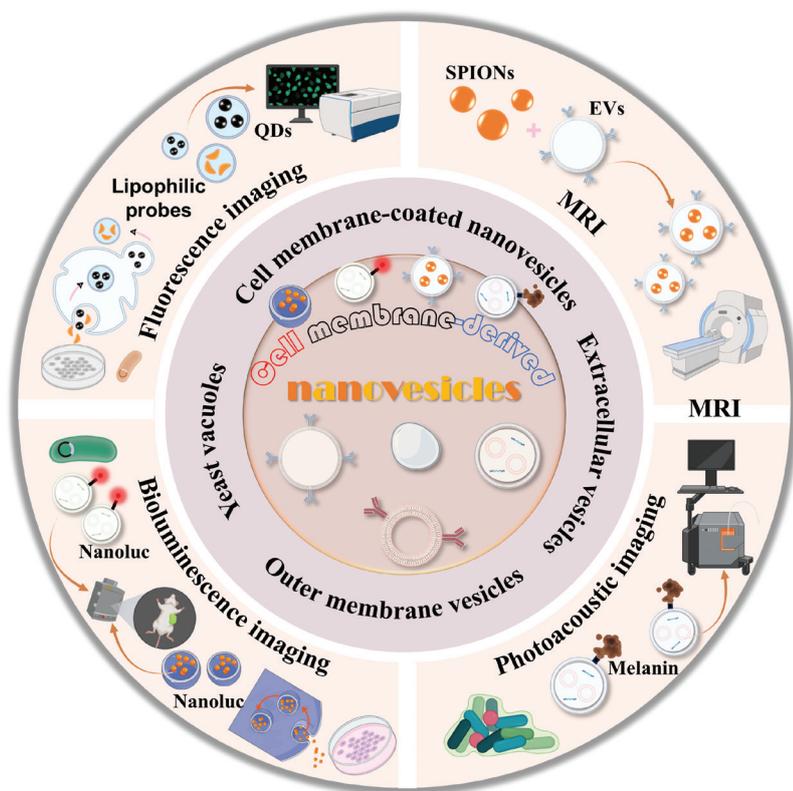


Harnessing Cell Membrane-Derived Nanovesicles for Enhanced Nanoprobes in Multimodal Imaging: Progress and Perspectives

Graphical abstract



Authors

Lingling Lei, Meng Du, Jianjian Zhang and Yuguang Mu

Correspondence

dumeng_work@126.com (M. Du);
zhangjj@ nwu.edu.cn (J. Zhang);
ygmu@ntu.edu.sg (Y. Mu)

In brief

CMNVs are biomimetic nanoplatforms overcoming rapid clearance and toxicity of traditional nanoprobes. Leveraging inherited membrane proteins or minimally disruptive modifications, CMNVs enhance targeting, prolong circulation, and improve imaging precision across fluorescence, bioluminescence, photoacoustic imaging, and MRI. Challenges in scalable production, stability, and standardization require resolution for clinical translation.

Highlights

- Cell membrane vesicles overcome rapid immune clearance of nanoprobes.
- Inherited biofunctionalization enables precise targeting and enhanced imaging.
- Unified platform for fluorescence/bioluminescence/MRI/photoacoustic imaging applications.
- Scalability and stability challenges limit clinical translation.

Harnessing Cell Membrane-Derived Nanovesicles for Enhanced Nanoprobes in Multimodal Imaging: Progress and Perspectives

Lingling Lei^{1,2,3}, Meng Du^{1,2,3,*}, Jianjian Zhang^{4,*} and Yuguang Mu^{5,*}

Abstract

Cell membrane-derived nanovesicles (CMNVs) are natural nanocarriers efficiently encapsulating bioactive molecules and imaging probes. Conventional nanoprobes face challenges, like rapid immune clearance, off-target accumulation, and long-term toxicity. CMNVs overcome these challenges by leveraging inherent biofunctionalization. CMNVs enhance encapsulated nanoprobe targeting, prolong circulation, boost specific tissue accumulation, and improve imaging precision through inherited membrane proteins or minimally disruptive exogenous modifications. CMNVs show significant promise across multiple imaging modalities, including fluorescence, bioluminescence, photoacoustic, and MRI. This review systematically evaluates the limitations of traditional nanoprobes, highlights the unique advantages of CMNVs in enhancing diverse probe performance, discusses current challenges in harnessing CMNVs for imaging, and provides insights for developing future high-precision imaging strategies.

Keywords

Bioluminescence, cell membrane-derived nanovesicles, MRI, nanoprobes, fluorescence, photoacoustic imaging.

Introduction

Nanoprobes, as nanoscale sensors for probing individual living cells, encompass organic and inorganic variants, such as fluorescent dyes, magnetic nanoparticles, quantum dots (QDs), and noble metal nanoparticles [1]. While the minimal serum protein coverage of nanoprobes enhances surface functionalization and cellular uptake, conventional nanoprobes face critical limitations in biomedical imaging [2, 3]. Challenges include rapid clearance by the reticuloendothelial system (RES), accumulation in off-target tissues (e.g., liver and spleen), and resultant low signal-to-noise ratios [SNRs] [4–7]. Moreover, persistent biocompatibility concerns, such as the slow clearance of QDs and the long-term toxicity, remain unresolved despite strategies, like PEGylation or antibody modifications [8–11]. These challenges highlight the pressing need for biocompatible platforms that can evade the RES to propel advances in nanoprobe-based imaging. Significantly, these vesicles function as self-contained

imaging platforms, which allows for the efficient encapsulation of reporter molecules and enhances the versatility and effectiveness of imaging applications [e.g., luciferase [12] and tyrosinase [13]] through genetic engineering of parent cells, thus eliminating manual cargo loading.

Cell membrane-derived nanovesicles (CMNVs) have emerged as a promising class of natural nanocarriers to overcome these barriers. CMNVs, including cell membrane-coated nanovesicles (CMCNVs), extracellular vesicles (EVs), bacterial outer membrane vesicles (OMVs), and yeast vesicles, serve as natural engineering substrates for nanoprobe design. CMNVs leverage inherent biofunctionalization, which is achieved through inherited membrane proteins or minimally disruptive exogenous modifications, to fundamentally redesign nanoprobe delivery [14–17]. This biomimetic strategy enhances targeting capability, prolongs systemic circulation, boosts specific tissue accumulation, and improves imaging precision across diverse pathologic contexts.

CMNVs demonstrate significant promise as versatile platforms for multiple imaging

¹Key Laboratory of Medical Imaging Precision Theranostics and Radiation Protection, College of Hunan Province, The Affiliated Changsha Central Hospital, Hengyang Medical School, University of South China, Changsha, China

²Department of Medical Imaging, The Affiliated Changsha Central Hospital, Hengyang Medical School, University of South China, Changsha, China

³Institute of Medical Imaging, Hengyang Medical School, University of South China, Hengyang 710127, China

⁴Key Laboratory of Synthetic and Natural Functional Molecule Chemistry of Ministry of Education, Key Lab of Modern Separation Science in Shaanxi Province, College of Chemistry and Materials Science, Northwest University, Xi'an, Shaanxi, China

⁵School of Biological Sciences, Nanyang Technological University, Singapore 637551, Singapore

*Correspondence: Meng Du, Changsha Central Hospital, 161 Shaoshan South Road, Changsha, China. E-mail: dumeng_work@126.com, Tel: +86-13570919798; Jianjian Zhang, Key Laboratory of Synthetic and Natural Functional Molecule Chemistry of Ministry of Education, Key Lab of Modern Separation Science in Shaanxi Province, College of Chemistry and Materials Science, Northwest University, Xi'an, Shaanxi 710127, China. E-mail: zhangjj@nwu.edu.cn; Yuguang Mu, School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore. E-mail: ygm@ntu.edu.sg

