Dean&Francis
ISSN 2959-409X

Small RNAs in Bacterial Outer Membrane Vesicles

Boxi Tan

Imperial College London, London, United Kingdom, SW7 2AZ
bt621@ic.ac.uk

Abstract:

Outer membrane vesicles (OMVs) from Gram-negative bacteria exhibit selective enrichment for small RNAs (sRNAs), including tRNA fragments, as opposed to randomly reflecting cellular RNA pools. Building on well-established RNase-detergent controls and ddRT-PCR quantification, we summarise delivery stoichiometry (≈1% of vesicle RNA reaching recipient cytoplasm) and propose reporting standards (vesicles per cell, RNA copies per vesicle, delivery per cell). Functionally, OMV RNAs signal via two main routes: cytosolic Argonaute-dependent silencing and endosomal TLR7/8 sensing in both pathogenic and commensal bacterial species. We highlight evidence that OMVs can travel along the gut–brain axis and activate astrocytic NF-κB, thereby exacerbating amyloid-associated pathology, and we outline the experimental controls needed to attribute these effects specifically to RNA cargo. Finally, we consider how these principles could guide the engineering of probiotic-derived OMVs to tune immune tone within a quantitative mechanistic framework. This review identifies key methodological gaps and puts forth quantitative frameworks for studying RNA delivery.

Keywords: outer membrane vesicles, small RNAs, tRNA fragments, TLR7/8 sensing, Argonaute-mediated silencing

1. Introduction

Outer membrane vesicles (OMVs) are nanoscale blebs that naturally bud from the outer membrane of Gram-negative bacteria, carrying lipids, proteins, and nucleic acids [1]. Unlike bulk cellular RNA, the RNA repertoire of OMVs is markedly enriched in short RNA species, indicating selective sorting rather than random carryover [2,3secreted biomolecules are not only involved in intra-species communication but they also play roles in inter-kingdom exchanges and pathogenicity. To date, released products, such as small molecules,\n DNA\n , peptides, and proteins, have been well studied in bacteria. However, the bacterial extracellular\n RNA\n complement has so far not been comprehensively characterized. Here, we have an-

ISSN 2959-409X

alyzed, using a combination of physical characterization and high-throughput sequencing, the extracellular\n RNA\n complement of both outer membrane vesicle (\n OMV\n]. Early causal evidence came from Pseudomonas aeruginosa, where an OMV-borne tRNA fragment (sRNA52320) reduced epithelial IL-8/KC signaling and neutrophil recruitment [4]. RNase protection with detergent controls establishes that much of the RNA is encapsulated within vesicles, while imaging and ddRT-PCR provide a quantitative anchor: only ~1% of vesicle RNA is delivered to target cells under typical in-vitro conditions [5].

Functionally, OMV RNAs act in two ways. First, they enter the cytosol and regulate host mRNAs at the post-transcriptional level, ultimately inducing gene silencing. Second, they stimulate endosomal receptors such as TLR7/8, which reprogram innate immune signaling pathways[1,3]. These activities occur in both pathogens and commensals: Bacteroides OMVs can trigger TLR2/4/7 and NOD1 signaling in epithelia [1]. OMVs also circulate beyond the gut, reach the brain parenchyma, and exacerbate amyloid-linked pathology with astrocyte NF-κB activation, outlining a plausible route from intestinal microbes to neuroinflammation [6,7]. However, quantitative standards for RNA delivery remain underdeveloped, leaving uncertainties in comparing studies and interpreting biological significance across systems. We aim to explore the mechanisms of sRNA packaging in OMVs, their delivery to host cells, and their functional impact on host gene expression and immune signalling. It will also discuss the potential implications of these findings for understanding the gut–brain axis and for developing therapeutic strategies targeting microbial RNA communication. Understanding these processes is significant because it links microbial vesicle biology with host immune modulation and neuroinflammation. Clarifying how bacterial sRNAs are selectively packaged and functionally delivered may help design probiotic-based or vesicle-based interventions to maintain immune balance and protect against neurodegenerative diseases. This review synthesises selective packaging, delivery stoichiometry, and these two lanes of action, and argues for quantitative standards to enable translational strategies on the gut–brain axis.

