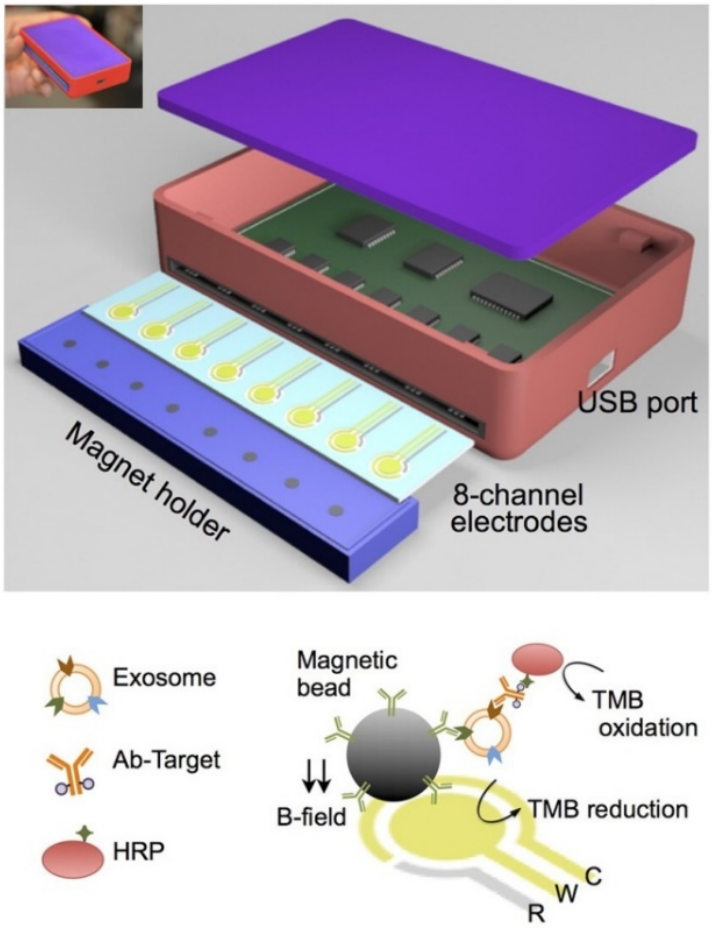

Figure 4. Schematic representation of iMEX and its exosome sensing mechanism. (Reproduced with permission from ref. 44. Copyright (2016) American Chemical Society.)

Working in this direction, Jeong and co-workers recently fabricated a hand-held electrochemical device for exosome analysis – integrated magneto-electrochemical exosome (iMEX) platform (Figure 4) [44]. A unique feature of the iMEX platform is the integration of exosome isolation and detection into a single platform. The workflow of the iMEX is as follows: exosomes in cell-free body fluids such as serum, plasma, and urine are first selectively captured by magnetic beads coated with an exosome-specific antibody – antitetraspanin transmembrane protein antibody. A signal unit – a secondary antibody tagged with HRP – is introduced to the captured exosomes after the magnetic beads are held on the sensing units and a quick washing. Amplified electrochemical detection is realized through the electroreduction of enzymatically oxidized 3,3′,5,5′-tetramethylbenzidine. The iMEX platform takes advantages of the highly specific capturing ability of IAC, the ease of magnetic separation, and enzymatically amplified electrochemical detection. Particularly, the use of the magnetic beads greatly simplifies the assay procedure since sample matrix and excess of reagents can be conveniently removed by a simple wash and the captured exosomes are exclusively concentrated on the sensing units. To enhance its capability, eight independent electrochemical sensing units (electrodes) are integrated in the iMEX platform with an overall cost of less than $50. It was demonstrated that the analytical performance of the iMAX platform is comparable to a commercial instrument with a correlation coefficient of 0.93. In addition, the iMEX platform is capable of completing the entire procedure within 60 min with a sensitivity of less than 10^4 exosomes in as little as 10 μL of samples [44]. The iMEX platform offers several attractive features such as its high portability, multiplexing capability, direct analysis of specific exosomes in pristine samples without any sample pretreatments, high selectivity through immunoaffinitive enrichment, and high sensitivity through amplified electrochemical detection. With further development, the iMEX platform could be a powerful platform for routine exosome analysis in clinical settings at point-of-care, thus deepening our understanding of cancer biology and offering a viable tool for cancer management.

In another attempt, Zhao et al. fabricated a microfluidic chip for fluorescent detection of exosomes after magneto-immunoaffinitive isolation [45]. Similar to the iMEX platform, exosomes in untreated cell-free biofluids are directed fished out by exosome-specific antibody coated magnetic beads. Instead of the HRP-tagged secondary antibody, this time fluorophore-tagged secondary antibodies are utilized to perform the readout of the captured exosomes. Similarly, the engagement of the magneto-immunoaffinitive exosome isolation greatly simplifies the assay procedure, enabling on-chip isolation and enrichment of exosomes with multiplexed detection. After a thorough optimization, the microfluidic chip delivered a detection limit of 7.5×10^5 exosomes/mL with a sampling capacity from 0.01 to 10 mL and a turnaround time of ~40 min. This microfluidic chip is an attractive candidate for further development with high probability of transforming into a simple, fast, and highly accessible

platform for rapid and specific quantification of exosomes in body fluids, thereby paving the way for the implantation of exosome analysis in clinical practice.

IAC is highly specific because it is based on exosome-specific antigen-antibody immune interaction. [34]. It is the only capture technique that is specific enough to differentiate exosome subtypes, such as tumor derived exosomes expressing specific antigens [32]. In ELISA, as demonstrated by Zarovni and co-workers, a conventional colorimetric ELISA has a sensitivity for common exosomal proteins down to subpicogram levels and a detection limit of below 3.5 pg/mL, which corresponds to 0.1 μg of total exosomal proteins [35]. It is also capable of rapidly handling large amounts of samples in parallel with high efficiency in term of cost and time since modern ELISA is designed in a 96-well plate format for multiplexing analysis [46].

