Counting of peripheral extracellular vesicles in Multiple Sclerosis patients by an improved nanoplasmonic assay and dynamic light scattering

Antonia Mallardi [a, 1], Nicoletta Nuzziello[b,c,1], Maria Liguori[b], Carlo Avolio[d], Gerardo Palazzo[e,f] *

[a]*CNR-IPCF, National Research Council of Italy, Institute for the Chemical Physics Processes, Division of Bari, Bari, Italy*
[b]*National Research Council of Italy, Institute of Biomedical Technologies, Section of Bari, Bari, Italy*
[c]*Department of Basic Sciences, Neurosciences and Sense Organs, University of Bari, Bari, Italy*
[d]*Department of Medical and Surgical Sciences, University of Foggia, Foggia, Italy*
[e]*Department of Chemistry and Center for colloid and surface science (CSGI), University of Bari, Bari, Italy*
[f]*CNR-NANOTEC, National Research Council of Italy, Istitute ofi Nanotecnology, Bari, Italy*

[1] A.M. and N.N. equally contributed to this work

* **Corresponding author**: Gerardo Palazzo
Dipartimento di Chimica, Università di Bari,
via Orabona 4, I-70125 Bari (Italy)
Tel: +39/080/5442028;
Fax: +39/080/5442128;
e-mail: gerardo.palazzo@uniba.it

Highlights

- The quantification of EVs released by cells is a crucial point for the development of EV-based clinical biomarkers.
- We propose an improved and robust analytical protocol to quantify the total phospholipid concentration (C_{PL}) and the EVs number.
- A treatment with proteinase K efficiently digests the large protein aggregates that coexist with the EXO preparations purified by differential ultracentrifugation.
- An aggregation index measured at three wavelength (Abs@520 nm/[Abs @650 nm + Abs@900 nm]) considerably reduces the LOD and the LOQ values associated to the C_{PL} concentration.
- The spread in EV size is also taken into account when the concentration of phospholipids is turned into concentration of vesicles.

ABSTRACT

Extracellular vesicles (EVs) are vesicles naturally secreted by the majority of human cells. Being composed by a closed phospholipid bilayer secluding proteins and RNAs they are used to transfer molecular information to other cells, thereby influencing the recipient cell functions. Despite the increasingly recognized relevance of EVs, the clarification of their physiological role is hampered by the lack of suitable analytical tools for their quantification and characterization.

In this study, we have implemented a nanoplasmonic assay, previously proposed for the purity of the EV fractions, to achieve a robust analytical protocol in order to quantify the total phospholipid concentration (C_{PL}) and the EVs number. We show how the coupling of the nanoplasmonic assay with serial dilutions of the unknown sample allows, by simple visual inspection, to detect deviations from the physiological EVs content. The use of a response that depends on the absorbance values at three wavelengths permits to reduce the limit of detection of C_{PL} to 5 μM (total) and the limit of quantification to 35 μM. We also propose a method that takes into account the spread in EV size when the concentration of phospholipids is turned into a concentration of vesicles. The proposed analytical protocol is successfully applied to a small cohort of Multiple Sclerosis patients examined in different stages of their clinical diseases.

Keywords: extracellular vesicles, exosomes, nanoplasmonic assay, gold nanoparticles, multiple sclerosis

1. Introduction

Extracellular vesicles (EVs) are a heterogeneous population of cell-to-cell shuttles released into the extracellular environment [1]. According to their name EVs are small membranous vesicles composed by a closed phospholipid bilayer decorated with membrane proteins and polysaccharides and secluding proteins and RNAs. They are classified depending on their size and biogenesis into apoptotic bodies, microvesicles (MVs) and exosomes (EXOs) [2]. Apoptotic bodies are the largest EV type, with size (diameter) ranging approximately from 0.5 μm to 5 μm, generated upon apoptosis by blebbing of the cell plasma membrane. MVs vary greatly in size (from 0.1 μm to 1 μm in diameter) and originate by direct outwards plasma membrane budding so that they preserve the orientation of membrane proteins. EXOs, released by exocytosis of multivesicular bodies, are the smallest subtype of EVs, ranging from 30 to 100 nm [3]. The difference in size allows a relatively

easy separation and collection of the three classes of EVs using suitable ultracentrifugation protocols.

The main importance of EVs consists in their capacity to transfer information to other cells, thereby influencing the recipient cell functions [4]. EVs contain cell specific biological information and their composition (proteins, lipids, DNAs, mRNAs, miRNAs and other non-coding RNAs) depends on cell type and its physiological data [5]. EVs can act as a signaling complex, can transfer membrane receptors between cells, deliver proteins to target cells and also modify the receiving cell phenotype by horizontal transfer of genetic information [6].

EVs populate different biological fluids such as serum, plasma, cerebral spinal fluid, urine, saliva, synovial fluid and breast milk, modulating biological processes also at remarkable distance from their site of origin. Peripheral blood represents an immense source of EVs. It has been reported that serum contains ~10^8 EXO per microliter [7]. EVs are released constitutively into the bloodstream and their concentration increases with cellular activation, as in many pathologic conditions [8]. Therefore, the analysis of EVs counts and subtype composition in peripheral blood has been considered as possible indicator of different aberrant processes, thus providing useful information in clinical settings [2].

