Kwansei Gakuin University
Report of Research Outcome

2026/03/13

To President

Department : Science and Technology
Position : Postdoctoral fellow
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I report the outcome of the research as follows.

Name of the Fund/Program	□Sabbatical leave with grant □Sabbatical leave with no grant □KGU Joint Research □Individual Special Research ☒Postdoctoral fellow ※Please report by designated form as for "International Research Collaboration".
Research Theme	Nanoscale Topographical and Chemical Characterization of Outer Membrane Vesicles (OMVs) using Atomic Force Microscopy and Raman Spectroscopy
Research Site/Venue	KGU KOBE-SANDA CAMPUS (BUILDING IV)
Research period	2025/04/01 ～ 2026/03/31 （12 month）

◆ **Summary of the research outcome** **(approx. 2,500 words)**

Please write down the outcomes in detail regarding the research theme above.

I. Introduction

Outer membrane vesicles (OMVs) are nanoscale spherical lipid bilayer structures naturally released from the outer membrane of Gram-negative bacteria during normal physiological processes. These vesicles encapsulate diverse biomolecular components, including membrane lipids, structural proteins, periplasmic proteins, and nucleic acids. Due to their structural stability, biological functionality, and intrinsic biocompatibility, OMVs have attracted increasing attention for their roles in bacterial communication, host-pathogen interactions, vaccine development, and nanobiotechnology applications [1, 2]. Because OMVs are nanometer-sized and compositionally complex, comprehensive characterization requires complementary analytical techniques capable of probing both their physical morphology and molecular composition. Structural visualization alone cannot confirm biochemical identity, while molecular analysis without morphological confirmation cannot distinguish vesicles from other nanoscale contaminants. Therefore, correlative multimodal characterization is essential for reliable OMV analysis.

In this study, Atomic Force Microscopy (AFM) and Raman spectroscopy were employed as complementary analytical tools to investigate the structural integrity, size

distribution, and biochemical properties of purified *Escherichia coli* OMVs. AFM provides high-resolution nanoscale topographical imaging that enables direct visualization of vesicle morphology and quantitative size analysis. Raman spectroscopy offers label-free vibrational characterization that reveals molecular fingerprints of lipids, proteins, and other biomolecules. The combination of these techniques enables comprehensive structural and biochemical evaluation of OMVs.

II. Methods Overview

OMVs were isolated or harvested from bacterial culture supernatants and purified using centrifugation-based protocols designed to remove cellular debris and medium contaminants while preserving vesicle integrity. For AFM analysis, vesicle suspensions were deposited onto plasma-cleaned glass substrates functionalized with 3-aminopropyltriethoxysilane (APTES). APTES treatment introduces positively charged amine groups onto the glass surface, promoting electrostatic adsorption of negatively charged OMV membranes and improving particle immobilization. After deposition, samples were gently rinsed to remove loosely bound materials and dried under controlled ambient conditions. AFM imaging was performed in air to evaluate surface morphology, vesicle distribution, and nanoscale structural features. Topographic height images were acquired and analyzed to identify vesicle-like particles. Vesicle size distributions were determined through cross-sectional height profiling of individual particles, as height measurements are less affected by tip convolution compared with lateral dimensions.

For Raman spectroscopic analysis, vesicle samples were also deposited onto clean functionalized glass substrates and measured using confocal Raman microscopy with a 532 nm excitation laser (4-8 mW). Spectra were collected from multiple spatial positions (approximately 23 measurement spots) to ensure representative sampling and reduce local heterogeneity. Raw spectra were preprocessed to remove substrate background contributions through glass subtraction, followed by baseline correction to eliminate fluorescence background and spectral drift using Igor Pro 64 software (WaveMetrics Inc., USA). Quantitative spectral comparisons were performed by calculating lipid-to-protein intensity ratios using local peak maxima of characteristic Raman band.

III. Results

AFM Characterization

AFM imaging revealed distinct differences between OMV preparation batches, reflecting variations in vesicle surface binding efficiency and size distribution. The initial sample displayed a highly uneven substrate surface with numerous irregular and aggregated structures. Vesicle-like particles were only intermittently visible and difficult to identify clearly. This limited visibility is likely associated with weak vesicle-substrate interactions, which may have caused partial detachment during washing procedures. Consequently, APTES substrate functionalization was introduced to improve vesicle immobilization and imaging reliability.

Following surface functionalization, AFM measurements demonstrated clearly distinguishable dome-shaped OMV-like nanostructures distributed across the substrate. Cross-sectional height profiling of the first OMV batch revealed vertical elevations reaching approximately 150-200 nm, indicating the presence of larger vesicle aggregates and suggesting a broad size distribution. However, quantitative height distribution analysis showed that the dominant vesicle population was concentrated between 33-69 nm, with decreasing frequency toward larger sizes. The distribution exhibited right-skewed characteristics, with a median vesicle height of 93 nm (n = 248), indicating

substantial heterogeneity in vesicle dimensions and coexistence of individual vesicles and aggregated structures.

