Chemical characterization of extracellular vesicles of mesenchymal stromal cells: TOF-SIMS and BCARS approach

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Abstract. Paracrine functions of mesenchymal stem (stromal) cells (MSCs) rely, at least partly, on membrane-bound extracellular vesicles (EVs) with rich composition of lipids, nucleic acids and signaling proteins. Elucidation the underlying chemistry could potentially lead to MSCs-free therapy. However, the secretome of MSCs (EVs' composition) is non-static and depends on many other factors including surrounding cells and medium. Thus, the research techniques must be able to provide not only bulk but microscopy-scale data within a reasonable time frame. Two of these label-free techniques are subject of this work toward the question of chemical composition of the EVs.

1. Introduction
Mesenchymal stem/stromal cells (MSC) are known as a subset of pluripotent cells that can differentiate into various cell types. Earlier experimental studies have shown the ability of MSCs to osteogenic, chondrogenic and adipogenic differentiation, which is currently one of the obligate criteria of MSCs [1]. Recent studies also show MSCs differentiating capability into cardiomyocytes, cells of skeletal, muscle and nervous tissues [2-4]. MSCs of multiple tissues, i.e. bone marrow, adipose tissue, umbilical cord can be isolated, amenable to ex vivo cultivation and subsequent cells transplantation [5] with initial idea to augment the structure and function of damaged or diseased tissues via direct cell replacement. Recent studies suggest that, in addition to direct differentiation, stem cells can regulate the reparative process through paracrine secretion of various growth factors, glycosaminoglycans, cytokines and extracellular vesicles (EVs) modulating immune response, apoptosis, proliferation, differentiation, angiogenesis [6,7].

In recent years, interest in the field of EVs has grown tremendously leading to some paradigm shift that EVs could be one of the main components of the cell’s secretome responsible for paracrine functions. Secreted by the majority of cell types, EVs can be divided into exosomes, microvesicles, apoptotic bodies, and other subsets of extracellular vesicles [8]. Smaller sized EVs, i.e. exosomes (30–100 nm) and microvesicles (up to 1000 nm), derived from MSCs have recently become known as promising agents for tissue regeneration and immunomodulation while their remarkable handling advantages can possibly lead to so-called 'cell therapy without cells' [9,10]. Exosomes and
microvesicles differ based on their origin within the cell. The formation of exosomes is associated with endosome biogenesis and directly depends on the multivesicular body while endosome maturation [11]. Unlike exosomes, microvesicles formed by direct cell membrane budding and contain the same characteristic lipids as exosomes [12]. Both exosomes and microvesicles carry functional cargo composed of nucleic acids (including microRNAs, messenger RNAs and DNAs) and proteins [13].

Quality and reproducibility of current EVs isolation methods are crucial for understanding the actual source of regenerative potential in the secretome of MSCs. Several isolation methods have been described to isolate EVs [14], including differential ultracentrifugation, density gradient ultracentrifugation with centrifugal forces more than 100,000×g, isolation reagents, exclusion chromatography. Some recommendations for the characterization of EV samples were given by International Society for Extracellular Vesicles (ISEV) [15]. In addition, the subtypes of EVs obtained from different tissues using several methods were summarized [9,16]. In view of importance of potential applications, various techniques were already employed for EVs characterization, including electron microscopy (SEM, TEM) [10]; PCR [17]; dynamic light scattering (DLS), spontaneous Raman scattering [18,19]; narrow band CARS, SHG [20]; Raman scattering with multispectral optical tweezers [21]; mass spectrometry [22]. In this work we study EVs using multimodal approach based on laser multicolor confocal fluorescence scanning microscopy (LSM) and modern label-free chemical imaging techniques which are: broadband CARS microspectroscopy (BCARS) of the fingerprint range and time-of-flight secondary ion mass spectrometry (TOF-SIMS). Particularly, both BCARS and TOF-SIMS are especially good at lipid imaging/characterization. The latter are essential molecular components of EVs, but the role of lipid content for EVs functioning is still not fully elucidated [23].