Several studies reported the importance of EVs in Central Nervous System (CNS) activities, since they seem to play important roles in CNS homeostasis, such as myelin formation, metabolic support and immune defense [9,10,11]. In the pathogenesis of multifactorial disease like Multiple Sclerosis (MS), EVs appear to be involved in the spreading of pro-inflammatory signals as in the damaging of the neuronal functions [1,12,13,14].

Despite the increasingly recognized relevance of EVs, the clarification of their physiological role and their possible applications in diagnostics (or even as targets of novel focused therapies) is hampered by the lack of suitable analytical tools for their quantification and characterization.

To gain insight on the EVs biological function, one requires a comprehensive analysis of EVs in terms of i) their biological content (i.e. amount and nature of proteins and nucleic acids) and ii) their physical properties (size and concentration *in primis* since they rule the efficacy of EVs as shuttle).

While the customary molecular biology protocols (immunoblotting, RNA analysis, etc.) can be relatively easily adapted to the screening of nucleic acids associated with the EVs [15], the physico-chemical characterization of the EVs remains challenging, in particular in the case of the small EXOs. Their small size, in fact, rules out the use of conventional flow-cytometry and also the mere determination of EXOs concentration and size is strongly complicated by the fact that protein complexes (e.g. Ago2-miRNA, high and low density lipoproteins, protein aggregates) share with EXOs the same size, refractive index and surface charge. In addition, also the trivial quantification

of phospholipids through the evaluation of the total phosphorus becomes impractical in the case of EVs containing nucleic acids.

A popular technique for determining the EV concentration is Nanoparticle Tracking Analysis (NTA), where the single EVs are counted and their Brownian motions are simultaneously tracked to extract the diffusion coefficient and thus the hydrodynamic size distribution [16]. Very recently also the Fluorescence Correlation Spectroscopy has been proposed as a tool that in principle allows the determination of both size and concentration of the EVs [17]. However, the risk of artifacts due to the low sampling statistics (in particular for NTA) and to the presence of non-EV particles (e.g. protein complexes) should be taken into account.

It becomes therefore of paramount importance to be sure that the EV sample is free from protein contaminants.

Recently a colorimetric nanoplasmonic assay has been proposed as a tool to assess the purity of the EV preparation [18,19,20].

In addition, the same assay has been proposed, once the sample has been purified from any protein contamination, as a method to quantify the phospholipids content. In the case of monodisperse vesicles the lipid concentration can be easily transformed in the EV concentration as long as the vesicle size is known.

Such a nanoplasmonic assay exploits, for analytical purposes, the interactions that exist among lipid bilayers, nanoparticles, and proteins. The tuning of such interactions is crucial in the incorporation of nanoparticles[21], nanoplexes [22], hydrophobic proteins [23, 24] in natural or artificial bilayers.

Compared with NTA and FCS the quantification of EVs by means of the nanoplasmonic assay is incomparably cheaper and simpler but suffers yet from some limitations. First, the EVs preparation are unavoidably polydisperse and can have sizes considerably different from the liposomes that are used as reference in the assay (e.g. in the case of the large MVs) and both these points must be taken into account in the evaluation of the vesicle concentration. Second, the analytical sensitivity of the assay is low and, for reasons that will be described in the following, reliable quantification can be achieved only in a limited range of concentrations that presently must be found trough a trial-and-error procedure.

In this contribution, we propose approaches that surmount these pitfalls developing standardized and reproducible methods for physico-chemical characterization of EVs capable of application on trials involving large number of subjects. To this aim, these methods have been tested on EVs obtained from a small cohort composed by patients with the first clinical episode suggestive of CNS demyelination (known as Clinically Isolated Syndrome, CIS), clinically definite Multiple Sclerosis (MS) patients and healthy control subjects (HC).

2. Materials and Methods

2.1. Ethic Statement and sample collection

In the paper the data resulted from a subset of 6 subjects, 2 CIS and 1 MS patients (all naïve for any disease-modifying therapy) and 3 age-matched HC, were analyzed.

Peripheral blood samples of patients and HCs were collected at the Department of Medical and Surgical Sciences, University of Foggia. After routine analysis, waste serum samples were coded, anonymized and frozen at − 80 °C. The study was conducted in accordance with the Declaration of Helsinki: informed consent was obtained from all individual participants included in the study (approved by the Ethical Committee of the University of Foggia, Italy—prot. 120/CE/2016).

2.2 Extracellular Vesicles Purification

MVs and EXOs were isolated from peripheral blood samples by standardized differential centrifugations (DC) [25,26]. Briefly, 10 ml of peripheral blood samples were collected in Vacutainer blood collection tubes (BDInc, Franklin Lakes, NJ) and centrifuged at 3,000×g for 7 min to separate serum fraction from blood clot. Each serum sample was diluted 1:1 with Phosphate Buffered Saline solution (PBS) and centrifuged at 2,000×g for 30 minutes at 4°C to eliminate cell debris and other particles. The supernatant was then filtered by gravity through 0.8 µm filters to remove particles >800 nm and centrifuged at 12.000×g for 45min at 4°C to collect MVs. The supernatant was collected and filtered through 0.22 µm filters. The fraction containing EXOs was pelleted by ultracentrifugation at 120.000×g for 70min at 4°C and resuspended in PBS.