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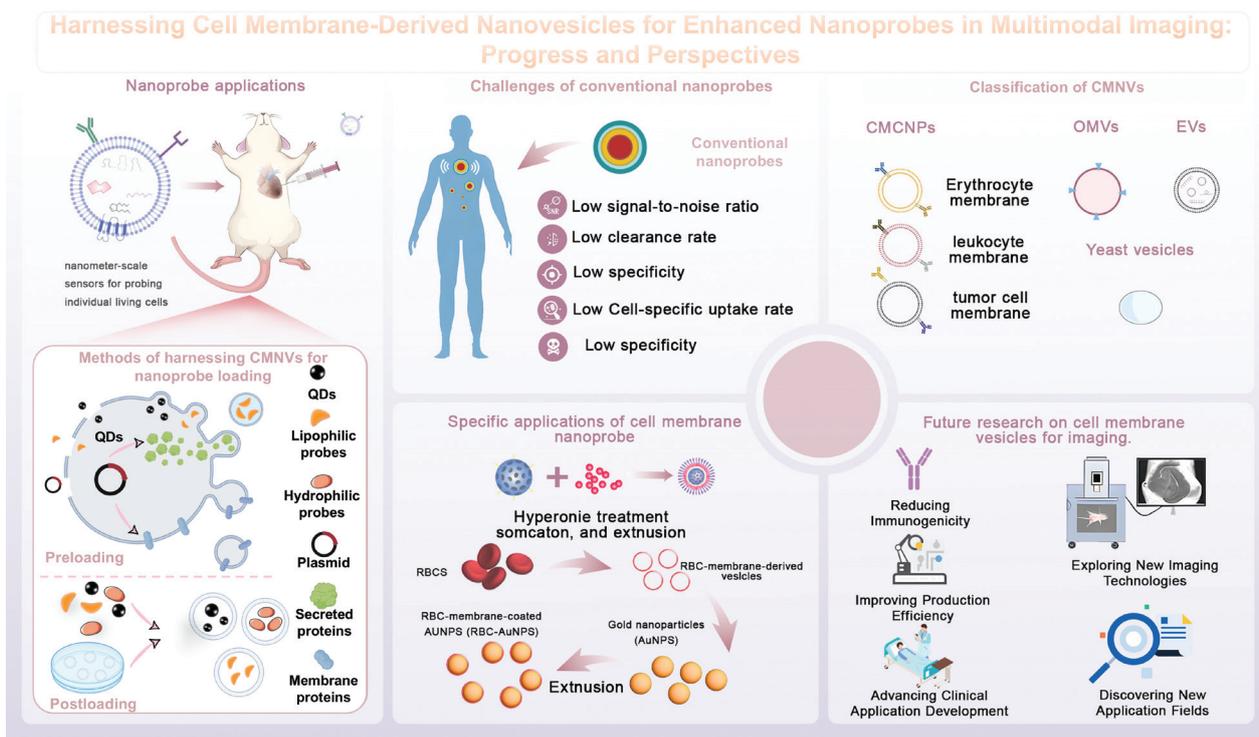


Figure 1 A summary of harnessing CMNVs in multimodal imaging, showing the design, applications, and future prospects of CMNVs as advanced platforms for enhancing nanoprobes in multimodal imaging.

modalities, most notably fluorescence imaging, bioluminescence imaging, photoacoustic imaging, and magnetic resonance imaging (MRI). The ability of CMNVs to integrate seamlessly with various contrast mechanisms positions CMNVs at the forefront of next-generation diagnostic tools. This review systematically evaluates the fundamental constraints of existing traditional nanoprobes and highlights the unique advantages of harnessing CMNVs for enhanced nanoprobes in multimodal imaging. Furthermore, we critically discuss current challenges in CMNVs-based imaging (spanning scalable production, standardization, and *in vivo* stability) and provide insights into future translational pathways. By synthesizing recent advances and unresolved questions, this work aims to catalyze the development of high-precision imaging strategies leveraging CMNV nanotechnology (**Figure 1**).

Challenges of conventional nanoprobes

Despite advantages, like enhanced brightness and stability, the clinical translation of nanoprobes faces the following critical barriers: low SNR; suboptimal clearance rates; and material toxicity. Based on these critical barriers, researchers have conducted numerous investigations.

Low SNR

SNR determines imaging clarity by measuring target-specific signals against background noise. Traditional nanoprobes

suffer from non-specific biodistribution and poor specific cellular uptake, which compromises imaging efficacy.

Non-specific biodistribution

Non-specific biodistribution of nanoprobes amplifies off-target signals. To address this issue, conjugating antibodies, ligands, or peptides enable selective tumor binding, leveraging biomarkers, like EGFR or folate receptors overexpressed in cancers. For example, gold nanorods conjugated to UM-A9 antibodies selectively bind squamous cell carcinoma biomarkers, enabling tumor differentiation via X-ray signal amplification [18]. Khademi et al. attached gold nanoparticles to folate-conjugated cysteamines (FA-Cys-AuNPs), which exploit folate receptor overexpression in tumors to detect lesions < 1 mm in size that are undetectable by conventional CT [19]. Polysaccharides have good water solubility and flexibility and can be used as standalone macromolecular nanocarriers. Ma et al. reported efficient targeting of visceral adipose tissue macrophages in obesity with polysaccharide nanocarriers for multimodal imaging or delivery of anti-inflammatory drugs [20].

Poor specific cellular uptake

Exogenous nanoprobes face rapid opsonization and sequestration by the RES (liver and spleen), which compromises the target tissue SNR. Consequently, it is crucial to simultaneously mitigate RES clearance, while promoting targeted cellular internalization. PEGylation enhances circulation

time through steric hindrance [21] but is limited by immunogenicity [pre-existing anti-PEG antibodies accelerate clearance and induce hypersensitivity] [22, 23]. Dextran coating clinically validated for iron oxide nanoparticles, provides dual protection against aggregation and macrophage uptake [24]. Chitosan-based systems can also evade clearance by the RES, prolong drug circulation in the bloodstream, and improve drug targeting and bioavailability [25].

Low clearance rate

Conventional nanoprobe face a critical clearance paradox. Specifically, excessive systemic retention induces potential toxicity, while rapid renal elimination compromises imaging efficacy through insufficient target accumulation. This dilemma stems from the static physicochemical properties that cannot dynamically balance circulation, persistence, and clearance efficiency. Size-variable designs resolve this conflict through stimulus-responsive structural transitions, maintaining > 100 nm hydrodynamic diameters for prolonged blood circulation, then disassembling into < 6 nm subunits (i.e., below the glomerular filtration threshold) post-targeting for prompt renal clearance [26]. Light/pH/enzyme-activated systems exemplify this spatiotemporal control [27–31], as demonstrated by Huang's oxygen-sensitive activatable polymeric nanoprobe for superoxide anion (APN_{so}) [160 nm → 4 nm transition, achieving a 12-d clearance] [32]. Ruan et al. also created ADN1 and ADN2, size-variable nano-emitters, that can be activated by superoxide anions (O²⁻) at disease sites, which enable dual imaging/urinalysis via renal-excreted fluorophores [33].

Material toxicity

Nanoprobe-induced oxidative damage constitutes a major clinical safety barrier with reactive oxygen species (ROS) generation triggering cascade biological destruction through

DNA strand breaks, membrane lipid peroxidation, and chronic inflammation [34]. Current mitigation strategies adopt dual approaches, as follows: accelerated clearance via renal-excretable designs; and intrinsic biocompatibility engineering. Yang's magnetic ions coordinated nanoplateform (MICN) exemplifies a synergistic theranostic system. Fe/Mn/Gd-coordinated nanoparticles undergo programmed biodegradation into renal-clearable metal ions (< 5 nm), avoiding persistent bioaccumulation risks, while maintaining multimodal imaging capacity (photoacoustic/MRI SNR enhancement) through controlled metal coordination dynamics [35].

Harnessing CMNVs for multimodal imaging

The biomimetic camouflage strategy addresses the critical limitations of conventional surface modification approaches [e.g., PEG-induced accelerated blood clearance] [36], while resolving the dual challenges of nanoprobe biocompatibility and functionality. Natural cell membrane encapsulation establishes the following three critical biological interfaces: antigenic camouflage for RES evasion and prolonged circulation via native surface markers [37]; toxicity isolation through biomaterial-membrane separation; and tissue-specific homing through membrane-inherited targeting motifs, like integrins/chemokine receptors. To facilitate a clear comparison of harnessing different types of CMNVs for imaging, the key characteristics of various CMNVs are systematically summarized in **Table 1**.

