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Viewpoint

Visualization of the Biogenesis, Dynamics, and Host Interactions of Bacterial Extracellular Vesicles

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The first discovery of bacterial Extracellular Vesicles (bEVs) was made in the Gram-negative bacterium *Escherichia coli* in the 1960s.[1] The secretion of vesicles by Gram-positive bacteria was not uncovered until the 1990s because the thick cell wall was seen as a physical barrier to their release.[2] It is now evident that in all domains of life, bacteria secrete spherical membrane vesicles ranging in diameter from 20 to 400 nm (Figure 1A). bEVs are found to be associated with numerous cellular factors, suggesting their pivotal role in bacterial homeostasis, survival, stress response and adaptation to environment.[3,4] bEVs are involved in communication with other microorganisms and with host cells. They can transport proteins, lipids, peptidoglycan fragments, DNA, RNA, phages and small signaling molecules. In addition, in pathogenic bacteria, bEVs carry and deliver toxins and virulence factors.[5,6] To date, researchers are able to purify and isolate them, but very few protocols allow the tracking of bEVs biogenesis and their fusion with host cells.

Due to their nanometric sizes, the reference method for characterizing bEVs remains transmission electron microscopy (TEM).[7] However, TEM is not suitable for visualizing bEVs in their native state since it requires fairly drastic sample pretreatment (Figure 1B). In contrast, super-resolution techniques including structured illumination (SIM), STED, and PALM/STORM microscopies have become tools of choice to image them. While their spatial resolution remains unmatched, label-free methods, such as ISCAT interferometry, have emerged recently, using arguably much simpler setups.[8] Optical phase microscopies have also evolved way beyond simple phase contrast imaging to provide quantitative phase imaging.[9,10] Here, we present the state-of-the-art methods and labeling strategies for imaging bEVs.

BIOGENESIS OF bEVs

In a recent report, Sartorio et al. employed an elegant dual marker system through the fusion of outer membrane and outer membrane vesicle markers with fluorescent proteins to visualize biogenesis of bEVs in the prominent member of the human intestinal microbiota *Bacteroides thetaiotaomicron*.[11] Using time-lapse (widefield) microscopy they showed that the production of bEVs in *Bacteroides* resulted in an orchestrated cellular process that assisted bacterial cells to gain a fitness (Figure 1C).

Previously unresolved bEV biogenesis in Gram-positive bacteria was addressed at the nanoscale by super-resolution STORM.[12] By staining *Staphylococcus aureus* (or *S. epidermidis*) with Nile red to label the plasma membrane, WGA to label cell walls, and antiprotein A or antienterotoxin B antibodies to label virulence factors, vesicles were observed at the surface of bacterial cells. The correlative analysis of STORM and scanning electron microscopy images of bEVs suggested that membrane blebbing and explosive cell lysis were the main biogenesis pathways (Figure 1D). Notably, this multicolor imaging showed that cell wall degradation plays a significant role in regulating bEV release in Gram-positive bacteria. In another study, live cell imaging and electron cryo-tomography revealed a mechanism for bEV formation in *Bacillus subtilis*.[13] Through the labeling of vesicles' membranes with fluorescent FM4-64 and their nucleic acid cargo with SYTOX green dye, it was shown that cell death induces biogenesis of bEVs in neighboring bacteria. Moreover, the release of vesicles was induced by genotoxic stress through the activation of the endolysin system. Indeed, the expression of a prophage-encoded endolysin in a subpopulation of *B. subtilis* induced the formation of holes in the peptidoglycan cell wall. These holes acted as open doors facilitating the release of bEVs. The involvement of phages in the biogenesis of bEVs was also observed in *E. coli*. Mandal et al. used phase-contrast and super-resolution microscopy (3D-SIM, Structured Illumination Microscopy) to study bacteriophage infection of *E. coli*.[14] Time-lapse image sequences of the plasma membrane labeled with the FM1−43X dye indicated that bEVs were generated under phage infection by explosive membrane blebbing and cell lysis (Figure 1E).