Unfortunately, since many antigens present on exosomes are also found on other cells or membrane vesicles, purification or size-based separation is often required [37]. In addition, off-target binding and masking of epitopes as a result of post-translational modifications on target antigen are potential issues to be tackled since they pose significant challenges to the performance of IAC [40]. Therefore, for optimal performance, one suggestion is to target multiple antigens, so that the loss of one epitope will not result in reduced efficiency in IAC [21]. As IAC is based on highly-specific antigen-antibody interaction, prior identification of a suitable surface antigen must be executed and its complementary antibody for exosome binding must be available. Moreover, due to tumor heterogeneity, there is a possibility that not all cells within a tumor have the target antigen, thus likely resulting in the underestimation of exosome concentration. To add to the complications, the performance of IAC is heavily dependent on antibody quality [46]. Furthermore, the long period of incubation required for antibody binding in ELISA, for example, raises contamination issues to the sample, which will likely affect the binding. Lastly, although IAC is a user-friendly technique, its performance is still dependent on users' skills and experience [47].

2.2 Asymmetrical-flow field flow fractionation (AF4) coupled with multidetection

AF4 is a subcategory of field-flow fractionation techniques which separates macromolecules and particles based on their diffusion coefficients [48]. As illustrated in Figure 5 [49], AF4 is conducted in a channel consisting of two plates separated by a spacer. The upper plate is impermeable while the bottom one is permeable with porous frits and covered by a semipermeable membrane with a known pore size [48]. In AF4, a cross-flow perpendicular to the down-channel flow pushes particles toward the membrane attached to the channel. However, counteracting diffusion (Brownian motion) causes the particles to move away from the membrane. Smaller particles with larger diffusion coefficients migrate further away from the membrane (nearer to the channel center) and are subjected to the faster flow stream of the parabolic flow profile. Larger particles with smaller diffusion coefficients migrate at the

region closer to the membrane are thus subjected to a slower flow. As such, the smaller particles travel down-channel more rapidly than the larger ones, and therefore elute out earlier [37].

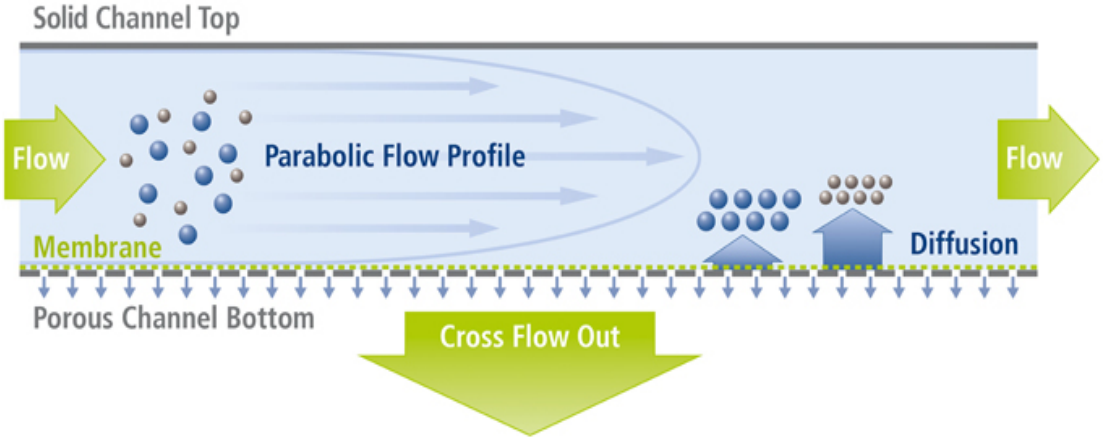

Figure 5. The separation principle of AF4 (Reproduced with permission from ref. 49.)

When AF4 is coupled with a multidetection system, such as dynamic light scattering (DLS) and multi-angle light scattering (MALS) detectors, it becomes a robust technique that can be used to characterize and quantify exosomes. DLS independently measures the hydrodynamic radius (R_h) of exosomes [37], while the MALS detector is able to obtain the size of exosomes without the need to concentrate the sample and increase its refractive index. In the study carried out by Sitar et. al., the size of exosomes was portrayed in two different radii, namely the root-mean-square radius (R_{rms}) and geometric radius (R_{geom}) [48]. The number of eluted exosomes is evaluated by a density template using a refractive index value of 1.39 [50]. The results obtained using AF4-MALS was shown to be reliable as they agreed well with those obtained by NTA [45].[45]

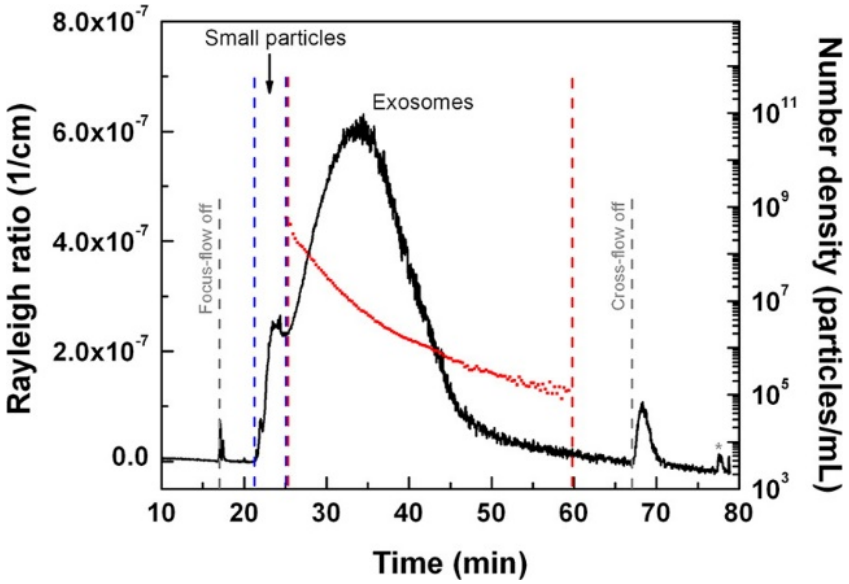

Figure 6. An AF4-MALS fractogram of an exosome sample and the calculated amount of particles (number density per mL) for larger exosome population as a function of elution time. (Reproduced with permission from ref. 48. Copyright (2015) American Chemical Society.)