Classification of CMNVs

CMNVs, which encompass entities, like EVs, OMVs, and yeast vesicles, have been harnessed for enhancing

Table 1 Summary of Cell Membrane-derived Vesicles as Nanoprobe Modification Strategies

CMNVs	Cargo Loading Strategies	Advantages	Disadvantages	References
CMCNVs	Co-extrusion Ultrasonication Microfluidic electroporation infiltration	Ease of production Highly biocompatible Multi-functional modifications possible	Functions only as nanoparticle surface modification and cannot replace artificial nanocarriers Potential for disease transmission Immunocompatibility concerns the possibility of immune reactions occurring	[38, 42]
EVs	Preloading: co-incubation, transfection Post-loading: co-extrusion, electroporation, sonication, co-incubation, saponins, and transfection	Ease of storage Mature cargo loading technology High biostability for long-distance transfer Homologous targeting capability Low immunogenicity	Production stability Low yield of EVs High difficulty and high cost of EV isolation and purification technology	[65, 68, 110, 173]
OMVS	Direct loading: co-incubation, electroporation, and extrusion Indirect loading: transfection	Intrinsic immunomodulatory properties Ease of industrialization Ease of customization	Lack of standardization Potential off-target effects Potential biosafety Ambiguous contents	[78, 79]
Yeast vacuoles	Preloading: transfection	Good tissue penetration and easy amplification	Ambiguous long-term stability, immunogenicity, and toxicity	[101, 104]

nanoprobes in multimodal imaging and studied in prokaryotic and eukaryotic cells [13].

Cell membrane-coated nanovesicles (CMCNVs)

CMCNVs are synthesized through three-stage precision engineering, encompassing membrane extraction, nanocarrier core fabrication, and membrane-core fusion via extrusion, sonication, or microfluidic electroporation [38] with classification based on membrane origin, including red blood cells (RBCs), white blood cells (WBCs), platelets, mesenchymal stem cells (MSCs), cancer cells, and hybrid membranes [39]. The membrane extraction process for nucleate mammalian cells, like RBCs and platelets, utilizes hypotonic lysis or freeze-thaw cycles, followed by differential centrifugation and extrusion [40, 41]. RBC membranes leverage CD47-mediated SIRP α signaling for immune evasion and prolonged circulation [16, 42], while early RBC-camouflaged nanoprobes rely on passive EPR targeting and are later enhanced by anti-EGFR modifications for active tumor targeting [43]. Platelet membranes offer low immunogenicity [44] and intrinsic affinity for circulating tumor cells, enabling pathologic site-specific delivery [45].

Eukaryotic cell membranes (WBCs, cancer cells, and MSCs) require complex isolation techniques, like sucrose gradient centrifugation and extrusion [46]. WBC membranes utilize endogenous chemotaxis for ligand-free tumor homing [42, 47], which has been demonstrated by therapeutic protein delivery using tumor-associated macrophage membranes [48]. MSC membranes offer scalable platforms due to expansion stability and tumor tropism, making MSC membrane-modified nanoprobes suitable for large-scale production [49–51].

Cancer cells exhibit homotypic adhesion, unlimited proliferation, and immune escape [52]. Cancer cell surface adhesion molecules (N-cadherin, galectin-3, and EpCAM) enable multicellular aggregation and can be exploited for nanoparticle functionalization. Cancer cell membrane-encapsulated nanoprobes are therefore promising for homologous targeting and specific imaging [53]. Lv et al. further utilized the ability of cancer cell membranes to evade phagocytosis and enable homotypic targeting for nanoprobe modification, resulting in excellent biocompatibility and imaging performance [15]. These probes show high recognition specificity and selective aggregation in homologous cancer cells, exhibiting a 16-fold increase in intracellular fluorescence intensity and strong *in vivo* signals.

While red blood cell membrane-camouflaged nanoparticles (RBCNPs) have a long circulation, RBCNPs often require added targeting ligands for tumor localization [54]. Fusing membranes from different sources provides multifunctionality, adapting CMCNVs to complex environments. Current hybrid-coated nanoparticle development focuses on RBC-based hybrids, particularly RBC-platelet fusion membranes. Thermodynamic treatment achieves membrane fusion [55], retaining dual functions and enhancing circulation and offering a natural alternative for post-synthesis functionalization.

Although harnessing CMCNVs offers advantages, such as prolonged circulation time, immune evasion, and active

targeting, the clinical translation of CMCNVs still faces several challenges. For example, the extraction of cancer cell membranes requires thorough removal of the nucleus and genetic material to reduce carcinogenic risks; related clinical trials are still under investigation [42]. Furthermore, developing standardized protocols is crucial for the large-scale production and clinical translation of cell membrane-modified nanoprobes. Membrane isolation typically requires at least 100 million cells and these cells must maintain phenotype, purity, and quality during passage. Developing standardized and mature cell culture protocols for each cell type is essential for large-scale production. In this regard, existing mature biomanufacturing platforms, such as the use of 3D bioreactors (e.g., stirred-tank and WAVE bioreactors), can be used for large-scale cell expansion [56]. Additionally, extrusion methods commonly used in laboratories have low production efficiency and are challenging to scale up for industrial manufacturing, which often requires good reproducibility and stable technical parameters [57]. Moreover, for some cell types, such as WBCs, surface membrane proteins exhibit heterogeneity, posing a risk of triggering hemolysis *in vivo*. Autologous cells are the most suitable option in this case and strict donor cell screening protocols must be established to prevent the use of allogeneic cells as membrane sources.

EVs

EVs are nanosized vesicles released from various cells, including reticulocytes, platelets, stem cells, dendritic cells, and tumor cells [58], and can be detected in body fluids, such as blood, cerebrospinal fluid, and urine [59, 60]. EVs were considered “waste material” released by cells until 1973 [61] but recent studies have shown that EVs are key messengers in intercellular communication [62–64]. EVs can be classified into apoptotic bodies (500 nm–5 μ m), microvesicles (150–500 nm), and EVs (40–150 nm) based on size and origin with EVs being the smallest and most suitable for nanoprobe modification [65].

EVs are composed of a lipid bilayer membrane encapsulating a hydrophilic core and carry ligands and receptors from parent cells. EVs offer lower cytotoxicity and immunogenicity, better resistance to degradation in circulation, and enhanced ability to evade phagocytosis by the mononuclear phagocyte system when harnessed for nanoprobe loading compared to synthetic nanocarriers [66–68]. Tumor-derived EVs can target specific tissues and protect nanoprobes from blood degradation [17] and can cross the blood-brain barrier, providing new strategies for drug delivery and diagnostic imaging in neurologic diseases [66]. Given the immense research potential of harness EVs to load nanoprobes for precise imaging, understanding the *in vivo* biodistribution of EVs is crucial for accelerating clinical translation. Relevant studies mainly use fluorescent probe labeling, transgenic parent cells expressing luciferase on the vesicle surface, and radioactive labeling to analyze EV behavior [69–71].

A key prerequisite for the widespread use of EVs in nanoprobe loading is the efficient preparation of high-purity vesicles with maintained structural integrity. Currently, common EV isolation methods include ultracentrifugation,

ultrafiltration, immunoaffinity separation, and polymer-based precipitation. Among these modalities, ultracentrifugation is the gold standard but faces challenges, such as being time-consuming, requiring expensive equipment, resulting in non-specific purification, and reducing biological activity, making ultracentrifugation unsuitable for large-scale production [72]. Both ultrafiltration and precipitation methods face the dilemma of low purity recovery, which hinders subsequent experiments [73]. Immunoaffinity separation is based on antibody recognition of unique surface markers of EVs, such as CD9, CD63, CD81, and Alix, and involves separating the EVs using antibodies fixed to magnetic beads, chromatographic column resins, porous plates, or microfluidic devices. Although this method effectively improves the purity and sensitivity of EV isolation, high cost makes immunoaffinity separation unsuitable for large-scale separation [74]. With the development of microfluidic chips and micro-nanofabrication technologies, new tools for high-sensitivity, high-accuracy EV separation, and detection are emerging [75].

