Improved extracellular vesicle detection and characterization

Ph.D. thesis

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Introduction

Extracellular vesicles (EVs) are a heterogeneous group of lipid bilayer enclosed particles found to be released by most, if not all, cells. To date EVs have been found to be present in most biological fluids as well as in the environment around us. These vesicles can efficiently carry and protect from degradation different biological molecules such as functionally active proteins, lipids, and nucleic acids. By transferring such molecules between cells, different EVs participate in a diversity of biological processes in health and disease such as inflammation, immune suppression, antigen presentation, tumor development, as well as in the transfer of genetic information, morphogens and signaling molecules.

Given their ability to participate in the above biological processes and to transfer molecules among cells, EVs have become increasingly attractive to researchers from various disciplines for the development of novel diagnostic and therapeutic tools. As a consequence, the field of EV research has seen tremendous growth and an exponential increase in number of publications year after year which has profoundly impacted our understanding of intercellular communication, tumor and stem cell biology, inflammation, virology, circulating extracellular RNA and DNA research among other fields.

With this rapid growth in the amount of reports about EV biology some classification and nomenclature of different subsets of EVs has emerged. Although there is still some debate over the nomenclature used, most in the field utilize the classification of different EVs based on their biogenesis and size ranges as exosomes (EXOs) of approximately 100 nm in size, microvesicles (MVs) of 100 to 1000 nm in size, and apoptotic bodies (APOs) of more than 1000 nm in size. These three important subsets are depicted in Figure 1 but it is important to note that other terms may also be encountered in EV literature. Some of the other used terms for EVs will be described later but in most cases they simply represent alternative nomenclature that actually corresponds to one of these previously described subtypes with size ranges similar to

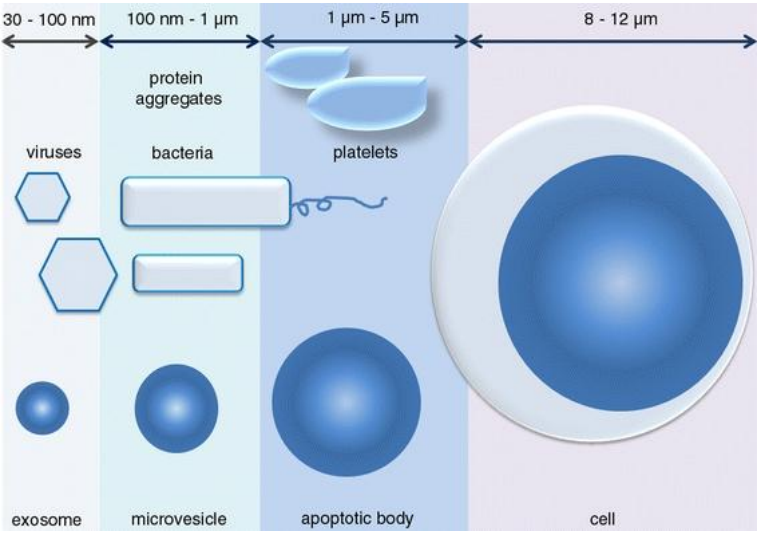

Figure 1. Size ranges of different EV subtypes. György et al 2011

EXOs, MVs, or APOs.

Despite of the fast growth experienced in the field of EVs there are still remain many questions to address about the fundamental characteristics of distinct EV subpopulations. One important limitation for the field is that there are still no universally accepted molecular markers with which to characterize EVs. Additionally, there are no gold standard EV isolation techniques. Because of these reasons some disparity exists in the literature in regards to the different isolation and characterization techniques used for various studies. Consequently, even if there is ample evidence clearly supporting the effects of EVs in many biological processes there are still some areas where the evidence is ambiguous due to dissimilar findings.

Considering these important limitations in the field, the present work focused on improving existing EV characterization and detection techniques by introducing to the field new approaches based on lipid properties with which to quantify and characterize EV preparations.

Objectives

In recent years the field of EV research has truly emerged as one of the fastest growing areas due to all these recent findings and more scientists from other areas continue to get interested and start exploring the functions of EVs in their respective fields. However, despite this rapid growth one concern is that in regards to some biological processes it seems that different reports have found discordant roles for EVs. Therefore, it might seem puzzling whether EVs are in fact beneficial or detrimental in various biological processes in health and disease.

Some of the observed discordance in biological roles reported for EVs can be explained by the inherent differences among various subtypes of EVs simultaneously found in the body which can also be released by different cells possibly in different activation states. Furthermore, the EVs and their cargo can also undergo various post-synthetic modifications or combine with other EVs or soluble molecules which can alter their biological effects. Thus, when comparing different studies showing effects of EVs in any biological process all these inherent biological differences should be accounted for as they may explain discordant results if each report is analyzing completely different EVs.

