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Unveiling Invisible Extracellular Vesicles: Cutting-Edge Technologies for Their in Vivo Visualization

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ABSTRACT

Extracellular vesicles (EVs), nanosized lipid bilayer vesicles released by nearly all types of cells, play pivotal roles as intercellular signaling mediators with diverse biological activities. Their adaptability has attracted interest in exploring their role as disease biomarker theranostics. However, the in vivo biodistribution and pharmacokinetic profiles of EVs, particularly following administration into living subjects, remain unclear. Thus, in vivo imaging is vital to enhance our understanding of the homing and retention patterns, blood and tissue half-life, and excretion pathways of exogenous EVs, thereby advancing real-time monitoring within biological systems and their therapeutic applications. This review examines state-of-the-art methods including EV labeling with various agents, including optical imaging, magnetic resonance imaging, and nuclear imaging. The strengths and weaknesses of each technique are comprehensively explored, emphasizing their clinical translation. Despite the potential of EVs as cancer theranostics, achieving a thorough understanding of their in vivo behavior is challenging. This review highlights the urgency of addressing current questions in the biology and therapeutic applications of EVs. It underscores the need for continued research to unravel the complexities surrounding EVs and their potential clinical implications. By identifying these challenges, this review contributes to ongoing efforts to optimize EV imaging techniques for clinical use. Ultimately, bridging the gap between research advancements and clinical applications will facilitate the integration of EV-based theranostics, marking a crucial step toward harnessing the full potential of EVs in medical practice.

Prakash Gangadaran, Fatima Khan, and Ramya Lakshmi Rajendran contributed equally to this article.

1 | Introduction

1.1 | Extracellular Vesicles

Extracellular vesicles (EVs) constitute small membranous structures released by various cellular entities, including prokaryotes and eukaryotes, under both physiological and pathological conditions. They serve as crucial mediators in intercellular communication, facilitating the transfer of bioactive molecules such as proteins, nucleic acids, and lipids between cells. This intricate exchange underscores their pivotal role in maintaining cellular communication, becoming promising candidates for therapeutic interventions (de Jong et al. 2019). EVs encompass three primary types: exosomes, microvesicles, also known as ectosomes, and apoptotic bodies, with exosomes and microvesicles being the most extensively studied (Jeppesen et al. 2023). Exosomes, originating from endosomes, typically have a diameter of 40–160 nm, formed through the invagination of the plasma membrane to create multivesicular bodies (MVBs) with diverse compositions (Edgar 2016). Conversely, microvesicles are generated through direct outward budding of the plasma membrane, yielding vesicles with a diameter ranging from approximately 50 nm to 1 μM (Kalluri and LeBleu 2020; Wang et al. 2019) Furthermore, apoptotic bodies, formed during apoptosis, have a diameter of 1–5 μM (Caruso and Poon 2018; Kakarla et al. 2020). The biogenesis of EVs involves complex and dynamic processes (Liu and Wang 2023).

1.2 | Biogenesis

Exosomes are created with the inward budding of the plasma membrane, forming early endosomes that mature into multivesicular bodies (MVBs). Intraluminal vesicles (ILVs) are formed within MVBs through the inward budding of the endosomal membrane, facilitated by the endosomal sorting complex required for transport (ESCRT) machinery, including ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III, and the vacuolar protein sorting 4 (Vps4) complex. These complexes mediate cargo sorting, ILV packaging, and vesicle release (Gurung et al. 2021; Umbaugh and Jaeschke 2021; Xu et al. 2023). Through an alternative ESCRT-independent mechanism, MVBs can fuse with lysosomes for degradation or traffic to the plasma membrane for exosome release. Then, exosomes are released into the extracellular space through MVB fusion with the plasma membrane regulated by molecular mechanisms involving such proteins as Rab GTPases, soluble N-ethylmaleimide-sensitive factor attachment protein receptors, and calcium ions (Kim, Lee, and Baek 2022; Xu et al. 2023).

In contrast, microvesicles form through outward budding and fission of the plasma membrane, which is regulated by small GTPases involved in various regulatory pathways. Mature microvesicles are released from the cell surface through controlled pinching and scission, directly discharging into the extracellular space (Blanc and Vidal 2018; Gurunathan et al. 2021; Tricarico, Clancy, and D'Souza-Schorey 2017). Figure 1 shows the processes involved in EV biogenesis.

Understanding the intricate mechanisms underlying EV biogenesis, including exosomes and microvesicles, is essential for unraveling their roles in various biological processes and developing novel therapeutic strategies. This knowledge not only sheds light on the fundamental processes governing intercellular communication but also holds great promise for the advancement of diagnostic and therapeutic applications in medicine and biology.

