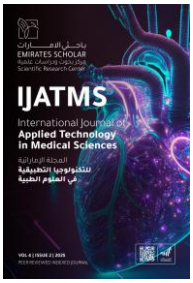

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Engineering fungal extracellular vesicles: the next phase of nanobiotechnology and biofabrication

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ABSTRACT

Fungal Extracellular vesicles (EVs) have emerged as a critical frontier in nanobiotechnology and biofabrication, offering a dynamic interface between microbial biology and engineered nanosystems. These membrane-bound particles, actively secreted by *fungi*, are now recognised as pivotal mediators of intercellular communication, molecular transport, and host-pathogen interactions. Recent advances have underscored their structural diversity and molecular cargo, including proteins, nucleic acids, lipids, and secondary metabolites, which confer them the ability to modulate both microbial physiology and host responses. While EVs were traditionally viewed as passive by-products, evolving research has reframed them as programmable biological entities with vast potential for bioengineering. This article examines the next phase in *fungal* EVs science: the deliberate engineering of vesicles to serve as tailored tools for drug delivery, vaccine development, biosensor integration, and regenerative applications. Drawing upon synthetic biology and gene editing platforms, researchers are now designing EVs with custom cargo profiles and surface functionalities, enhancing their utility across clinical and industrial domains. Moreover, the biofabrication potential of engineered *fungal* EVs offers new pathways for creating sustainable nanomaterials and biologically active scaffolds. Despite these advances, significant hurdles persist, particularly concerning biosafety, immunogenicity, standardisation of production processes, and translational scalability. As the field evolves, interdisciplinary convergence with artificial intelligence (AI), multi-omics technologies, and microfluidics is set to amplify the precision and throughput of EVs engineering. Engineered *fungal* EVs are thus positioned not merely as passive nanocarriers, but as intelligent, responsive platforms within the broader nanobiotechnological ecosystem.

Keywords: *Fungal Extracellular vesicles (EVs), Engineered nanovesicles in biotechnology, Biofabrication using fungi, Synthetic biology and fungal EVs, Nanobiotechnology drug delivery platforms.*

1. Introduction

Fungal EVs have emerged as pivotal mediators in the physiology and pathogenicity of *fungi*, functioning as intricate carriers of a broad spectrum of bioactive substances. These include proteins, lipids, nucleic acids, and polysaccharides, all of which facilitate molecular exchange and modulate both intra and interspecies interactions, particularly in host-pathogen dynamics. First identified in *Cryptococcus neoformans*, EVs have since been documented across a wide range of *fungal* taxa, encompassing both pathogenic and non-pathogenic species, thereby underscoring their evolutionary preservation and biological significance throughout the *fungal* kingdom (Oliveira *et al,* 2020).

The functional repertoire of *fungal* EVs is diverse, encompassing roles in the construction and remodelling of the cell wall, responses to environmental stressors, and the establishment of biofilms each essential to *fungal* viability and virulence (*Liebana-Jordan et al, 2021; Brandt et al, 2024).* In pathogenic contexts, these vesicles are instrumental in evading or modulating host immune responses by delivering virulence-associated determinants directly to host tissues, which can significantly influence the trajectory and severity of infection (*Herkert et al, 2019; Ullah et al, 2023).*

Beyond their physiological relevance within microbial systems, *fungal* EVs have garnered substantial attention for their translational potential in biomedical science. They are being actively investigated as innovative platforms for antigen delivery in vaccine development, as vectors for targeted drug transport, and as biomarkers for early and non-invasive diagnostic applications (*Rizzo et al, 2017*; *Nenciarini and Cavalieri, 2023*). The inherent structural diversity and molecular specificity of these vesicles allow them to facilitate complex interkingdom communications, further enhancing their appeal as bioengineered systems in therapeutic and synthetic biology frameworks (*Rizzo et al, 2020; Rizzo et al, 2021*).

Despite these promising attributes, significant gaps remain in the elucidation of EVs biogenesis pathways, the mechanisms governing selective cargo incorporation, and the full spectrum of their contributions to *fungal* pathogenesis. Addressing these knowledge deficits through targeted research will be essential for leveraging *fungal* EVs in both clinical and industrial domains, particularly in the development of next-generation biotechnological interventions (Nenciarini and Cavalieri, 2023; Rutter and Innes, 2023).

Fungal EVs represent an increasingly recognised modality of intercellular communication and biomolecular transport within the *fungal* kingdom. These membrane-bound nanostructures, first identified in *Cryptococcus neoformans*, have since been observed across a wide range of pathogenic and non-pathogenic *fungi*, fundamentally shifting our understanding of *fungal* biology and host pathogen interactions (*Rodrigues* et al, *2007; Brown* et al, *2015*). Their ability to package a diverse array of bioactive molecules, including lipids, proteins, nucleic acids, and polysaccharides, positions them as complex delivery systems with applications that extend beyond microbial physiology into the realms of biomedical engineering and synthetic biology (*Bleackley* et al, *2019).*

Fungal EVs are increasingly recognised as innovative platforms for nanobiotechnological applications, owing to their distinctive characteristics, including intrinsic biocompatibility, customisable molecular payloads, and compatibility with scalable microbial fermentation systems. Produced by a wide spectrum of fungal taxa, these vesicles are implicated in a variety of biological phenomena, such as cell-to-cell communication, host-pathogen crosstalk, and immunological modulation (*Nenciarini and Cavalieri, 2023; Jiang et al, 2023; Rizzo et al, 2020*). In contrast to synthetic vesicles such as liposomes, *fungal* EVs exhibit evolutionarily refined mechanisms that enhance their resilience in extracellular milieus, promote selective host cell targeting, and facilitate immune system evasion, thereby rendering them particularly suitable for advanced biofabrication strategies (*Herkert et al, 2019; Ikeda et al, 2023*).

Their utility spans drug delivery, vaccine formulation, and regenerative scaffolding, where they act as natural conveyors of therapeutic agents and immunogenic components (*Nenciarini and Cavalieri, 2023*). Their inherent capability to encapsulate a wide range of bioactive substances including proteins, lipids, nucleic acids, and virulence-associated factors further consolidates their relevance in clinical and therapeutic settings (*Ikeda et al, 2024; Ullah et al, 2023*). Recent empirical findings have underscored their immunostimulatory potential, particularly in the development of vaccines that harness EVs' native capacity to elicit protective host responses (*Honorato et al, no date; Brandt et al, 2024)*. Nevertheless, despite these advances, current understanding of *fungal* EVs biogenesis, cargo selection, and their mechanistic involvement in disease progression and therapeutic efficacy remains incomplete. Continued investigation into these pathways is essential for the translational realisation of *fungal* EVs based technologies in clinical and industrial domains (*Silva et al, 2019; Brandt et al, 2024)*.

Advances in synthetic biology and molecular engineering have markedly expanded the capacity to manipulate EVs biosynthetic pathways within *fungi*, enabling their transformation into programmable entities with refined functionalities. These technological strides have been significantly accelerated by integrating high-throughput omics approaches such as next-generation sequencing and proteomics with artificial intelligence-driven design models, collectively shifting *fungal* EVs from niche biological curiosities to critical components in nanobiotechnology. These lipid-based nanoscale structures have been found to harbour diverse molecular cargoes, including regulatory proteins, signalling lipids, genomic and non-genomic nucleic acids, as well as small metabolites, which together facilitate vital processes such as microbial signalling and host colonisation (*Rizzo et al, 2020; Rizzo et al, 2021; Bleackley et al, 2019*).

The implications for therapeutic innovation are significant, particularly given their application in the targeted delivery of drugs, vaccine development, and regenerative medicine (*Nenciarini and Cavalieri, 2023*; *Wiklander et al, 2019*). Through genetic and metabolic engineering, *fungal* species can now be modulated to yield EVs enriched with bespoke cargoes and functional ligands, thereby enhancing the specificity and efficacy of these nanostructures in therapeutic settings (*Valiante, 2023; Martins-Santana et al, 2018*). Multi-omics platforms have been instrumental in uncovering the molecular underpinnings of EVs generation, composition, and bioactivity, providing critical insights into their roles in antifungal resistance and biomarker discovery (*Zamith-Miranda et al, 2021*). Engineered EVs, characterised by low immunogenicity and high compatibility with biological tissues, have been explored as intelligent nanocarriers in medicine, representing a promising frontier in drug delivery research ("Engineered extracellular vesicles as drug delivery systems for the next generation of nanomedicine", 2023; *Levy et al, 2024*). These developments exemplify the transformative potential of *fungal* EVs in contemporary nanobiotechnology, driven by the convergence of synthetic biology, systems biology, and AI-informed molecular design.

Simultaneously, the deployment of 3D cell culture systems, enabled by engineered biomaterials, has demonstrated enhanced yield and consistency of EVs production, pointing to the need for scalable and reproducible manufacturing protocols to support future translational and industrial applications (Stone and Wang, 2023). In parallel, research into fungal nanotechnology is exploring the capacity of *fungi* as sustainable biofactories for nanoparticle generation, with implications extending across therapeutic, agricultural, and environmental domains ("Fungal nanobionics: Principle, advances and applications", 2023; *Shaheen et al, 2021*).

The integration of *fungal* mycelium into engineered living materials (ELMs) represents another frontier, wherein the self-repairing and responsive nature of *fungal* biomass can be harnessed to develop durable, multifunctional biocomposites (Elsacker *et al,* 2023; "Three-dimensional Printing of Mycelium Hydrogels into Living Complex Materials", 2022). Taken together, these advancements signal a new era in

biomedical engineering, where the synergy between *fungal* biotechnology, 3D bioprinting, and nanotechnology may yield unprecedented innovations in tissue repair and targeted therapeutics. However, realising this vision will require sustained research into the biological and engineering principles that govern EV behaviour and integration across diverse application platforms.