2. Selective Packaging of sRNAs

Multiple transcriptomic analyses have demonstrated that RNAs encapsulated within OMVs are not randomly derived from the cytoplasmic transcriptome. They exhibit a distinct size distribution, typically ranging from 15 to 40 nucleotides in length [3,8bacterial RNAs comparable in size to eukaryotic miRNAs (18–22 nucleotides]. Among these, transfer RNA fragments (tRFs) and defined small regulatory RNAs are consistently enriched, while full-length messenger RNAs form only a minor proportion of the total vesicular RNA content [5]. Such skewed distributions persist even after stringent removal of external contaminants using RNase protection and density-gradient purification, suggesting that these RNAs are selectively incorporated into vesicles rather than randomly adsorbed onto their surface.

Environmental factors appear to play an active role in shaping OMV RNA composition. In Salmonella enterica, for example, culture under Salmonella Pathogenicity Island (SPI)-inducing conditions dramatically alters the small RNA profile of OMVs, implying that RNA packaging is linked to bacterial physiological state and virulence regulation [4]. Pathogenic bacteria such as Salmonella enterica tend to reshape the RNA composition of their vesicles when exposed to virulence-related stress. By contrast, commensal species like Bacteroides fragilis maintain more stable RNA packaging. These B. fragilis OMVs can transfer nucleic acids to intestinal epithelial cells, activating pattern-recognition receptors including TLR4, TLR7 and NOD1. At the same time, polysaccharide A within the vesicles engages TLR2 on dendritic cells and promotes anti-inflammatory responses [1,9]. These examples point to two main regulatory patterns: stress-induced packaging in pathogens and steady-state packaging in commensals. This distinction suggests that selective RNA export is a general feature of bacteria, not limited to pathogens.

Several non-exclusive mechanisms have been proposed to explain how certain RNAs are preferentially loaded into OMVs. One hypothesis emphasises the role of RNA-binding proteins (RBPs) that recognise specific sequence or structural motifs and co-segregate with their bound RNAs into vesicle-forming membrane microdomains[3]. Another model suggests that tRNA processing produces stable fragments that accumulate within OMVs[2secreted biomolecules are not only involved in intra-species communication but they also play roles in inter-kingdom exchanges and pathogenicity. To date, released products, such as small molecules,\n DNA\n , peptides, and proteins, have been well studied in bacteria. However, the bacterial extracellular\n RNA\n complement has so far not been comprehensively characterized.

Here, we have analyzed, using a combination of physical characterization and high-throughput sequencing, the extracellular\n RNA\n complement of both outer membrane vesicle (\n OMV\n]. A third explanation is that vesicles bud from specific lipid–protein regions, where certain ribonucleoprotein complexes become enclosed during formation. Finally, OMVs may serve as a form of RNA quality control, enabling bacteria to export misfolded or potentially deleterious RNAs—an apparent waste-disposal mechanism that inadvertently facilitates intercellular signaling[10].

Collectively, these findings suggest that selective packaging may involve RNA-protein interactions and lipid-RNA affinities that become accentuated under SPI-like or other stress-induced conditions, coordinating RNA export with bacterial physiology. Current evidence therefore supports the view that OMV RNA cargo reflects active and regulated selection rather than stochastic inclusion. Reproducible enrichment across species and environments suggests that OMV RNA profiles are not the product of random debris. To investigate the mechanism, it will be important to pair crosslinking–immunoprecipitation of bacterial RNA-binding proteins with well-characterised OMV fractionation and single-vesicle RNA quantification assays. These approaches can show which RBPs are responsible for RNA loading, what sequence or structural features they recognise, and how many RNA molecules each vesicle typically carries. Defining these details will make it possible to separate true regulatory secretion from waste export and to understand how bacterial vesicles shape host responses, including communication along the gut-brain axis.