1.3 | Contents (Cargo) and EV-to-Cell Communication

The composition of EVs is highly heterogeneous, including proteins, nucleic acids (such as messenger RNAs, microRNAs, and long noncoding RNAs), lipids, and even organelles (Jeppesen et al. 2023). This diverse composition is influenced by cell type, physiological condition, and pathological state, reflecting the dynamic nature of EVs across different biological contexts. Vesiclepedia (http://www.microvesicles.org) serves as a valuable resource offering information on DNA, RNA, proteins, lipids, and metabolites associated with EVs and extracellular particles (Chitti et al. 2023), reflecting the growing interest in EV research. EVs are selectively packaged into vesicles through specific sorting mechanisms, ensuring the precise delivery of bioactive molecules to target cells and tissues (Chen et al. 2021).

EVs are crucial mediators of intercellular communication in both physiological and pathological contexts (Liu and Wang 2023). They play critical roles in immune modulation, tissue regeneration, cancer progression, and neurodegenerative disease pathogenesis (Bahmani and Ullah 2022), underscoring their significance in various biological processes. Different cell types, including immune cells, stem cells, and cancer cells, release EVs, highlighting the widespread involvement of EVs in cell-to-cell communication (Khan et al. 2024; Liu et al. 2019). This communication is essential for regulating cellular responses such as proliferation, differentiation, and immune responses by signaling molecules carried by EVs (Liu et al. 2019). Figure 2 shows the primary structural components of EVs, providing a visual representation of their complexity.

1.4 | Interaction, Internalization, Cargo Delivery, and Functions

EVs employ various interaction mechanisms, including membrane receptor binding, fusion, and endocytosis, to come into close contact with target cells (Liu et al. 2019). Once interaction occurs, EVs can be internalized by recipient cells, releasing their cargo comprising miRNAs, mRNAs, proteins, and lipids. This cargo delivery mechanism represents a form of cell-to-cell communication. EV internalization predominantly occurs through endocytosis, when recipient cells engulf the vesicles by invaginating their plasma membrane, involving clathrin-dependent or independent pathways. Clathrin-mediated internalization involves receptors such as low-density lipoprotein and transferrin, forming clathrin-coated pits via activating protein 2 adaptor complexes. Clathrin proteins coat EVs, while dynamin pinches off the plasma membrane with support from actin filaments and phosphoinositide 3-kinases (PI3Ks) activity. Then, EV loses its coat through ATPase heat shock cognate 70 and auxilin, enabling intracellular trafficking and