bEVs IN MODULATING MICROBIAL ECOSYSTEMS

bEVs facilitate interactions between bacterial cells of the same species but also play an essential role in interactions between different bacterial species, influencing competition, cooperation, and overall community dynamics within microbial ecosystems.[15] Recently, Meyer and Nodwell used fluorescence transfer of lipid soluble dyes and confocal microscopy to assess the delivery of *Streptomyces* bEV content to different bacteria

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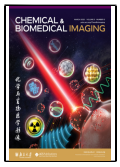

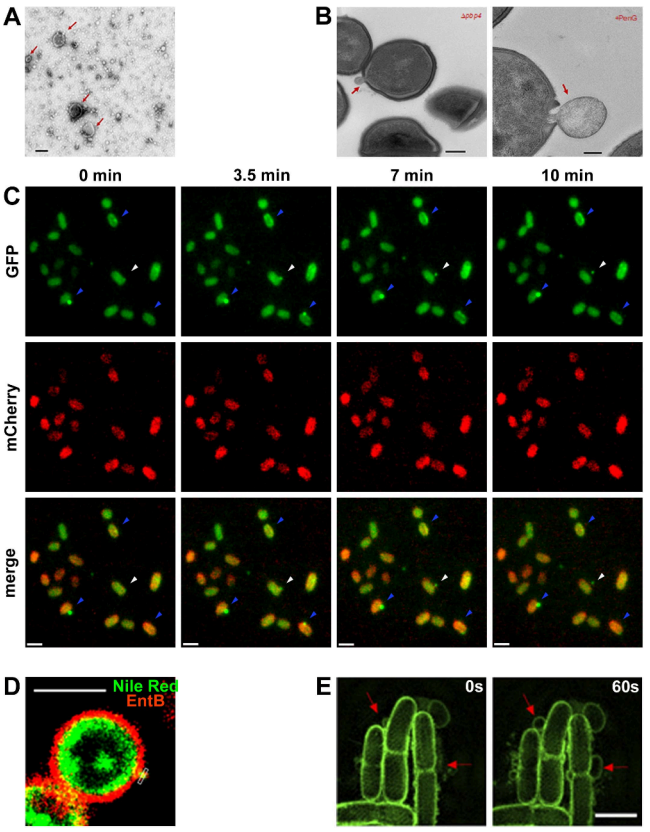

Figure 1. Monitoring of bEVs secretion. (A) Ultrathin sections of bEVs (red arrows) from *S. aureus* examined by TEM. Bar: 100 nm. (B) Production of *S. aureus* bEVs increased when peptidoglycan cross-linking was reduced. Bar: 200 nm. Reprinted from ref 7. Copyright 2018 Springer Nature Ltd. (C) Confocal time-lapse of bEVs formation in Bacteroides coexpressing Inulinase-GFP and OmpF-mCherry. Bar: 2 μm. Reprinted from ref 11. Copyright 2023 NAS. (D) Representative multicolor STORM image of *S. aureus* secreting bEVs colabeled for membrane (Nile red, green) and Enterotoxin B (EntB, red). Bar: 1 μm. Reprinted from ref 12. Copyright 2022 Springer Nature Ltd. (E) Time-lapse image sequence (3D-SIM) of membrane blebbing in *E. coli* under phage infection and stained with the membrane dye FM1−43X (green). Red arrows indicate blebs. Bar: 2 μm Reprinted from ref 14. Copyright 2021 Microbiological Society.