Even if it may be that many of the discordances can be explained by biological differences among the studies EVs there are still some examples where various reports supposedly investigating the same

particles do not obtain comparable results. One such example can be found with reports of EVs released during remote ischemic preconditioning (RIPC) which were observed to either reduce or to have no effect in infarct sizes (Jeanneteau et al. 2012, Giricz et al. 2014, Yamaguchi et al. 2015). Based on this problem, we believe that in such a rapidly growing field it is also very important to have sound methodology and controls with which to characterize the material that is being studied so that researchers can easily compare their results to each other. As the field currently stands that is not always the case as many groups utilize different isolation and characterization methods and there are still no universally accepted protocols and molecular markers for EVs.

For these reasons, the work undertaken during this Ph.D. had the following objectives.

I. Improve existing EV methodology.

II. Introduce novel assays for EV analysis.

III. Develop a workflow for the quantification of EV lipid content.

IV. Investigate the protein to lipid ratio of the different EV subpopulations.

V. Characterize the membrane lipid order of the different EV subpopulations.

VI. Establish the detergent lysis sensitivity of EVs.

By pursuing these objectives we attempt to develop methods which we believe will expand the existing toolkit for EV research and

hopefully will allow researchers to better characterize the material under study.

Methods

Jurkat (TIB-152) human T cell lymphoma, THP-1 (TIB202) human acute monocytic leukemia, U937 (CRL-1593.2) human histiocytic lymphoma, BV-2 murine microglia, and MH-S murine alveolar macrophage cell lines were cultured in RPMI-1640 or DMEM media containing 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, and 1% Antibiotic Antimycotic Solution (Ab/AM) at 37°C in 5% CO2/air and placed in serum free conditions 24 prior to EV isolation. Cell viability was always monitored by flow cytometry to be >90-95% as confirmed by staining with Annexin V FITC and propidium iodide (both from BD Biosciences, Franklin Lakes, NJ). Acid-citrate dextrose (ACD) anti-coagulated plasma as well as expiring platelet concentrates were also submitted for microvesicle and exosome isolation after removal of platelets.

Three different EV subpopulations were isolated including APOs, MVs, and EXOs by the combination of gravity driven filtration and differential centrifugation. Initially cells were removed by centrifugation at 300 g for 10 mins, and then the supernatant was filtered by gravity through a 5 μm filter (Millipore) and submitted to a 2,000 g centrifugation for 10 mins to pellet APOs. The supernatant was next filtered by gravity through a 0.8 μm filter (Millipore) and centrifuged at 12,600 g for 30 mins to pellet MVs. Finally, the supernatant was ultracentrifuged in an Optima MAX-XP bench top ultracentrifuge with MLA-55 rotor (Beckman Coulter Inc, Brea, CA) at 100,000 g for 70 mins to pellet EXOs. To remove co-isolated materials

and attain higher purity, each EV pellet was resuspended once in PBS and recentrifuged under the same conditions that were originally used for pelleting. A simplified schematic representation of the EV isolation workflow is shown in the below Figure 2.

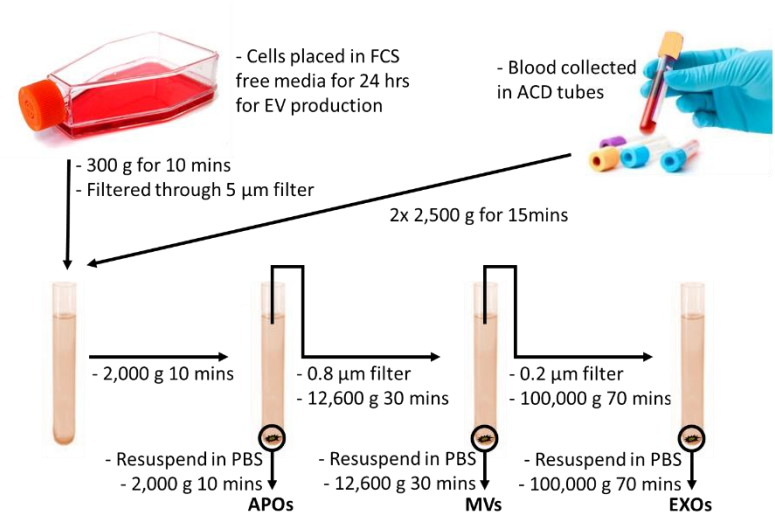

Figure 2. Schematic summary of the EV isolation workflow

To asses EV concentration and size, preparations as well ascell-depleted conditioned media were submitted to tunable resistive pulse sensing (TRPS) analysis using a qNano instrument (IZON Science, New Zealand).