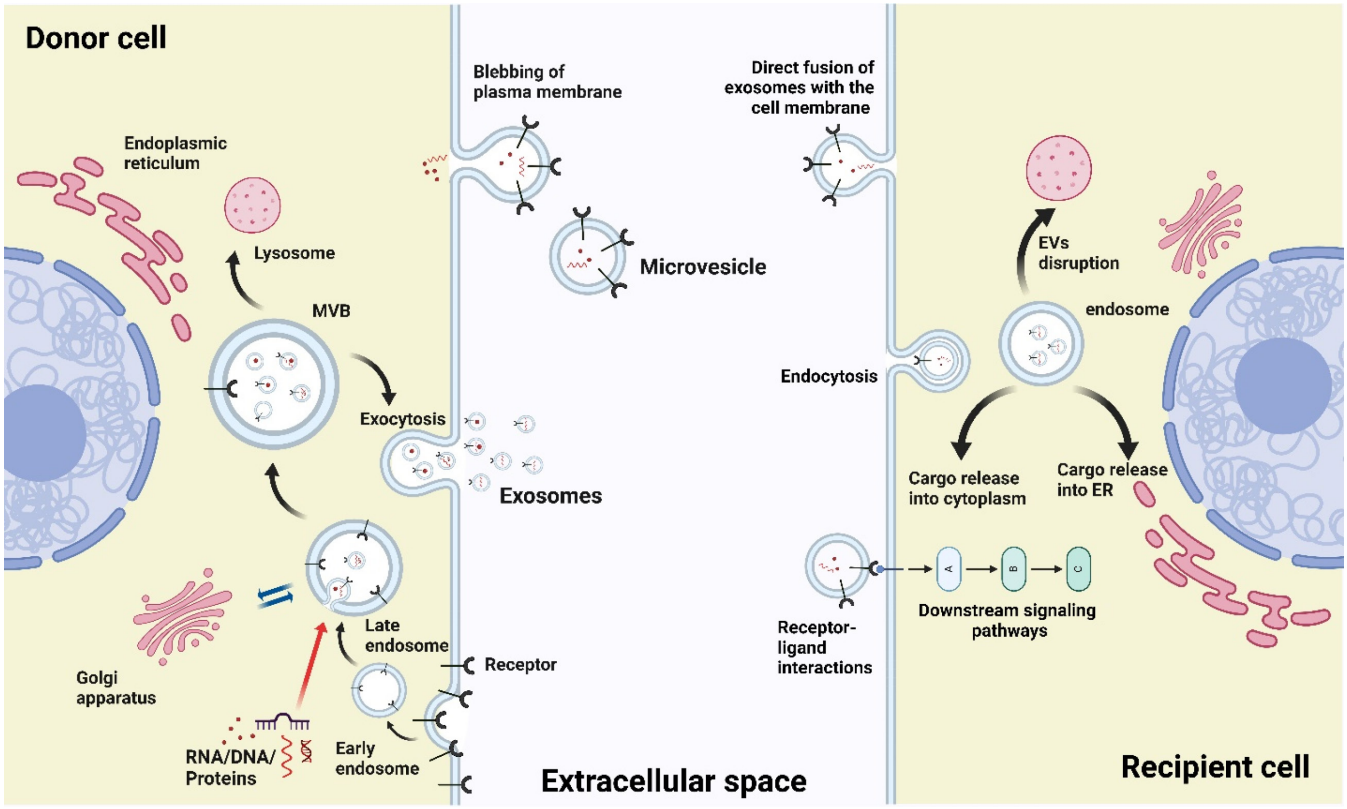

FIGURE 1 | Biogenesis and release of EVs from donor cells and internalization of EVs into recipient cells. EV biogenesis involves the inward budding during endocytosis. Exosomes containing specific cargo (proteins, lipids, RNAs, and genetic materials) are formed within MVBs, where early and late sorting endosomes are assembled. EVs enter recipient cells through various mechanisms, including direct fusion with the cell membrane, receptor-ligand interactions, and endocytosis. Upon cellular internalization, EVs transport their contents and release them into the cytoplasm or endoplasmic reticulum. Then, EVs undergo degradation in lysosomes. EV, extracellular vesicle; MVB, multivesicular bodies. Created with BioRender.com.

receptor recycling. In contrast, clathrin-independent endocytosis involves lipid raft domains and GTPases, internalizing various cargos without clathrin (Gandek, van der Koog, and Nagelkerke 2023; Mulcahy, Pink, and Carter 2014). Figure 1 shows EV internalization into a recipient cell. In some cases, particularly with larger microvesicles, EV internalization can involve phagocytosis, when the recipient cell engulfs the vesicles using pseudopodia. Additionally, EVs can directly fuse with the recipient cell's plasma membrane, releasing their cargo directly into the cytoplasm.

Once internalized, EVs undergo intracellular trafficking within endosomes, lysosomes, or other vesicular compartments, depending on the specific cargo and biological context (O'Brien et al. 2022). EVs release their cargo within the recipient cell, exerting various effects. For example, EV-delivered miRNAs can regulate gene expression (Bahmani and Ullah 2022), whereas EV-delivered proteins can participate in intracellular signaling pathways (Roefs, Sluijter, and Vader 2020). Furthermore, EV-delivered lipids can influence membrane composition (Ghadami and Dellinger 2023). These processes play a crucial role physiologically and pathologically. Thus, understanding the interaction, internalization, and cargo delivery mechanisms of EVs is vital to exploring their roles in health and disease. Researchers are actively investigating these processes to harness the potentials of EVs for drug delivery, regenerative medicine, and diagnostics, as well as to gain insights into the roles of EVs in various diseases, including neoplastic, neurodegenerative, and immune-related disorders (Herrmann, Wood, and Fuhrmann 2021).

1.5 | Functionalizing EVs With Tumor-Targeting Moieties

The surface of EVs can be conveniently modified with molecules to enhance their targeting, offering several advantages over modifying donor cells for targeted drug delivery. The chemical and genetic modification of EVs enables highly controlled attachment of ligands, enhancing the precision of drug delivery, which may not be achievable through donor cell modification. Chemical modifications of EVs involve introducing various functional groups or moieties (peptides, proteins, lipids, aptamers, small molecules, and polymers). Chemical modifications are achieved through specific chemical reactions targeting lipids or membrane-bound proteins, or lipid–lipid interactions within the membrane. For genetic modification, ligands or homing peptides are fused with transmembrane proteins expressed on the surface of EVs. Then, donor cells transfected with plasmids encoding these fusion proteins secrete engineered EVs with targeting ligands on their surface. These methods provide a promising avenue for developing targeted therapies with potential applications in personalized medicine.