and yeast through membrane fusion.[16] They demonstrated that antimicrobial containing vesicles achieve direct delivery of the cargo to target microbes. For instance, FM4−64-, Vancomycin-Bodipy-Fl-, or CFSE (5-(and-6)-Carboxyfluorescein Diacetate Succinimidyl Ester)-stained bEVs were able to transfer red fluorescence to stationary or actively growing *B. subtilis*, within less than 15 min. Moreover, bEVs delivered their multiple cargo specifically to the plasma membrane or the cytoplasm of surrounding bacterial cells[13] (Figure 2A). Elsewhere, self-quenching octadecyl rhodamine B chloride (R18)-labeled bEVs released by *S. aureus* were mixed with *Pseudomonas aeruginosa* cells, and the fluorescence signal was observed over time under a fluorescence microscope.[17] The fusion of bEVs and *P. aeruginosa* membranes was assessed, revealing that *S. aureus* bEVs may promote *P. aeruginosa* pathogenicity.

bEV-based interbacterial communication is of particular interest in the case of hydrophobic signal molecules transport, notably for bacteria living in aqueous environments. For instance, the hydrophobic signal *N*-hexadecanoyl-L-homoserine lactone is solubilized and released from *Paracoccus* sp. by the aid of bEVs.[18] Using epifluorescence microscopy and FM4−64-labeled *Paracoccus*-delivered bEVs, it was observed that vesicles preferentially targeted surrounding cells of the same strain rather than other bacteria. This suggests that bEVs may recognize particular cell types. The bEV carriage indispensable requirement for a cargo's function in cell−cell communication was also observed for hydrophobic bacteriocin micrococcin P1 (MP1) that plays an important role in bacterial interaction within the human microbiome.[19] While free MP1 is poorly active due to its low solubility, both its antimicrobial activity and its delivery to target bacteria depend on its incorporation into bEVs as demonstrated by confocal imaging of bEVs labeled using PKH26, a red fluorescent dye, or the self-quenching fluorescent dye R18.

■ bEVs IN HOST−MICROBE INTERACTION

By acting as efficient and protective carriers for communication signals and genetic material, bEVs allow bacteria to coordinate their mutual interaction, but also allow exchanges with the host by delivering virulence factors modulating the immune responses or promoting infection of the host. Most studies used bEVs labeled with lipid-specific fluorescent dyes DiD and PKH_2 or lipophilic dyes Vybrant DiI and DiO to monitor vesicles interaction with host cells. Sahr et al. have shown that *Legionella pneumophila* used bEVs to translocate its small RNAs into host cells.[6] Automated conformal imaging showed internalization of purified DiD-labeled bEVs into host cells which then acted on host defense signaling pathways. This activity is proposed to be a general mechanism employed by *L. pneumophila* to interact with eukaryotic cells. Similarly, the SYTO RNASelect dye-labeled small RNA cargo of DiD-labeled bEVs from *S. aureus* was shown to influence both microbe−microbe and host−pathogen interactions[20] (Figure 2B).

Besides RNA, bEVs can translocate DNA into the host cells. Bitto et al. visualized a packaging of chromosomal DNA in bEVs from Gram-negative bacteria.[21] They observed the internalization of colabeled bEVs with the membrane-permeant DNA dye SYTO-61 and the membrane dye DiO or DiI into the epithelial human lung epithelial cells (A549) by confocal microscopy. The transported DNA encoded proteins involved in virulence, stress response, antibiotic resistance and metabolism. Similarly, in *S. aureus*, bEVs transport immunostimulatory DNA, RNA and peptidoglycan fragments (Figure 2C and D) that activate innate immune receptors and induce autophagy of host cells.[22,23]

Codemo et al. stimulated A549 cells and human monocyte-derived dendritic cells with pneumococcal bEVs to characterize their immunomodulatory effect.[25] The analysis of high-resolution immunofluorescence microscopy images of host cells upon incubation with bEVs, labeled with an antipneumolysin antibody, demonstrated intracellular localization of vesicles. Internalized bEVs triggered proinflammatory cytokine responses regardless of the presence of the cytosolic pore-forming toxin pneumolysin indicating that other virulence factors were also transferred. Interestingly, the heterogeneity in the presence of toxins within bEVs was also observed in *Aggregatibacter actinomycetemcomitans*, a bacterium implicated in periodontal disease.[26] The modulation of host cellular functions by bEVs was shown during infection through the monitoring of bEVs labeled with DiO and internalized in human macrophages by dynamin-dependent endocytosis.[27]