Microfluidic technology, with its advantages of low sample consumption, high throughput, and easy integration, can precisely control micro-scale reaction conditions and enhance EV operation efficiency. In particular, integrated microfluidic systems combining two or more separation technologies, such as the integrated magnetic-electrochemical EV device, which combines electrochemical EV detection methods with magnetic-based EV separation methods to achieve high-throughput EVs detection, better meet the needs of clinical research [76]. Nanoplasmonic biosensors, such as surface plasmon resonance, localized surface plasmon resonance, and surface-enhanced Raman scattering, enable label-free, high-sensitivity detection of EVs through the optical properties of metal nanostructures [75].

OMVs

OMVs are natural vesicles secreted by Gram-negative bacteria [77]. Some studies in recent years have shown that some Gram-positive bacteria also produce vesicles. Only OMVs produced by Gram-negative bacteria will be discussed herein. An important role of OMVs in nature is to deliver biomolecules to specific distant sites for parent bacteria [78]. OMVs have been developed as bacterial vaccines, adjuvants, tumor immunotherapeutic reagents, drug delivery carriers, and anti-bacterial adherence reagents [79], which are nanolipid bilayer vesicles with a particle size between 50 and 200 nm, and are mainly composed of lipids, proteins, and various pathogen-associated molecular patterns (PAMPs), including LPS, lipoproteins, peptidoglycan, DNA, and RNA [80]. Unlike the above-mentioned modifications, these PAMPs make OMVs easily detectable and internalized by phagocytes. Li et al. demonstrated that 83.4% of attenuated OMVs-loaded nanoparticles are internalized by neutrophils compared to 9.6% internalization by monocytes and by piggybacking on neutrophils, OMVs can efficiently target the nanoprobe to the deeper parts of inflamed tumor tissue [81].

Harnessing OMVs to load nanoprobe offers several advantages. Genetic engineering of the parent bacterium enables facile targeted delivery. Chen et al. developed a modular

method embedding original cell-expressed luciferase within OMVs, while displaying specific antibodies externally that bypassed complex purification or attachment steps for versatile applications, like live-cell imaging [82]. Furthermore, the intrinsic OMV composition promotes efficient neutrophil uptake and “piggybacking” transport to deeper inflammatory sites, like tumors, with rapid neutrophil release contrasting slower erythrocyte and monocyte release involving digestion, which facilitates specific neutrophil imaging. The rigid OMV membrane also protects encapsulated nanoprobe from degradation en route to targets. Additionally, OMVs possess inherent anti-tumor activity by inducing anti-tumor cytokines (CXCL10 and interferon [IFN]- γ), inhibiting tumor growth [83]. Thus, harnessing OMVs to load nanoprobe is expected to achieve theranostics.

Despite several successful applications, the safety of OMVs requires ongoing scrutiny. Surface LPS activates macrophages, endothelial cells, and inflammatory cascades, potentially causing tissue damage or septic shock [78, 84]. The toxicity resides primarily in lipid A, biosynthesized through nine essential enzymes [85]. Genetic engineering mitigates this risk. Deleting *lpxL* or *lpxM/msbB* genes or introducing Hp0021 generates OMVs containing exclusively monophosphorylated lipid A, which significantly reduces toxicity and inflammation [86, 87].

Low production efficiency further impedes OMV applications. OMV production efficiency is dynamically regulated by environmental signals, growth phase, and genetic factors [88]. Peak production occurs during late-exponential to stationary phases, even though harvesting during the late-exponential phase prevents contamination from bacterial lysis [89]. Environmental modulation is equally crucial. Iron limitation boosts production in *Mycobacterium tuberculosis* and *Helicobacter pylori*, while suppressing *VacA* virulence factor expression [90, 91]. Hypoxia increases *Pseudomonas aeruginosa* vesicle output sixfold [92]. Detergents, metal chelators, or H₂O₂ induce oxidative stress to promote vesiculation [93–95]. Mutations in any gene of the *Escherichia coli* Tol-Pal membrane complex (*tolQ*, *tolR*, *tolA*, *tolB*, and *pal*) significantly increase yields [86, 96, 97]. Periplasmic expression of colicin A/E3 or phage G3p translocation domains similarly enhances vesiculation [86]. Nevertheless, OMV purification remains a bottleneck due to reliance on time-consuming ultracentrifugation and filtration. Advanced technologies are therefore imperative for scalable production.

Yeast vacuoles

With the discovery of biologically derived, nano-sized vesicles, an increasing number of researchers are focusing on the study of biologically derived nanocarriers. Yeast vacuoles, as one of the biologically derived nanocarriers, are derived from yeast cells and are highly dynamic degradative single-membrane organelles used to break down abnormal intra- or extra-cellular substrates and to recover the stored nutrients [98]. Yeast vacuoles have lower immunogenicity and stronger tissue penetration compared to the uncertain immunogenicity of bacterial exocysts from genetically engineered bacteria

and the high technical difficulty of purifying EVs from mammalian cell sources and yeast vacuole production technology is simpler than EVs [99]. Moreover, yeast vacuoles possess a lipid bilayer similar to the composition of mammalian cell membranes, which improves the fusion efficiency with the plasma membrane or endolysosomes and ultimately facilitates the release of the drug into the target cells or tissues [100]. In general, obtaining yeast vesicles for use as bio-nanocarriers requires genetically engineered yeast cells for modification. There are usually 2–5 large vesicles with a diameter of approximately 1 μm inside the yeast cells. Size is a key factor that determines the vesicle drug encapsulation rate and half-life with smaller vesicles having more chances to evade phagocytosis for uptake [101]. Rab GTPase is a prerequisite for homotypic vesicle fusion. The major Rab GTPase that promotes fusion of yeast vesicles is yeast protein transfer protein7 (YPT7), the overexpression of which causes membrane expansion of yeast vesicles. Thus, setting up YPT7 protein knockdown results in many fragmented, nanoscale vesicles [102, 103]. Engineering to increase the specific targeting of yeast vesicles has also garnered interest in transfecting some tissue-specific ligands into yeast cells so that the surface of yeast vesicles carries tissue-specific targeting ligands [104].

Yeast cells that have been used as vectors for testing include *Saccharomyces cerevisiae*, a non-polluting, non-pathogenic yeast that has been used in fermentation products for thousands of years [105]. Unlike bacteria, the lipid composition of *S. cerevisiae* is like that of mammalian cell membranes, potentially increasing the efficiency of fusion with the plasma membrane or endolysosomes and thereby facilitating drug release into targeted cells or tissues [100]. Taking advantage of these properties, Gujrati et al. genetically engineered *S. cerevisiae* to express human epidermal growth factor receptor 2 (HER2)-specific affinities on vesicular membranes, which target the HER2 receptor that is expressed on a variety of cancers [99]. The anti-HER2 affinity-expressing vacuole (Affi_{HER2} vacuole) was further loaded with the chemotherapeutic drug, adriamycin (Dox), which is widely used for the treatment of solid tumors. Active administration of Affi_{HER2} vacuole^{Dox} had greater accumulation and penetration in tumor tissues, reflecting significant tumor growth inhibition. Although vacuole^{Dox} exhibited relatively low cellular uptake due to passive targeting, the enhanced distribution, like that of Affi_{HER2} vacuole^{Dox}, ensured the effectiveness of the yeast vesicle biocarrier for deep tissue penetration. The above-mentioned results, as well as the low toxicity of the yeast vesicle vector, suggest that the bioengineered vesicles have the potential to be used as drug delivery carriers for the treatment of cancer.