3. Delivery and Functional Impact

3.1 Entry and quantification

The internalization of bacterial OMV-derived RNA into host cells has been visualized using lipid-labeled vesicles and metabolically labeled RNA, such as 5-ethynyluridine (5-EU). These approaches have revealed that vesicular RNA can localize to both the cytoplasm and, in certain cases, the nucleus of epithelial cells [4,8bacterial RNAs comparable in size to eukaryotic miRNAs (18–22 nucleotides]. To distinguish truly internalised RNA from material adhering to the cell surface, researchers have applied a nuclease-detergent logic test: treatment with RNase alone leaves vesicle-encapsulated RNA intact, whereas combining RNase with a detergent that disrupts the membrane abolishes the RNA signal [11]. This simple but rigorous control ensures that detected RNA reflects bona fide delivery rather than surface adsorption.

Quantification of delivery efficiency has improved with the use of digital droplet RT-PCR (ddRT-PCR) combined with spike-in standards, which allows absolute counting of RNA copies[12]. According to MISEV2023, quantitative standards for RNA delivery and OMV purity assessment have recently been proposed, which can serve as methodological benchmarks for OMV studies[13]. Studies converge on an estimate that roughly one percent of total OMV RNA ultimately reaches the recipient cytoplasm—a small fraction in physical terms, but one that can have considerable biological impact if the transferred molecules are functionally potent and directed to specific pathways [4]. Quantitative comparability can be improved by reporting vesicles per cell, RNA copies per vesicle, and the estimated delivery per cell [13]. Establishing this standardized stoichiometric framework would facilitate the development of meaningful dose-response models and enhance reproducibility in the expanding field of microbial RNA communication.

3.2 Two-lane model: silencing versus sensing

The functional impact of delivered bacterial RNA appears to follow two principal “lanes”. The first is the silencing lane, in which vesicular RNA enters the host cytoplasm and directly modulates gene expression through Argonaute (AGO)-dependent pathways. A well-characterised example involves a *Pseudomonas aeruginosa* transfer RNA fragment, tRF 52320, which downregulates the epithelial chemokine interleukin-8 (IL-8, also known as KC in mice) via targeting MAPK-associated transcripts [4]. Reporter assays demonstrate sequence-specific target repression, while rescue experiments with synthetic mimics and Argonaute pull-downs detecting bacterial RNA fragments collectively support this mode of action [2secreted biomolecules are not only involved in intra-species communication but they also play roles in inter-kingdom exchanges and pathogenicity. To date, released products, such as small molecules,\n DNA\n , peptides, and proteins, have been well studied in bacteria. However, the bacterial extracellular\n RNA\n complement has so far not been comprehensively characterized. Here, we have analyzed, using a combination of physical characterization and high-throughput sequencing, the extracellular\n RNA\n complement of both outer membrane vesicle (\n OMV\n].

ISSN 2959-409X

The minimal experimental dataset to validate this mechanism should include evidence of intracellular RNA delivery, quantifiable target repression, and complementary loss- and gain-of-function tests using RNA-depleted and RNA-supplemented OMVs, respectively.

The second, functional sensing lane, entails the recognition of vesicular RNA by endosomal pattern-recognition receptors. In macrophages, RNA packaged within OMVs from oral pathogens such as *Aggregatibacter actinomycetemcomitans* activates TLR8, leading to NF-κB signalling and the release of TNF-α [14]. Human TLR8 selectively recognises uridine-rich bacterial RNA motifs, which accounts for its responsiveness to OMV-derived transcripts [15]. Commensal *Bacteroides fragilis* likewise produces OMVs that engage several innate immune receptors—including TLR2, TLR4, and TLR7—and deliver peptidoglycan that triggers the cytosolic sensor NOD1, indicating coordinated signaling between endosomal and cytosolic pathways. Evidence supporting this sensing mechanism typically derives from experiments utilizing receptor-specific antagonists, genetic knockout models of adaptor proteins, or inhibitors of endosomal acidification—all of which attenuate cytokine induction. To rigorously confirm RNA dependence, signals must be shown to be RNase-sensitive but resistant to protease or lipase treatment. This excludes contributions from non-RNA components (e.g., proteins or lipids), thereby establishing RNA as the primary driver of the observed biological response.