Figure 6 illustrates an example of the quantification of exosomes using AF4-MALS [48]. It was shown that the larger exosome subpopulation has a number density of ~1.1 x 10^{10} exosomes/mL. However, in this work, the particle number density for the smaller exosome subpopulation could not be measured, probably due to parameter setting issues.

AF4 has very a powerful separation capability with a wide separation range from micrometers down to nanometers [48]. In an investigation reported by Petersen and co-workers, AF4 was demonstrated to effectively separate exosomes into vesicle subpopulations based on their size [37]. AF4 coupled with the multidetection system has a high potential to achieve a more detailed exosome analysis since it has the capability of subtyping exosomes. Moreover, the working principle of AF4 is simple and straightforward. Since it is capable of subtyping exosomes, no complicated and expensive exosome labeling is involved [37], thus very little or almost no sample pre-treatment prior to analysis is required.

Unfortunately, despite its impressive capability to differentiate small and large exosome subtypes, this approach is still under development. Very little work has been carried out to optimize the field-flow fractionation in characterizing and separating exosomes [37]. It is highly likely that AF4 will be a promising and reliable technique for exosome size characterization and quantification after thorough optimization and standardization [51].

2.3 Nanoparticle tracking analysis (NTA)

NTA is a light-scattering technique designed to characterize nanoparticles and was found to be well suited for the analysis of exosomes [36]. For nanoparticles suspended in liquids such as exosomes in body fluids, there is a direct correlation between the rate of Brownian motion of the nanoparticles and their size [52]. Capitalizing on this established correlation, NTA is able to visualize exosomes by tracking their Brownian motion in a suspension and generate their size and concentration data. NTA positively identifies and tracks exosomes in real-time and produces frequency and size distribution data [53]. When coupled with the information obtained on temperature and viscosity, NTA can provide an accurate exosome size estimation with the engagement of the Stokes-Einstein equation [54]. NTA is only capable of monitoring two-dimensional motions, but Brownian motion of exosomes is three-dimensional in nature. Such difference is mitigated by the use of the Stokes-Einstein equation [55].

A simple setup of NTA can be seen in Figure 7, where a laser beam is first directed to the suspension at a fixed angle and the scattered light is captured by an optical microscope and subsequently directed to a camera sensor to produce real-time data. First, the laser beam is directed through a glass filter within the sample cell. The glass filter is designed in such a way that the incident light is refracted at the glass-liquid interface and gives a resultant beam of higher power density. This beam of higher power density then penetrates through and hits exosomes in suspension, which, in turn, leads to an increment in scattered light intensities [56]. This facilitates easy visualization of the

exosomes which scattered light along the incident path of light upon magnification by the optical microscope fitted with an appropriate magnifying objective. The camera sensor that is connected to the microscope is most commonly found to be either a highly sensitive complementary metal-oxide-semiconductor camera, an electron multiplied charge-coupled device, or a charge-coupled device (CCD), all of which register the light scattered by the exosomes in a dynamic video format, as opposed to the conventional static imaging. This allows users to monitor any changes in the suspension or movement of the exosomes and make adjustments accordingly so as to simultaneously analyze different exosome subpopulations under optimized settings. The size of the exosomes can then be discerned and their concentration information can also be obtained based on the frame-by-frame analysis of the exosomes recorded. To better understand this technique, an in-depth description of its operating principle and performance in various applications can be found in previous work [57-59].

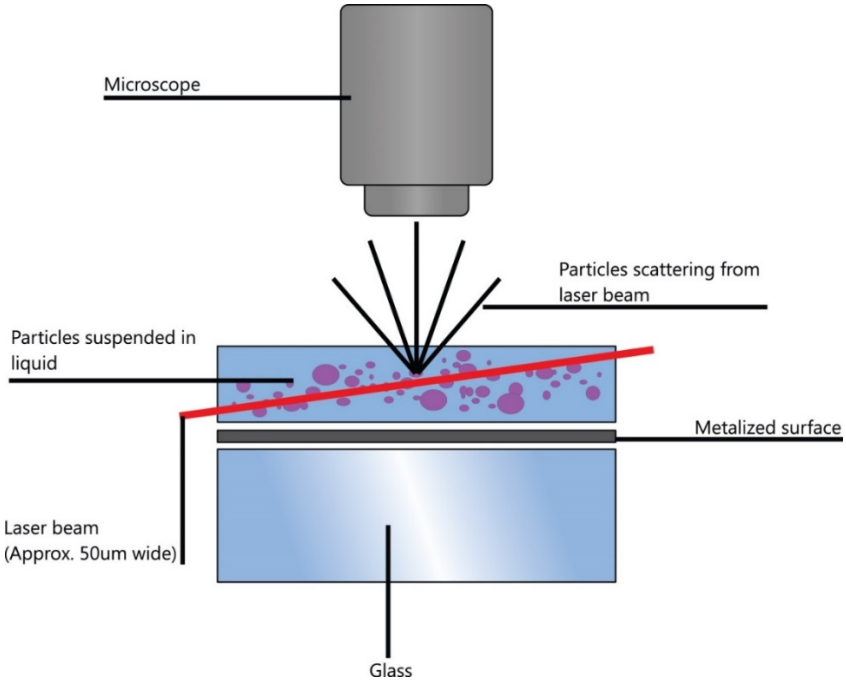

Figure 7. A simplified schematic diagram of NTA. (Reproduced with kind permission of Malvern Instruments Limited.)