This article presents a comprehensive exploration of the engineering potential of *fungal* EVs, examining their biogenesis, compositional complexity, and diverse applications in nanobiotechnology and biofabrication. It also critically addresses current limitations and future directions for the field, emphasising the interdisciplinary strategies required to harness these naturally derived nanostructures in therapeutic and industrial contexts.

2. Biogenesis and Molecular Composition of *Fungal* EVs

The synthesis of EVs in *fungi* represents a highly regulated and evolutionarily conserved biological process, enabling the translocation of a broad array of functional macromolecules through the complex and multilayered *fungal* cell wall. In contrast to mammalian systems, where the mechanisms of EVs biogenesis are relatively well characterised, the molecular machinery underpinning this phenomenon in *fungi* remains comparatively obscure, owing to the distinctive structural barriers and taxonomic diversity intrinsic to *fungal* species. Nevertheless, accumulating evidence has challenged earlier assumptions that *fungal* EVs are incidental by products of cellular stress, instead positioning them as deliberate and biologically purposeful nanostructures that underpin essential physiological functions such as intercellular signalling, environmental adaptation, and virulence expression (*Herkert et al, 2019; Rizzo et al, 2020*).

Fungal EVs have been implicated in the transport of various bioactive constituents, including proteins, lipids, toxins, polysaccharides, and nucleic acids, which contribute to metabolic regulation, signal transmission, and the establishment of pathogenic potential. These vesicles have further been shown to participate in immune modulation through the delivery of virulence factors, allergens, and antigens, thereby playing crucial roles in host-pathogen interactions and environmental sensing (*Herkert et al, 2019; Liebana-Jordan et al, 2021*). Although the complete molecular details of EVs biogenesis in *fungi* remain under investigation, current findings suggest that vesicle formation occurs within the cytoplasm and at the plasma membrane, with vesicles either released into the extracellular milieu or retained within the periplasmic space (*Oliveira et al, n.d.; Liebana-Jordan et al, 2021).*

The compositional heterogeneity and molecular intricacy of *fungal* EVs present substantial analytical challenges, particularly in understanding their role during infection and pathogenesis (Rizzo *et al,* 2021). Nonetheless, advances in high-throughput omics technologies have been instrumental in deconstructing the cargo profiles of these vesicles. These approaches have revealed that *fungal* EVs carry components integral to cell wall synthesis and virulence, thereby broadening their relevance in both basic mycology and translational medicine (*Zamith-Miranda et al, 2021*). Such insights have fostered growing interest in the clinical exploitation of *fungal* EVs as platforms for novel diagnostic assays, adjuvants in vaccine formulations, and targets for antifungal therapeutics (*Silva et al, 2019; Ullah et al, 2023*). However, much remains to be elucidated concerning the genetic determinants and molecular regulators orchestrating vesicle production and their precise interactions with host systems (*Oliveira et al, n.d.; Silva et al, 2019*).

Emerging evidence indicates that *fungal* EVs formation is governed by multiple biogenetic pathways, encompassing both ESCRT (endosomal sorting complexes required for transport)-dependent and ESCRT-independent mechanisms. The ESCRT system, particularly proteins such as Vps23 and Snf7, plays a central role in vesicle scission events from multivesicular bodies (MVBs), particularly in model organisms such as *Saccharomyces cerevisiae* and *Cryptococcus*

neoformans. This machinery orchestrates endosomal membrane invagination and is vital for membrane fission, remodelling, and the accurate sorting of vesicle cargo (*Hanson and Jackson, 2016; Madan, 2023; Wang et al, 2023; "ESCRTing around the Cell", 2023*). In addition to this canonical route, alternative pathways have been described in species such as *Candida albicans* and *Histoplasma capsulatum*, involving Golgi-derived vesicles, lipid raft microdomains, and autophagosome like intermediates, all of which point to a complex network of cellular interactions that contribute to EVs biogenesis (*Motaung et al, 2023; Oliveira et al, 2010*).

In *Aspergillus fumigatus*, specific vesicle trafficking genes including *sec6* and *sso1* have been linked to the flexibility of the secretory pathway under pathogenic stress, suggesting that dynamic modulation of secretion is critical for EVs production in response to environmental cues (Oliveira *et al,* 2010). The ESCRT machinery's functionality is not restricted to EVs formation; it also plays essential roles in cellular homeostasis processes such as cytokinesis, membrane repair, and viral particle release, thereby reflecting its evolutionary versatility (Schuh and Audhya, 2014; Carlton, 2010). Despite the significant strides in delineating these mechanisms, the integrative molecular architecture by which diverse biogenetic routes converge to facilitate EVs formation in *fungi* remains incompletely understood. Ongoing investigations are now directed at mapping the regulatory networks and protein interactions that govern this intricate process, with the objective of unlocking new strategies for therapeutic intervention and fungal disease management (*Banjade et al, 2019; Henne et al, 2013).*

Fungal EVs carry a diverse repertoire of proteins, RNA species, lipids, and metabolites, which collectively reflect the physiological state of the producing organism and its interaction with the extracellular environment. Proteomic investigations have repeatedly revealed that *fungal EVs* contain a highly conserved set of functional proteins, including molecular chaperones such as Hsp70 and Hsp90, antioxidant enzymes like superoxide dismutase, various peptidases, and enzymes involved in cell wall remodelling, notably β-glucanases, mannosidases, and chitin synthases (*Bleackley et al, 2019; Herkert et al, 2019; Medina-Castellanos et al, 2022*). These vesicular proteins are indispensable for *fungi* to establish infection, survive environmental stress, and invade host tissues, indicating that EV-mediated secretion is a critical determinant of virulence regulation (*Silva et al, 2019; Xu et al, 2024*). For example, in *Candida albicans*, EVs influence the host's immune defences, suggesting a sophisticated means of immune evasion and pathogenesis (Vargas *et al,* 2015). In the domain of plant pathology, *Fusarium graminearum* EVs deliver protein effectors essential for virulence, exemplifying a mechanism of cross-kingdom communication that facilitates infection (García-Cerón *et al,* 2021). The detection of these enzymes within EVs supports the notion of unconventional secretion pathways for virulence factors, independent of classical signal peptides (García-Cerón *et al,* 2021). Additionally, EVs enable bidirectional *fungal* cell signalling, coordinating collective pathogenic behaviour during host colonisation (Herkert *et al,* 2019). Despite considerable progress in characterising *fungal* EVs, their full exploitation in diagnostics, vaccination, and anti*fungal* therapy remains limited, as the field is still nascent (Silva *et al,* 2019; Liu & Hu, 2023). Overall, *fungal* EVs constitute essential vectors for the transport of virulence determinants and bioactive molecules, underpinning the pathogenic capabilities and adaptability of *fungi* (Silva *et al,* 2014; Joffe *et al,* 2016).

EVs derived from species such as *Paracoccidioides brasiliensis* and *Candida auris* are gaining recognition for their roles in inter-kingdom communication through their RNA contents, which include messenger RNAs, microRNAs, transfer RNAs and small interfering RNAs. These RNA species are capable of modulating gene expression in both fungal cells and host tissues, thereby affecting infection dynamics and immune responses (*Bitencourt et al, 2022; Octaviano et al, 2022*) The selective packaging of these RNA molecules into EVs is believed to be orchestrated by RNA-binding proteins (RBPs) bearing domains such as RRM, zinc finger and KH motifs, which are

prevalent within *fungal* EVs populations and play pivotal roles in RNA metabolism, stability and translation (*Dallastella et al, 2023*). The sophisticated nature of this sorting process, though not yet fully understood, is evidenced by the presence of these RBPs (*Dallastella et al, 2023; Zamith-Miranda et al, 2021).*

In *P. brasiliensis*, EVs harbour functional mRNAs and small non-coding RNAs capable of reprogramming the transcriptome of murine monocyte-derived dendritic cells, underscoring their role in communication across biological kingdoms (Silva *et al,* 2019). Likewise, *C. auris* EVs have been shown to alter their RNA cargo in response to antifungal agents, shedding light on their potential involvement in antifungal resistance and virulence regulation (*Rocha et al, n.d.*). These findings emphasise the therapeutic and diagnostic promise of *fungal* EVs, suggesting their value as vehicles for bioactive cargos that mediate pathogenicity and host response modulation (*Herkert et al, 2019; Ullah et al, 2023).*

Lipidomic profiling of *fungal* EVs has demonstrated a complex complement of molecules crucial for fungal function and virulence. The vesicle membranes are enriched in neutral lipids such as ergosterol and phospholipids including phosphatidylcholine and phosphatidylserine, which confer membrane curvature and stability (*Vallejo et al, 2012; Rizzo et al, 2021*). These lipids not only maintain vesicle structure but also facilitate fusion with host membranes, contributing to pathogenic processes (*Rizzo et al, 2021; Ullah et al, 2023*). In *P. brasiliensis*, vesicular lipids incorporate sterols such as brassicasterol and ergosterol, with isolate-dependent variation suggesting a role in virulence (*Vallejo et al, 2012*).

Moreover, pathogenic *fungi* EVs contain sphingolipids and ceramides, compounds implicated in immune evasion and pro-inflammatory signalling, thereby supporting survival and persistence in host environments (*Lattif et al, 2011; Hans et al, 2021*). In *C. albicans*, lipid rafts in EVs are instrumental in biofilm development and contribute to antifungal resistance (*Lattif et al, 2011*). Disruption of sphingolipid biosynthesis has been shown to hinder biofilm formation and attenuate virulence, presenting a potential therapeutic target (*Lattif et al, 2011; Hans et al, 2021*). Collectively, the lipidomic composition of *fungal* EVs reveals their central role in *fungal* biology and pathogenesis, emphasising them as promising targets for antifungal intervention (*Ullah et al, 2023; Liébana-Jordan et al, 2021).*

Fungal EVs function as carriers of melanin precursors, mycotoxins and quorum sensing molecules, all of which are instrumental in chemical communication, biofilm establishment and host immune modulation. In *Cryptococcus neoformans*, EVs enriched with laccase catalyse extracellular melanin synthesis, a virulence mechanism that protects against phagocytic clearance (*Eisenman et al, 2009*). In *Aspergillus* spp., EVs transport aflatoxins and gliotoxins, which exacerbate host tissue damage and provoke immune responses (*Rizzo et al, 2021*).