Methods of harnessing CMNVs for nanoprobe loading

The methodologies for nanoprobe loading into CMNVs are fundamentally classified into pre- and post-loading strategies [106]. Pre-loading approaches accomplish probe incorporation during CMNV biogenesis through two primary mechanisms [genetic engineering involving transfection of plasmids encoding reporter molecules (e.g., fluorescent

proteins and luciferases) into parental cells (bacteria, fungi, tumor cells, macrophages, or stem cells) for endogenous expression and subsequent packaging into secreted vesicles [107] or cellular uptake, where lipophilic probes (organic dyes, quantum dots) internalize via co-incubation and integrate during membrane assembly] [108, 109]. Conversely, post-loading strategies use physicochemical techniques, including co-incubation, electroporation, sonication, extrusion, and freeze-thaw cycling, to modify isolated CMNVs, which offers enhanced flexibility in controlling encapsulation efficiency (EE%) and nanoprobe payload [110] without requiring parental cell modification. This strategy renders post-loading particularly suitable for hard-to-transfect cells (erythrocytes and primary cells) or biofluid-derived vesicles. Lipophilic probes (dialkylcarbocyanines and cyanine dyes) spontaneously embed into lipid bilayers through co-incubation, as exemplified by DSPE-PEG2000-Cy5/DSPE-PEG2000-N³ incubation with EVs forming Cy5-EV-N³ within 30 min at room temperature [111, 112]. However, hydrophilic probes (polar dyes and ultrasmall superparamagnetic iron oxide nanoparticles) require assisted loading methods, such as electroporation or freeze-thaw cycling, although these probes may compromise vesicle integrity by altering physicochemical properties or morphology [113, 114] (Figure 2).

Harnessing CMNVs advances in multimodal imaging

Harnessing CMNVs in fluorescence imaging

Fluorescence imaging, recognized for operational simplicity and cost-effectiveness, has demonstrated significant advancements in sensitivity, spatiotemporal resolution, and rapid response capabilities [115]. Driven by growing clinical demand for enhanced clarity and deeper penetration in *in vivo* imaging, which is crucial for early diagnosis and prognosis, researchers are actively exploring novel methodologies with superior bioimaging performance. The near-infrared-II (NIR-II [1000–1700 nm]) spectral window offers substantial advantages over conventional NIR-I wavelengths, enabling greater tissue penetration depth, a SNR, and improved spatiotemporal resolution [116, 117]. Consequently, inorganic and carbon nanomaterial-based NIR-II probes have been developed, including single-walled carbon nanotubes [SWCNTs] [118, 119], QDs [120, 121], and rare earth-doped nanoparticles [122, 123].

QDs exhibit broad absorption spectra, high quantum yields (QYs), exceptional photostability, and strong fluorescence intensity, which facilitates application in disease imaging and diagnosis [124, 125]. However, inadequate targeting often leads to off-site accumulation and rapid clearance by the RES, which compromises tumor-specific imaging efficacy. For example, intravenous administration of Ag²S QDs results in predominant fluorescence signals in the bladder and urine within 1 h with negligible tumor detection, indicating rapid renal clearance. By 12 h, only weak fluorescence

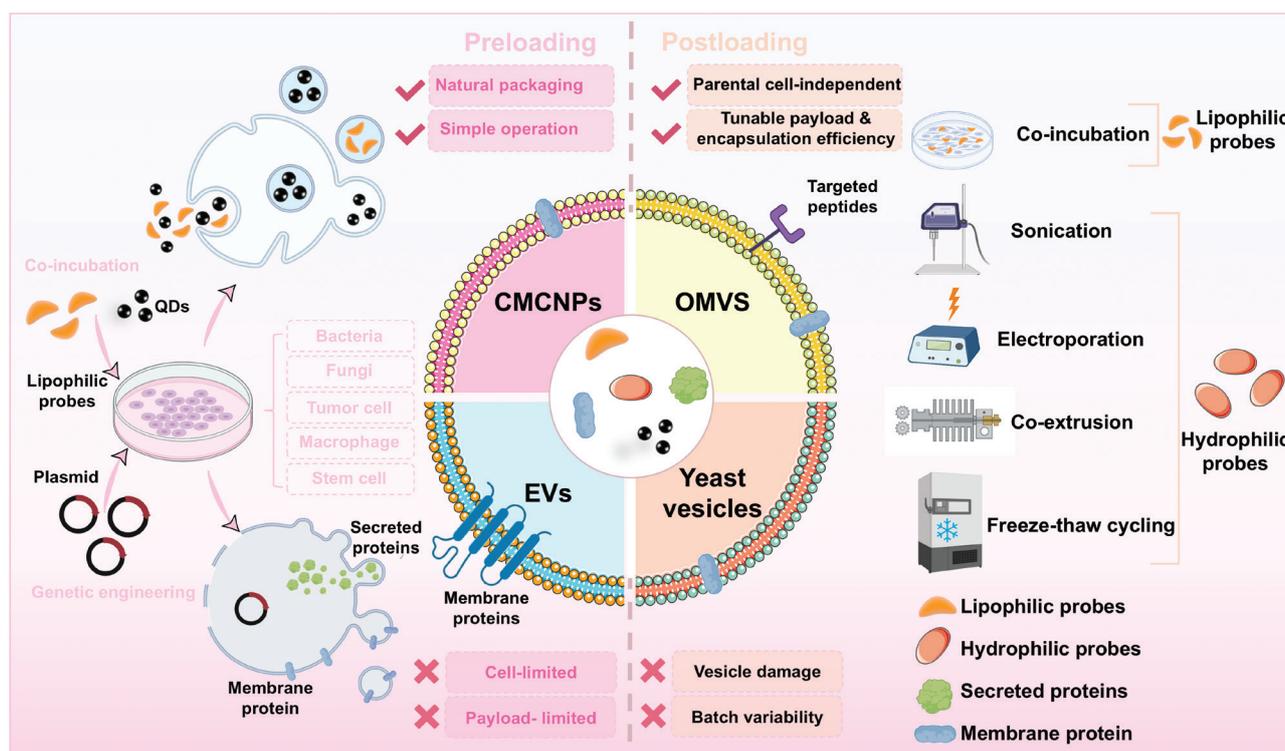


Figure 2 Schematic overview of pre- and post-loading strategies for integrating nanoprobes into CMNVs. Nanoprobe (QDs and lipophilic probes) and plasmids encoding fluorescent proteins or luciferase can be pre-loaded into parental cells via co-incubation or genetic engineering. Post-isolation, lipophilic probes are incorporated into purified CMNVs via co-incubation, whereas hydrophilic probes require active loading methods (e.g., sonication, electroporation, co-extrusion, or freeze-thaw cycling).

appears at tumor peripheries without deep tissue penetration. Free QDs become undetectable in blood after 24 h and bladder fluorescence nearly disappears by 48 h [126].

Surface modification strategies to enhance QD hydrophilicity and targeting may reduce fluorescence QY and stability, which diminish imaging signals [127]. To address this issue, macrophage-secreted EVs were used to deliver Ag²S QDs (MV@QDs&DOX). MV@QDs&DOX generates intense tumor fluorescence 12 h post-injection, peaking at 24 h with sustained detection until 48 h compared to free QDs. Near-infrared light-triggered EV disruption releases QDs, yielding uniform signal distribution throughout tumors with an 85- μ m penetration depth and full coverage of 9-mm diameter lesions. This biomimetic carrier prolongs circulation, enhances tumor accumulation, and improves penetration via light-responsive release, effectively overcoming the limitations of free QDs and offering a promising strategy for efficient, low-toxicity theranostics [126]. Nevertheless, the targeting mechanism relies on macrophage-derived exosomal membrane proteins, while cumbersome purification and low yield hinder scalability. Consequently, alternative biomimetic vesicular carriers for QD delivery are being explored. Choi et al. engineered erythrocyte membrane-derived nanoparticles (EDNs) incorporating QDs within the lipid bilayers and conjugated the EDNs with tumor-specific anti-EGFR antibodies. The natural erythrocyte membrane structure significantly improved circulatory stability with detectable QD signals in blood 48 h post-administration [43].

Conventional chemical synthesis of QDs often involves toxic organic solvents and heavy metal ions, compromising

biocompatibility and clinical translation [127]. Emerging biosynthesis approaches leverage endogenous metabolic pathways, such as glutathione (GSH) and thioredoxin (TRX) systems in *S. cerevisiae*, *Staphylococcus aureus*, and MCF-7 cells, to synthesize CdSe QDs under mild conditions. This strategy confers intrinsic biostability and biocompatibility without requiring additional ligand exchange or encapsulation, establishing a green synthesis route for biocompatible QDs [128, 129]. Intracellularly synthesized QDs, exemplified by CdSe QDs in MCF-7 cells, can integrate into EVs via natural secretory pathways, enabling autonomous vesicular loading [130]. Expanding this approach to macrophages could facilitate mass production of fluorescent probes combining prolonged circulation, high targeting specificity, and exceptional biocompatibility (Figure 3).