Importantly, these two lanes are not mutually exclusive. A single OMV population can deliver distinct RNA cargos that act through both routes—some RNAs are sensed by endosomal TLRs to initiate innate immune signalling, while others escape into the cytosol to participate in AGO-mediated post-transcriptional regulation. To distinguish the relative contributions of these pathways, experiments that monitor endosomal and cytosolic responses separately are required. This objective can be accomplished using receptor-knockout models in combination with Argonaute perturbation or compartment-specific reporters. Clarifying these parallel mechanisms will help establish when OMV RNAs primarily function as immunostimulatory "danger signals" versus when they serve as fine-tuning regulators of host gene expression programs.

4. Gut–Brain Axis and Systemic Implications

Recent biodistribution studies have shown that OMVs derived from the gut microbiota can enter the systemic circulation, thereby reaching distant organs, including the brain. *Helicobacter pylori* OMVs have been detected in the brain parenchyma of amyloid-prone mouse models. Their accumulation accelerates amyloid-β accumulation and cognitive decline [6]. In vivo studies also report that OMVs activate NF-κB signalling in astrocytes and lead to neuronal injury, which suggests that microbial vesicles directly contribute to neuroinflammatory processes [7]. These findings support that OMVs function as messengers carrying pro-inflammatory cues from the gut to the central nervous system. However, the extent to which OMVs enter systemic circulation and cross the blood-brain barrier remains debated, highlighting the need for in vivo imaging and quantitative tracing assays.

Although the pathological effects of OMVs are gaining growing recognition, the specific molecular cargo responsible for OMV-mediated neurotoxicity has yet to be definitively identified. While lipopolysaccharides (LPS) and outer-membrane proteins have been implicated as potential contributors, vesicle-associated RNA has been proposed as a candidate mediator of microbial communication with host tissues [11]. However, contamination from LPS or co-purified protein factors represents a prominent confounder in the interpretation of vesicle-induced immune activation. To rigorously attribute causality to the RNA fraction rather than to proteins or LPS, a set of targeted experiments is required. First, selective degradation of intraluminal RNA via RNase treatment—either with or without mild detergent—can establish whether the integrity of intraluminal RNA is indispensable for the observed neuroinflammatory effects. Second, the use of RNA-deficient OMVs—either generated from bacterial strains expressing periplasmic RNases or produced through controlled vesicle permeabilisation—would clarify whether RNA-free vesicles lose their ability to induce neuroinflammation. Third, reintroduction of purified RNAs into RNA-depleted vesicles could test sufficiency by rescuing the phenotype. Finally, cell-type-specific assays such as Argonaute immunoprecipitation (AGO-IP) or Toll-like receptor (TLR) reporter systems in microglia or astrocytes could help identify which recognition pathways are activated [14].

These system-level observations give rise to several testable hypotheses about how OMV-borne RNA might influence the brain. One possibility is that particular sRNA or tRFs act as pathogen-associated molecular patterns (PAMPs) that bind to TLR7 or TLR8 on glial

cells, thereby inducing the secretion of cytokines such as TNF-α and IL-6 [15]. A plausible scenario is that a subset of these RNAs escapes endosomal degradation, enters the cytoplasm, and directly disrupts the neuronal or glial gene expression via post-transcriptional gene silencing (PTGS) mechanisms [8bacterial RNAs comparable in size to eukaryotic miRNAs (18–22 nucleotides]. A third, more chronic outcome could be that repeated or long-term exposure to OMV-RNA fine-tunes neuroimmune set-points, ultimately increasing susceptibility to neurodegenerative diseases such as Alzheimer's or Parkinson's [9].

To determine whether RNA is the primary driver of these effects, future work will need to test both its necessity and sufficiency using controlled dosing and receptor-knockout models. Such studies could help separate the contributions of TLR-mediated immune signalling from those of cytosolic RNA-silencing pathways, thereby clarifying the mechanisms by which bacterial vesicles interact with neural and glial cells. A more comprehensive understanding of these mechanisms could shed light on how long-term perturbations in the gut microbiota modulate neuroimmune balance, and could ultimately guide new therapeutic strategies targeting microbe-derived vesicles in neurodegenerative disease.