In order to characterize the morphology and size of the different EV preparations, EV pellets were submitted for transmission electron microscopy analysis using a Hitachi 7100 electron microscope

(Hitachi Ltd., Japan) equipped with a Megaview II (lower resolution, Soft Imaging System, Germany) digital camera.

For characterization of molecular markers present on EVs, APOs, MVs, and bead boud EXOs were analyzed by flow cytometry after incubation with different fluorochrome conjugated antibodies and affinity reagents. These included cholera toxin subunit B and di-4-ANEPPDHQ, an anti-cholesterol antibody as well as annexin V, anti-CD9, and anti-CD63 antibodies. To verify the vesicular nature of EVs, and to exclude the presence of antibody aggregates, we added Triton X-100 to 0.05 % final concentration to the samples.

Total protein content of EV preparations was determined using the commercially available bicinchoninic acid assay (BCA), Micro BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL), following the manufacturer's specifications.

To measure total lipid content of EVs, the colorimetric reaction of sulfuric acid and phosphovanilin with lipids (Chabrol and Charonnat 1937) was adapted for use in a 96 well plate format sulfophosphovanilin (SPV) assay using menhaden fish oil as a lipid standard.

In order to investigate the membrane lipid bilayer order of EV preparations, EVs were stained with the membrane probe Di-4-ANEPPDHQ (Life Technologies) and analyzed by confocal microscopy (IX81 inverted microscope based Fluoview500 laser scanning confocal system and software; Olympus Europe, Hamburg, Germany) at 60x magnification and also assessed by flow cytometry.

Results

Since one very important limitation when working with EVs is that typically the yields are much smaller than most other biological samples we first evaluated whether EV preparations could be detected in a 96 well plate adaptation of the SPV reaction using menhaden fish oil as a lipid standard. We found that with this adapted format the assay sensitivity is adequate for EV studies measuring samples containing as little as 0.5μg EVs either in dry pellet or in up to 50μL volume. Furthermore, we found that many of the compounds commonly found in cell line supernatants or biofluids from which EVs are isolated didn't interfere strongly with the SPV and BCA assays. Notably, tryptophan interfered with the BCA assay and glucose interfered with both SPV and BCA assays. We also assessed the accuracy and variability of the SPV assay and compared it to those of the commonly used BCA assay finding that SPV assay compares favorably to the BCA assay in terms of accuracy, although showing slightly higher intra and inter-assay variabilities. However, at concentrations >50 μg/mL lipid for SPV or protein for Micro BCA, both assays show low variability and good accuracy.

Following isolation we characterized EV preparations by electron microscopy, flow cytometry, and tunable resistive pulse sensing finding typical morphology, size, and molecular markers.

We next determined the lipid content of the EV preparations in parallel with protein determination by BCA assay. Using the values

obtained from BCA and SPV assessment, we calculated protein to lipid ratios of EV subpopulations isolated from different cell lines and human blood plasma and found this ratio to be characteristic of each respective EV subpopulation regardless of their cell origin. APOs were consistently characterized by the highest protein to lipid ratio followed by an intermediate ratio for MVs and the lowest ratio was in all cases found for EXOs.

To test the applicability of protein to lipid ratio for quality control assessment we measured EVs in different conditions with suspected poor quality. Firstly, EVs from adenosine diphosphate induced platelets were tested. The low speed (20,000 g) MV pellets were characterized by the expected protein to lipid ratios and the presence of MVs was confirmed by transmission electron microscopy. However, the protein to lipid ratios of the high speed "EXO" (100,000 g) pellets were found to be abnormally elevated and electron microscopy showed the lack of any vesicular structures, suggesting that these "EXO" pellets were mostly composed of proteins. This was in line with the previous finding that ADP is a poor inducer of EXO production (Aatonen et al. 2014). Secondly, freshly isolated MVs incubated overnight at 37 ^{o}C, a condition that we have found earlier to damage MV preparations. Protein to lipid ratios were determined before and after overnight incubation. The overnight incubated and re-pelleted MV preparations were characterized by abnormal protein to lipid ratios compared to the typical MV protein to lipid ratios measured for freshly isolated MVs. This was in agreement with the electron microscopy

showing vesicular damage following the overnight incubation. Finally MV preparations derived from red blood cell concentrates were compared after 2, 3, 4 or 5 consecutive washes showing a shift from elevated protein to lipid ratios to those expected for MVs after the third washing step suggesting the removal of contaminating proteins.