Engineered EVs can facilitate the successful delivery of EV cargo through the blood–brain barrier in a mouse model. For example, multifunctional peptides containing therapeutic Lys-Leu-Ala (KLA) and targeted low-density lipoprotein (LDL) are used to target LDL receptors on glioblastoma cells, amplifying chemotherapy effectiveness in challenging conditions such as glioblastoma (Ye et al. 2018). Chemical modification techniques, such as copper-catalyzed azide-alkyne cycloaddition "click" reactions, can be utilized to nonspecifically modify EVs (Ye et al. 2018). This demonstrates the potential of chemically modified EVs for precise drug delivery in glioma treatment. In another study, researchers bioorthogonally conjugated cyclic peptide arginine-glycine-aspartic acid-(spacer)-lysine (c[RGDyK]) to the surface of EVs. This modification resulted in curcumin-carrying c(RGDyK) EVs, which efficiently penetrated the blood–brain barrier. These modified EVs could effectively mitigate inflammatory responses and cellular apoptosis in a murine model of transient middle cerebral artery occlusion (Tian et al. 2018). Bioorthogonal click chemistry, exemplified by the conjugation of peptide c(RGDyK) to EVs, showcases the viability of targeted drug delivery via EVs in neurological disorders (Tian et al. 2018).

Amphipathic molecules such as DSPE-PEG can modify EV membranes, enabling targeted drug delivery. For instance, chemically altered RGD EVs with DSPE-PEG accumulate in tumors with folate, offering potential in photothermal therapy and chemotherapy for proangiogenic treatment. Additionally, EVs conjugated with DSPE-PEG and a sigma ligand effectively target lung cancer expressing sigma receptors (Jung et al. 2024; Kim et al. 2018). DSPE-PEG, an FDA-approved material, holds significant potential for modifying EV membranes, thereby enhancing their clinical potential for various medical purposes (Y. Liang et al. 2021). Researchers developed immune checkpoint blockers to disrupt the interaction of CD47-SIRPα, thereby enhancing macrophage-mediated phagocytosis of tumor cells. This innovative approach, exemplified by cancer EVs fused with cRGD-decorated liposomes, enhances the natural homing ability of exosomes and efficiently delivers chemotherapy drugs in vitro and in vivo (Koh et al. 2017; Q. Liu et al. 2022b; Nie et al. 2020; Sato et al. 2016; Zhang et al. 2023). In another study, an exosome-liposome hybrid fusing MSC exosomes with polypyrrole-loaded liposomes was used to treat diabetic peripheral neuropathy, demonstrating the potential for stem cell therapy combined with targeted electrical stimulation (Singh et al. 2021).

The acidic nature of the tumor microenvironment (TME), primarily due to increased glycolysis and lactate production, significantly impacts the behavior and modification of engineered EVs. This unique characteristic of the TME can be harnessed to enhance the targeting and efficacy of therapeutically loaded EVs. Ran et al. (2020) demonstrated the potential of tetraspanin-mediated exosome modification by fusing the myostatin propeptide with CD63, using tetraspanins such as CD63, CD81, and CD9 and thereby enhancing delivery efficacy and serum stability in a Duchenne muscular dystrophy mouse model. This method can be adapted for cancer therapy to improve the stability and delivery of therapeutic agents (Ran et al. 2020). For instance, antitumor immunity can be further enhanced by genetically incorporating αCD3 UCHT1 and scFv fragments of αEGFR cetuximab antibodies into exosomes, promoting cross-linking of T cells with EGFR-expressing breast cancer cells (Cheng et al. 2018). Surface functionalization with PDGFR was also utilized to improve exosomal targeting capabilities (Nguyen Cao et al. 2022). This functionalization significantly improves the specificity and efficacy of exosome-mediated drug delivery to cancer cells. An innovative approach by Nguyen Cao et al. involved sodium bicarbonate ($NaHCO_3$)-loaded EVs, which generate CO_2 bubbles upon endocytosis by cancer cells, efficiently releasing paclitaxel. This technique leverages the acidic TME to trigger drug release, enhancing the delivery and therapeutic effect of anticancer agents. Additionally, photo-activatable EVs loaded with siRNA against PAK4 (siPAK4) and a reactive oxygen species (ROS)-sensitive polymer, were camouflaged with M1-macrophage-derived vesicles, enabling ROS-responsive release of siRNA, silencing PAK4, and modulating the TME for enhanced cancer immunotherapy (Wang et al. 2020). Figure 3 shows EVs with tumor-targeting moieties. Lactate dehydrogenase A (LDHA)-containing EVs can increase glycolysis, proliferation, and survival of glioblastoma cells (Khan et al. 2024). These EVs potentially increase the acidity of the TME, improving the targeting efficiency and therapeutic effect of engineered EVs in glioblastoma treatment. Furthermore, inhibition of LDHA-mediated interactions markedly suppresses tumor progression and macrophage infiltration in glioblastoma mouse models, highlighting the potential of targeting metabolic interactions between tumor cells and macrophages to impede tumor growth and modulate the TME (Khan et al. 2024).