5. Future Directions and Applications

A key challenge in advancing OMVs for therapeutic or diagnostic applications is the absence of standardized methods for their quantitative analysis. Variations in purification and characterization protocols across laboratories frequently lead to inconsistent measurements of RNA content, biological activity, and delivery efficiency. Establishing reproducible pharmacological baselines is therefore an important next step for the field. Currently, OMV-based RNA therapies remain conceptual and at the preclinical stage, with no clinical trials yet reported. Recent advances in purification have improved the reliability of OMV studies. Combining density-gradient ultracentrifugation or size-exclusion chromatography with viability and integrity assessments has improved the reliability of OMV purification. These approaches help to reduce contamination from cell debris and free nucleic acids, resulting in cleaner and more consistent vesicle preparations [3]. In addition, controlled nuclease and detergent treatments can be used to distinguish RNA species enclosed within OMVs from those loosely associated with their surface, enabling a more accurate characterisation of vesicle-protected RNA transcripts [4]. Complementary use of digital-droplet RT-PCR with spike-in standards facilitates absolute quantification of RNA delivery—expressed as copies per recipient cell—thereby enabling the evaluation of OMV dosing to be evaluated with pharmacological precision [5].

Recent studies have focused on engineering OMVs as controllable platforms for microbial–host communication and immune modulation. Probiotic-derived OMVs enriched with anti-inflammatory small RNAs have been shown to attenuate Toll-like receptor signalling in intestinal and neural tissues [1,4]. Another strategy involves constructing RNA decoy vesicles that bind TLR7/8-recognising motifs, thereby reducing neuroinflammatory activity while preserving normal immune function [6,7]. Together, these studies suggest that OMVs hold the potential to function both as targeted delivery tools and as regulatory components that aid in stabilising immune responses in mucosal and neural tissues.

Future progress will likely depend on the integration of multi-omics profiling with computational modeling approaches to guide OMV design. Lipidomic and transcriptomic analyses offer a means to identify the molecular factors that modulate vesicle stability, targeting, and RNA packaging, while proteomic studies provide insight into host-receptor interactions that regulate tissue distribution. Integrating these datasets with machine learning could enable the prediction of OMV behaviour in different biological contexts, supporting more systematic optimisation of their pharmacokinetic properties and tissue-targeting capabilities. In the long term, such approaches may allow the rational development of OMVs with defined RNA cargos and controllable immune activity, moving them closer to clinical application as reliable biological therapeutics. The establishment of standardised RNA isolation and quantification pipelines is critical to advancing reproducible translational progress.

6. Conclusion

In summary, recent advances have transformed the study of bacterial OMVs from descriptive transcript profiling toward the definition of causal roles for vesicle-associated small RNAs in modulating host responses. A robust conceptual framework now distinguishes two primary modes of action—cytosolic gene silencing and endosomal immune sensing—collectively explaining observations across both pathogenic and commensal species. To move the field forward, rigorous methodological standards

ISSN 2959-409X

are imperative—including standardized RNase and detergent controls, receptor or Argonaute knockouts, and quantitative benchmarks such as vesicle dose, RNA copy number, and delivery efficiency. Within the gut–brain axis, bacterial OMVs have been demonstrated to elicit neuroinflammatory responses, supporting a potential link between microbial RNAs and neurodegenerative disease mechanisms. However, the precise requirement and sufficiency of these RNAs to drive such pathological effects remain unresolved. Clarifying this causality represents a pivotal priority for future research. A deeper understanding may ultimately facilitate the design of therapeutic OMV-derived small RNAs that suppress inflammation or the development of strategies to block vesicular RNAs that drive disease pathogenesis. Establishing standardised RNA quantification pipelines is therefore fundamental to advancing OMV-based therapeutic applications.

References

[1] Gilmore, W. J., Johnston, E. L., Bitto, N. J., Zavan, L., O'Brien-Simpson, N., Hill, A. F., & Kaparakis-Liaskos, M. (2022). Bacteroides fragilis outer membrane vesicles preferentially activate innate immune receptors compared to their parent bacteria. Frontiers in Immunology, 13, 970725.