Organic small molecule fluorophores represent promising NIR-II imaging agents due to excellent biocompatibility and pharmacokinetic profiles. However, most small molecule fluorophores exhibit low QYs and brightness. To overcome this issue, researchers have developed innovative strategies to enhance fluorescence QY and spatiotemporal resolution. One effective approach utilizes natural nanoplateforms to integrate multiple NIR-II fluorophores with tumor-targeting moieties, creating targeted fluorescent nanoprobes with optimal biocompatibility and emissive properties. For example, PEGylated CH1055, which is noted for high water solubility, was incorporated by Hong et al. into small extracellular vesicles (sEVs [140 nm diameter]) along with EGFR-targeting GE11 peptides to construct the sEV-CH1055-GE11 nanoprobe. This assembly preserved the characteristic absorption

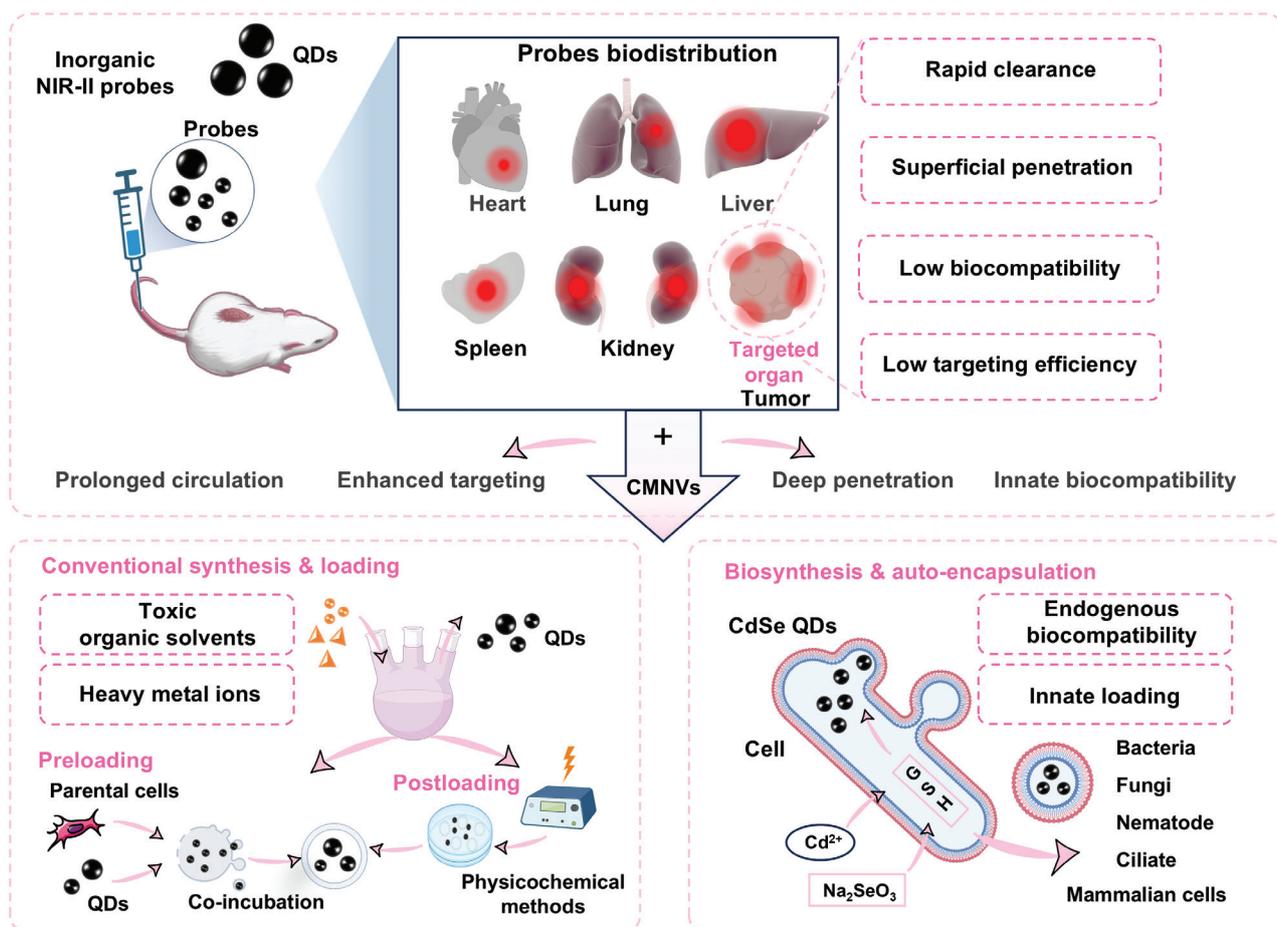


Figure 3 Application of CMNVs to overcome inherent limitations of conventional QDs in biomedical fluorescence imaging. Conventionally synthesized QDs loaded into CMNVs via pre- or post-loading strategies exhibit prolonged circulation, enhanced targeting, and deeper penetration. Biosynthetic QDs, generated via intracellular biogenesis, further improve biocompatibility when integrated into this framework.

(720 nm) and emission (1050 nm) profiles of CH1055-PEG, while delivering high NIR-II brightness and enhanced tumor targeting. The tumor-to-background ratio exceeded the Rose criterion of 5 at 48 h and reached 10.43 by 72 h post-injection. Cell viability, hematologic analysis, and hematoxylin and eosin (H&E) staining confirmed excellent biocompatibility. This platform enables non-invasive, high-contrast imaging of EGFR-overexpressing tumors, demonstrating how natural nanocarriers can optimize precision and reliability in fluorescence imaging [131].

Harnessing CMNVs in bioluminescence imaging

Bioluminescence is a type of optical imaging that utilizes luciferase enzymes to react with substrates. Bioluminescence offers high sensitivity, resolution, and a low background signal. Unlike fluorescence imaging, bioluminescence does not require external light excitation because animals lack autoluminescence [132]. Luciferase reporter imaging is the representative technique in bioluminescence imaging with bacterial luciferase, firefly luciferase, and flower bug luciferase being commonly used reporter genes. Recently, a new platform (NanoLuc [NLuc]) has emerged, which is more stable, smaller in size, > 150 times more efficient in

luminescence, and unaffected by ATP. NLuc also has substrates with higher stability and lower background activity, opening new possibilities for bioluminescence imaging [133]. Another advantage of NLuc is that NLuc does not require post-translational modification in mammalian cells, which allows for low-level expression of reporter genes that better mimic physiologic conditions [134]. Mammals can be made bioluminescent by introducing luciferase genes or via infection with a recombinant adeno-associated virus (rAAV) to enable stable expression of the required enzymes for bioluminescence imaging [135]. Bioluminescence imaging is non-toxic, making bioluminescence suitable for live cell applications that do not require high spatial and temporal resolution, particularly for long-term studies involving slow kinetics or photosensitive components [136].

Intercellular communication is a dynamic process involving cellular activity. Tumor-derived EVs can induce various biological responses, including oncogenic responses, proliferation, cell movement, and stemness, ultimately leading to metastasis [137–139]. Studies have shown that melanomas can utilize an efficient exosomal messenger mechanism to prepare for metastasis through tumor exosomal precursors to pre-metastatic lymph nodes [140]. Therefore, monitoring the distribution of EVs in tumor cells would be valuable for studying tumor metastasis. Tracking the *in vivo* distribution of EVs is crucial and several studies have attempted

to engineer EVs to express luciferase for tracing purposes. Gangadaran et al. stably transduced the *Rluc* gene into a human undifferentiated thyroid cancer cell line (CAL-62 cells [CAL-62/Rluc]) and a human breast cancer cell line (MDA-MB-231) by lentivirus [141]. EVs with Rluc were successfully prepared and the reaction with the substrate, luminalin, produced optical radiation at wavelengths of 475–480 nm, which was used to trace the distribution of EVs *in vivo*. This study also demonstrated for the first time that the transduction of BLI reporter protein expression into CAL-62 and MDA-MB-231 cells did not significantly alter EV properties. This study also demonstrated for the first time that transduction of BLI reporter protein expression in CAL-62 and MDA-MB-231 cells did not significantly alter EV properties. However, this approach, which requires *in vitro* gene transfection of tumor cells, isolation, and purification, and then import into mice for tracing of the EVs, may not faithfully reflect the concentration, location, and transport dynamics of endogenous EVs.