The modification of exosomes for targeted tumor therapy offers significant advantages, including the ability to carry diverse targeting moieties that enhance specificity for cancer cells and allow using exosomes as a diagnostic method. Various chemical tools can be employed to conjugate therapeutic and diagnostic agents to EVs, increasing their functionality and effectiveness. However, ensuring the stability and integrity of the EV membrane during and after modification, preventing aggregation, and achieving the optimal density of targeting ligands on EVs to maximize targeting effectiveness without compromising stability are challenging. Ongoing research continues to enhance the efficacy and specificity of these nanoscale delivery systems, paving the way for advanced cancer treatments.

2 | The Importance of EV in Vivo Imaging

The in vivo imaging of EVs is an essential area of research with several potential applications. This technique offers valuable insights into EV biology and function by observing its behavior within living organisms (Yanez-Mo et al. 2015). In vivo imaging elucidates how EVs function in various physiological and pathological processes, enhancing our understanding of their roles in intercellular communication and disease progression. EVs are implicated in various diseases, including oncologic, neurodegenerative, and cardiovascular disorders (Shah, Patel, and Freedman 2018). Observing EV behavior in vivo can elucidate their role in these diseases, facilitating early detection, diagnosis, and monitoring. In vivo imaging

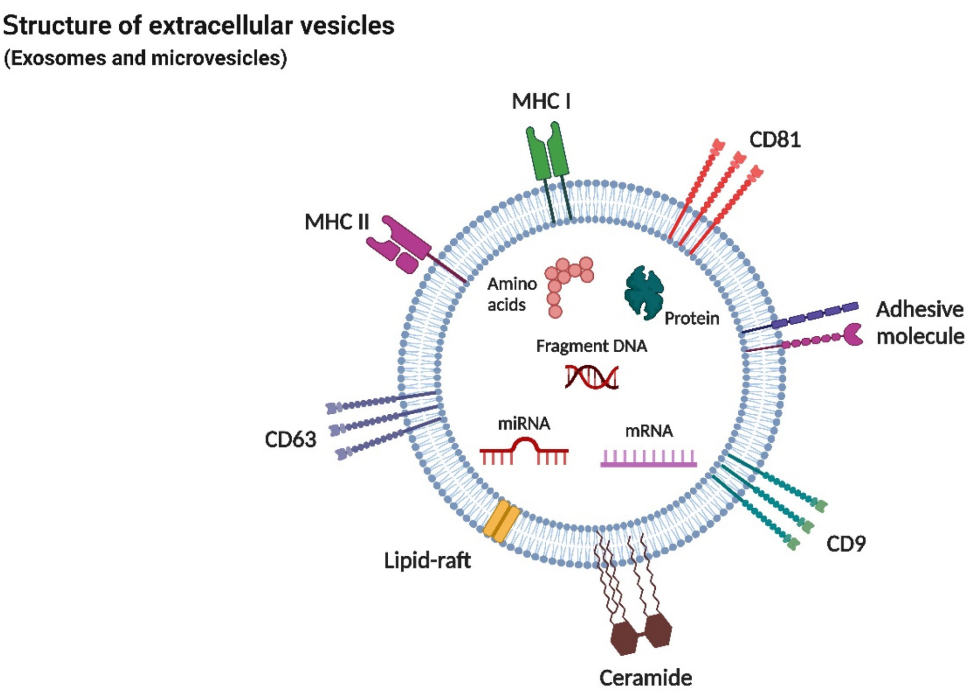

FIGURE 2 | EV structure. EVs are small lipid bilayer vesicles containing proteins, lipids, RNAs, and genetic materials. EVs are composed of diverse proteins, including transmembrane or lipid-bound surface proteins (e.g., CD63, CD9, and CD81). Additionally, EVs contain lipids (e.g., ceramide) and RNAs (e.g., mRNA, miRNA, and DNA fragments). EV, extracellular vesicle; mRNA, messenger RNA; miRNA, microRNA. Created with BioRender.com.

can track the distribution and behavior of disease-associated EVs, which is crucial for monitoring disease progression and response to treatment. EVs are being explored as potential drug delivery vehicles (de Jong et al. 2019; Gangadaran et al. 2018). This imaging technique allows researchers to track the biodistribution of drug-loaded EVs, ensuring they reach their target tissues and cells, which has significant implications for personalized medicine and targeted therapies (Beetler et al. 2023). Additionally, in vivo imaging aids in developing therapeutic strategies that target EVs, tracking the effects of EV-based therapies, and assessing their safety and efficacy (Gangadaran, Hong, and Ahn 2018).