These vesicles not only transmit toxins but also instigate inflammatory cascades, potentially subverting host immunity (*Kwaku et al, 2025; Joffe et al, 2016*). The inclusion of quorum signalling molecules in fungal EVs enhances biofilm formation, thereby increasing resistance to stressors and antifungal treatments (Estrela & Abraham, 2016). Due to their central role in mediating such multifaceted interactions, EVs remain at the forefront of research into *fungal* pathogenesis and are recognised as attractive targets for novel antifungal therapies and vaccine design (*Herkert et al, 2019; Brandt et al, 2024; Ullah et al, 2023; Ikeda et al, 2024*).

Fungal EVs constitute dynamic lipid bilayer assemblies whose biogenesis is tightly controlled by cellular machinery and environmental factors. They encapsulate biomolecules in a highly selective manner, reflecting their specialised functions in stress adaptation, nutrient acquisition, virulence modulation and inter-organismal communication (Ullah *et al,* 2023; Zamith-Miranda *et al,* 2021; Bleackley *et al,* 2019). Although the mechanistic foundations of EVs formation which can occur via intracellular compartments or at the plasma membrane–

remain incompletely resolved (Oliveira *et al,* n.d.), multi-omics methodologies (proteomics, transcriptomics, metabolomics and lipidomics) have been invaluable in elucidating their roles in cell wall biosynthesis, virulence and antifungal resistance (*Zamith-Miranda et al, 2021*).

Through the mediation of immune reactions and biofilm formation, EVs exert profound influence over *fungal* infection outcomes (Ullah *et al,* 2023; Brandt *et al,* 2024). Their biotechnological potential is expansive; research into their utilisation in diagnostics, vaccine adjuvants and targeted drug delivery is advancing rapidly (*Nenciarini & Cavalieri, 2023; Silva et al, 2019)*. Nonetheless, the discipline remains in its early stages and further elucidation of EVs biogenesis, cargo selection and secretion are imperative to exploit their therapeutic and industrial promise fully (*Nenciarini & Cavalieri, 2023; Silva et al, 2019*). Given the economic significance of *fungi* in the production of nutraceuticals and pharmaceuticals, enhancing our understanding of fungal EVs could pave the way for engineered strains capable of precise cargo delivery for medical applications (Liébana-Jordan *et al,* 2021; Nenciarini & Cavalieri, 2023).

Table 1: Representative Molecular Cargo of Fungal EVs

Molecular Class	Examples Identified in Fungal EVs	Biological Functions
Proteins	Hsp70, glucanases, chitin synthases, peptidases	Cell wall re-modelling, virulence, stress response
RNAs	mRNA, tRNA, miRNA, siRNA	Intracellular regulation, trans-kingdom gene expression
Lipids	Ergosterol, phosphatidylcholine, phosphatidylserine	Membrane stability, host interaction, and vesicle fusion
Metabolites	Melanin precursors, gliotoxin, aflatoxin	Immune modulation, signalling, and pathogenesis

3. Engineering Approaches for *Fungal* EVs

Engineering *fungal* EVs comprises an intricate interplay of molecular biology, synthetic engineering, and tailored bioprocessing. These methodologies empower precise manipulation of vesicle cargo and surface properties, making them highly versatile for applications such as targeted drug delivery, biosensing, immunotherapy, and sustainable material production. Moreover, the design of reproducible manufacturing pipelines underscores the potential of fungal EVs to meet clinical and industrial regulatory benchmarks.

Advancements in CRISPR/Cas9 methodology have revolutionised genetic engineering in filamentous *fungi*, particularly in *Aspergillus niger*, by enabling precise genomic edits previously hindered by the inefficiencies of classical gene targeting techniques. The CRISPR/Cas9 system has been ingeniously modified to address the inherent challenges of gene integration in filamentous *fungi*, which is pivotal for both industrial biotechnology and environmental applications (*Ullah et al, 2020*; Wang & Coleman, 2019). This approach allows the seamless insertion of coding sequences into native cellular pathways, including vesicle-sorting mechanisms, thereby amplifying the capacity of *fungi* to generate commercially significant metabolites and recombinant proteins (*Shi et al, 2017; Kumar et al, 2021*).

Nevertheless, scaling these modifications within filamentous *fungi* must overcome several obstacles, such as limited transformation efficiencies, the complex morphology of hyphae, and issues with plasmid maintenance (*Song et al, 2019; Shen et al, 2024*). In contrast to unicellular yeast like *Saccharomyces cerevisiae*, which relies on promoter-driven plasmid vectors for heterologous protein expression, filamentous

fungi necessitate more robust systems owing to their intricate cellular architecture and the predominance of non-homologous end-joining DNA repair pathways (*Nødvig et al., 2015; Shen et al., 2023*).

To this end, bespoke CRISPR/Cas9 platforms have been engineered specifically for filamentous species, featuring AMA1-based autonomously replicating plasmids and Cas9 ribonucleoprotein (RNP) delivery systems, which have already demonstrated enhanced transformation success and plasmid stability (*Rozhkova & Kislitsin, 2021; Jin et al., 2022*). These refined methodologies not only bolster the industrial utility of *fungi* but also open avenues for more refined genetic engineering strategies, such as multiplex genome editing and sophisticated transcriptional control, essential for maximising the biosynthesis of bioactive molecules and therapeutic agents (*Kumar et al, 2021; Shen et al, 2024).* Continued efforts to resolve these technological barriers will be instrumental in unlocking the full scope of filamentous *fungi* applications within the realms of biotechnology and synthetic biology (*Song et al., 2019; Nødvig et al., 2015).*

Synthetic biology has propelled the development of modular systems for vesicular modulation, exemplified by the utilisation of self-assembling protein nanocages and engineered transcriptional circuits. These constructs permit the conditional release of cargo in response to external stimuli such as environmental alterations or metabolite concentrations. By adapting motifs that commandeer ESCRT-mediated pathways - originally described in mammalian systems for use in *fungi*, researchers have produced 'enveloped protein nanocages' capable of programmable cargo secretion (*Patterson et al, 2014; Vázquez & Villaverde, 2010*).

Complementing these strategies, synthetic transcriptional designs employing inducible promoters, riboswitches and logical gating orchestrate extracellular vesicle production in response to cues such as pH variations, offering precise temporal control over vesicle-mediated biofabrication (*Hai, 2012; Elani et al, 2024).* Additionally, thermo responsive synthetic cells have been devised to trigger protein synthesis and cargo release at defined temperatures, signifying potential biotechnological and medical applications (*Elani et al, 2024*).

The incorporation of channel proteins into vesicle membranes creates membrane-based AND logic gates, enabling the regulation of vesicle permeability and cargo discharge through Boolean logic (*Hilburger et al, 2019*). Bottom-up assembly of synthetic extracellular vesicles, with controlled combinations of lipids, proteins and RNA, demonstrates their capacity to emulate natural vesicles and facilitates programmable therapeutic delivery systems (*Staufer et al, 2021*). Collectively, these innovations illustrate the capacity of synthetic biology to engineer highly sophisticated, programmable systems capable of targeted and controlled biological functions across diverse applications (*Cheng et al., 2019; Hirschi et al., 2016; Huang & Nikel, 2019*).

Within the sphere of extracellular vesicle engineering, cargo loading mechanisms are central to improving delivery specificity and efficacy. Passive loading, relying on high intracellular expression and random vesicle encapsulation, often proves suboptimal, prompting the development of active loading techniques. These methods include the integration of RNA aptamer motifs or scaffold proteins to enrich vesicles with therapeutic cargo. A notable example is the use of aptamer-guided encapsulation to load therapeutic mRNAs into engineered vesicles, which has yielded effective outcomes in models of inflammatory bowel disease and adipose tissue browning (Zhang *et al,* 2021).

Additionally, the Targeted and Modular EVs Loading (TAMEL) platform, leveraging MS2 bacteriophage coat protein fused to vesicle-associated proteins, has markedly increased RNA encapsulation efficiency, reportedly by up to 40-fold (Hung & Leonard, 2016). In *fungi*, targeting sequences derived from ESCRT-associated proteins or GRASP-mediated secretion pathways have been exploited to enrich vesicles with specific proteins or RNAs, with genetic manipulations of VPS, GRASP, and lipid-flippase

genes supporting enhanced cargo loading (*Han et al., 2021*). Furthermore, fusion constructs such as hCD9.hAGO2 have been demonstrated to bolster the loading of small RNAs, including miRNAs and shRNAs, into extracellular vesicles, thereby improving their delivery to recipient cells (*Es-haghi et al., 2023*). These advances highlight the versatility of engineered vesicles as delivery platforms for a spectrum of biomolecules, ranging from mRNA to proteins, and suggest promising routes for clinical translation (*Peruzzi et al., 2024; Zickler et al., 2023*). In sum, the integration of active loading mechanisms into vesicle engineering represents a significant step towards overcoming the limitations of conventional drug delivery systems, offering tangible progress in gene therapy and beyond.