In recent years, NTA is considered one of the most popular direct-counting techniques that has been used to quantify exosomes owing to its numerous advantages. For example, it allows real-time visualization of exosome activities, thus facilitating the characterization of protein aggregation or dissolution [60]. This is especially crucial if further quantification of genetic materials is warranted as the aggregation can trigger undesirable chemical reactions which may compromise the quality of the sample or degenerate the genetic materials within exosomes, thus impeding downstream analysis [61]. NTA is also able to provide a far more accurate estimation of the exosome population counts as compared to DLS with minimal sample preparation [62], which is paramount to safeguard the integrity of the sample and reduce the time required to process each sample [63]. As biological samples are

usually poly-dispersive, NTA allows simultaneous analysis of exosomes of varying sizes under different settings within a single recording at high-resolution in the absence of any pretreatments [64]. Tedious procedures to purify samples that involve the introduction of foreign agents to the samples can be omitted, thereby greatly minimizing the possibility of contamination.

Though it is greatly optimized to be a powerful tool for the quantification of exosomes ranging from 30 to 1000 nm [65], NTA, however, has its limitations. Being a relatively new exosome quantification technique [66], NTA lacks the standardization required for exosome analysis which substantially undermines its compatibility under different conditions [67]. For instance, some experiments mandate sample pretreatments to remove possible interfering cells and other extracellular vesicles, while other experiments are only simply carried out a serial dilution to prevent clustering. Reproducibility of these experiments will thus be compromised. Very often, if sample treatments are required, they are far more aggressive than those engaged in DLS, where ultracentrifugation and other methods are used to isolate the exosomes of interest prior to analysis and resuspension has to be carried out with the introduction of more agents [68]. As the analysis with NTA requires manual adjustments to both instrumental and software settings to eliminate deviations in the measurements, it is time-consuming and prone to human error [69]. It is also difficult to achieve highly reproducible results by different users due to the high level of experience and skills required to work with NTA.

Fortunately, substantial improvements have been made to NTA which significantly boost its performance in the quantification and characterization of exosomes. Steps to advance the standardization of NTA have also been underway in recent years which aim to promote good practice and provide the essential information required to optimize various aspects of data analysis [70].As mentioned earlier, many cells secrete a multitude of exosomes for intercellular communications which make the standardization highly challenging. One key issue is to identify or synthesize a reference material that is sufficiently generic and yet specific at the same time to be used as a compatible standard in all NTA analysis. Conventionally, polystyrene microspheres are used as the standard for calibration and as a size reference [71]. Though they are not entirely an ideal standard since they have a higher refractive index than that of exosomes [72], their consistency and availability make them a favorable option over the years. Due to the noticeable difference in refractive index, Mie theory predicts that the polystyrene microspheres can scatter at least four folds as much light as exosomes of identical size do which severely compromises the accuracy of exosome size determination by NTA. In one of the more recent reports, it was found that the replacement of the polystyrene microspheres with silica microspheres as the standard for exosome size and concentration measurement is preferred due to their comparable refractive index value to that of exosomes of similar size. The amount of light scattered by exosomes is better approximated which eliminates the overestimation of exosome population per frame recorded [73]. Nonetheless, a biological standard is still highly sought after, but there are many challenges and more research and development are required at current stage. For

instance, the identification of an existing standard material that is generic (in terms of size, shape, and composition), stable, and exhibits predictable behavior is both time-consuming and experimentally-challenging. The same applies to the synthesis of an artificial material to serve as the standard. Apart from the above-mentioned problems, some other issues such as storage, detection efficiency, and sample compatibility persist [74]. Until a standard that can overcome these restrictions and limitations has been developed, the silica microspheres will remain the next best alternative.

Conventional NTA is capable of resolving exosomes with their size and concentration measured simultaneously, but it cannot positively differentiate the phenotypes that are present in a poly-dispersive sample [75]. Additional mechanism has to be engaged in order to introduce phenotyping capability to NTA. For example, it was found that with the usage of fluorophore-labeled antibodies or tracker peptides that specifically interact with the membrane proteins of exosomes, subpopulations can be distinguished [76]. This will greatly enhance the quantification power of NTA [77]. Although no generic surface biomarkers have been identified thus far, future prospects remain promising as a number of biomarkers such as CD81, tetraspanins, and CD63 show a great potential and have gained much attention since their discovery [78].

2.4 Dynamic light scattering (DLS)

DLS is a light-scattering technique used to characterize particle-size distribution based on fluctuations in scattered light intensities detected at a fixed angle provided the particles are small compared to the wavelength of light [79]. When the particles in three-dimensional Brownian motion are hit by a monochromatic light, the light scatters in all directions due to the constant changes in charge positions such that its intensity fluctuates over time [80]. Based on these time-correlated fluctuations, the structural and dynamic information of the particles can be obtained [81]. As schematically illustrated in Figure 8, a laser beam is first directed to the sample cell where the scattered light is collected by a photomultipler at an angle which generates a speckle pattern on screen and passes on the fluctuating intensities registered to an autocorrelator for particle-size determination [82]. This is made possible due to the Brownian motion of the particles which directly correlates the scattered light intensity to their size. For mono-dispersive particles, the autocorrelation function used will be a simplified exponential decay, where it is proportional to the hydrodynamic diameter of the particles. For poly-dispersive particles, the autocorrelation function used will be the summation of all exponential decays contributed by each subpopulation of the particles. Specific mathematical algorithms will then be employed to generate the particle-size distribution of subpopulations. DLS is especially sensitive in the analysis of relatively large particles, as the intensity of scattered light varies proportionally to the sixth order of the diameter of the particles [83].