Understanding the life cycle of EVs in the body, including their clearance from circulation (Nguyen et al. 2020), is essential for assessing their safety and potential for long-term use. Since EVs represent a heterogeneous population (Willms et al. 2018), in vivo imaging can help study the diversity of EV subtypes and their distinct functions. Overall, the in vivo imaging of EVs is a valuable tool for basic scientific research and their integration into the clinic. It provides a dynamic view of EVs within living organisms, advancing our understanding of their roles in health and disease. This technology holds promise for improving disease diagnosis, monitoring, and the development of targeted therapies.

3 | Labeling Strategies in Vivo Imaging of EVs

The labeling strategies for in vivo imaging of EVs can be categorized into direct and indirect labeling. These strategies enable the visualization and tracking of EVs within living organisms.

3.1 | Direct Labeling

Direct labeling is a straightforward process for controlling the concentration of labeling agents. This involves attaching or encapsulating a contrast agent or fluorophore to EVs or within the EV core, respectively, enabling their direct visualization.

Quantum dots (QDs) are nanoscale semiconductors that emit light under specific conditions. Unlike traditional dyes, they offer stability, efficient labeling, and multiplexing ability (Medintz et al. 2005; Singh et al. 2020). Carbonaceous dyes (e.g., PKH67, PKH26, 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine [DiR], 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine [DiD], and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate [DiI]) are lipophilic fluorescent dyes for staining cell membranes and other lipid-soluble biological components, such as EV membranes. These dyes significantly increase their fluorescence upon binding to lipid membranes. Once the lipid bilayer is stained, these dyes disperse across the entire membrane, visualizing the complete membrane structure of EVs (Askenasy and Farkas 2002; Fick et al. 1995; Heinrich et al. 2007; Wiklander et al. 2015). However, these dyes exhibit prolonged in vivo half-lives, possibly causing an inaccurate distribution in longitudinal EV studies (Kuffler 1990; Teare et al. 1991).

Magnetic resonance imaging (MRI) is among the key technologies used in clinical imaging. MRI contrast agents, such as superparamagnetic iron oxide (SPIO), can diminish the T2 signal in tissues, enhancing the signal-to-noise ratio and lesion detectability (Lu and Hsiao 2023). SPIO can be loaded into EVs through electroporation, generating a magnetic signal detectable