A separate OMV preparation (second batch) exhibited markedly improved structural uniformity. AFM topography revealed more homogeneous surface coverage with clearly defined rounded nanostructures distributed evenly across the substrate. Three dimensional renderings confirmed well-resolved vesicle morphology with minimal overlapping features. Height profiling demonstrated smaller vesicles with vertical dimensions up to approximately 60 nm. The corresponding height distribution revealed a dominant vesicle population between 30-55 nm with a mildly right-skewed profile. The median vesicle height was 44 nm (n = 265), indicating a narrower and more uniform size distribution compared with the previous batch. This improved uniformity suggests more consistent vesicle formation and purification efficiency.

Control experiments using LB medium processed under identical purification procedures primarily exhibited irregular aggregated surface features rather than vesicle-like nanostructures. Although occasional small spherical particles (~5-10 nm) were observed, their dimensions were significantly smaller than typical OMVs and are therefore attributed to medium residues or nanoscale debris. These control results confirm that the observed nanostructures originate from purified OMVs rather than preparation artifacts.

Raman Spectroscopic Characterization

Raman spectroscopy revealed characteristic vibrational signatures corresponding to key membrane biomolecules. Prominent lipid-associated bands were observed from CH_2 bending vibrations near ~1450 cm^{-1}, indicating contributions from fatty acyl chains within membrane phospholipids. Strong protein amide I bands appeared around ~1660 cm^{-1}, corresponding to C=O stretching vibrations of peptide backbones and confirming the presence of structural membrane proteins. Distinct aromatic amino acid signals, including the phenylalanine ring-breathing mode at ~1003 cm^{-1}, further supported the proteinaceous composition of the vesicles. Additionally, bands in the fingerprint region reflected combined lipid and protein molecular contributions. The CH-stretching region (~2850-2950 cm^{-1}) exhibited combined lipid and protein vibrational features typical of biological membranes.

Stacked Raman spectra collected from multiple measurement positions displayed highly consistent peak patterns and relative intensities, demonstrating strong measurement reproducibility and uniform biochemical characteristics across the sample. The spectral similarity indicates stable vesicle composition and minimal spatial heterogeneity. Quantitative analysis using intensity ratios further supported these observations. Lipid-to-protein ratios provided relative indicators of membrane lipid and protein spectral contributions, while phenylalanine-to-amide I ratios reflected aromatic amino acid contributions relative to overall protein structure. The distributions of these ratios remained stable across measurements, supporting reliable comparative biochemical characterization. These ratios represent relative spectral indicators rather than absolute compositional quantification due to inherent differences in Raman scattering efficiencies among molecular groups.

Correlative Interpretation

AFM and Raman analyses provide complementary confirmation of OMV properties. AFM directly visualizes vesicle morphology, size distribution, and aggregation behavior, while Raman spectroscopy verifies molecular composition and biochemical reproducibility. The convergence of structural and molecular evidence supports successful OMV isolation and reliable multimodal characterization.

Effect of Frozen Storage

AFM imaging of OMVs stored at -81°C for one week revealed increased vesicle clustering and aggregation compared to freshly prepared samples. This aggregation is consistent with freeze-induced membrane adhesion and vesicle-vesicle interactions commonly observed for lipid vesicles during freezing and thawing processes. Despite clustering, vesicle-like structures remained distinguishable, indicating preservation of overall morphology. Preliminary Raman inspection suggests that stored samples exhibit spectral features comparable to freshly prepared OMVs, indicating preservation of major biochemical components. However, further quantitative Raman analysis is required to evaluate potential subtle biochemical changes induced by storage.

IV. Conclusions

Combined AFM and Raman spectroscopy provide a reliable and complementary approach for structural and biochemical characterization of *Escherichia coli* OMVs. AFM confirms vesicle morphology and reveals batch-dependent size variations, with median vesicle heights of 93 nm and 44 nm observed for different preparations. Control experiments verify that observed nanostructures originate from vesicles rather than medium artifacts. Raman spectroscopy validates characteristic lipid and protein molecular signatures and demonstrates highly reproducible spectral profiles. Stable intensity ratios further support reliable relative biochemical assessment. Together, these findings confirm successful OMV isolation and robust correlative characterization.

References

1. Zhang, X.M., et al., *Bacterial outer membrane vesicles as intrinsically immunogenic and highly modifiable nanocarriers for precision tumor therapy.* Mol Biol Rep, 2026. **53**(1).
2. Munoz-Echeverri, L.M., et al., *Bacterial extracellular vesicles: biotechnological perspective for enhanced productivity.* World J Microbiol Biotechnol, 2024. **40**(6): p. 174.