In order to have EXO vesicles free from protein contaminant, the partially purified EXO pellet obtained from DC was treated with 1 mg/mL proteinase K (PK) for 1 h at 37°C and then ultracentrifuged at 120,000×g for 70 minutes at 4°C. The containing EXO pellet was recovered and resuspended in PBS. PBS is a high ionic strength buffer (~ 0.16 M), this imply that in all described experiments vesicles are always in a high ionic strength medium.

2.3. Dynamic light scattering

DLS measurements were performed using a Zetasizer-Nano S from Malvern operating with a 4 mW He-Ne laser (633 nm wavelength) and a fixed detector angle of 173° (non-invasive backscattering geometry NIBS™) and with the cell holder maintained at 25 °C by means of a Peltier element. DLS technique analyzes the diffusion distribution of particle moving under Brownian motion by measuring the fluctuations of scattered light intensity [27,28] at a fixed angle (173°).

Data were collected leaving the instrument free to optimize the instrumental parameters (attenuator, optics position and number of runs) [29,30]. Usually the time autocorrelation function (ACF) of scattered light intensity was the average of 12-16 consecutive runs of 10 s each.

The ACF of scattered light intensity was converted into the ACF of scattered electric field. This last quantity is the Laplace transform of the intensity weighted size distribution function that has been retrieved using a standard regularised non-negative least squares analysis, operated by means of the software implemented by the manufacturer. The obtained intensity weighted size distribution function represents the fraction of the light intensity scattered by particles of different size.

For DLS measurements, 10 µl of EVs were diluted in 990 µl of PBS (1:100) and then gently mixed to provide a homogenous solution. At least three measurements were acquired for each sample.

2.4. Nanoplasmonic colorimetric Assay

EV samples were checked for purity by adapting the assay developed by Maiolo et al. [18].

For the assay, EVs were resuspended in PBS at the desired concentration and were incubated for 20 minutes with AuNPs (at 2 nM final concentration). The UV-VIS spectrum of AuNPs in the region between 400 and 900 nm was registered, using a JASCO V-530 spectrophotometer. For the EVs quantification a calibration curve using liposomes (as synthetic mimetic system of EV) made of phosphatidylcholine was used. For calibration, 2 nM AuNPs and liposome solutions with a lipid concentration ranging from 0.3 to 400 µM were used. For the lipid concentration calculation a phosphatidylcholine average molecular weight of 800 g/mol was assumed.

The AuNPs were synthesized in Milli-Q water following the classical Turkevich protocol [31]. Briefly, 2 ml of 1% Na-citrate aqueous solution were rapidly added to 20 ml of boiling 1 mM $HAuCl_4$ under fast stirring. After few minutes the reaction was stopped cooling down the AuNPs solution by means of a water-ice bath. The AuNPs concentration was determined recording the absorption a 519 nm ($\varepsilon = 4x10^8\ M^{-1}cm^{-1}$).

For liposome preparation, the proper amount of soybean phosphatidylcholine was dissolved in a small volume of chloroform, inside an Eppendorf tube, dried under a nitrogen stream and then kept under vacuum for 60 minutes. Lipids were rehydrated in PBS (1 ml), sonicated on ice (3 minutes) and extruded through a polycarbonate filter with 100 nm pore size using an Avanti Polar mini extruder. Like purified EVs, liposomes also are then always in a high ionic strength medium.

2.5. Total RNA Isolation

Total RNA was extracted using Total Exosome RNA and Protein Isolation Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. Ten picogramms of

synthetic miRNA spike-in controls (cel-miR-39 and UniSp6) were added to the respective lysis/denaturant buffer. RNA samples were stored at -80 °C until used. RNA quantity and quality were assessed by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) with total RNA 6000 Nano Chip and Small RNA chip following the manufacturer's instructions.

3. Results and Discussion

3.1. Quantification of the EV in terms of the phospholipid concentration

The nanoplasmonic colorimetric assay described in [18] probes the presence of protein contaminations because proteins preferentially adsorb on the AuNP surface eventually forming a true nanoparticle-protein corona complex. Such a compact protein coating prevents the nanoparticle-nanoparticle contact even at high ionic strength (e.g. in PBS solution, which has an ionic strength of ~ 0.16 M) and therefore the gold plasmon remains almost unaffected with a maximum at ~520 nm and the solution appears yet pink. At variance, in the absence of proteins, the AuNPs at high ionic strength coagulate because salt screens the repulsive interparticle interactions and the ubiquitous van der Waals interactions yet set an attractive potential between particles. The AuNP aggregation leads to structureless plasmon in the red region of the spectrum (the solution turns blue and subsequently gray) and eventually they precipitate.