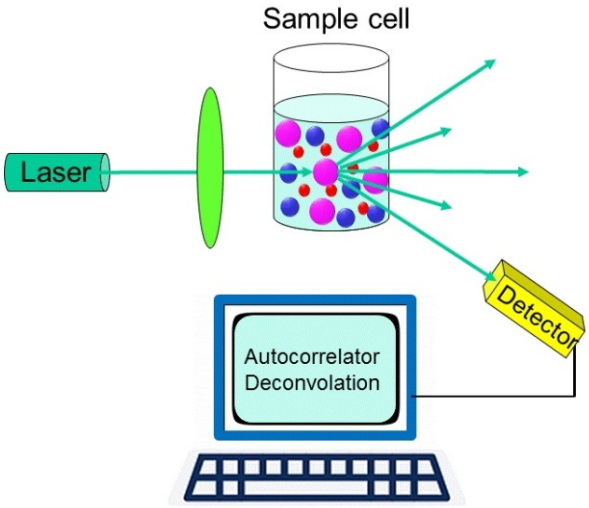

Figure 8. A simplified schematic illustration of DLS.

DLS is popular and widely employed for the characterization of nanoparticles due to its ease of use and quantitative nature. It is non-invasive and allows the measurement to be performed in situ and in real-time, thus offering more flexibility than conventional techniques and greatly facilitating the analysis of kinetic processes [84,85]' and the visualization of transient states [86-87].' Moreover, DLS is user-friendly and automated which makes it less susceptible to human errors and does not require a large sample volume for analysis. Therefore, it is widely used for fast and accurate determination of particle-size distribution [88-90]. It also confers great sensitivity in the analysis of homogenous samples [91] and is highly compatible to other techniques such as size-exclusion chromatography (SEC) and Bradford assay. It has greater consistency and stability as compared to NTA, since it does not require frequent manual adjustments, which can impact data collection and interpretation.

DLS can measure the particle-size distribution based on the fluctuations in scattered light intensities, but it is not able to determine the particle concentration by itself in the absence of algorithm analysis [92]. As such, coupling with other techniques is warranted to complete the quantification and characterization of exosomes in order to provide concentration information. While DLS has good compatibility with other techniques, this will nevertheless introduce greater variability and may compromise accuracy.

Though highly sensitive in the analysis of homogenous samples, the presence of dust particles or aggregates of larger size in the suspension can impair accurate size determination [93]. This is due to the fact that the data obtained by DLS are biased towards larger particles as the scattered light intensities are likely to mask those of smaller particles [94]. This poses a challenge in the analysis of poly-dispersive samples using DLS since the multitude of particle subpopulations undermine the accuracy and efficiency in size determination [95]. In such instances, NTA is better equipped to analyze poly-dispersive samples [96]. DLS also has a lower peak resolution, where only particles that differ in size by a factor of three can be resolved [97]. However, it was shown that by adapting a cross-

correlation approach, undesirable scattered light from contaminants can be suppressed [98], implying that novel analytical techniques can be developed to mitigate these limitations. The analysis of particles with high refractive index values has improved accuracy as well.

As mentioned above, DLS is susceptible to signal contribution from its environment that impairs its capability to analyze poly-dispersive samples. It is especially so if the interfering particles are larger in size as their signals are too significant to be ignored [99]. In the absence of selective suppression, this will lead to an inaccurate interpretation of DLS data [100,101]. However in a recent report, it was observed that DLS in a depolarized configuration (DDLS) can render signal contribution from surrounding particles in poly-dispersive samples negligible with scattered light information originated from the particles of interest selectively detected [102]. This opens up the possibility of applications to the quantification of exosomes with greater accuracy and specificity. A simplified illustration of selective suppression by DDLS is shown Figure 9 [102].

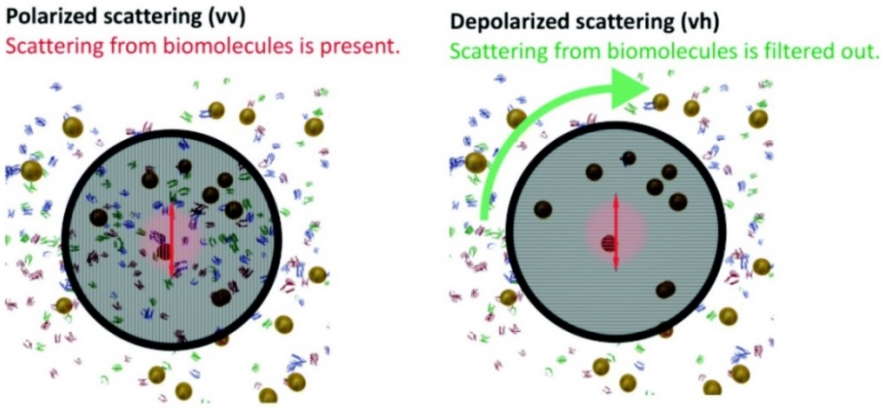

Figure 9. Schematic illustration of selective suppression by DDLS.
(Reproduced with permission from ref. 102. Copyright (2015) Royal Society of Chemistry.)

To compensate for its inability to distinguish small particles from the large ones, it was found that DLS can provide quantitative characterization of exosomes with their shape and size examined [103]. It was also found that an experimental hyphenation of DLS with Bradford assay shows a great potential in the quantification of exosomes as well as their protein contents. A comparison made across the concentration measurements obtained by DLS and protein concentrations obtained by Bradford assay exhibited a direct correlation that can facilitate quality checks, contamination evaluation, and protein recovery. Since protein contamination is detrimental to subsequent proteomic studies [5,104], this hyphenation greatly facilitates downstream analysis.