Therefore, Luo et al. developed a mouse model that can be non-invasively and quantitatively labeled to monitor endogenous EVs [12]. A mouse model expressing TG- α MHC-STOP-CD63NanoLuc was successfully established by performing prokaryotic microinjection. Upstream of the CD63NanoLuc reporter gene is not expressed due to the STOP sequence but can be driven by the cardiomyocyte-specific α -MHC promoter, and for the purpose of controllable labeling, this is the first genetic mouse model capable of tracking endogenous EVs. Compared to fluorescently labeled EVs, CD63-NanoLuc-labeled EVs are more specific and show a strong bioluminescent signal only in the cardiac region of transgenic animals. In addition, CD63NanoLuc mRNA and protein were not induced to be expressed in cardiac fibroblasts, indicating that this EV tracking system is highly cell-specific and strictly controllable. NanoLuc bioluminescence is highly sensitive, stable, and non-invasive *in vivo*, and the fusion of NanoLuc and CD63 has no deleterious effect on cardiac EV biosynthesis. This mouse model provides a useful tool for tracking the dynamics of cardiac EVs *in vivo*.

Hikita et al. previously designed CD63NanoLuc for EV tracking in a cancer cell line to directly quantify EVs without purification [142]. However, only purified exotics could be tracked but by adding a tumor cell-specific promoter upstream of CD63NanoLuc, the exosomal behavior of tumor cells *in vivo* could be monitored.

The synthesis of NanoLuc-expressing OMVs by genetically engineered and edited *E. coli* became a good choice due to the difficulty of introducing the luciferase gene into mammalian eukaryotic cells and the low yield of EVs. Chen et al. reported a one-pot synthesis method to design multifunctional sensors based on OMVs, where the lipoprotein, Sly B, specifically directs the bacterial in-house expressed nanoluciferase into the interior of OMVs. The ice nucleation protein (INP) was used to decorate the vesicle surface with the Z-domain via a trifunctional scaffold composed of cohesion domains from *Clostridium cellulolyticum* (CC), *C. thermocellum* (CT), and *Ruminococcus flavefaciens* (RF). This approach facilitates vesicle binding

to the antibody. This engineered OMV emitted > 105 times as many photons as control OMVs after co-incubation with substrate, displaying highly sensitive luminescent signals and creating a virtually infinite combination of capture and reporter sections [82].

In 2019 the team was able to detect the most intense bioluminescence shortly after substrate injection by isolating OMVs containing NanoLuc and Z structural domains from genetically engineered *E. coli* and injecting the OMVs subcutaneously into mice under mild substrate reaction conditions (Figure 4). The bacterial OMVs had low cytotoxicity and high biocompatibility. In addition, the bacterial OMVs used in this study could be further functionalized with dockerin-tagged foreign proteins by adding dockerin-tagged foreign proteins to further functionalize the OMVs with relevant antibodies to recognize biomarkers on cancer cells for bioluminescence-based tumor-targeted imaging. The unique properties of engineered multifunctional OMVs can be used for molecular imaging [143].

Harnessing CMNVs in photoacoustic (PA) imaging

PA imaging is a non-ionizing, non-invasive mode that combines optical and ultrasonic imaging modalities [144]. When a pulsed laser irradiates tissue, light energy is transformed into heat energy by endogenous or exogenous contrast agents, resulting in an instantaneous increase in local tissue temperature, followed by thermoelastic expansion and the generation of sound waves. The acoustic waves are captured by an ultrasound transducer and data processing based on the arrival time, which transforms the acoustic waves into PA images [145]. PA imaging provides a high spatial resolution and a significant contrast compared to optical and ultrasound imaging [146]. PA imaging has been widely used for breast, skin, blood vessel, and adipose tissue imaging. Although endogenous contrast agents, such as hemoglobin, can produce PA imaging signals, PA imaging does not provide further tissue information in the absence of exogenous contrast agents. Therefore, it is critical to produce exogenous contrast agents. Some small organic molecules, such as cyano dyes, are limited in practical application because of rapid degradation in water, poor photostability, a short *in vivo* half-life, a tendency to bind to serum in the blood, and rapid clearance after intravenous injection [147]. One way to increase the PA signal intensity of this type of contrast agent is to increase the accumulation of the probe in the tissue of interest. The use of cell membrane-derived vesicles for the transport of such contrast agents can achieve this goal. For example, ICG-loaded PLGA camouflaged on A549 cell membranes encapsulating perfluorocarbons can be actively targeted to tumor tissues for homologous targeting [148]. The biocarrier can also be genetically modified in the original cells of the vesicle or directly modify the vesicle with ligands so that the biocarrier can spontaneously encapsulate the endogenous contrast agent and possess active targeting properties during the formation process.

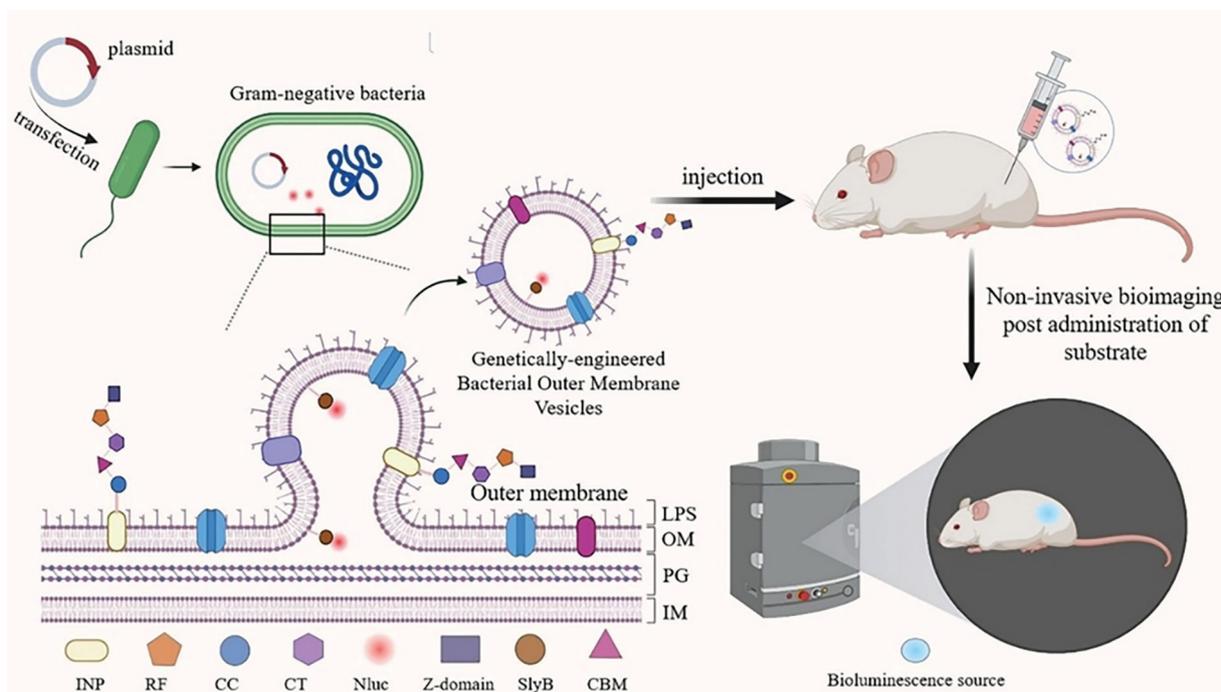


Figure 4 Engineered OMVs enable targeted tumor bioluminescence imaging. Gram-negative bacteria were transfected with plasmids encoding nanoluciferase (Nluc) and a targeting moiety. SlyB lipoprotein transported intracellularly expressed Nluc into OMVs. An INP-based scaffold incorporating CC, CT, and RF adhesin domains display surface Z-domains for antibody conjugation. Subcutaneously injected engineered OMVs generated strong bioluminescent signals post-substrate administration, demonstrating low cytotoxicity, high biocompatibility, and sensitive tumor-targeting capability. Figure 4 was created in BioRender. (2025) <https://BioRender.com/6tkqcb>.