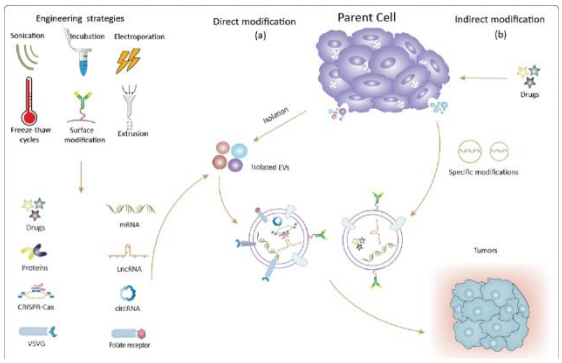

Figure 1. Workflow for engineering *fungal* extracellular vesicles (EVs). (Zhang, F., Guo, J., Zhang, Z. *et al.* Application of engineered extracellular vesicles for targeted tumor therapy. *J Biomed Sci* 29, 14 (2022).

The entire workflow crystallises in a seamless, iterative pipeline, as illustrated in Figure 1. Commencing with the choice of a fungal host and an appropriate secretion pathway, researchers proceed through genetic modification, promoter engineering, bioreactor-based cultivation, vesicle isolation, post-isolation functionalisation, and ultimately rigorous quality control. Vesicle purification employs methodologies such as ultracentrifugation, tangential-flow filtration or size exclusion chromatography to ensure both purity and scalability. Subsequently, surface engineering through techniques such as PEGylation or ligand conjugation serves to enhance vesicle stability and targeting efficacy. Quality control is comprehensive, utilising nanoparticle tracking analysis alongside proteomic and lipidomic profiling, as well as functional bioassays to verify delivery efficiency and biocompatibility.

EVs have emerged as versatile delivery vehicles for a broad spectrum of therapeutic cargos, including small-molecule drugs, proteins, mRNAs, long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs). EVs, being inherently capable of mediating intercellular communication, have been further engineered to amplify their therapeutic potential particularly within oncology. These vesicles can be loaded with chemical entities, proteins and nucleic acids such as siRNA, miRNA, mRNA and CRISPR/Cas9 components to selectively target and destroy tumour cells more effectively (*Zhang et al., 2021; Han et al., 2021; Lu et al., 2023; Shi et al., 2020*).

Surface modifications, including the addition of tumour-targeting peptides or folate receptors, refine their specificity for malignant cells (*Tang et al., 2023*). EVs have been employed to facilitate the direct delivery of chemotherapeutic drugs to tumour sites, regulate gene expression, and stimulate anti-tumour immune responses, thereby enhancing therapeutic efficacy (*Chulpanova et al., 2018; Han et al., 2021*). Beyond oncology, EVs have demonstrated efficacy in mRNA vaccine delivery—such as in COVID-19 applications and in treating genetic and inflammatory disorders (*Picon et al., 2023; Shi et al., 2020*). Their biocompatibility, structural stability and ability to traverse biological barriers render EVs promising clinical candidates, despite ongoing challenges related to production costs and regulatory approval (*Tang et al, 2023*).

The development and purification pipeline for EVs from *fungal* hosts comprises several meticulous stages, each essential for ensuring the quality and functionality of the final product. Initially, the selection of a suitable fungal strain and secretion pathway is critical, given that fungi are proficient producers of nanoparticles due to their enzymatic systems, which facilitate nanoparticle biosynthesis with unique properties (*Rami et al., 2024*). Genetic modification and promoter

optimisation are employed to augment EV secretion, followed by culture in controlled bioreactor environments. Isolation of EVs utilises tangential-flow filtration (TFF) and size exclusion chromatography (SEC), which are favoured for their efficiency in removing contaminants while preserving vesicle integrity (*Yuan et al., 2023; Kawai-Harada et al., 2024*).

TFF offers scalability and the capacity to separate macromolecular complexes while minimising stress on vesicles (*Kawai-Harada et al., 2024*). Post-isolation, surface engineering via PEGylation or ligand conjugation is employed to enhance vesicle stability and targeting attributes that are crucial for drug delivery applications (*Zia et al., 2010*). Quality control remains stringent, incorporating nanoparticle tracking analysis, proteomic and lipidomic profiling, and functional bioassays to confirm delivery performance and biocompatibility (*Zamith-Miranda et al, 2021; Xu et al, 2024).* In the context of anti*fungal* therapy, EVs offer advantages such as enhanced drug penetration, reduced toxicity, and improved targeting capacity (Wang & Li, 2023). The integration of omics approaches further elucidates *fungal* EVs biogenesis and function, enhancing their potential as therapeutic carriers and diagnostic tools (*Zamith-Miranda et al., 2021; Herkert et al., 2019).*

Advancements in imaging technologies have markedly improved the characterisation of EVs derived from both yeasts and filamentous *fungi.* Cryo-electron microscopy (cryo-EM) has been pivotal in revealing the structural diversity of EVs, including actin-like filaments suggestive of motility, thereby challenging the paradigm of EVs as static particles (*Cvjetkovic et al., 2017*). The combination of fluorescent cargo labelling, high-content imaging, and super-resolution microscopy enables precise quantification of vesicle morphology and uptake kinetics at the single-vesicle level (*Isogai et al, 2024; Verweij et al, 2021*). These methods have been exploited to investigate EV-mediated processes such as intercellular communication and pathogenesis in fungal systems (*Rutter & Innes, 2023; Rodrigues et al., 2016*). For example, EVs have been observed to transport protein effectors and metabolites that modulate host pathogen interactions in fungal pathogens (Rutter & Innes, 2023). The development of genetically encoded fluorescent proteins and advances in vivo imaging strategies have further deepened understanding of EVs physiological roles and therapeutic potential (*Chuo et al., 2018; Visser et al, 2024*). Such imaging tools are driving EVs research forward, broadening insight into their multifaceted roles in cellular communication and disease.

As *fungal* EVs advance towards clinical and ecological applications, biosafety and regulatory considerations assume critical importance. Purification must rigorously remove immunogenic cell-wall residues to mitigate the risk of immune activation, given the diverse biochemical cargo these vesicles carry, including proteins and lipids that can influence virulence (*Herkert et al., 2019*). Stability testing must adhere to Good Manufacturing Practice (GMP) standards, examining storage buffers and conditions. Recent studies indicate that phosphate-buffered saline supplemented with human albumin and trehalose (PBS-HAT) presents a superior medium for maintaining EVs integrity over time (Görgens et al., 2022).

Immunological profiling of lipid and protein content is essential to prevent unintended immune responses, a frequent obstacle in biopharmaceutical development (Vandivort et al., 2020). In agricultural or environmental contexts, environmental safety must also be assured; this includes evaluating the potential for EVs to interact with non-target organisms or ecosystems (*Liu & Hu, 2023; Octaviano et al., 2022).* The establishment of standardised protocols for EVs purification and characterisation combining multiple methods to optimise purity and yield is vital to unlock the therapeutic promise of *fungal* EVs while addressing biosafety and regulatory imperatives (*Simon et al., 2020*).

In summary, the convergence of genetic engineering, synthetic biology, advanced purification techniques and thorough functional characterisation establishes a formidable platform for engineering *fungal* EVs. These

nanobiotechnological agents are rapidly approaching real-world applications in drug delivery, responsive biosensing and sustainable biofabrication, heralding the advent of programmable, functional mycelial nanomachines.

4. Applications in Nanobiotechnology and Biofabrication

The potential of engineered *fungal* EVs is being realised across medical, industrial, and biotechnological domains. Their natural composition, cargo versatility, and structural resilience make them promising vehicles for disease therapies, enzymatic catalysis, and biosensor integration.

4.1 Medical Applications

In contemporary biomedical research, engineered EVs derived from *fungi* have garnered attention for their potential in vaccine development, precision drug delivery, and the mitigation of antimicrobial resistance. Investigations into vaccine design have identified EVs from species such as *Candida albicans* and *Cryptococcus neoformans* as carriers of a diverse repertoire of immunogenic proteins including enolase, glucosylceramides, and heat-shock proteins. These components have been shown to elicit both humoral and cellular immune responses in murine models, even in immunocompromised systems (*Freitas et al, 2019; Vargas et al, 2020; Piffer et al, 2021*). The inherent immunostimulatory capacity of these vesicles positions them as promising candidates for next-generation anti*fungal* and antiviral vaccines, offering an alternative to conventional adjuvants, which are often associated with undesirable reactogenic effects.

Beyond immunisation, *fungal*-derived EVs have exhibited considerable promise in the field of targeted therapeutics. For instance, EVs isolated from *Saccharomyces cerevisiae* have demonstrated efficacy in encapsulating and delivering small RNA molecules, including microRNAs and messenger RNAs, within in vivo tumour models. These studies reveal not only significant inhibition of tumour progression but also confirm the biocompatibility and non-immunogenic nature of the vesicles (*Yuan et al, 2024*). Compared to synthetic delivery systems, *fungal* EVs offer unique advantages owing to their structural biocompatibility, ease of bioengineering, and intrinsic biodegradability. These properties enable the controlled, tissue-specific delivery of therapeutic agents while minimising systemic toxicity and immunogenicity (Yuan *et al,* 2024).

Moreover, the role of *fungal* EVs in combating antimicrobial resistance is increasingly recognised. Through bioengineering, EVs can be tailored to transport resistance-modifying enzymes such as β-glucosidase, which act on resistant bacterial phenotypes by facilitating the intracellular delivery of antimicrobial adjuvants or inhibitors. This strategy has been effective in restoring susceptibility in resistant strains and enhancing the efficacy of conventional antimicrobials (Herkert *et al,* 2019). Parallel developments in exosome engineering further refine these delivery mechanisms, incorporating surface modifications and cargo-loading enhancements to optimise vesicle stability, targeting specificity, and trans-barrier delivery (Liang *et al,* 2021; Ahmed *et al,* 2024).