Another hyphenation of DLS with SEC also presents the potential to remove contaminants and allows accurate size determination at the same time [105]. SEC separates the exosome subpopulations by size and positively identifies the species of interest based on UV absorption and elution time, while DLS distinguishes exosomes from plasma proteins based on their scattered light intensity which is directly related to their size. This hyphenation mitigates the detrimental effect of signal masking that

can lead to under- or overestimation of exosome counts since the exosomes are well separated prior to the measurements that can aid in better approximation of size distribution and concentration. Such a hyphenation is favorable since SEC has already been widely used in the characterization of liposomes, which is similar to exosomes in terms of size and morphological information [106,107].' Additionally, positive identification of subpopulations can be realized by means of UV absorption measurements during SEC which can have further applications in exosome quality control.

Much like the DLS-Bradford assay and DLS-SEC hyphenation, it is conceivable to hyphenate DLS with other techniques to bring about the quantification of genetic materials that exosomes carry. Alternatively, a recent report discussed the use of DLS for the characterization of gold nanocluster-functionalized DNA [108]. It is possible that by capitalizing on the interaction of the gold nanoclusters to genetic materials, the genetic materials carried by exosomes can be examined.

2.5 Surface plasmon resonance (SPR)

When the frequency of an incident light matches the intrinsic frequency of delocalized surface electrons at an interface between two materials of negative and positive permittivity, resonant oscillation of the delocalized surface electrons occurs stimulated by the incident light. This oscillation, in the form of non-radiative electromagnetic waves, propagate in the direction parallel to the material interface having negative permittivity, usually a thin metal film (i.e. the interface between the thin metal film and the external medium – analytes containing medium). Since this oscillation is highly sensitive to any changes at the interface, the resonant frequency can be utilized as an analytical signal to evaluate traces of mass brought onto the thin metal film.

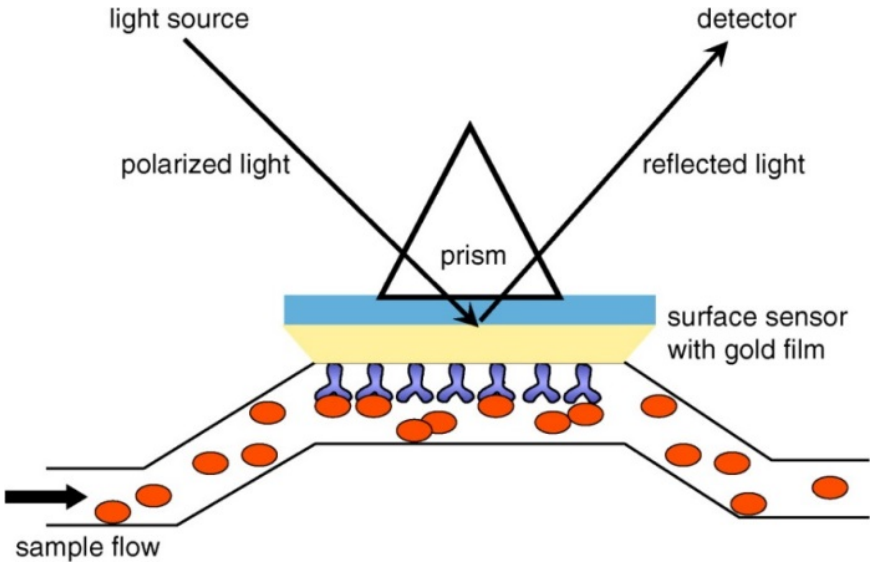

Figure 10. A typical setup of SPR. The red circles and inverted "Y" labels within the flow represent sample and ligand, respectively. (Reproduced with permission from ref. 110. Copyright (2013) American Chemical Society.)

Figure 10 shows the working principle of SPR [109]. Firstly, the setup requires an optical light source. In most cases, it's a near-infrared light-emitting diode. The second component is the thin metal film which is usually a thin gold film coated onto a glass slide.

The incident light must be located at the opposite side of the metal surface so that it will not directly excite the adsorbent on the surface that it hits. The sample flow and interaction take place on the other side of the metal film. The last important part is a detection system which is usually a CCD camera. With a narrow defined angle, the incident light interacts with the delocalized electrons on the metal film which decreases the intensity of the reflected light. When the adsorbent is excited upon the matching of the energy of the photons, the photons are absorbed and consequently results in a dip in the reflected light at the specific angle. This specific angle is exclusively dependent on the refractive index of the adsorbent. The difference between the values of refractive index of the buffer and the adsorbent is used to deduce the absorbed mass and thickness of the adsorbent.

SPR is becoming more and more popular owing to its exceptional capability in real-time investigations of interactions between molecules [110]. It can be used to explore the specificity of interaction, dissociation and association, as well as many parameters of a reaction, like entropy, enthalpy, and even activation energy. Moreover, it has been successfully utilized to detect the interactions between biomolecules, like protein-protein [111], protein-ligand [112], protein-DNA [113], protein-membrane interaction [114]. Therefore, SPR can also be applied in the quantification of exosomes since it is a surface sensitive optical detection technique, which is particularly suitable for label-free mass-uptake determination [115]. It works more efficiently than fluorescence-based techniques for quantifying biomolecular interactions. The main reasons leading to its popularity in biological science [116] are (1) its label-free and real-time nature, (2) its high signal-to-noise ratio [117], (3) its good compatibility with different sample matrices [118], and (4) its ease and diverse modifications of the surface [119]. The last advantage allows the introduction of binding partners on the metal surface and minimizes nonspecific interactions at the same time. In most cases, SPR plays a key role in the study of binding events that occur within the range between 0 and 200 nm of the active surface [120]. Therefore, exosomes, with the size around 100 nm, fit very well with the working range of SPR.