To asses membrane lipid order of EVs, the GP value was calculated from the fluorescence intensities at 560-600 nm and 660 nm of samples stained by Di-4-ANEPPDHQ resulting in values between -1 and +1 with higher GP values reflecting higher membrane lipid order (low liquid disordered, high liquid ordered structure). APOs and MVs were characterized by intermediate and partially overlapping lipid orders with GP values of -0.15 ± 0.15 and 0.00 ± 0.21 (median ± SEM), respectively. In contrast, EXOs displayed a GP value of 0.36 ± 0.29 (median ± SEM), reflecting a higher degree of lipid order than either MVs or APOs (Figure 12). The observed GP values were also assessed by flow cytometry and the results were close to those of the confocal microscope measurement. The GP values measured by flow cytometry were -0.14, -0.14, and 0.25 for APOs, MVs, and EXOs, respectively (Figure 13). The differences between the GP values measured by confocal microscopy and flow cytometry could be ascribed to the small differences in the available filters in each instrument (560-600 nm and >660 nm in the confocal microscope as opposed to 585 ± 21 nm and 616 ± 12 in the flow cytometer). However, the pattern of higher order in EXOs was observed by both instruments.

Detergent sensitivity of EV subpopulations was assessed by treatment with sodium dodecyl sulphate (SDS), Triton X-100, Tween 20 and deoxycholate. All four detergents were found to lyse EVs albeit at different concentration ranges. EXOs were found to be the least sensitive to detergent lysis while APOs and MVs showed higher sensitivity to detergent lysis (and were similar to each other). Out of the four detergents, SDS was found to reduce APO and MV counts at the lowest concentrations (0.01 %) while Triton X-100 lysed EXOs at the lowest concentration (0.075 %). Interestingly, SDS lysed EXOs at least at an order of magnitude higher concentration (0.125 %) than required for lysing MVs and APOs (0.01 %). In contrast, EVs were the most resistant to treatment with Tween 20 which was found to reduce EV counts at very high concentrations (10-15 %) only. Similarly to Triton X-100, the bile acid deoxycholate had moderate efficiency to disrupt all EV subpopulations.

Discussion

The work of this Ph.D. dissertation was undertaken to establish for the first time a simple lipid assay for EVs and to find good quality control parameters to characterize EV preparations that might be utilized both in basic research and in clinical laboratory settings. To this end, we used and optimized the SPV total lipid assay for EV studies and combined this lipid assay with the conventional microBCA protein determination that is used widely in the EV field. Furthermore, we showed that if used under the optimized conditions described in this dissertation, the SPV assay sensitivity is adequate for EV studies. Using these two assays, we introduced protein to lipid ratio as a novel parameter to characterize EV preparations enriched in EXOs, MVs, and APOs. This parameter

The spectral ratiometric approach of this study provides evidence that EV subpopulations can also be distinguished based on the difference in their membrane lipid order. Our data suggest that EXOs are characterized by highest membrane lipid order, while APOs and MVs showed low to intermediate, partially overlapping lipid order reflecting important differences in membrane lipid composition. A high degree of membrane lipid order and the relatively high content of cholesterol in EXOs may be important factors explaining their distinguished role in intercellular signaling as this is one of the most important biophysical parameters of membranes since in low ordered membrane domains the probability of protein-protein interactions is decreased (Owen et al. 2012).

Based on previous findings of our group, we also carried out a comprehensive analysis of the effect of detergents on size-based EV subpopulations. This work provides a practical tool to confirm the vesicular nature of particles present in biological samples using either TRPS or flow cytometry. Given that both TRPS and flow cytometry are commonly used methods to characterize EVs, the use of detergent control in EV analysis may prove useful in a wide variety of experimental settings and may also be combined with other techniques. We found that similar detergent concentrations lysed both APOs and MVs while higher concentrations were needed to lyse EXOs.

These results point to the similarity of the lipid membrane composition of APOs and MVs as opposed to EXOs which were found to be more liquid ordered while both APOs and MVs had similar and lower membrane liquid orders. Accordingly, several studies show that liquid ordered as opposed to liquid-disordered membranes are resistant to detergents (Schroeder et al. 1994, Brown 1998).

In conclusion, for the first time our work introduced a simple lipid assay and novel EV characterization parameter as well as the appropriate concentrations of different detergents to lyse EVs. We demonstrated that different size-based subpopulations of EVs secreted by highly diverse human cell lines show similar protein to lipid ratios, membrane lipid orders, and detergent sensitivity patterns. Taken together, our data validate the use of the SPV lipid assay, membrane lipid order assessment, and detergent lysis as integral components of the EV detection toolbox.

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