Melanin, as a natural light absorber with high absorption capacity in the visible and near-infrared windows, is ideal for enhancing contrast in PA imaging [149, 150]. In addition, melanin has a high photothermal conversion efficiency, making melanin ideal for use in photothermal therapy, in which light is used to selectively irradiate melanin-containing tissues and induce localized heating that kills tumor cells. The ability of melanin to act as a contrast agent and a therapeutic agent potentially makes melanin clinically integrable.

Gujrati et al. thus developed a molecular approach to encapsulate melanin in cell membrane-derived nanocarriers. *E. coli* expressing a tyrosinase transgene was transformed into a low endotoxin strain (OMV^{ΔmsbB}) by inactivating the *msb* gene [13]. The melanin produced by this strain passively binds to the cytoplasm and cell membrane, resulting in the formation of bacterial OMVs encapsulating the biopolymer-melanin (OMV^{Mel}). Experimental results have shown that OMV^{Mel} produces a strong MSOT signal both mimicking and *in vivo*. Exposure to a pulsed near-infrared light source produces local heating that inhibits tumor growth *in vivo*, suggesting that melanin-coated OMVs is a promising therapeutic agent for PA imaging.

The use of cell membrane-derived vesicles of mammalian origin is more biologically safe compared to the uncertainty of immunogenicity of bacterial exocysts. Exocystic vesicles from EVs isolated from tumors were used as nanocarriers of the photosensitizer, Chlorin e6 (Ce6), for effective targeted PA imaging of malignant tumors. In addition, EVs-loaded Ce6 showed a stronger PA signal in imaging compared to free or liposome-loaded Ce6 [151].

Harnessing CMNVs in MRI

MRI, as a non-invasive clinical modality, provides exceptional spatial resolution and soft tissue contrast. The image contrast primarily depends on tissue magnetic properties, including proton density and T¹/T² relaxation times of water protons. However, insufficient magnetic heterogeneity between tumors and normal tissues often compromises MRI contrast. To address this limitation, paramagnetic metal ions and paramagnetic metal ion chelates serve as contrast agents that modulate tissue relaxation times to enhance imaging contrast [152]. Gadolinium-based contrast agents and superparamagnetic iron oxide nanoparticles (SPIONs) represent widely used MRI contrast materials. SPIONs exhibit super-paramagnetism, high saturation magnetization, favorable biocompatibility, and low toxicity, functioning as negative MRI contrast agents with significant biomedical utility [153–155]. Nevertheless, inadequate targeting remains a major challenge for SPIONs. Specifically, sub-micromolar concentrations in target tissues often fall below MRI detection thresholds. EV-derived carriers offer a promising solution by providing enhanced targeting specificity and biosafety [156].

In 2014 Hu et al. first demonstrated MRI detection of EVs loaded with super-paramagnetic iron oxide nanoparticles (SPIONs), establishing applicability in neurologic disease theranostics [157]. Wang et al. subsequently conjugated stem cell-derived EVs with SPIONs, achieving targeted brain MRI in rats through significant T² relaxation time shortening and enhanced tissue contrast [158]. Further advancing this approach, astrocyte-derived EVs carrying ultra-small SPIONs (USPIOs) were efficiently delivered to the brain via

nasal administration with significantly elevated ΔR^2 values detectable on day 1 and USPIOs particles observed in 85.7% of astrocytes, validating utility as blood-brain barrier (BBB)-traversing carriers for brain disorders [159].

EVs lacking innate targeting specificity, such as those derived from macrophages, have attracted considerable interest due to low immunogenicity, scalable production, and inherent BBB penetration capability. Surface engineering strategies, including covalent modification and ligand-receptor interactions, enable active targeting for precision delivery [160, 161]. Engineering EVs with arginine (R)-glycine (G)-glutamic acid (E) [RGE] peptides, which are specific ligands for neuropilin-1 overexpressed in gliomas, yielded an RGE-Exo-SPION/Cur system in which MRI revealed substantially higher contrast-to-noise ratios (CNRs) and sharper tumor boundaries compared to controls [162–164]. Genetic engineering approaches further enhanced vesicle-based MRI performance. Galisova et al. used CRISPR/Cas9 to generate HEK293 cells stably expressing the SARS-CoV-2 spike receptor-binding domain (RBD), secreting RBD-retaining EVs (EVs^{RBD}). Loading SPIONs into EVs^{RBD} produced a 40-fold greater CNR enhancement in ACE2-positive tumors than free SPIONs within 4 h post-IV injection, enabling non-invasive monitoring of RBD-ACE2 binding dynamics for tracking viral variants and evaluating therapeutic sensitivity [165].

Optimizing contrast agent size constitutes a critical design parameter for EV-based MRI probes. Studies have indicated that USPIOs fail to generate detectable MR signals, even at maximum feasible concentrations (5 mgFe/mL), which limits utility for *in vivo* imaging [166]. Conversely, clinically approved large-particle agents, like ferucarbotran (Resovist[®]), demonstrate superior performance, including higher SNRs than USPIOs [167], efficient EV loading without compromising structural integrity, and distinct signal attenuation in T²-weighted imaging. Intramuscular injection experiments revealed concentrated Resovist[®]-labeled EV signals at target sites (muscle tissue), whereas USPIO-labeled counterparts diffused into subcutaneous fat, confirming that SPIONs > 50 nm enhance tissue retention and local targeting precision [168]. The established safety profile of Resovist[®], biocompatibility, and good manufacturing practices (GMP)-compliant manufacturing synergize with EV carriers to accelerate clinical translation of this integrated platform.

Discussion

Nanoprobes offer superior stability, imaging quality, and specificity when compared to traditional probes. CMNVs are a key platform for advanced imaging, supporting nanoprobe design, and loading. CMNV biocompatibility and low immunogenicity enhance targeting and address key limitations in high-quality imaging. However, clinical translation requires overcoming critical challenges, such as heterogeneity control, scalable purification, and safety and immunogenicity balance for long-term safety.

Verifying long-term safety is essential due to parental cell endotoxicity for OMV-based probes or vaccines. The MenPF1 OMV vaccine from *Neisseria meningitidis* B H44/76 is safe

and tolerable in healthy adults [169]. A phase I trial showed increased serum IgG but poor memory B cell response and antibody persistence [170], suggesting engineered OMVs offer long-term safety potential for immunotherapy, although novel adjuvants or nanocarriers are needed to boost antigen presentation. Progress has also been achieved for EV long-term safety. While prolonged EV circulation may be unnecessary, preclinical studies have shown rapid intravenous clearance in minutes [171]. Routine plasma transfusions transfer trillions of allogeneic EVs without adverse events, supporting biocompatibility [172]. However, CMNVs face heterogeneity and standardization challenges. Size and composition vary significantly with parent cell growth stage, culture conditions, and isolation methods (e.g., ultracentrifugation), causing batch-to-batch differences in potency and immunogenicity. Thus, standardized assays for purity, antigen integrity, unified production, isolation, characterization guidelines, and technologies, like microfluidic chips for automated modification, are urgently needed to ensure study reproducibility [79].

CMNVs should prioritize low immunogenicity and ease of sourcing for nanoprobe design in the future. As excellent natural carriers, genetically engineering source cells to intrinsically carry reporter molecules or therapeutics could enable theranostic integration.

Data availability statement

All data generated and/or analyzed during this study are included in this published article.

Author contributions

Lingling Lei: Investigation, Formal analysis, Writing - Original Draft. Meng Du: Conceptualization, Methodology, Supervision, Writing - Review & Editing. Jianjian Zhang: Conceptualization, Resources, Supervision, Writing - Review & Editing. Yuguang Mu: Validation, Supervision, Writing - Review & Editing.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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