Journal of
Nanomedicine & Nanotechnology

Sharma and Gimzewski, J Nanomed Nanotechnol 2015, 6:6
DOI: 10.4172/2157-7439.1000e135

Editorial Open Access

Exosomes: Nanoscale Packages Contain the Health-state [Status Quo] of the Cells that Secrete them

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Editorial

How cells communicate over large or small distances has only recently become known (Figure 1). The body uses nanometer-sized exosomes, also called Extracellular vesicles [EVs], to facilitate cell communication and send signals to distant cells throughout the body [1]. Mounting evidence indicates that programmed/triggered secretion and targeted migration of exosomes to distant cells is a fundamental aspect of cell biology that is ubiquitous in diseased and normal cells. Exosome transport occurs through extracellular body fluids including blood, cerebrospinal fluid, saliva, milk, and urine. Characterizing this fascinating class of nanovesicles offers exciting and unique insights into how intercellular biomolecular machinery remotely orchestrates physiological and pathological events at a distance. They have been described as a cellular FedEx system. Furthermore, it is being increasingly suggested that diseases such as cancer, Alzheimer's disease, and AIDS can propagate throughout the body by hijacking exosomes and disguising themselves like wolves in sheep's clothing to sneak into healthy cells [2]. Thus, EVs are intriguing for cell biology research, their importance in various diseases, and as models for a new class of pharmaceuticals.

From the nanotechnology perspective, exosomes are truly amazing multifunctional nanoparticles. Quantitative high-speed and high-resolution single-vesicle methods for isolation and characterization of exosomes derived from various biological specimens, are essential for understanding exosomes. Applications of nanotechnology tools are important to facilitate knowledge of exosome disease biomarkers, and for new therapeutics that go beyond current bulk proteomic, genomic, or lipidomic assays. Those assays often target the biomolecular complex that constitutes the exosomes with unique signatures from the cells of origin, or explore the functional effects of exosome-mediated intercellular communication. Efforts have now focused on investigating exosomal content, which is rich in mRNA, microRNA, ncRNA and double- and single-stranded DNA signatures. This includes disordered genes, lipids or proteins [membrane, soluble, cytoplasmic, and perhaps nuclear] and protein modifications such as phosphorylation relating to cancerous, neurodegenerative, or other disease pathways.

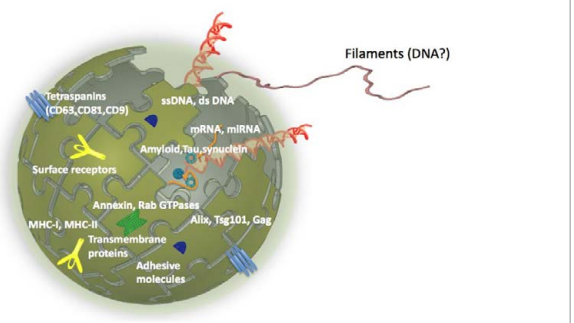

Figure 1: Schematic illustration of an exosome: A nano-jigsaw puzzle. Structurally, exosomes arespherical lipid bilayer vesicles, shown here in green. The presence, density and organization of an exosome's surface and luminal biomolecules is still a major puzzle.

Opportunities include signatures and characterization of therapeutic response, identifying cellular [stem] cell subpopulations and cellular states, and Epithelial-Mesenchymal transitions to identify disease detection, progression, and treatment modalities. EVpedia [3], a public database for exosome research shows 172,080 vesicular components identified from 263 high-throughput datasets.

Our current knowledge of single exosomes is, however, desperately lacking, despite the wealth of methods applied thus far for their characterization [4]. Basic questions concerning morphology, size, phenotype, internal or external location of constituent components, and even concentration levels remain to be elucidated using semi-quantitative and quantitative characterization methodologies. These factors are hampered by two main challenges related to their nanometer dimensions and heterogeneity.

It is evident that characterizing these "super-enriched information" particles at the vesicular and sub-vesicular scale has tremendous potential for understanding, diagnosing, and identifying new approaches to combat brain and other cancers, Alzheimer's disease, and other potentially exosome-mediated infectious and non-infectious diseases. In particular, more efficient nanoparticle sizing, enumeration, and phenotyping methods that provide quantitative, sensitive, and specific "visualization" of isolated EV preparation can expand and complement the much-needed confirmation of EV purity, distinct differentiation from other smaller cells, and aggregates. This will help standardize any potential methods for downstream studies in disease-associated exosome genomics, proteomics, and lipidomics. To better understand the role of exosomes in health and disease, we first need accurate individual or simultaneous measurements of exosome size, composition, concentration, and cell of origin.