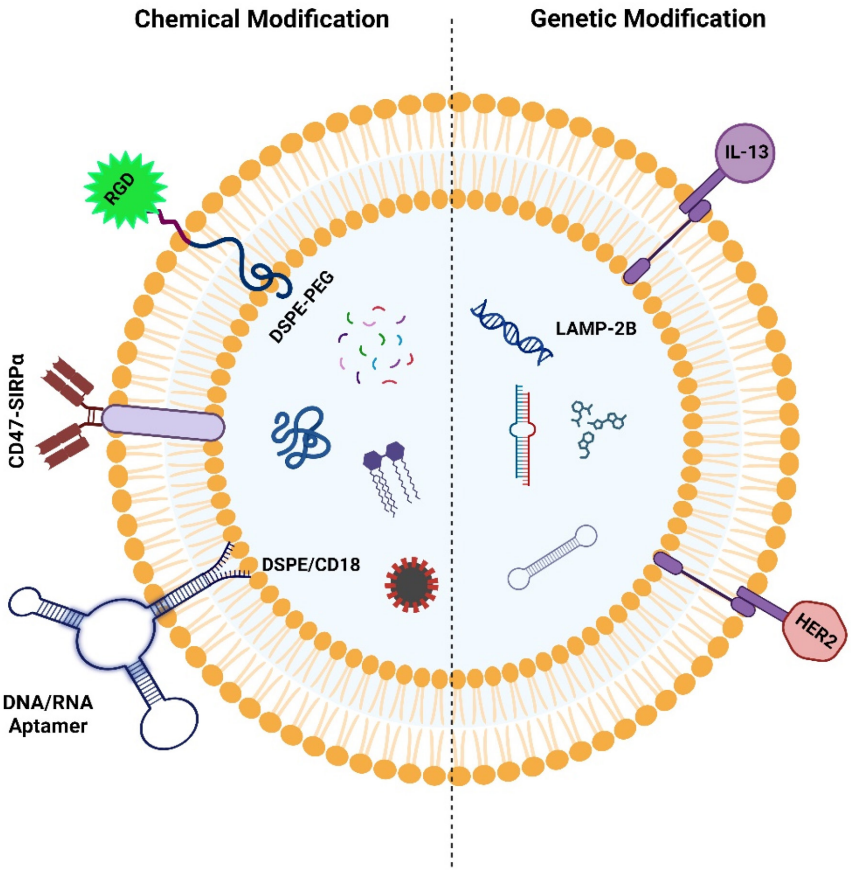

FIGURE 3 | EV modification strategies using tumor-targeting moieties to enhance targeted drug delivery. The EV surface can be modified with various molecules, offering advantages for targeted drug delivery. Chemical and genetic modifications enable precise ligand attachment, surpassing the limitations of naïve EVs from donor cells. EVs targeting peptides, antibodies/nanobodies, multifunctional peptides, and chemically modified EVs are being explored to allow precise drug delivery. EV, extracellular vesicle; DSPE-PEG, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol; CD, cluster of differentiation; SIRPα, signal regulatory protein alpha; IL, interleukin; HER2, human epidermal growth factor receptor-2; RGD, arginylglycylaspartic acid; LAMP-2B, lysosomal-associated membrane protein-2B. Created with BioRender.com.

by MRI. However, electroporation may cause EV fusion and affect its morphology (Hu, Wickline, and Hood 2015).

Various radionuclides, such as technetium-99 m (^{99m}Tc; half-life of 6 h), indium-111 (^{111}In; half-life of 2.8 days), fluorine-18 (half-life of 109 min), and copper-64 (half-life of 12 h), can be used to label EVs. Labeling strategies include covalent binding (Morishita et al. 2015; Royo et al. 2019; Varga et al. 2016), encapsulation or intraluminal labeling (Gangadaran et al. 2018; Hwang et al. 2015; Smyth et al. 2015), and membrane radiolabeling (Banerjee et al. 2019; Faruqu et al. 2019). Radionuclide-labeled EVs can be monitored using certain imaging modalities (e.g., single photon emission computed tomography [SPECT] or positron emission tomography [PET]) for periods spanning from hours to days based on the half-lives of these radionuclides. This approach provides high sensitivity but requires caution because of radiation exposure concerns.

SPECT, PET, and MRI systems offer substantial imaging depth. However, labeling compounds used in MRI may exhibit signals even after the degradation of EVs. Furthermore, labeling radionuclides used in SPECT or PET may not produce signals after periods longer than half-lives of labeled radionuclides (Jiang, Nie, and Xie 2022).

3.2 | Indirect Labeling

In indirect labeling, imaging agents (e.g., nanoparticles) are incorporated within the EV core after incubating with donor cells, and genetically modified EV-producing donor cells to express a reporter molecule for in vivo imaging. EV biomarkers, such as tetraspanins, heat shock proteins, lactadherins, and donor cell-specific proteins, such as viral envelope protein VSVG5, are found on the EV surface (Jeppesen et al. 2019; Meyer et al. 2017). Genetic engineering techniques can integrate coding sequences of fluorescent proteins or luciferases with EV biomarkers, creating fusion proteins to label and track EVs under specific conditions.

Ultrasmall SPIO (USPIO) overcomes this issue by accumulating in MVBs, being released as USPIO-labeled EVs (Busato et al. 2016). Radionuclides label cells before transplanting them into living subjects and can label EVs. Fluorescent proteins are commonly used as reporter proteins emitting a fluorescent signal when excited by light of a particular wavelength. After genetically modifying cells to express green fluorescent protein (GFP) or other fluorescent proteins, the EVs produced by these cells carry the same fluorescent label, enabling their long-term tracking (Valbuena et al. 2020). A study linked enhanced GFP and tandem dimer Tomato (tdTomato) at the NH_2 termini with a palmitoylation signal (PalmGFP,

PalmtdTomato) to label EV membranes, facilitating the imaging and monitoring of EV release, uptake, and exchange among cell populations in vitro and in vivo (Lai et al. 2015). However, while GFP generates a robust signal, its effectiveness diminishes with tissue penetration due to absorption and refraction, limiting its applicability to labeling tissues of < 2 mm depth within an animal's body (Ochiai et al. 2012).

Genetically engineered cells can also express luciferases (Firefly luciferase [Fluc], Renilla luciferase [Rluc], and Gaussia luciferase [Gluc])—enzymes catalyzing the emission of bioluminescent light detectable by bioluminescence imaging (BLI) systems. A model expressing a Gluc-lactadherin fusion protein in mouse melanoma cells was used to study the in vivo biodistribution of EVs (Yuki Takahashi et al. 2013). Another study utilized Rluc-transduced thyroid and breast cancer cells to produce Rluc-expressing cancer-derived EVs and investigated their in vivo biodistribution (Gangadaran et al. 2017a). Furthermore, bioluminescent labeling was advanced by tethering five different luciferase enzymes (NanoLuc, ThermoLuc, Fluc, Super RLuc8, and CBRLuc) to CD63 for tracking EVs in vitro and in vivo (Gupta et al. 2020). The genetically engineered labeling technique offers specificity, flexibility, and stability, minimally impacting EVs and surpassing traditional membrane dye labeling. Figure 4 shows direct and indirect labeling of EVs. Each labeling strategy has its advantages and disadvantages. Thus, the choice of strategy depends on the specific experimental requirements, imaging modalities, and properties of EVs and host cells.