[2] Ghosal, A., Upadhyaya, B. B., Fritz, J. V., Heintz-Buschart, A., Desai, M. S., Yusuf, D., Huang, D., Baumuratov, A., Wang, K., Galas, D., & Wilmes, P. (2015). The extracellular RNA complement of Escherichia coli. MicrobiologyOpen, 4(2), 252–266. https://doi.org/10.1002/mbo3.235

[3] Malabirade, A., Habier, J., et al. (2018). The RNA Complement of Outer Membrane Vesicles From Salmonella enterica Serovar Typhimurium Under Distinct Culture Conditions. Frontiers in Microbiology, 9, 2015. https://doi.org/10.3389/fmicb.2018.02015

[4] Koeppen, K., Hampton, T. H., et al. (2016). A Novel Mechanism of Host-Pathogen Interaction through sRNA in Bacterial Outer Membrane Vesicles. PLOS Pathogens, 12(6), e1005672.

[5] Blenkiron, C., Simonov, D., et al. (2016). Uropathogenic Escherichia coli Releases Extracellular Vesicles That Are Associated with RNA. PLOS ONE, 11(8), e0160440.

[6] Meng, D., Lai, Y., Zhang, L., et al. (2024). Helicobacter pylori outer membrane vesicles directly promote Aβ aggregation and enhance Aβ toxicity in APP/PS1 mice. Communications Biology, 7(1), 1474. https://doi.org/10.1038/s42003-024-07125-1

[7] Palacios, E., Lobos-González, L., Guerrero, S., Kogan, M. J., Shao, B., Heinecke, J. W., Quest, A. F. G., Leyton, L., & Valenzuela-Valderrama, M. (2023). Helicobacter pylori outer membrane vesicles induce astrocyte reactivity through nuclear factor-κappa B activation and cause neuronal damage in vivo in a murine model. Journal of Neuroinflammation, 20(1), 66. https://doi.org/10.1186/s12974-023-02728-7

[8] Choi, J.-W., Kim, S.-C., Hong, S.-H., & Lee, H.-J. (2017). Secretable Small RNAs via Outer Membrane Vesicles in Periodontal Pathogens. Journal of Dental Research, 96(4), 458–466.

[9] Shen, Y., Torchia, M. L. G., Lawson, G. W., Karp, C. L., Ashwell, J. D., & Mazmanian, S. K. (2012). Outer Membrane Vesicles of a Human Commensal Mediate Immune Regulation and Disease Protection. Cell Host & Microbe, 12(4), 509–520. https://doi.org/10.1016/j.chom.2012.08.004

[10] Kulp, A., & Kuehn, M. J. (2010). Biological Functions and Biogenesis of Secreted Bacterial Outer Membrane Vesicles. Annual Review of Microbiology, 64(1), 163–184.

[11] Tsatsaronis, J. A., Franch-Arroyo, S., Resch, U., & Charpentier, E. (2018). Extracellular Vesicle RNA: A Universal Mediator of Microbial Communication? Trends in Microbiology, 26(5), 401–410.

[12] Stein, E. V., et al. (2017). Steps to achieve quantitative measurements of microRNA using two step droplet digital PCR. PLOS ONE, 12(11), e0188085.

[13] Welsh, J. A., Goberdhan, D. C. I., et al. (2024). Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. Journal of Extracellular Vesicles, 13(2), e12404.

[14] Han, E., Choi, S., Lee, Y., Park, J., Hong, S., & Lee, H. (2019). Extracellular RNAs in periodontopathogenic outer membrane vesicles promote TNF-α production in human macrophages and cross the blood-brain barrier in mice. The FASEB Journal, 33(12), 13412–13422.

[15] Krüger, A., Oldenburg, M., Chebrolu, C., Beisser, D., Kolter, J., Sigmund, A. M., Steinmann, J., Schäfer, S., Hochrein, H., Rahmann, S., Wagner, H., Henneke, P., Hornung, V., Buer, J., & Kirschning, C. J. (2015). Human TLR 8 senses UR / URR motifs in bacterial and mitochondrial RNA. EMBO Reports, 16(12), 1656–1663. https://doi.org/10.15252/embr.201540861