In the presence of phospholipidic surfaces, as in the case of EV or liposome solutions, the AuNPs can interact with the phospholipid headgroups as well. An attractive interaction takes place only at high enough ionic strength and it has been demonstrated that the non-specific interactions between AuNPs and phospholipid do not favor an uniform distribution of the nanoparticles but instead favors a surface clustering that can eventually lead to the formation of AuNP crust [32]. This surface clustering results in a progressive red shift of the gold plasmon so that the solution can turn blue also in such a case. The actual absorbance spectrum, and thus the optical appearance, depends on the ratio between AuNPs and the vesicles (either natural or artificial). At high ratios, the AuNPs make clusters and the solution becomes blue while at low ratios the AuNPs adsorbed on the phospholipid membrane are so far apart that the plasmon remains unchanged and the sample retains its pink color. The situation is summarized in the **Figure 1A** where the solutions obtained leaving fixed the AuNP concentration at 2 nM and varying the concentration of liposome (dispersed in PBS) are shown. The corresponding spectra are shown in **Figure 1B** where, for comparison purpose, also the spectra of AuNPs in water and in PBS are reported. It is clear that, at high

phospholipid concentration, the spectra show a plasmon band centered close to the wavelength found in pure water. When the lipid concentration decreases, the spectra gradually evolve showing a second band in the long-wavelength region with a maximum around 650-750 nm.

Summarizing, the test for protein contamination of EVs preparation is straightforward and robust: one prepare two or three dilutions of the same sample (always in PBS, thus at high ionic strength), then add the same amount of AuNPs. If the solutions remain pink there are proteins, while if at least one of the dilution turns blue the sample is essentially protein-free. Using as probe AuNP in the nM range, it is possible to probe a concentration of proteins down to 5 ng/μL at which the transition in color from pink to blue take place [18].

An example of this is shown in the inset of **Figure 1B** where the presence of protein has been assayed in MV and EXO samples obtained from an HC serum by differential centrifugation (DC), a protocol suffering from low purity of EXOs for the presence of protein contaminants [33]. After the addition of AuNPs to both samples, in the case of MVs the color solution turns blue while in the case of the EXOs it remains pink. Dilution of the EXO-enriched sample doesn't change the result, thus confirming that differential centrifugation easily removes circulating proteins from the MV fraction while leaves proteins still present in the EXO fraction. Such a contamination is likely in the form of protein aggregates with sizes of the same order of magnitude of that of EXO.

In the attempt to remove the protein contamination from the EXO-containing fractions, we have treated several samples (obtained by DC) with Proteinase K (PK) as described in section 2.2. It should be noted that we have skipped the final step of PK thermal inactivation (customary in the molecular biology practice) because this destabilizes the EXOs inducing the formation of micron-sized aggregates that preclude the DLS analysis (see next section). The PK treatment cleaves the peptide bonds thus digesting the protein aggregates to small polypeptides that can be separated from the EXO by the final ultracentrifugation step. The protein content, quantified according to the Bradford's assay, is around 20 mg/mL in the EXO fractions before the PK treatment and drops to below 0.3 mg/mL after the treatment. Considering that the phospholipids itself can give rise to a response in the Bradford's assay [18], this suggests that we are in the presence of an almost protein-free sample. One must be aware that the PK treatment could also affect the membrane proteins protruding on the external side of the EV. This does not have implications on the EVs' quantifications, but must be taken into account when searching for exosomal proteins as biomarkers.

The response of the nanoplasmonic colorimetric assay to the EXO samples obtained after PK treatment is very informative. The loading of AuNPs to the as purified EXOs (in PBS) leaves the pink color for all the solutions but if the EXOs are diluted enough before the mixing, the final

solution turns blue. This indicates that the EXO samples after the PK treatment are truly protein free and the onset of turning depends on the EXO concentration.

The effect, in some cases, is so marked that it might furnish a preliminary visual screening of anomalous condition. In **Figure 2** are compared the responses to dilution of EXO preparations, after PK treatment, in the cases of healthy control (HC) and two different clinically isolated syndrome (CIS) patients. **Figure 2** shows the solutions obtained by the nanoplasmonic assay performed on the as-purified EXOs (first cuvette), a 1/5 dilution (second cuvette), a 1/10 dilution (third cuvette) and (when present a fourth cuvette) a 1/20 dilution. The visual comparison easily indicates that the EXO concentration in the CIS1 patient is higher than in HC because to achieve in the assay the same nuance of violet and blue the control has dilutions 1/5 and 1/10, while the CIS1 must be diluted 1/10 and 1/20 respectively. Similarly, the assay performed on the EXOs from the CIS2 patient furnishes blue solution already after the first 1/5 dilution indicating a lower concentration compared to the healthy control.

In the first seminal paper on the nanoplasmonic colorimetric assay, the authors explained that, in the absence of protein contamination, the nuance of the solution depends on the extent of clustering of the AuNP on the EV surface and thus the assay responds to the exposed phospholipid surface [18]. They proposed a ratiometric approach in which an aggregation index (AI) was used to somehow quantify the level of AuNPs aggregation. The AI they proposed was the ratio of the absorbance at two wavelengths ($AI_{2\lambda}$=Abs at 520 nm /Abs at 560 nm). In **Figure 3** (open blue circles), the corresponding calibration line obtained from the data of the spectra in **Figure 1B** is shown.

Unfortunately, this approach to the quantification is much less robust than the test for protein contamination. The precision of such a phospholipid quantification is particularly poor at very low and very high concentration of vesicles for reasons that can be fully understood by examining the representative spectra in **Figure 1B**.

Moving towards lower vesicles concentration, the phospholipids available to adsorb the AuNPs decrease (the experiments are performed at fixed nanoparticle concentration) and this trigger the clustering of AuNPs on the vesicle surface. However, for too low concentration of vesicles the AuNPs direct coagulation in the bulk of the salt solution becomes competitive. This process too leads to a red-shift of the gold surface plasmon.