Despite these advancements, challenges persist. Standardised methodologies for EVs production, isolation, and characterisation are not yet universally adopted, which hampers reproducibility and scalability for clinical translation (*Yuan et al, 2024*). However, progress in isolation technologies, such as differential ultracentrifugation, size exclusion chromatography, and microfluidic separation, continues to support the advancement of EVs research towards therapeutic application.

Furthermore, recent innovations have demonstrated the use of EVs from *Saccharomyces cerevisiae* and *S. boulardii* in encapsulating chemotherapeutics and small RNAs. These vesicles protect their cargo under serum conditions and promote enhanced cellular uptake in targeted tissues (Bachurska *et al,* 2023). This is particularly relevant in oncology, where EV-mediated delivery systems can bypass systemic

barriers and provide sustained, localised drug release. Concurrently, EVs engineered to carry resistance-modulating enzymes continue to play a pivotal role in addressing the growing global threat of antimicrobial resistance.

Taken together, these findings underscore the immense potential of *fungal* EVs as multifunctional platforms in modern medicine. Their integration into drug delivery frameworks represents a transformative approach to enhancing therapeutic precision, minimising off-target effects, and addressing the pressing challenge of antimicrobial resistance.

4.2 Industrial Applications

Engineered EVs derived from *fungi* demonstrate notable potential in the realm of industrial biotechnology, particularly with respect to enzyme delivery, the development of sustainable biocatalytic systems, and the degradation of organic waste. Notably, *Trichoderma reesei*, a filamentous fungus recognised for its prolific secretion of lignocellulolytic enzymes, has been reported to generate EVs exhibiting significant cellulolytic activity. These vesicles are especially enriched with filter paper-degrading enzymes and β-glucosidase when cultivated in cellulose-rich environments, suggesting their utility as natural vectors for enzyme transport in biomass processing (Paula *et al,* 2019; Seiboth *et al,* 2011). Such findings reinforce the candidacy of *T. reesei* EVs as efficient mediators of enzymatic hydrolysis in biofuel manufacturing and the pulp industry.

Historically, *T. reesei* emerged as a pivotal organism during World War II due to its cellulolytic capabilities, a legacy that has since underpinned its extensive application in modern biofuel production. Its enzymatic repertoire plays a critical role in the saccharification of cellulosic substrates into fermentable monosaccharides, thus contributing to the development of sustainable bioenergy systems (Fischer *et al,* 2021; Derntl *et al,* 2014). Concurrently, species within the genus *Aspergillus* are being investigated for their proficiency in secreting industrially relevant lipases. These enzymes serve integral functions in sectors such as detergent formulation, leather treatment, and biodiesel synthesis. The possibility of utilising EV-mediated delivery for these lipases opens the door to creating recyclable and immobilised enzyme systems with improved catalytic performance and reusability (Soliman *et al,* 2013).

Moreover, the ability of fungal EVs to transport lignin-degrading enzymes presents an ecologically viable strategy for the decomposition of industrial pollutants and recalcitrant organic materials. This capability aligns with the tenets of mycoremediation, where fungal organisms contribute to environmental detoxification via enzymatic degradation pathways (Druzhinina and Kubicek, 2017). The incorporation of such vesicles into waste management protocols may significantly enhance enzymatic efficacy while reducing ecological burden.

Incorporating fungal EVs into industrial applications not only improves the precision and efficiency of enzyme delivery but also fosters the transition toward greener and more sustainable bioprocesses. This approach is integral to the evolution of a circular, bio-based economy that relies on renewable biological systems (Sharma and Salwan, 2019; Ferreira *et al,* 2014). However, challenges remain in optimising enzyme production, maintaining catalytic activity under industrial conditions, and ensuring structural stability of the vesicles. Continued research is essential to address these limitations and to facilitate the scalable integration of fungal EVs into commercial biotechnology pipelines (Chaudhary *et al,* 1985; Mishra *et al,* 2019).

4.3 Biotechnological Applications

EVs derived from *fungi* are increasingly recognised as critical elements in the advancement of cutting-edge biotechnological innovations, especially within the domains of biosensing, scaffold engineering, and regenerative medicine. These vesicles transport a diverse array of biologically active constituents—including enzymes, polysaccharides, and conductive compounds that can be functionalised for integration into biosensor platforms. Their incorporation has significantly enhanced the

electrochemical and optoelectronic performance of devices such as wearable sensors and bio-interactive electronic skins. These systems exploit the dynamic properties of *fungal* EVs to achieve responsive, adaptive behaviour in real-time monitoring applications (Kaltenbrunner, 2023; Danninger *et al,* 2022).

One of the defining features of *fungal* EVs in biosensing lies in their ability to ferry proteins and enzymes with high substrate specificity. Upon immobilisation on biosensor surfaces, these molecules significantly improve the sensitivity and detection thresholds of environmental and pathogen biosensors. Such enhancements enable more accurate detection of microbial contaminants and environmental toxins, expanding the practical utility of biosensing systems in public health and environmental surveillance (Dolatabadi & Manjulakumari, 2012; *Nsairat et al, 2021; Singh et al, 2020).*

In tissue scaffold design, 3D-printed mycelium-based hydrogels incorporating *fungal* EVs represent a sustainable and innovative material paradigm. These constructs exhibit self-healing and stimuli-responsive characteristics while providing a biocompatible matrix for cellular colonisation and tissue integration. Notably, the resilience and adaptability of EV-infused mycelial scaffolds render them suitable for use in dynamic tissue environments, making them valuable for applications in tissue engineering and regenerative medicine (Kaltenbrunner, 2023; *Ganzeboom et al, 2024).*

From a regenerative perspective, *fungal*-derived EVs embedded within polysaccharide-rich hydrogels have demonstrated capacity to support key physiological processes including cellular proliferation, extracellular matrix synthesis, and neovascularisation. These features are especially relevant in the context of soft tissue repair and biofabrication, where a supportive microenvironment is essential for effective tissue regeneration (Herkert *et al,* 2019; Rodrigues *et al,* 2018). The bioactivity and molecular cargo of *fungal* EVs allow them to modulate the local cellular milieu, facilitating targeted interactions and signalling pathways critical to tissue healing.

Collectively, the multifunctionality of *fungal* EVs across biosensor development, scaffold engineering, and regenerative therapeutics highlights their strategic importance in contemporary biotechnology. Their eco-compatible origin, capacity for molecular customisation, and biointeractive properties offer a robust foundation for the creation of next-generation solutions in health monitoring, environmental diagnostics, and medical rehabilitation (Herkert *et al,* 2019; Mayne, 2023).

Table 2: Applications of engineered fungal EVs across domains

Domain	Application	Examples and Outcomes
Medical	Vaccine development	*C. albicans* EVs induced strong IgG and T-cell responses; protective in murine models (Freitas *et al,* 2019; Piffer *et al,* 2021)
	Targeted drug delivery	*S. boulardii* EVs delivered doxorubicin to intestinal cells with low immunogenicity (Bachurska *et al,* 2023)
	Antimicrobial resistance	EVs engineered to carry resistance-modifying enzymes restore the efficacy of antifungal drugs
Industrial	Enzyme delivery & biocatalysts	*T. reesei* EVs carry cellulases; potential for biomass conversion (Extracellular vesicles carry cellulases…, 2019)
	Sustainable waste	EVs transporting lipases from *A. carneus* for biodiesel or dye

	degradation	removal; ligninolytic EVs support mycoremediation (Plant-associated endophyte..., 2022; Lignin-modifying enzyme, 2024)
Biotechnological	Biosensors	Fungal EV-based conductive elements integrated into textiles and wearables (Reactive fungal wearable, 2020; Fungal electronics, 2021)
	Scaffold formation & regenerative medicine	Mycelium-EV hydrogels enable self-healing scaffolds, cell colonisation, and soft tissue repair (3D printing..., 2022; Frontiers, 2023)

Engineered EVs derived from *fungi* represent a rapidly emerging innovation in nanobiotechnology, offering expansive utility across medical, industrial, and biotechnological domains. In medical science, fungal EVs are being actively investigated as next-generation vehicles for drug delivery due to their intrinsic biocompatibility, minimal immunogenicity, and tunable surface characteristics. These attributes make them particularly effective in the treatment of complex diseases such as malignancies, cardiovascular conditions, and persistent infections (Wang *et al,* 2023; Claridge *et al,* 2021; Riaz *et al,* 2023). Their immunomodulatory capacities, including anti-inflammatory potential, further elevate their therapeutic relevance, particularly in modulating host immune responses and delivering immunotherapies (Levy *et al,* 2024).

From an industrial perspective, *fungal*-derived EVs support environmentally sustainable practices, most notably through their application in the green synthesis of nanoparticles. These biosynthesised particles exhibit catalytic and reductive properties essential for processes such as pollutant degradation and heavy metal remediation, thus contributing to eco-friendly industrial solutions (Shaheen *et al,* 2021; Iqbal *et al,* 2023). In biotechnology, EVs serve as dynamic platforms for the development of responsive biomaterials and biosensors. Their innate roles in intercellular communication and selective molecular transport make them ideal for functionalising adaptive sensor networks and smart biointerfaces (Herkert *et al,* 2019; Stranford and Leonard, 2017).

Nonetheless, despite their multifaceted potential, several challenges must be addressed to translate *fungal* EVs into scalable technologies. These include the development of robust, reproducible protocols for large-scale vesicle production, advanced strategies for cargo loading and surface functionalisation, and compliance with evolving regulatory frameworks governing biological nanocarriers (Claridge *et al,* 2021; Stawarska *et al,* 2024). High-resolution analytical techniques—such as mass spectrometry-based proteomics and lipidomics—are increasingly indispensable for dissecting the molecular composition of EVs. These methods facilitate the identification of bioactive constituents, improve our understanding of vesicle functionality, and enable targeted applications in precision therapeutics and diagnostics (Rizzo *et al,* 2020; Stawarska *et al,* 2024).