2. Materials and methods

2.1. Samples
Adipose tissue derived MSCs (d122, kindly provided by Koltsov Institute of Developmental Biology, Moscow, Russia) were prepared using a standard methodology [24]. Culturing continued until ~90-95% confluence density was reached, followed by serum-free cultivation for 2 days. EVs isolation procedure included multi-stage centrifugation to obtain debris-free media followed by ultracentrifugation. The pellet containing exosomes/microvesicles was re-suspended in DPBS (Gibco).

2.2. TOF-SIMS measurements
Mass spectrometry studies of EVs were performed on the TOF.SIMS.5 instrument (ION-TOF, Germany). Bismuth cluster ions (Bi$_3^+$) with the energy 30 keV were used as an ion source. An analysis area was 300×300 μm (64×64 pixels) and the primary ion dose density was kept below 6×10$^{11}$ ions/cm$^2$ for each measurement. Electron flood gun was activated to avoid charging. Since TOF-SIMS requires high vacuum conditions, EVs have to be analyzed in the dehydrated state. Dehydration procedure was as follows. 10 μL of EVs solution in mQ water was applied on the surface of cleaned silicon wafer and dried in the argon atmosphere.

2.3. LSM measurements
LSM studies of live MSCs cells were performed on the LSM-980 multicolor scanning microscope with stage top incubator (Zeiss, Germany). Live cell staining fluorescent dyes used in experiments: 1) CellMask™ Deep Red Plasma membrane Stain (5 mg/ml, excitation at 639 nm, emission range 650-680 nm); 2) Hoechst 33342 (1 mg/ml, excitation at 405 nm, emission range 415-480 nm); 3) BioTracker 488 Green Microtubule Cytoskeleton Dye (1 mg/ml, excitation at 488 nm, emission range 510-550 nm). Incubation time with dyes was 30 min at 37 °C followed by multiple washing with DMEM. Finally, nutrient mixture DMEM/F-12 (Gibco) was added.

2.4. Broadband CARS measurements
Broadband coherent anti-Stokes Raman scattering (BCARS) microspectrometer implemented as a part of femtosecond laser complex [25] representing two pulses collinear scheme for CARS generation.
Narrowband (FWHM < 10 l/cm) sub-picosecond pump pulses centred at 730 nm to tune for vibrational bands of the fingerprint range: 700-1800 l/cm. Bell-shaped Stokes pulse (FWHM ~500 l/cm) at 800 nm was transformed to flat-top pulse (FWHM ~1000 l/cm) using real-time 1D-SLM/spectrometer optimization algorithm; additionally, negatively chirped was supplied to 1D-SLM to provide dispersion compensated pulse (~50 fs) at microscope area of the setup. Measured spectra of biological samples were related to ones of sapphire glass to exclude non-resonant component of CARS. Finally, the related spectra transformed to Raman bands using CARS-variation of MEM described earlier [26].

3. Results and discussion
The result of NTA (nanoparticles tracking analysis) shows (figure 1a) the isolated EVs solution contains both exosomes (< 100 nm) and microvesicles (< 1000 nm) with average particle size ~120 nm. Although bright field microscopy also shows large particles (> 1000 nm), they generally tend to uneven distribution over the thickness of the solution and accumulate mostly in the upper part of the solution (as shown in figure 1b). In general, the fraction of such particles is small. Overall concentration of all EVs, according to NTA is ~10^10 particles/ml.

Figure 1. Size distribution of isolated EVs (a) bright field microscopy of EVs (b).

Figure 2 represents typical lipid components presented in the mass spectrum of EVs. While lipid ions are clearly seen, they are not dominant in mass spectrum. The majority of intense peaks are likely attributed to the inorganic salts presented in EVs solutions. However, despite significant complication of mass spectra caused by presence of the salts the same lipid peaks were prominent for all samples. Phosphatidylcholine (PC), sphingomyelin (SM) and cholesterol fingerprint fragment ions were detected in the positive ion mode. Negative ion spectra show peaks of myristic (C14:0), palmitoleic (C16:1), palmitic (C16:0), linolenic (C18:3), linoleic (C18:2), oleic (C18:1), stearic (C18:0) acids. It should be noted that fatty acids ions likely formed under bismuth bombardment of phospholipids (at least PC and SM) or acylglycerols. In general, formation of these lipid ions corresponds to the lipid content of the cell plasma membrane [27,28]. However, peaks intensity was significantly lower due to low concentration of EVs. Therefore, only the most intense bands of the corresponding lipids are clearly visible (Figure 2).
Figure 2. TOF-SIMS spectrum of isolated EVs.