SPR is an attractive technique as compared to the traditional immunoassays for multiplexed profiling of exosomes since exosomes with much larger masses can generate larger signals. Therefore, SPR allows greater detection sensitivity than those competing techniques. Additionally, SPR is a label-free real-time detection technique which can deliver fast analysis and has no need of tedious and time-consuming sample preparation steps. SPR is also non-destructive so the sample can be used for further analysis. Versatility is another attractive feature of SPR because of its diverse surface modifications. Furthermore, SPR opens the door for clinicians to obtain cancer-specific exosomes in a fully-automated

format by using a very small amount of blood – only a few microliters, thus taking the diagnosis of cancer and personalized medicine one step further.

On the other hand, one major disadvantage of SPR is that any artefactual change in refractive index other than those from the interaction also produces a signal, like the injection of sample between flow cells, or a different amount of materials immobilized. Another issue is that the regeneration of ligand surface seems impractical. The goal of regeneration is to allow SPR chips to be re-used many times, saving both time and money. But in reality, establishing ideal regeneration conditions can be very time-consuming and the regenerated chip can only be used for a few times. Lastly, as the thickness of the metal film also plays an important role, a fine control over it can be problematic to some degree.

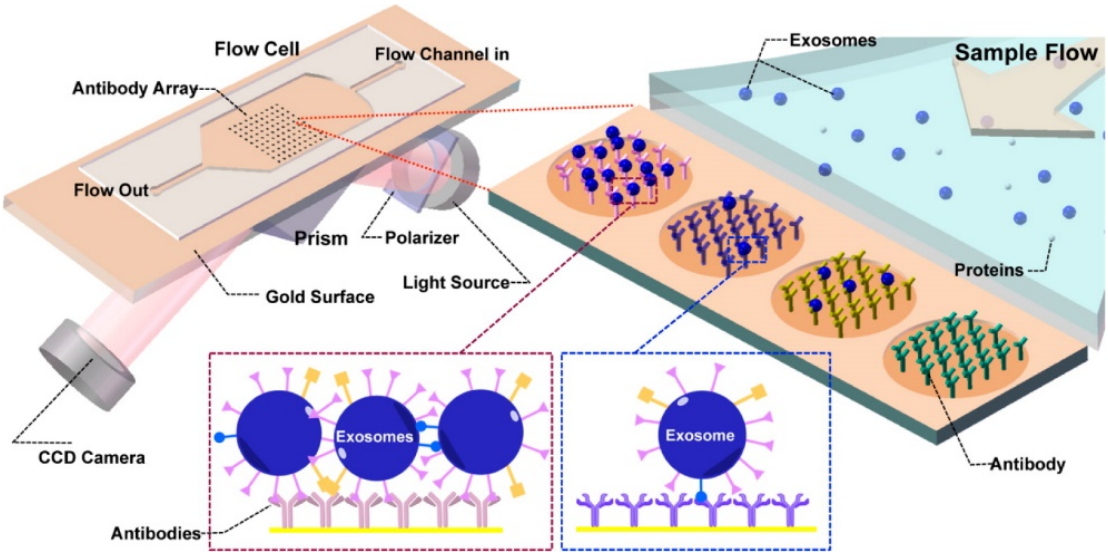

Figure 11. A schematic view of a SPRi chip. (Reproduced with permission form ref. 121. Copyright (2014) American Chemical Society.)

The development of SPR imaging (SPRi) technique, a modified version of SPR, opens a new door for SPR analysis [121]. It is a sensitive and label-free technique for visualizing the entire SPR chip via a video CCD camera. This design allows the SPRi chip to be made in an array format with each active site (spot) providing SPR information simultaneously. Figure 11 illustrates the configuration of the SPRi chip which is in combination with an antibody microarray to bind and detect exosomes in a cell culture supernatant.[121] The antibodies that are specific to exosome transmembrane proteins are printed on the chip. The optical path originating from the laser, at a fixed angle of incidence, passes through the coupling prism, and the CCD camera records the reflection. By injecting a sample into the flow cell, exosomes in the sample are captured by the antibodies on the chip, thereby leading to refractive index changes and consequently the reflection intensity changes are captured by the CCD camera.

SPRi provides simultaneous detections of the interactions of analytes with several hundred to several thousand ligands pre-immobilized on the chip. Some commercial SPRi instruments even feature

several flow cells and lines of different ligands, thus enabling many more interactions to be detected simultaneously and presenting the possibility of multimodal analysis.

3. Comparison of Exosome Quantification Techniques

Table 1 details the analytical characteristics of the five techniques discussed. From Table 1, it can be seen that all the five techniques are based on optical phenomena and they can be broadly categorized into two groups – those solely based on the physical properties of exosomes like size and density and those involving immunoaffinitive interactions. Being immunoaffinity-based techniques, ICA and SPR offer the best specificity. Nonetheless, by coupling with immunoaffinitive interactions, all the five techniques are capable of achieving excellent specificity. Regarding cost issues, AF4, NTA, and DLS are relatively more affordable.

Table 1. Comparison of commercial techniques used in exosomes quantification

Detection technique	Signal	Detection range	Specificity	Time and cost
ICA (ELISA)	Light transmission	0.01–0.1 ng	High, because of exosome specific antigen-antibody interactions	High cost, moderate running time
AF4-MALS/DLS	Light scattering	nm–μm	Low, but can be enhanced by coupling with ICA	Relatively low-cost, short running time
NTA	Light scattering	10 nm–2 μm	Low, but possible enhancement by coupling to immune reactions	Low cost, long running time
DLS/DDLS	Light scattering	10 nm–1 μm	Low when uses alone, but can be enhanced by coupling with immune reactions	Relatively low cost, long running time
SPR	Light reflection	<300 nm	High, because of exosome specific antigen-antibody interactions	High-cost, short running time

