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The Quest for Characterizing Exosomes: Circulating Nano-Sized Vesicles

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Atomic Force Microscopy Imaging of Exosomes- Opens the Way towards Higher Resolution, Quantitative Single Vesicle Information

Research on secreted membrane vesicles, in particular exosomes, is a very exciting field. Exosomes are 50-100 nm particles secreted into the extracellular environment by a wide range of normal mammalian cells. Exosomes are formed by exocytosis through an inward budding of the cell membrane [1] and possess cell type-specific lipid-bilayer membrane (containing cytosolic proteins, m-RNA, mi-RNA). Although their physiological functions are yet unclear, exosomes are known to play a role in specialized functions such as cell antigen presentation [2], intercellular communication [3], shuttling m-RNA/mi-RNA [4] or infectious agents [5].

For over two decades after their discovery, exosome secretion was mostly considered to be a process whereby cells continually discarded unwanted cellular components. But recent new studies recognized possible use of exosomes secreted into easily assessable bodily fluids such as saliva [6,7], blood [8] and urine [9] to gain insights about distant cells that release them under normal physiology or diseased states. Isolating exosomes and studying their structural, biochemical and molecular characteristics can provide novel clues about specific cells and tumors. Apparently, exosomes have gained significance as diagnostic biomarkers for different types of cancers [10] and infectious diseases [11]. Malignancy and other diseases cause elevated exosome secretion and tumour-antigen enrichment of exosomes associated with cancer cells [12,13]. Cancer cells may use exosomes as extracellular signaling messengers to modify the tumor microenvironment and promote metastasis. Thus, specifically targeting cancer exosomes may prove to be an attractive target for cancer therapy.

Although, the relevance of exosomes is increasingly recognized, for diagnostic and therapeutic applications, yet there is an urgent need for better understanding of structure, biomechanics and biochemistry of what exosomes are and how they function. One of the main obstacles to unraveling single exosome "form and function" has been a lack of tools to characterize these nano-sized vesicles.

Electron Microscopy (EM) has provided a wealth of information regarding the shape and size characteristics of exosomes including immunolabeling for endosomal markers. Exosomes were first observed by EM in the 1980s [14]. EM still remains the classical method to characterize exosomes and validate purification methodologies invariably due to the sub-100 nm size of these particles, below the detection sensitivity of diffraction-limited optical techniques such as FACS (200-300 nm). EM imaging of numerous exosomes has revealed round cup-shaped membrane vesicles of 50 to 90 nm vesicles but with limited substructure details [15]. The potential influence of chemical fixation and/or embedding and electron dense staining on the observed structure of exosomes however remains a limitation.

Nanotechnology tools such as AFM [16] can overcome some of the limitations of current methods and play a crucial role by enabling sensitive and quantitative characterization of these nanoscale (30-100 nm) vesicles. AFM is a powerful biophysical nanoscale characterization technique [17-19] that offers unique capability for direct 3D imaging of single exosomes without electron dense staining, fixation, extreme temperatures, and with imaging resolution comparable to that of EMs. Additionally, AFM is a versatile technique that also provides information about the structure, mechanical properties and specific bio-molecular composition of exosomes including under physiological buffer conditions [20,21].

Recently, AFM imaging has been successfully applied to investigate the ultrastructure of exosomes isolated from saliva, establishing AFM as a valuable tool for single exosome analysis. Human saliva exosomes morphology was studied using correlative AFM and FESEM (Field Emission Scanning Electron Microscopy) approaches [22]. As a contact microscope, AFM does not suffer from diffraction limits, unlike optical microscopes, enabling it to reach sub-nm resolution. AFM imaging revealed distinct tri-lobed ultrastructure of single saliva exosomes [22]. Further, force nanomanipulation during AFM imaging can detect exosome mechanics, local deformation and rupture characteristics. AFM imaging at low forces revealed three-dimensional round spherical shaped exosomes. High magnification (at low voltage) FESEM images also show spherical surface, revealing that exosomes possess unflattened, round morphology unless an outside force is exerted on them (*e.g.,* when imaged at higher forces under AFM). Based on the AFM and FESEM observations, the likelihood of EM sample perturbation resulting in the commonly observed "cup-shaped" morphology of exosomes seems very likely.