Nanoscale imaging techniques have captured biological processes ranging from dynamic events such as endocytosis, cancer cells, and subcellular structures to malfunctioning protein structures associated with Alzheimer and Parkinson's disease. While much progress has

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Received September 23, 2015; **Accepted** September 25, 2015; **Published** October 07, 2015

Citation: Sharma S, Gimzewski JK (2015) Exosomes: Nanoscale Packages Contain the Health-state [Status Quo] of the Cells that Secrete them. J Nanomed Nanotechnol 6: e135. doi:10.4172/2157-7439.1000e135

been made in vesicular imaging since the discovery of exosomes using electron microscopy [EM] [5] in the mid-1980s, we need to continue to push the limits of microscopy and imaging for exosome research. The availability of new and advanced tools with correlative techniques have made quantitative, high-resolution information on EV size, morphology, phenotype, and biomolecular characteristics of single exosomes possible. Imaging modalities such as cryogenic-transmission electron microscopy [cryo-TEM], scanning electron microscopy [SEM], Field-Emission SEM [FESEM] and atomic force microscopy [AFM] have been used to assess morphology and quality of exosome preparation. AFM and FESEM studies [6] have challenged the previous model of "cup-shaped" exosomes originally proposed from stained samples studied with TEM. Recent advances in cutting-edge nanotechnologies, and physical and biochemical know-how, are facilitating improved detection, visualization, and characterization of individual vesicles. The aim of this progress is to combine advantages of high-resolution imaging technologies with molecular phenotyping of exosomes.

Correlative microscopy

Correlative Microscopy uses different complementary forms of microscopy to create multidimensional information, such as combining optical and electron microscopy. This approach enables detailed spatially resolved information that is greater than the sum of its parts and is becoming increasingly useful in biology.

Cryo-EM

While a common method for imaging exosomes, it is acknowledged that conventional EM observations are inadequate for characterizing the structure of exosomes due to artifacts introduced by sample preparation and staining strategies [7]. More advanced modes [7], such as the cryo-TEM, are powerful tools for investigation of exosomes in their native aqueous environment without staining or added fixatives. Cryo-EM [8] also enables 3D tomography, enabling spatial visualization of more complex structures. Although Cryo-EM studies of viruses are widespread [9-11], it has only recently been used for imaging exosomes [12]. Cryo-EM and immuno-gold labeling with secondary antibodies also allow detailed characterization of subpopulations of exosomes for more accurate phenotyping and enumeration in complex biofluidic environments [13]. Using field-emission cathodes in SEMs [FESEM] provides narrower probing beams at low and high electron energies. This improves spatial resolution, minimizes sample charging, and minimizes sample damage, and has been successful for illustrating morphological characteristics of individual exosomes [6]. FESEM provides lower electrostatic distortion, enabling a spatial resolution ~1.5 nm. High-quality, low-voltage images are obtained with negligible electrical charging of samples with accelerating voltages ranging from 0.5 to 30 kV.

Atomic force microscopy [AFM]

Atomic force microscopy [AFM] has emerged as a successful method for studying the morphology, size, and phenotype of exosomes [6]. AFM enables imaging of isolated vesicles under physiological buffers to achieve nanoscale morphology, size, and exosome count information for populations and subpopulations. Using either functionalized nanobeads or a functional probe tip [single molecule force spectroscopy [SMFS], AFM enables complementary phenotyping of exosome subpopulations at the single-vesicle level [14].