Ultimately, the advancement of fungal EV-based nanobiotechnologies will require coordinated, interdisciplinary efforts that bridge microbiology, materials science, synthetic biology, and regulatory science. Through such integration, fungal EVs are poised to become foundational components in next generation biofabrication, precision medicine, and industrial bioprocessing.

5. Challenges in EVs Engineering and Biosafety

Engineering *fungal* EVs holds immense promise for revolutionising therapeutic delivery, diagnostics, and biotechnological innovation. However, despite considerable advancements, the field continues to grapple with a number of formidable challenges that must be systematically

addressed before large-scale clinical and industrial translation can be realised. Chief among these are biosafety concerns, variability in standardisation protocols, unpredictable immune interactions, cytotoxicity risks, and manufacturing constraints. Unlike mammalian-derived EVs, *fungal* EVs are often secreted in the context of highly variable morphologies, environmental conditions, and genetic backgrounds, all of which significantly influence their composition and functional properties (Brown *et al,* 2015; Oliveira *et al,* 2010). This variability presents obstacles in achieving reproducible yields and consistent biological activity, which are essential criteria for regulatory approval under current good manufacturing practices (cGMP) (Wiklander *et al,* 2019). Furthermore, the potential immunogenicity of *fungal*-derived vesicles—especially those produced by opportunistic pathogens such as *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*—raises valid concerns regarding host immune modulation, inflammation, and off-target effects (Peres da Silva *et al,* 2015; Vargas *et al,* 2015).

Without rigorous purification and characterisation strategies, including removal of contaminating proteins, nucleic acids, and immunostimulatory lipids, such EVs may trigger adverse immune responses or exhibit cytotoxicity in sensitive tissues (Coelho *et al,* 2019). On the technical front, the lack of standardised protocols for EV isolation, quantification, and quality control complicates comparative studies and hinders reproducibility across laboratories (Théry *et al*, 2018). Additionally, scalable manufacturing remains a major bottleneck, as current culture systems and downstream processing techniques are not yet optimised for cost-effective, high-volume EVs production without compromising vesicle integrity or bioactivity (Willms *et al*, 2018). Addressing these multifaceted issues is not only critical to ensuring that engineered EV platforms are safe and effective but also vital to establishing the foundational standards that will guide future innovation, regulation, and widespread adoption across biomedical, industrial, and environmental domains.

5.1 Biosafety and Standardisation

Achieving biosafe and standardised production of *fungal* EVs presents foundational challenges that must be addressed to facilitate their translation into therapeutic, industrial, and environmental applications. A primary issue arises from the heterogeneity of EV populations, driven by variations in fungal strain, growth conditions, and upstream culture systems. As Thomas *et al.* (2023) emphasise, even minor alterations in medium composition, vessel type, or nutrient availability can profoundly influence EV yield and cargo profile. This is particularly problematic when comparing data across research laboratories or attempting to scale up for industrial production. The variability not only hinders reproducibility but also raises safety concerns, as EVs may inadvertently contain virulence determinants or immunologically active polysaccharides endemic to pathogens such as *Candida albicans* or *Cryptococcus neoformans* (Oliveira *et al*, 2010; Peres da Silva *et al,* 2015).

The lack of standardised purification protocols further exacerbates the problem. Commonly used methods such as differential ultracentrifugation, density-gradient separation, and tangential-flow filtration yield EVs fractions of varying purity, efficiency, and cargo preservation. Recent comparative analyses in human cell systems have shown that tangential-flow filtration combined with size-exclusion chromatography offers superior yield and batch consistency compared to ultracentrifugation alone, producing 10-20-fold higher EVs recovery with significantly reduced protein contamination (Lobb *et al*, 2015; Busatto *et al*, 2018; turn0search0). While similar trends are anticipated in fungal systems, rigorous validation in fungal culture matrices is still required.

To ensure biosafety in clinical or environmental contexts, rigorous downstream processing is indispensable. Purification procedures must effectively remove endotoxins, immunogenic cell-wall fragments, and other contaminants without compromising EVs integrity. Quality control workflows should include nanoparticle tracking analysis to verify size distribution and concentration, alongside proteomic and polysaccharide profiling to affirm vesicle

composition. For pathogenic species, it is essential to develop assays for detecting virulence-related molecules, as their unintended presence could evoke adverse immune responses or disrupt host-microbe homeostasis (Vargas *et al*, 2015).

Regulated manufacturing will also necessitate transparent reporting of EVs preparation conditions a practice encouraged by the International Society for Extracellular Vesicles (MISEV guidelines), which highlight the need for detailed metadata on sample sourcing, isolation steps, and characterisation methods (Théry *et al*, 2018). Adopting such standards in *fungal* EVs research will be fundamental to achieving reproducibility and facilitating cross-study comparisons. Ultimately, the design of harmonised protocols, encompassing defined *fungal* strains, precise media formulations, calibrated isolation pipelines, and rigorous quality control, is vital to unlocking the full translational potential of fungal EVs across biomedical, industrial, and ecological arenas.

5.2 Immune Interactions and Cytotoxicity

The complex interplay between engineered *fungal* EVs and the host immune system presents both therapeutic promise and critical concerns. Notably, EVs derived from *Cryptococcus neoformans* and *Candida albicans* have been shown to interact dynamically with macrophages, inducing secretion of both pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and anti-inflammatory mediators including interleukin-10 (IL-10) (Vargas *et al,* 2015; de Paula *et al,* 2019). This dual response is largely dependent on vesicle composition, cargo payload, and dosing regimen. Furthermore, distinct EV subpopulations can polarise immune responses towards either Th1 (cell-mediated immunity) or Th2 (humoral immunity) phenotypes, which may result in functional protection or inadvertent immunosuppression (*de Paula* et al*, 2019*). Such polarisation has significant implications for EV-based therapeutics, especially in immunocompromised or sensitised populations.

In addition to modulating cytokine networks, evidence has emerged that specific *fungal* EVs components, such as melanin precursors, glucans, and fungal toxins, can exert direct cytotoxic effects on mammalian cells. For example, EVs contaminated with melanin intermediates like DOPA and 5, 6-dihydroxyindole have been associated with HepG2 (hepatic) and HEK293 (renal) cell death via oxidative mechanisms (*Zhao* et al*, 2024; Nappi* & Ottaviani, 2000). Similarly, melanised EVs from *Exophiala dermatitidis* demonstrated significant cytotoxicity in neuroblastoma-derived SH-SY5Y lines, in contrast to non-melanised vesicles, which were biocompatible (Scherlach *et al*, 2024). The cytotoxic potential of pigment-matched EVs may facilitate anti-cancer applications, y*et al*so raises questions of unintended toxicity in healthy tissues, particularly when systemic delivery is employed.

These observations highlight the importance of rigorous safety profiling. Systematic in vitro cytotoxicity assays should be complemented by in vivo studies in multiple organ systems, including hepatic, renal, and neuronal models, to delineate dose-dependent toxicity thresholds. Furthermore, immunophenotyping of primary immune cells is required to detect skewing towards Th1, Th2, or regulatory T-cell subsets. The presence of IL-10-dominant EVs responses, for instance, may undermine desired therapeutic immunity by promoting an immunosuppressive milieu (de Paula *et al*, 2019). Incorporating surface modifications, such as PEGylation or receptor-targeting ligands, may ameliorate off-target immune activation and enhance cellular specificity, though this warrants comprehensive pharmacodynamic evaluation (Busatto *et al*, 2018).

Ultimately, the dual immunomodulatory and cytotoxic potential of *fungal* EVs demands carefully calibrated engineering strategies. Balancing immune activation against cytotoxic risk will require refined cargo selection, surface engineering tactics, and standardised preclinical testing that conforms to regulatory frameworks. This vigilance is essential to harness their biomedical potential without compromising safety or therapeutic efficacy.

5.3 Manufacturing and Scalability

Achieving large-scale production of *fungal* EVs presents significant technical and economic challenges, particularly when translation into industrial or clinical contexts is considered. Current EV manufacturing platforms are characterised by low yield and high variability, which are problematic even in well-established mammalian systems; the shift from adherent cultures to bioreactor formats often leads to issues such as cellular senescence, culture heterogeneity, and medium contamination (*Andriolo* et al*, 2018; Kawai-Harada* et al*, 2024*). In the context of *fungal* EVs, similar hurdles emerge, compounded by morphological complexity and sensitivity to environmental fluctuations.

Initial scaling efforts rely heavily on high-density culture systems, such as 3D bioreactors or hollow fibre arrays, which maximise surface area and productivity compared with traditional flasks (Kawai-Harada *et al*, 2024; Andriolo *et al*, 2018). While these systems can increase EVs yield multiple-fold, they demand meticulous control of culture parameters, including oxygen tension, shear stress, and nutrient flow, due to the high sensitivity of *fungal* secretome profiles to environmental conditions (Lobb *et al*, 2015; Kawai-Harada *et al*, 2024).

Downstream processing represents another bottleneck: isolation techniques such as differential ultracentrifugation, density-gradient separation, and tangential flow filtration (TFF) are variably employed, yet each carries trade-offs in yield, purity, and reproducibility (Busatto *et al*, 2018; Kawai-Harada *et al*, 2024). Notably, TFF followed by size-exclusion chromatography (SEC) has demonstrated superior recovery rates, structural integrity retention, and batch consistency in mammalian EV studies, suggesting similar applicability to *fungal* systems (Busatto *et al*, 2018; Kawai-Harada *et al*, 2024).

Furthermore, the reproducibility of EV preparations is influenced by both upstream and downstream variability. Slight deviations in fungal strain genetics, medium composition, or bioreactor operation can lead to significant changes in vesicle phenotype, yield, and cargo composition, complicating regulatory approval and quality assurance efforts (Andriolo *et al*, 2018; Lobb *et al*, 2015).