The consequence of this is that in the absence of any vesicle (EV or liposomes) we have still a blue solution with spectral features similar to the case of highly diluted vesicle solution (see spectrum in Figure 1B). Actually, the ratio of the absorbances at 520 nm and at 650 nm is higher for AuNPs coagulated in PBS (without any vesicles) respect to that found in solutions of low phospholipid concentration (see the blue circles close to the origin in the graph of **Figure3**). This has an

important impact in the analytic figures of merit of the assay. The Limit of Detection (LOD) is evaluated (according to the IUPAC recommendations, from the mean and its standard deviation of several independently prepared blank samples), as the concentration corresponding to a response that is three times the standard deviation above the mean of the blank [34]. In the present case the blank is the AuNPs in PBS solution without any liposome and, since the AI found for the blank itself is above the signal measured for low concentrations, one evaluates an high LOD ~25 μM (in phospholipid concentration) although the blank standard deviation remains relatively low (4%). Analogously, the limit of quantification (LOQ), operatively estimated as the concentration corresponding to a signal that is ten standard deviations above the mean blank signal, is LOQ~55 μM when the ratio $AI_{2\lambda}$ is taken as aggregation index.

In order to have an aggregation index that monotonically decreases passing from high to null phospholipids concentration we propose to divide the absorbance at 520 nm (reflecting monomeric AuNP) by the sum of absorbance values at 650 nm (reflecting mainly oligomers) and at 900 nm (mainly due to large aggregates formed during bulk coagulation);

$$AI_{3\lambda} = \frac{Abs\ at\ 520\ nm}{Abs\ at\ 650\ nm + Abs\ at\ 900\ nm} \qquad (1)$$

The plot of this aggregation index based on three wavelengths as a function of the phospholipid concentration is shown in **Figure 3** as red triangles; the LOD levels for the two AIs are shown as dashed lines and the corresponding concentrations are indicated by arrows. The use of the $AI_{3\lambda}$ furnishes a monotonic calibration line with improved LOD ~ 5μM and LOQ ~ 35 μM.

However, there is another critical issue in the orthodox use of the calibration curve.

Consider the samples obtained by serial dilution of the purified EXO CIS1 fraction of **Figure** 2:

Physically these samples must contain phospholipids in concentrations that halve consecutively but if we use the calibration line to evaluate their concentration we found a very different situation. In **Figure 3** the stars denote the intercept of the $AI_{3\lambda}$ value obtained for the three dilutions with the calibration curve (the use of $AI_{2\lambda}$ gives even worse results). According to **Figure 3** the first 1/2 dilution gives an apparent concentration that is ~1/4 of the initial and a further 1/2 dilution behaves as an additional 1/6 dilution.

What is wrong? At high phospholipid concentration, the absorption spectrum of the AuNPs resembles that observed in pure water with a very low absorbance at 650 nm. However, the target EV solutions are dispersions of colloidal particles that strongly scatter the light. Even worse, the scattering power depends dramatically on the size (see next section) and on the refractive index of the vesicles (remember that for EV the lumen contains proteins and RNAs). Any mismatch in the scattering properties between the liposomes used to build the calibration curve and the target EV

strongly impacts the evaluation of the AI in a regime where the denominator is very small and induces large errors in the concentration evaluation. On the other hand, at low phospholipid concentration we observe the AuNP clustering with the development of a true strong plasmon resonance band around 650 nm. In this regime, the scattering artifacts are less important but, likely, one is dangerously close to the LOQ.

To reduce the error in the quantification of the phospholipid concentration we suggest to globally exploit the response of the sample at all the dilutions tested according to the following approach.

The calibration curve is built as function not of the phospholipid concentration but of the dilution (defined as the ratio between the initial V° and the actual volume V) of their initial concentration. Assuming a linear relation between the AI and the phospholipid concentration one has:

$$AI_{3\lambda} = a + b\frac{V^\circ}{V}C^\circ \qquad (2)$$

Where C° is the known initial phospholipid concentration used for the calibration. An example of this approach is shown in **Figure 4** where two calibration experiments, performed using different stocks of AuNPs and liposomes, are compared. As long as the concentrations of phospholipid and AuNPs are fixed the reproducibility of the calibration line is good. The data can be therefore fitted to equation 2 keeping fixed the known C° and obtaining as best-fit parameters the intercept *a* and the slope factor *b*.

The aggregation index values measured for a given sample along a dilution path (at least three points) can be subsequently reported in a $AI_{3\lambda}$ vs. dilution plot as those shown in **Figure 5** and fitted to equation 2 keeping constant *a* and *b* to the values obtained from the calibration and leaving as adjustable parameter the initial concentration C°. Such a procedure takes into accounts all the information present in the measurements performed on samples at different dilution and considerably reduces the error associated to the phospholipid determination. As an example, the evaluation of the phospholipid concentration in the blood by canonical comparison of the AI with the calibration line of figure 3 furnishes the following values depending on the dilution chosen: 2.0±0.2 mM, 0.8±0.8 mM and 0.3±1.6 mM. At variance the use, here proposed, of the calibration line in dilution (**Figure 4**) and of all the three values of AI obtained along a dilution path gives a phospholipid concentration of 1.25±0.17 mM.