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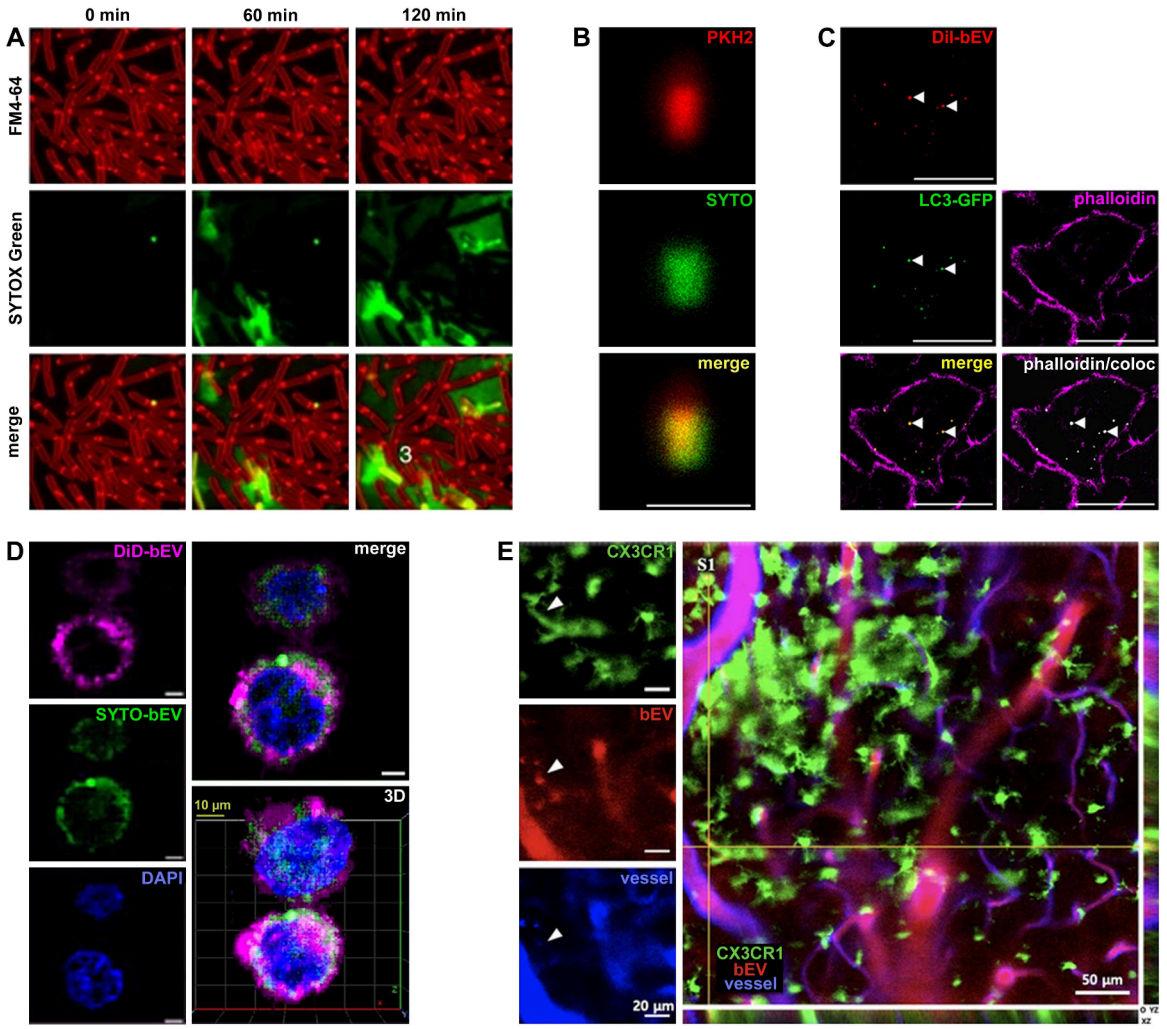