Emerging solutions include EV-mimetic particles generated via membrane extrusion, which offer higher yields but may differ in composition and functionality from native EVs, raising questions about their suitability for regulatory approval and therapeutic use (Andriolo *et al*, 2018; Andriolo *et al*, 2018). The design of closed, automated manufacturing systems integrating upstream culture and downstream purification, similar to those used in mammalian EV production, could offer a path to compliant, scalable manufacturing (Andriolo *et al*, 2018; Kawai-Harada *et al*, 2024).

However, the advancement of *fungal* EVs engineering into viable platforms for biomedical, industrial, and environmental applications necessitates an integrated approach that addresses critical biosafety, immunological, and manufacturing limitations. These vesicles, while promising in their natural versatility, encounter several translational bottlenecks that, if left unaddressed, could hinder their regulatory approval and practical deployment. As research in EVs bioengineering progresses, the implementation of robust, reproducible, and scalable production systems remains an essential prerequisite for clinical and industrial viability.

From a biosafety perspective, a central challenge lies in the biological complexity and heterogeneity of *fungal* EVs. Their cargo, which includes a diverse array of lipids, proteins, nucleic acids, and potential virulence factors, varies significantly with culture conditions, strain genotype, and environmental stimuli (Rizzo *et al,* 2020). This variability compromises standardisation, particularly in clinical contexts where consistency of therapeutic payload and immunogenicity must meet stringent regulatory thresholds. Establishing validated protocols for vesicle isolation, such as tangential flow filtration or density gradient centrifugation, combined with advanced characterisation using nanoparticle tracking analysis (NTA), proteomics, and lipidomics, is imperative for ensuring safety and

batch-to-batch fidelity (Busatto *et al,* 2018; Andriolo *et al,* 2018).

Moreover, the immunological profile of *fungal* EVs presents a double-edged sword. On the one hand, their intrinsic immunomodulatory properties render them attractive for vaccine delivery and immune adjuvancy. On the other hand, uncontrolled immune interactions may lead to adverse responses including inflammation, cytokine imbalance, or off-target effects (Joffe *et al,* 2016). To mitigate these risks, comprehensive immunotoxicology testing, spanning *in vitro* macrophage activation assays to *in vivo* cytokine profiling, must be conducted under good laboratory practice (GLP) conditions. The identification of non-immunogenic or engineered 'stealth' vesicles may also play a critical role in improving safety while preserving functional efficacy.

Manufacturing and scalability constitute further barriers to the deployment of *fungal* EVs in real-world settings. Compared to mammalian systems, fungal cultures offer advantages in terms of cost, growth rate, and biomass accumulation. However, the optimisation of large-scale bioreactor systems for vesicle production remains in its infancy. High-density fermentation technologies, including fed-batch or perfusion systems, can improve vesicle yield, but require precise environmental control to avoid metabolic drift and vesicle degradation (Lener *et al,* 2015). Furthermore, downstream processing workflows must be tailored to balance high recovery with structural preservation and low endotoxin levels. Emerging synthetic approaches, such as the generation of EV-mimetic nanoparticles through membrane extrusion or bio-orthogonal conjugation, provide alternatives that may enhance reproducibility, although questions remain regarding their bioequivalence and regulatory classification (Rizzo *et al,* 2020).

To navigate these multi-dimensional challenges, a strategic and collaborative framework is necessary. Interdisciplinary efforts combining molecular biology, bioengineering, immunology, and regulatory science are key to addressing knowledge gaps and accelerating the design of reproducible and safe EV-based systems. Alignment with international standards including those of the International Society for Extracellular Vesicles (ISEV), the European Medicines Agency (EMA), and the U.S. Food and Drug Administration (FDA) - will help ensure regulatory readiness of EV-based therapeutics and diagnostics (Lener *et al,* 2015).

Overall, the strategic future of engineered *fungal* EVs depends not only on technological innovation but also on infrastructural readiness and cross-sector collaboration. As the field matures, the establishment of global biobanks, data-sharing consortia, and open-access EV characterisation databases could significantly accelerate progress. In parallel, clear regulatory pathways and public-private partnerships will support the translation of laboratory discoveries into scalable, safe, and commercially viable EV-based technologies. Through such coordinated approaches, *fungal* EVs may emerge as one of the most adaptable and transformative platforms in the next phase of nanobiotechnology and biofabrication.

6. Future Outlook and Technological Convergence

The trajectory of research into EVs derived from *fungi* is set to intersect with a suite of transformative technological disciplines that promise to revolutionise their design, production, and application. With advancements in artificial intelligence (AI), synthetic biology, microfluidics, and multi-omics integration, the scope of *fungal* EVs in therapeutic, industrial, and biotechnological frameworks is poised to evolve beyond proof-of-concept studies into fully operational systems. The convergence of these technologies will be instrumental in refining vesicle bioengineering and ensuring their translation into scalable, safe, and efficacious products fit for clinical and commercial deployment.

One of the most compelling avenues of innovation involves the incorporation of AI and machine learning algorithms into EVs research pipelines. By integrating datasets generated from transcriptomic, proteomic, lipidomic, and metabolomic analyses, AI systems can identify predictive signatures for optimal EV cargo

loading, surface characteristics, and therapeutic efficacy. Computational models have already demonstrated capacity to classify vesicle subtypes, predict vesicle-cell interactions, and streamline vesicle design for targeted drug delivery (Lyu *et al,* 2021). In the context of *fungi*, where vesicle biogenesis pathways are still being elucidated, AI can be deployed to reverse-engineer vesicle formation and cargo sorting mechanisms through data-mined sequence motifs, structural models, and protein-protein interaction networks. Such predictive frameworks will dramatically shorten experimental cycles, reduce reagent costs, and enhance the precision of bioengineering interventions.

Parallel advances in microfluidic technologies are enabling the development of automated, high-throughput platforms for EVs production, isolation, and analysis. These miniaturised systems allow for continuous flow processing of culture supernatants, reducing shear stress and contamination risk while maintaining vesicle integrity (Busatto *et al,* 2018). Moreover, microfluidic chips embedded with immunoaffinity capture surfaces or acoustophoretic modules can sort vesicles with unprecedented specificity and speed, allowing the isolation of functionally distinct EV subpopulations from complex *fungal* cultures. By integrating biosensors directly onto these platforms, real-time monitoring of vesicle size, charge, and biomolecular composition becomes feasible, facilitating quality control that aligns with regulatory compliance for clinical-grade EVs. These technologies not only offer scalability but also significantly reduce variability and the need for large, energy-intensive laboratory infrastructure.

The integration of multi-omics approaches represents a cornerstone in understanding and manipulating the functional repertoire of *fungal* EVs. Whereas genomics alone provides limited insight into vesicle functionality, multi-omics platforms can map the dynamic relationships between genomic alterations, protein expression, lipid metabolism, and RNA content within the vesicles. Such integrative data are vital for designing EVs with tailored immunological, metabolic, or structural properties. For instance, transcriptomic and proteomic analyses of *Cryptococcus neoformans* and *Candida albicans* have revealed distinct cargo profiles linked to pathogenicity and host interactions (Rizzo *et al,* 2020), offering templates for therapeutic vesicle design. Future frameworks may incorporate single-vesicle sequencing, spatial proteomics, and lipidomics under a unified analytic pipeline, enabling the generation of 'designer vesicles' whose composition is precisely matched to intended clinical functions.

Another vital development concerns the transition from laboratory-scale EVs generation to industrial scale biomanufacturing. Traditional flask cultures and ultracentrifugation methods lack the efficiency and reproducibility required for commercial deployment. Emerging systems based on modular bioreactors with real-time biosensors, adaptive feeding strategies, and continuous harvest loops are currently being trialled to produce vesicles at the scale and quality needed for therapeutic applications (Lener *et al,* 2015). These systems, when combined with automation and AI-controlled feedback loops, can maintain vesicle yield and functionality across extended production runs. In the context of *fungal* EVs, which may be naturally produced in higher yields than mammalian counterparts, such biomanufacturing platforms offer a compelling alternative to mammalian cell lines, particularly when environmental sustainability and cost-efficiency are factored into development pipelines.

Moreover, the convergence of synthetic biology and nanotechnology is giving rise to hybrid vesicle systems that incorporate engineered lipids, peptides, and synthetic scaffolds into *fungal* EVs membranes. These hybrid structures can be programmed for enhanced cellular targeting, controlled release kinetics, or environmental responsiveness. Early studies using polyethylene glycol (PEG)-modified vesicles and engineered display of peptide ligands have shown that such surface modifications significantly enhance biodistribution and bioavailability (Piffoux *et al,* 2022). The ability to tune surface biophysics using synthetic modules will allow for customisation of vesicle-host interactions, improved

pharmacokinetics, and better payload protection in circulation.

Looking forward, regulatory preparedness and ethical considerations will also shape the future of *fungal* EV technologies. Standardised frameworks for vesicle characterisation, safety testing, and efficacy validation must be developed in alignment with international guidelines set by the EMA, FDA, and WHO. As EV-based products approach clinical testing and commercial release, these frameworks will underpin not only product safety but also public trust and market acceptance.

The future trajectory of engineered EVs derived from *fungi* is undergoing substantial transformation, underpinned by the incorporation of cutting-edge analytical platforms, algorithmic modelling, and scalable manufacturing frameworks. Recognised not only for their function in intercellular signalling but also for their capacity to transport bioactive molecules, *fungal* EVs are being repurposed across a spectrum of applications, ranging from biomedical therapeutics to industrial biotechnology and environmental remediation (Liebana-Jordan *et al,* 2021; Rizzo *et al,* 2020).