In addition, Single Molecule Force Spectroscopy (SMFS) can target specific surface receptors on individual vesicle surfaces. Unlike 2D gel analysis where trans-membrane proteins are difficult to analyze, SMFS enables easy distinction between lumenal and cytoplasmic exosomal proteins. The proof-of-concept has been demonstrated by quantitative detection of CD63 receptors, on individual exosomes from human saliva via targeted antibody tip coated force spectroscopy [23] and antibody-labeled gold beads [22]. The ability to measure and quantify single receptors using SMFS can open exciting new avenues in early detection of tumors by assessing changes in the receptor densities of tumor-associated biomolecules expressed on exosomal membranes, where conventional methods may prove ineffective due to sensitivity limitations.

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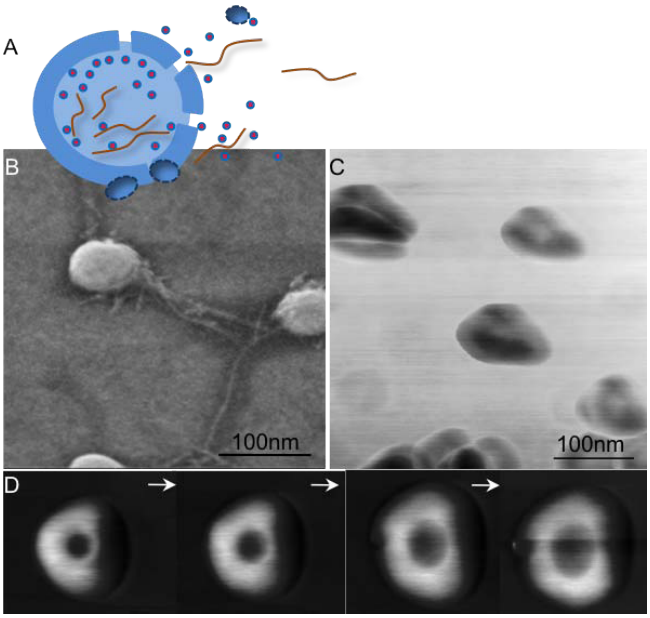

Figure 1: Structural characteristics of exosomes (A) Schematics of MV membrane rupture releasing exosomes along with intervesicular filaments (B) FESEM image of saliva derived exosomes showing round bulging vesicles and intervesicular filaments (C) AFM image showing trilobed exosome ultrastructure and (D) AFM image of single exosome showing increase in size with increased applied force (left to right). Adapted with permission from Sharma, S., et al., ACS Nano, 2010. 4(4): p. 1921-6, Copyright 2010 American Chemical Society and Sharma, S., et al., Langmuir, 2011. 27(23): p. 14394-400, Copyright 2011 American Chemical Society.

Despite the success of AFM imaging of exosomes several questions remain to be addressed. AFM images revealed for the first time, exosomes surrounded by framework of elongated intervesicular nano-filaments (Figure 1) both within the Multivesicular (MV) bodies as well as outside the extra-vesicular milieu. The origins and biological function of these filaments are relatively unknown. It is tempting to speculate the possibility of membrane bilayer, DNA, mRNA or microRNA constituting all or part of these structures, specifically due to the implications of exosomes as messengers for intercellular communication between distant cells. The filaments have not been observed elsewhere and need further confirmatory work such as labeling with proteomic and genomic markers and imaging in physiological buffer conditions.

Nevertheless, advances in nano-enabled and nano-enhanced tools to study exosomes, promises novel exosome diagnostic and therapeutic applications in the coming years. AFM can prove to be a useful alternative and complementary tool for exosome characterization together with EM and fluorescence microscopy [24]. Additionally, as naturally occurring nanostructures with inherent biocompatibility and nanoarchitecture, understanding the biophysical and biomechanical properties of individual exosomes may prove critical to encapsulate, mimic and enhance drug delivery systems.

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