Figure 2. Monitoring the interactions of bEVs with their targets. (A) Confocal time-lapse of cell death and extracellular DNA (SYTOX, green) of *B. subtilis* (labeled with FM4−64, red) induced bEVs release in neighboring bacteria. Numbers (3, merge 120 min) indicate the progression of cell death. Bar: 5 μm. Reprinted from ref 13. Copyright 2017 Springer Nature Ltd. (B) Confocal microscopy of bEVs costained for membrane (PKH2, red) and RNA (SYTO RNASelect, green). Bar: 1 μm. Reprinted from ref 20. Copyright 2021 Joshi, Singh, Nadeem, Askarian, Wai, Johannessen and Hegstad. (C) Single confocal plane showing the internalization of bEVs (DiI, red) from *H. pylori* in A549 cells (LC3-GFP, green and phalloidin, magenta). Arrowheads indicate colocalized LC3-GFP puncta with DiI-labeled bEVs (merge, yellow) with actin. Bar: 20 μm. Reprinted from ref 22. Copyright 2021 Wiley-VCH. (D) *S. aureus* bEVs, (DiD, magenta and SYTO RNA, green) delivered into macrophages stained with DAPI (blue). Bar: 10 μm Reprinted from ref.[23] Copyright 2023 Springer Nature Ltd. (E) Intravital confocal imaging of tail-vein-injected bEVs from *A. actinomycetemcomitans* in the cortex of CX3CR1-GFP mice. Arrowheads point to the colocalization of blood brain barrier-crossed DiD-stained bEVs (red) and CX3CR1-positive microglial cells (green) localized in the exterior of vessels (Alexa Fluor 555-conjugated anti-CD31 antibody, blue). Scale bar: 50 μm; magnified scale: 20 μm. Reprinted from ref 24. Copyright 2019 Wiley-VCH.

Finally, bEVs distribution after uptake can be monitored in whole animals. Han et al. administrated colabeled bEVs from *A. actinomycetemcomitans* with DiD and SYTO RNA-Select to mice by cardiac injection.[28] Cleared mouse brains were then imaged using two-dimensional light-sheet fluorescence microscopy, providing evidence that bEVs can cross the blood-brain barrier and that their RNA content promoted the expression of the proinflammatory cytokine TNFα in the cortex[24] (Figure 2E). The same group used laser scanning intravital confocal microscopy to show that DiD-stained bEVs went earlier in meningeal macrophages than in cortex microglial cells after intravenous injection in CX3CR1-GFP transgenic mice (Figure 2E). *In vivo* biodistribution of *E. coli*-delivered bEVs with the SpyCatcher protein anchored to the membrane in mice was flowed through PET/MRI imaging via synthetic SpyTag-NODAGA conjugates combined with radiolabeling (^{64}Cu).[29] This approach showed that the liver and spleen had the highest bEV uptake following intravenous administration.

■ CONCLUDING REMARKS

BEVs play a crucial role in bacterial cell−cell communication and bacterial−host interaction by serving as carriers for various biomolecules. Imaging bEVs presents still several technical challenges due to their small size, structural complexity, and dynamic nature. Electron microscopy can visualize detailed structures but does not provide dynamic or functional insights while conventional light microscopy has the resolution limit closes to bEV diameters (20−400 nm). Advanced techniques, such as super-resolution microscopy and specific labeling of bEVs, have provided deeper insights into their biogenesis, the molecules they transport, and the mechanisms by which they support bacterial survival and pathogenicity.

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Author Contributions

J.V. defined the scope and S.T. defined the structure of the Viewpoint article. All the authors prepared the manuscript by writing the initial draft, reviewing and editing.

Notes

The authors declare no competing financial interest.

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