Technological convergence is accelerating this shift. The synergistic application of artificial intelligence (AI), microfluidics, and integrated multi-omics approaches - encompassing transcriptomics, proteomics, and lipidomics—has significantly expanded the scope of EVs research (*Zamith-Miranda et al, 2021; Piffoux et al, 2022*). These high-resolution methodologies have elucidated the complex molecular architecture of *fungal* EVs, thereby reinforcing their potential in clinical diagnostics and targeted therapies.

Microfluidic systems have emerged as pivotal in refining EVs production, offering precise control over vesicle yield, uniformity, and functional loading. These systems address critical limitations related to scalability and standardization, parameters essential for clinical grade biomanufacturing (Piffoux *et al,* 2022; Salmon, 2022). Concurrently, engineering strategies are being developed to augment the cargo-carrying capabilities and specificity of *fungal* EVs. Innovations such as stimuli-responsive modules are being integrated to enable controlled, context-dependent cargo release, transforming EVs into programmable nanoscale delivery platforms ("Engineered extracellular vesicles as drug delivery systems for the next generation of nanomedicine", 2023).

This paradigm shift redefines *fungal* EVs not merely as biological by-products but as engineered bio-nanomachines central to synthetic biology and precision nanotechnology (Gurunathan *et al,* 2021; Rai *et al,* 2024). As these platforms continue to evolve, they are poised to drive translational advances in mycobiotechnology, creating novel pathways for innovation in sectors spanning personalised medicine, environmental detoxification, and sustainable biofabrication (Shaheen *et al,* 2021; "Fungal nanobionics: Principle, advances and applications", 2023).

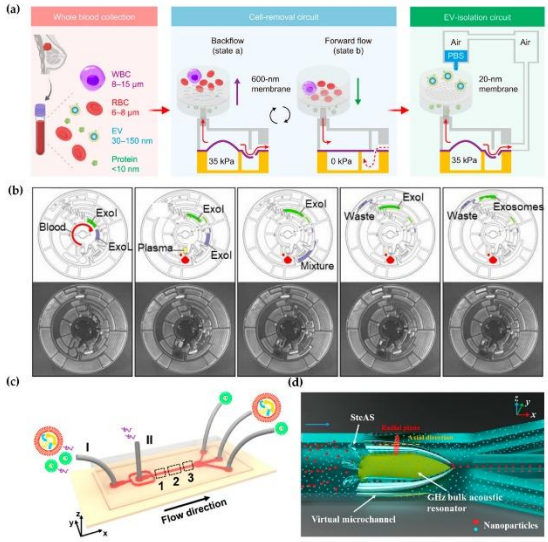

Figure 2: Emerging future landscape of fungal EVs in synthetic biology (Adapted from Chen, J., Zheng, M., Xiao, Q., Wang, H., Chi, C., Lin, T., Wang, Y., Yi, X., & Zhu, L. (2024).

Figure 2 illustrates the transformative potential of fungal EVs within the expanding discipline of synthetic biology. These vesicles, characterised by their lipid bilayer membranes, are integral to the transport of a wide spectrum of biomolecules—ranging from proteins and lipids to nucleic acids—

across the fungal cell wall, thereby mediating intercellular communication and host-pathogen dynamics (*Herkert et al,* 2019; *Rizzo et al,* 2017). In recent years, synthetic biology has accelerated the engineering of fungal strains to enhance the biosynthesis of high-value compounds, such as industrial enzymes, pigments, and antimicrobial metabolites, by refining both metabolic networks and secretory mechanisms (*Tiwari and Dufossé*, 2023; *Martins-Santana et al,* 2018).

The convergence of synthetic biology and *fungal* EVs research offers a promising platform for sustainable biofabrication. These vesicles act as natural delivery vehicles, capable of encapsulating and transporting biologically active molecules in a controlled manner, thus improving the precision and efficiency of biotechnological applications (*Fernández-Rhodes et al,* 2024; *Herkert et al,* 2019). Further support for these developments is found in the emergence of modular genetic engineering platforms, such as *Fungal* Braid, which allow for the standardised manipulation of filamentous *fungi*. These systems facilitate the integration of synthetic biological parts, enabling customisable and scalable bioproduction systems (*Giménez et al,* 2023).

Additionally, the bidirectional trafficking capability of *fungal* EVs, which includes their ability to absorb host-derived vesicles, reveals complex regulatory mechanisms that influence fungal physiology and adaptability (*Rodrigues et al,* 2018). This feature expands their utility in diverse biotechnological settings, including environmental bioremediation and precision fermentation. Nonetheless, significant challenges remain, particularly concerning the optimisation of EVs yield, the elucidation of biogenesis mechanisms, and the standardisation of isolation techniques (*Oliveira et al,* 2013).

In summary, the incorporation of *fungal* EVs into synthetic biology not only provides innovative strategies for enhancing biological production systems but also paves the way for environmentally responsible solutions in therapeutics, biomaterials, and industrial biosynthesis (*Zou et al,* 2024; *Jo et al,* 2023).

7. Conclusion

Recent advances in the exploration and engineering of EVs derived from *fungi* have significantly fostered the convergence of nanobiotechnology, therapeutic innovation, and environmental sustainability. These membranous vesicular organelles are recognised for their capacity to transport diverse biomolecules—such as proteins, nucleic acids, lipids, and metabolites imparting advantages over synthetic carriers through improved biocompatibility and inherent targeting capabilities (*Liebana-Jordan et al., 2021; Nenciarini & Cavalieri, 2023*). Their fundamental role in intercellular communication underpins both physiological and pathological processes, and they have emerged as promising candidates for drug delivery owing to their low immunogenicity and ability to traverse biological barriers (*Teng et al., 2021; Uddin et al., 2024*).

The deployment of genetic engineering platforms, notably CRISPR/Cas9, has enabled precise manipulation of EV cargo and surface properties, facilitating the production of bespoke vesicles tailored for applications in vaccines and targeted therapies (Huang & McNeill, 2023; *Zhang et al., 2024*). Such engineered EVs offer viable alternatives to traditional lipid nanoparticle-mRNA-mRNA systems. Despite ongoing challenges in scalable EVs isolation and effective cargo loading, the natural origin and favourable biocompatibility of *fungal* EVs position them as superior to their synthetic analogues, opening avenues for novel therapeutic strategies (*Uddin et al., 2024; Wang et al., 2023*). Furthermore, the economic significance of *fungi* in the manufacture of nutritional and pharmaceutical products highlights the industrial potential of EVs in applications ranging from environmental remediation to advanced nanotherapeutics and biosensors (*Liebana-Jordan et al., 2021*).

Nevertheless, the translation of *fungal* EVs into clinical and industrial settings is constrained by substantial hurdles. These include heterogeneity arising from *fungal* strain variability, culture conditions and production methodologies, which complicates reproducibility, compliance, and safety protocols (Clemmens & Lambert, 2018;

Davies & Rafiq, 2017; Rizzo et al., 2021). EVs may exert immunomodulatory effects that are beneficial or detrimental, depending on their engineering; careful design is therefore essential to harness therapeutic potential without eliciting adverse host responses (*Li et al., 2020; Ullah et al., 2023*). Manufacturing scale-up is further hampered by issues in maintaining cargo consistency, vesicle integrity, and contaminant removal, necessitating robust quality control frameworks aligned with Good Manufacturing Practice (GMP) requirements (Davies & Rafiq, 2017; *Lai et al., 2024; Paganini et al., 2019*).

A multidisciplinary strategy is imperative to surmount these challenges. Implementation of high-yield culture systems, complemented by scalable purification techniques such as tangential flow filtration and density gradients adapted from mammalian EV protocols, can improve output consistency (Corso, 2019; *Tiwari et al., 2021*). Comprehensive analytical pipelines, including nanoparticle tracking, lipidomic and proteomic profiling, and immunoassays, are critical to validate quality and functionality (Corso, 2019; *Nix et al, 2024; Tiwari et al., 2021*; *Zhou et al., 2024*). Adherence to guidelines from authoritative bodies such as the International Society for Extracellular Vesicles (ISEV) is crucial to regulatory acceptance (Corso, 2019). Moreover, integrative omics and advanced technologies including microfluidics and artificial intelligence can provide granular insights into EV biogenesis and function, thereby enhancing their translational utility in diagnostics, immunotherapies and biosensing (*Liu & Hu, 2023; Rizzo et al., 2021; Zamith-Miranda et al., 2021; Wang et al., 2024*).

Finally, *fungal* EVs represent a promising frontier in sustainable agriculture, industrial biotechnology and environmental applications. *Fungal* species act as natural nanofactories, synthesising bioactive compounds and nanoparticles useful for crop protection and phytoregulation (Karaila et al., 2024; Rao et al., 2017). These vesicles can mediate interkingdom communication, delivering small RNAs that modulate gene expression in plant pathogens, thereby offering novel crop protection strategies (*Martínez-Chávez et al., 2024; Xi et al., 2024*). Developments in EV-mimetic systems, combined with nanosensors and nano-fertilisers, support precision agriculture by enhancing nutrient delivery and ecological monitoring (*Sharma et al., 2024; Yadav et al., 2023*). The successful realisation of these technologies depends on the integration of molecular engineering, scalable production platforms and regulatory alignment (Kansotia et al., 2024). As these technologies mature, fungal EVs could emerge as programmable nanobiotechnological tools with significant impact on agriculture, environmental management and broader societal challenges ("Nanotechnology - Big impact: How nanotechnology is changing the future of agriculture?", 2022; *Kansotia et al., 2024*).

In conclusion, while *fungal* EVs face formidable scientific, technical and regulatory challenges, the continued evolution of bioengineering, analytical and manufacturing innovations heralds their emergence as versatile agents for therapeutic delivery, biosensing, sustainable agriculture and environmental stewardship.

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