3.2. From the phospholipids concentration to the EV concentration

The concentration obtained through the nanoplasmonic assay reflects the exposed area of the EV and thus is a measure of the overall concentration of the phospholipids (C_{PL}) making up the EV.

However, the most useful piece of information is the amount of the EVs itself that is related to C_{PL} by the EV size. For an idealized ensemble of monodisperse EV (i.e. all sharing the very same size) the total exposed phospholipid surface is equal to the number of EV (N_{EV}) multiplied by the surface of a single vesicle according to

$$V \cdot C_{PL} \cdot \alpha = 2\, N_{EV} 4\pi\, R^2 \qquad (3)$$

Where V is the sample volume, α the phospholipid polar head area, R the vesicle radius and the term 2 in the RHS is because the vesicles have two surfaces (inner and outer). Here, for the sake of simplicity, we are neglecting in the calculation the thickness of the phospholipid layer (~4 nm) compared to the vesicle radius.

In the case of size polydispersity one can yet generalize equation 3 accounting for the size distribution function $P_n(R)$ (where the subfix *n* highlight the fact that this a distribution function on a *number*-basis)

$$V \cdot C_{PL} = \frac{N_{EV} 8\pi}{\alpha} \int R^2 P_n(R) dR \qquad (4)$$

Rearranging the above equation one finds:

$$N_{EV} = \frac{V \cdot C_{PL} \cdot \alpha}{8\pi \int R^2 P_n(R) dR} = \frac{V \cdot C_{PL} \cdot \alpha}{8\pi R_{eq}^2} \qquad (5)$$

Where $R_{eq} = \sqrt{\int R^2 P_n(R) dR}$ is the equivalent radius, i.e. the size of the monodisperse system that has the same number of vesicles and phospholipids.

According to equation 5 one can evaluate the EV number as long as he knows the phospholipids concentration (C_{PL}) and the (*number*-weighted) size distribution function ($P_n(R)$).

The dynamic light scattering (DLS) is one of the most applied technique to determine particle size distribution because the measurements are fast and furnish a reliable sampling of the whole system [35]. In DLS the fluctuations of the scattered light intensity are collected and used to evaluate the field autocorrelation function $g^1(\tau)$ that, for a polydisperse sample, is the Laplace transform of the *intensity*-weighted size distribution $P_I(R)$ describing the distribution of the fraction of intensity scattered in by particles with hydrodynamic radius between R and R+dR.

$$g^1(\tau) = \int_0^\infty P_I(R)\, e^{-\Gamma(R)\tau} d\Gamma \qquad (6)$$

Where the term

$$\Gamma = \frac{kT}{6\pi\eta R} q^2 \qquad (7)$$

is the decay rate of a particle of radius R diffusing in a medium of viscosity η and q is the scattering vector that depends on the light wavelength λ, on the scattering angle θ and on the sample refractive index *n* according to

$$q = \frac{4\pi n}{\lambda} \sin\frac{\theta}{2} \qquad (8)$$

Inversion of the Laplace transform (equation 6) gives the $P_I(R)$ that accounts for the number of particles of radius R (per unit volume) and for their size-dependent scattering power.

Commercially available DLS instrumentation customarily evaluates the $P_I(R)$ from the autocorrelation function using suitable numerical routines [36].

According to the Rayleigh approximation, the light scattered scales with the particle mass raised to the second power. For homogenous objects, this implies that the contribution to the scattered light scales as the sixth power of the particle size and retrieving pieces of information on the size distribution on a number basis from the $P_I(R)$ is a process that is strongly vulnerable to the presence of contaminations. On the other hand, the mass of phospholipids in a vesicle is proportional to the bilayer volume $4\pi\delta R^2$ (where δ is the bilayer thickness) so that the scattered light scales as the fourth power of the particle size and the evaluation of $P_n(R)$ from the experimental $P_I(R)$ become more robust.

In addition, for particles with size of the same order of magnitude of q^{-1} the particle inner structure matters and this is accounted for the so called form factor F(qR) that depends on shape and size of the particles and in the specific case of spherical monolamellar vesicles is: [37]

$$F(qR) = \left(\frac{sin(qR)}{qR}\right)^2 \qquad (9)$$

that for $qR \ll 1$ reduces to $F(qR) \approx 1$.

In the case of vesicles, the *number*-weighted size distribution function $P_n(R)$ can be calculated from the *intensity*-weighted size distribution $P_I(R)$ normalizing for the vesicles mass and shape according to

$$P_n(R) = \frac{A \cdot P_I(R)}{R^4 F(qR)} \qquad (10)$$

where A is a normalization constant.