Traditionally, the determination of the concentration of exosomes relies on the colorimetric determination of the total mass of proteins. This approach can achieve accurate quantifications only under conditions that exosome suspensions do not contain other kinds of extracellular vesicles and soluble proteins, and hence demand high stringencies of the procedures of sample preparation. Moreover, since there are differences in protein contents in exosomes, this approach is at its best regarded as a rough estimation. Recently, techniques based on direct particle counting have become more popular. NTA and DLS can provide relatively more accurate quantification of exosomes. On the other hand, both NTA and DLS also face the disadvantages of low resolution and the quality of data strongly depends on the software settings and even the operator. Although NTA solely relies on the real-time imaging of light scattered by individual exosomes that are freely moving in solution [36], its quantification accuracy may differ from ill-defined probe volumes, uncertainties regarding reliable exosome counting threshold settings, and a risk of failing to count exosomes beyond a certain size range or optical density range [74]. Another disadvantage of NTA is that, in most cases, it fails to provide an opportunity to selectively detect specific exosome subpopulations. In other words, the

absence of exosome selectivity will not allow the assessment of different populations of exosomes, but this information may be important in clinical diagnosis. Thus, the techniques that are capable of specifically quantifying the subpopulations of exosomes require a combination of both exosome counting and immunostaining. In this case, exosomes are typically visualized by using fluorophore-labeled antibodies against selected membrane-bound biomarkers. Direct counting of individual exosomes can be obtained by using NTA operating in the fluorescence mode. But this strategy is not widely used as it requires sufficient biomarkers on the exosome surface to grant individual exosomes detectable fluorescence signals. In addition, the sample preparation steps involved to purify exosomes from unbound fluorescent antibodies usually are prone to the loss of exosomal materials.

In principle, these potential limitations can be alleviated by applying surface-based exosome detection techniques because specific molecular recognition probes, for example antibodies, immobilized on a surface can specifically recognize and capture exosomes. The obtained surface coverage can then be utilized to derive the content of a specific subpopulation of exosomes in a sample. For those antibodies of having high affinity, only a few copies of specific molecular markers per exosome are needed to bind exosomes to antibody-modified surfaces without involving extensive time-consuming exosome isolation procedures. The surface-based capture in combination with a fluorescence-based readout for the quantification of exosomal materials has been well researched for proteins [122] and nucleic acids [123]. As for exosome quantification, both ICA and SPR have been developed [38]. For example, SPR allows for label-free mass-uptake quantification, no needs to engage fluorescent labels, and usually behaves better than fluorescence-based methods in quantifying exosomes and their subpopulations.

4. Conclusions and Prospects

As discussed in this article, because of their extremely small size, there have been a unique set of technical issues to translate exosome research into clinical applications. One of the major problems that hinders the progress of accurate exosome quantification is the effective sample preparation procedures for isolating exosomes from complex media such as blood, ascetic fluid, urine, milk, amniotic fluids, and cell culture medium. Exosome isolation procedures established thus far are based on ultracentrifugation, ultrafiltration, precipitation, or immunoaffinity-based exosome isolation. Unfortunately, their tedious and time-consuming protocols greatly impede this translation process. To overcome these issues, the development of fully-integrated microfluidics-based devices that are capable of efficiently isolating as well as sensitively and specifically quantifying exosomes may eventually offer a plausible solution. Some encouraging results have recently been reported. Notable examples are those described by Lee et al. [124] and Im et al.[125]. For example, it was observed that intact extracellular vesicles including exosomes can be efficiently separated according to their size and density in a non-contact manner by a force exerted on them in an acoustic field [124]. Also, the

quantification of exosomes and their contents can be realized by a plasmonic platform integrated with a microfluidic chip [125]. Since no one technique is able to handle all tasks associated with exosome isolation and quantification, comprehensive approaches are the best way to quantify exosomes accurately and specifically. Therefore, the approaches that deserve further developments are the hyphenation of the detection techniques with exosome isolation platforms and possible integration and automation. Besides the five techniques discussed above, hyphenation with other techniques like atomic force microscopy, mass spectrometry, and electron microscopy should also be actively pursued. This will allow further investigation with regard to the specific properties and features of exosomes. By leveraging on the attractive traits of the two worlds, it is anticipated that exosome quantification devices and instruments of an unprecedented accuracy will definitely be realizable in the near future. The realization of highly efficient and translatable exosome isolations platforms in conjugation with accurate exosome quantification techniques will undoubtedly offer invaluable assistance in our understanding of exosome biology and revolutionize clinical diagnosis of many medical conditions and diseases, particularly various types of cancer. Further efforts and innovations in exosome quantification are in great demands, so that exosomes can be easily and accurately quantified. Provided that the cost of exosome affinitive agents and antibodies in particular can be lowered significantly or be replaced by much more affordable agents with similar affinity such as aptamers and SOMAmers, the magneto-affinitive approach deserves further work since it offers a highly specific means for the isolation and quantification of exosomes in addition to the simple and easy separation of specific exosomes from sample matrices.

The released cellular vesicles play major roles in the pathogenesis of diseases, for example, a critical role that exosomes play is the signaling mediated by specific interactions between tumors and their target cells. Knowledge of the molecular fingerprints of exosomes of different cellular origins is instrumental in the identification of their pathogenic roles and to their potential use as diagnostic tools. In addition, early detection of cancer is imperative to improve the survival rate of cancer patients. This assumes that cancer biomarkers are identified and clinically validated. Even though the number of potential biomarkers identified for many types of cancer is fast-increasing, the reality is that the actual number of biomarkers used in clinic is rather limited because of difficulties encountered during the validation step of those potential biomarkers. In current clinical practice, the diagnosis and prognosis of solid tumors require invasive tissue biopsies; exosomes isolated from body fluids and blood in particular offer a minimally invasive route. However, there is an urgent need to reliably identify and validate exosome biomarkers to be employed in diagnosis, prognosis, and treatment of cancer and other diseases. Nonetheless, the fact that exosomes are being recognized as next-generation biomarkers and therapeutic vehicles with interests in areas as diverse as basic research, clinical diagnosis, drug delivery, and molecular therapy implies that research on exosomes will continue to grow and the future of exosome research and application remains bright.

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