An attempt to increase the concentration by applying several drops to the same site showed no improvement in lipid sensitivity due to the presence of salts in the solution. Besides lipids, the detection of following phosphate ion peaks (H$_2$PO$_4^-$, PO$_4^{3-}$, PO$_2^{3-}$), as well as many fragments of carbohydrate ions (CH$_3$O$^+$, C$_2$H$_5$O$^+$, C$_3$H$_5$O$^+$, C$_5$H$_5$O$^+$, C$_5$H$_7$O$_3^+$, C$_5$H$_9$O$_3^+$) can also be an evidence of nucleic acid (NA) content of EVs [29].

Figure 3. LSM images of live MSCs (Blue: Hoechst 33342, Green: cytoskeleton dye; Red: Deep Red Plasma membrane stain)

Spatial resolution of LSM images shown in Figure 3 is of order of 200 nm (Airy scan mode of LSM-980). Since concentration of lipid content in the vesicles is locally high, the red (membrane dye) spots of the scan can be treated as microvesicles (exosomes are beyond the device resolution). Additionally, we believe that the shape of the microvesicles should be close to spherical due to the membrane structure of the vesicles [10]. In this sense, one can say the surface density of EVs over the
cell membrane can have essentially changes, forming large EVs clusters in some areas over the cell (Figure 3b). One of that clusters is probably also detected during label-free BCARS measurements (with band coverage reduced for image mode). Each point of the BCARS scan show integral value over the band of ~1440 1/cm. Locally increased value of the integral (by the factor of 5) can indicate that molecules of the same chemical class are concentrated at the corresponding scan points. The band of ~1440 1/cm is one of the main bands of lipids in the fingerprint range [30].

![Integration range: 1420-1480 1/cm](image)

**Figure 4.** BCARS image of live MSC: integration over the band of ~1440 1/cm.

The lipid content of isolated EVs samples can be recognized by a typical spectral profile which is reflected in following main bands of lipids: ~1442 1/cm (CH2 scissoring mode), ~1660 1/cm (C=C stretching mode), ~1300 1/cm (in phase CH3 twisting mode), 1060–1090 and 1110–1180 1/cm (C–C stretching vibrations) including gauche C–C stretching (~1086 1/cm) and trans C–C (~1120 1/cm), ~1260 1/cm (=C–H deformations), ~1740 (C=O stretching of ester groups) [30].

![BCARS (transformed to Raman bands) spectrum of isolated EVs. Blue, magenta and green markers represent typical bands of lipids, nucleic acids and proteins, respectively.](image)

**Figure 5.** BCARS (transformed to Raman bands) spectrum of isolated EVs. Blue, magenta and green markers represent typical bands of lipids, nucleic acids and proteins, respectively.

The band of ~1740 1/cm is characteristic of triacylglycerols, cholesteryl esters and complex phospholipids like phosphatidylcholine (PC) and phosphatidylethanolamine (PE) while stretching vibrations of the choline N+(CH3)3 group (PC and sphingomyelin, SM) is at ~720 1/cm; cholesterol (CHL) and cholesteryl esters band at ~703 1/cm is also detected (figure 5). The bands of nucleic acids are mainly seen at ~1575 1/cm (ring breathing modes), ~780 1/cm (various breathing modes of U,T,C) [31].
4. Conclusion
EVs derived from MSCs have recently become known as promising agents for tissue regeneration and immunomodulation while their remarkable handling advantages can possibly lead to new type of therapy based on paracrine functions of the EVs. Elucidating relationship between cell secretome chemistry and response of the target objects can significantly expand our knowledge of the MSCs paracrine status. Advanced femtosecond microspectroscopy and mass spectrometry techniques like BCARS and TOF-SIMS can be effectively used to determine the main features of complex chemical composition of the EVs as well as for MSCs imaging.

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