By combining equations 5 and 10 we can evaluate the equivalent radius required to evaluate the number of vesicles as

$$R_{eq} = \sqrt{\int R^2 P_n(R) dR} = \sqrt{\int A^{-1} R^{-2} P_I(R) dR} \qquad (11)$$

Accordingly, the function relevant for the evaluation of R_{eq} is not $P_n(R)$ but instead $R^2P_n(R)$ meaning that, in order to calculate the EV concentration, the $P_I(R)$ obtained in a DLS experiment must be corrected by the term R^{-2}. This is a relatively mild correction making the overall procedure of quantification of the EVs number reliable and robust. This approach has been first applied in the case of artificial liposomes in **Figure 6A**. The liposomes prepared through extrusion are relatively

monodisperse and accordingly the *intensity*-weighted size distribution $P_I(R)$ is monomodal with a maximum at 65 nm of radius. The corresponding *number*-weighted size distribution $P_n(R)$ was evaluated according to equation 10 and is dominated by the smaller vesicles resulting in a sharp distribution with a mode at 36 nm. However, the relevant function is $R^2P_n(R)$ whose mean value, (i.e. the R_{eq} of equations 5 and 11) is R_{eq} = 50 nm. All the EV preparations give results similar to those obtained for the liposomes provided the pellets have been duly suspended after the post PK ultracentrifuge run. When the large MV are examined as in **Figure 6B**, qR~1 and the correction due to the form factor become important. This can be appreciated in **Figure 6B** where are compared the $P_n(R)$ evaluated neglecting and taking into account the F(qR) correction (asterisks and open triangles, respectively). The function $R^2P_n(R)$ in the case of large MVs is not very different from the $P_I(R)$ directly obtained from the DLS measurement. With respect to the determination of the EVs concentration according to equations 5 and 11, we have found that the use of the mean radius of the $P_I(R)$ instead of the correct R_{eq} induces a bias that is below 50% if the EV have large sizes (mean size above 300 nm) but can be larger than 100% for small vesicles. Therefore, if such levels of uncertainty are tolerable one can use in the calculations the intensity-averaged radius or equivalently the z-average obtained from cumulant analysis.

3.3. Application to MS patients examined in different stages of their clinical diseases

The data collected in this pilot study are summarized in Table 1 in terms of total phospholipid concentration, equivalent radius and number of EV (in one μL). In addition, for each fraction (MV or EXO) the total RNA concentration ([RNA]) was quantified by standard methods. This allows evaluating the average intra-vesicle RNA concentration as the ratio between the RNA amount and the average volume enclosed by the vesicles:

$$[RNA]_{EV} = \frac{[RNA]}{N_{EV}\frac{4}{3}\pi R_{eq}^3}. \qquad (12)$$

The EXO concentration values in the blood of three healthy control subjects are mutually close with an average value of $(6.5\pm0.5)\cdot10^8$ exosome/μL, a value that is in good agreement with the data of a recent study performed using NTA and several different purification procedures that gives an average of $(1.4\pm0.3)\cdot10^8$ exosome/μL) [7]. Also the RNA concentration and the size of the EXO are relatively constant among the HCs resulting in a narrow spread of the intra-exosome RNA concentration; the average $[RNA]_{EV}$ is 0.016±0.002 g/mL. With respect to the MV, their concentration is drastically lower in terms of phospholipid amount (25 fold less than EXO) and this, taking into account the large size of MVs, corresponds to an amount of vesicles ~ 10^6. The spread in

size is, however, quite large already among the HCs and the C_{PL} quantified is very close to the LOQ so the MVs are not promising as potential marker of diseases.

Considering the examined subjects, Table 1 reveals that a significant difference for EXOs content is present in CIS and MS patients compared to HC. This finding is consistent with results previously published by Marcos-Ramiro et al. [38] showing different concentrations of human plasma EVs in patients presenting CIS suggestive of MS or in clinically definite MS patients compared to HCs. Since the size of the vesicles remains almost constant between the HCs and the patients, the differences in the EXO concentration can be quantified simply in terms of total phospholipids concentration. If trials involving a larger number of subjects will confirm this point, one could propose the colorimetric quantification (even by visual inspection as suggested by **Figure 2**) as preliminary screening for MS.

Particularly interesting is, in our view, the discrepancy observed between the two samples CIS1 and CIS2, both derived from patients who suffer for the very early clinical presentation of MS. CIS1 has the largest concentration of EXOs observed in this study; it is fivefold that found in HCs and two fold that found in the clinically definite MS patient. At variance, CIS2 has the smaller concentration of EXOs observed in this study; only a seventh of that found in HC. Such a difference might reflect individual differences of the 2 CIS subjects, e.g. in the time interval occurring to the clinically definite MS. A longitudinal follow-up of the clinical and neuroradiological (MRI) features of these patients will possibly give answer to this issue.

4. Conclusions

The quantification of EVs released by human cells is essential for the development of EV-based clinical biomarkers.

In this study, we have implemented a previously proposed assay for the purity of the EV fractions to achieve a robust analytical protocol to quantify the total phospholipid concentration (C_{PL}) and the EVs number. In particular, we have demonstrated that the treatment with proteinase K efficiently digests the large protein aggregates that coexist with the EXOs purified by differential ultracentrifugation. Subsequent ultracentrifugation allows the separation of EXOs (in the pellet) from the peptides (in the surnatant). For these protein-free samples, the nanoplasmonic assay can serve as a robust analytical method to quantify the total phospholipids if applied to serial dilutions of the original sample. The simple visual inspection of the sample color along the dilution path allows capturing deviations from the physiological EXO content. Quantification of the phospholipid content is achieved by comparing the unknown responses along the dilution path with the response upon dilution of a solution of liposomes of known concentration. The use as response (aggregation

index) of the ratio of the absorbance values at three wavelength (Abs@520 nm/[Abs @650 nm + Abs@900 nm]) considerably reduces the LOD (5μM) and the LOQ (35 μM) associated to the C_{PL} concentration. Finally we describe how to take into account the spread in EV size when transforming the concentration of phospholipids into a concentration of vesicles.
The proposed analytical protocol was here successfully applied on a small subset of MS patients and HC subjects, so its use in studies investigating the role of EVs e.g. in MS pathogenesis and its phenotypic characterization/monitoring can be applied in larger samples.