Advanced AFM methods such as peak-force mapping enable simultaneous evaluation of 3D morphology and physicochemical properties [elasticity and adhesion] of the nanoparticles at sub-nm resolution with pico-Newton [pN] sensitivity [15]. Several examples of integrated fluorescence and atomic force correlative microscopy have been reported in recent years. AFM has been integrated with a total internal reflection fluorescence microscope [TIRFM] [16] and a confocal optical microscope [17,18]. Diffraction limits lens-based optical microscopes at around half the optical wavelength, which is incompatible with imaging nano-sized particles. However, stimulated emission depletion [STED] microscopy [19] and stochastic optical reconstruction microscopy [STORM] achieve high lateral resolution down to several tens of nanometers, comparable to AFM measurements. Recently, an integrated imaging system combining STED and AFM was reported [20], enabling simultaneous imaging of morphological features and biomechanical parameters of fluorescently stained cells or vesicles. High-resolution information obtained by STED requires high illumination intensity, which may cause phototoxicity. STORM uses less intense radiation, but resolution is determined by data acquisition time. STED and STORM are scanning technologies, so the time resolution for larger samples is low. Live-cell imaging using STED is limited to smaller, less dynamic processes unlike real-time exocytosis of exosomes.

Despite limitations and complexity of multicolor imaging, the resolution of STED and STORM are highly attractive for vesicle research and we expect that integrated super-resolution/AFM systems will offer vesicle characterization with nanoscale resolution.

Combined flow cytometry and flow imaging

Exosomes have been analyzed by conventional flow cytometry via bead-based assays [21,22] where they are captured nonspecifically on the surfaces of microspheres [via chemical conjugation] or specifically [via antibody capture], then detected using a fluorescent antibody [or antibody combinations] against exosome surface markers. The beads are readily detected using light-scatter triggering, and the amount of exosomes captured is estimated based on the reflected fluorescence intensity of the bead-bound reporter antibody. This approach provides information on a population average of all of the exosomes captured on the beads. Because of their nanoscale size and inability of most flow cytometers to detect particles under 500 nm across, the accurate assessment of individual nanoparticles is difficult. Custom instruments with improved sensitivity and calibration [23,24] show promise for providing accurate estimates of size and antigen number on individual exosomes. Swarm detection [25] of exosomes remains useful for detection of smaller [<500 nm] microparticles. Here multiple vesicles are simultaneously illuminated by laser beam and counted as a single event, which results in significantly underestimating the concentration of measured vesicles in a sample [26]. As with conventional flow cytometers, protein aggregates and other vesicles or cellular components may limit the performance of novel generation flow cytometers. Combining exosome flow cytometry with imaging eliminates some of these limitations while providing morphological confirmation and ability to distinguish true single events from aggregates and cell debris [27]. Typically, flow cytometry generates exosome-sizing data exclusively based on fluorescence and scatter signals without the ability to confirm the source of these signals. Adding imaging to flow cytometry offers way to identify "true" vs. "false" positive events and enables higher sensitivity over conventional flow cytometry for exosome analysis by giving a better "view" of what is quantified in exosome preparations.

SEM coupled with raman spectroscopy

Methods such as Raman microspectroscopy have the potential to obtain biochemical information, such as cellular origin, on the level of single vesicles, directly in suspension, without labeling. Recently, Raman spectroscopy and surface-enhanced Raman spectroscopy [SERS] were applied to study the compositional differences between exosomes derived from ovarian cancer cells [28]. SEM/Raman spectroscopy can provide detailed exosome biochemical information with relative size distribution and morphology. SEM enables nanoscale imaging of exosomes, while Raman spectroscopy can acquire molecular fingerprints of individual particles. Raman spectroscopy can provide a direct measure of chemical component ratios like exosome's DNA-to-RNA, proteins-to-lipids, etc., and does not require molecular labels. Thus, it is a powerful technique for unbiased biomarker detection in cells, and is complementary to fluorescence labeling strategies [29].

For our knowledge of vesicles to leap forward, detection limits need to be pushed further by combining or developing new technologies. With more sensitivity, we expect to gain a growing insight into composition, and biological and clinical relevance, of vesicles in health and disease. The rapid development of nanoscale microscopy techniques over the last two decades positions us to gain insights into a detailed model of how exosomes work and how they can be manipulated to control diseases from HIV and Alzheimer disease to brain cancer.

Such control will certainly open a pathway for new drugs and could revolutionize medicine.

Acknowledgments

The authors would like to acknowledge support from California NanoSystems Institute at UCLA and the International Center for Materials Nanoarchitectonics Satellite [MANA], National Institute for Materials Science [NIMS], Tsukuba, Japan. Some parts of schematic adapted from Grabcad.

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