Conflicts of interest: Authors declare no conflicts of interest.

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Table 1. Phospholipid and RNA concentration, equivalent vesicle radius, number of EV per μL of blood and average RNA concentration within one EV.

		C_{PL} /μM	R_{eq} /nm	[RNA]ng/μl	EV/μL	$[RNA]_{EV}$ g/mL
HC1	MV	36±10	640	3.0±0.1	$(1.5\pm0.5)\times10^{5}$	0.019±0.006
	EXO	900±50	50	4.5±0.1	$(6.0\pm0.3)\times10^{8}$	0.014±0.001
HC2	MV	30±10	210	4.0±0.1	$(1.4\pm0.4)\times10^{6}$	0.08±0.03
	EXO	1100±200	51	5.9±0.1	$(7\pm1)\times10^{8}$	0.015±0.002
HC3	MV	32±16	99	4.3±0.1	$(5\pm3)\times10^{6}$	0.2±0.1
	EXO	990±100	50	6.1±0.1	$(6.6\pm0.8)\times10^{8}$	0.018±0.002
CIS1	MV	n.d	670	4.3±0.1	--	--
	EXO	5000±700	52	3.8±0.1	$(3.1\pm0.4)\times10^{9}$	0.0021±0.0003
CIS2	MV	44±12	105	5.2±0.1	$(7\pm2)\times10^{6}$	0.16±0.04
	EXO	144±8	51	2.9±0.1	$(9.5\pm0.5)\times10^{7}$	0.057±0.004
MS	MV	56±12	350	12.8±0.1	$(6\pm1)\times10^{5}$	0.09±0.02
	EXO	2280±160	51	6.9±0.1	$(1.5\pm0.1)\times10^{9}$	0.008±0.001

FIGURE CAPTIONS

Fig. 1. (A) Liposomes at different concentrations (in PBS), ranging from 400 to 0.3 mM, after 2 nM AuNP addition. AuNPs in pure water and PBS are also shown. (B) The absorbance spectra of the samples in (A). The vertical dotted lines evidence 520, 650 and 900 nm. **Inset.:** As purified MV and EXO samples in PBS, obtained from serum by Differential Centrifugation, after 2 nM AuNPs addition.

Fig. 2. Solutions obtained by the nanoplasmonic assay performed on the as-purified EXOs and their serial dilutions for a healty control (HC1) and two different patients (CIS1 and CIS2). For all the cases the first (closest to the observer) cuvette is the as-prepared sample, the second cuvette a 1/5 dilution, the third cuvette a 1/10 dilution and the fourth cuvette (present only for CIS1) a 1/20 dilution.

Fig. 3. Calibration lines obtained by plotting the two aggregation indexes AIs versus liposomes concentration; open blue circle correspond to $AI_{2\lambda}$ and red closed triangles to $AI_{3\lambda}$ as defined by equation 1 (see text for details). AI data refer to AuNPs spectra shown in Fig.1. The black stars denote the intercept with the calibration line of the $AI_{3\lambda}$ value obtained for the last three dilutions of sample CIS1 of Fig. 2. The dotted lines are the AI values corresponding to the LOD in the case of

$AI_{2\lambda}$ and $AI_{3\lambda}$ while the arrows indicate on the concentration scale the corresponding LOD values (in blue and red, respectively).

Fig. 4. Calibration curves obtained plotting AI versus liposome dilution. The initial phospholipid concentration used was 300 mM. Two different stocks of AuNPs and liposomes were used. Solid line represents the best fit to Equation 3 (reported also in the inset), keeping fixed the known C° and obtaining as best-fit parameters the intercept *a* and the slope factor *b*.

Fig. 5. Dilution plot of purified EXOs from HC1, CIS1 and MS1 subjects. Solid lines represent the best fit to Equation 3 keeping constant *a* and *b* to the values obtained from the calibration lineand leaving as adjustable parameter the initial concentration C°.

Fig. 6. Different types of size distribution function described in the text for two representative samples. Filled squares denote $P_I(R)$ *i.e.* the intensity-weighted size distribution function directly obtained from the experimental autocorrelation function measured in a DLS experiment. Asterisks denote $P_n(R)$ the number-weighted size distribution function obtained from $P_I(R)$ according to equation 10. Open triangles denote the number-weighted size distribution function ($P_n(R)$) obtained by neglecting in equation 10 the intra-vesicle interferences, i.e. for F(qR)=1; in the case of small vesicles the two number-weighted size distribution functions are indistinguishable. Gray circles denote the function $R^2P_n(R)$ whose mean value gives the square of the equivalent radius needed to evaluate the vesicles number according to equations 5 and 11.
Panel A) refers to data for a solution of artificial liposomes. Panel B) refers to data for a solution of MVs.

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