4 | In Vivo Imaging of EVs

4.1 | Overview of Imaging Modalities

Fluorescence imaging is a highly sensitive technique used to visualize biological processes using fluorescent materials (Liu et al. 2021). In vivo fluorescence imaging with living organisms operates similarly to fluorescence microscopy but on a macroscopic level, utilizing a low-light camera and proper filters to gather emission light from samples or animals (Rao, Dragulescu-Andrasi, and Yao 2007). This method offers the advantages of fast acquisition time, high spatial resolution, and easy implementation without additional substrates, unlike bioluminescence imaging (BLI) (Liu et al. 2021; Zelmer and Ward 2013). Fluorescent reporters include lanthanide-doped upconversion nanoparticles, QDs, organic dyes, fluorescent proteins, and aggregation-induced emission luminogen (Gioux, Choi, and Frangioni 2010; Kwok et al. 2015; Michalet et al. 2005; Park et al. 2015; Rodriguez et al. 2017; Xu et al. 2016). Although these reporters emit light in the visible range (400–650 nm), their use in vivo is limited by light scattering, attenuation, and autofluorescence (Zhao et al. 2018). Autofluorescence from endogenous substances, such as hemoglobin and water, interferes with imaging (Zhao et al. 2018). Near-infrared (NIR) fluorescence imaging, detecting emissions at 650–900 nm, addresses these issues, enhancing tissue penetration depth (W. J. Liang, He, and Wu 2022; Yan et al. 2019).

BLI is another sensitive technique, producing light through luciferase enzyme reactions in living organisms (Syed and Anderson 2021). This method involves genetically engineered bioluminescent reporter genes that emit light from animals. BLI is characterized by high sensitivity, specificity, signal-to-background ratio, and ability to repeatedly detect signals without autofluorescence, making it suitable for deep spatial resolution imaging (Gangadaran et al. 2017a; Zinn et al. 2008). Widely used bioluminescent reporters include Fluc, Rluc, and Gluc. Fluc, derived from the North American firefly (*Photinus pyralis*), reacts with D-luciferin and requires ATP, Mg^{2+}, and O_2, emitting light in the 550–570 nm range with glow kinetics (Fraga 2008; Kim, Kalimuthu, and Ahn 2015). Rluc, purified from sea pansy (*Renilla reniformis*), catalyzes the non-ATP-dependent oxidation of the substrate coelenterazine, causing emission (maximum emission 482 nm) (Bhaumik and Gambhir 2002; Kim, Kalimuthu, and Ahn 2015). Gluc, the smallest luciferase cloned (18 kDa), offers several advantages compared with commonly used bioluminescent reporters: its sensitivity is 2000 and 20,000 times greater than that of Fluc and Rluc, respectively (Badr et al. 2007; Tannous et al. 2005).

MRI can characterize soft tissue contrast with high spatial resolution (B. C. Ahn 2014; Wu and Shu 2018). MRI can be categorized into direct and indirect detection methods. Direct detection uses several nuclear species (e.g., ^{23}Na, ^{19}F, or ^{13}C) to detect metabolic and biochemical changes (Albers et al. 2005; Babsky et al. 2005; Hu et al. 2017; Mahon et al. 2004). In contrast, indirect detection observes the effects of an agent on the hydrogen nuclei (protons) in tissue water either by introducing new pathways for magnetization transfer or by changing the water relaxation rate (Gore et al. 2011). Accordingly, functional multimodal iron oxide nanoparticles have been developed for targeted atherothrombosis using chemical and biological conjugation techniques (Ta et al. 2017).

SPECT and PET are nuclear imaging techniques measuring molecular targets and biochemical changes, providing early diagnostic capabilities before physiological changes occur (Wu and Shu 2018). Additionally, they have high sensitivity, excellent quantification potential, feasible possibility for clinical translation, and high resolution with excellent tissue penetration depth (B. C. Ahn 2014). PET detects two time-coincident high energy photons (511 keV) by positron annihilation (emitted by decaying radionuclides) (Volpe et al. 2021). Meanwhile, SPECT detects various gamma rays produced by the decay of radionuclides (Wu and Shu 2018). Therefore, SPECT imaging is advantageous for simultaneous multi-tracer studies compared with PET imaging (Volpe et al. 2021).

Currently, various molecular imaging techniques are available for the noninvasive monitoring of externally administered EVs within living organisms (Verweij et al. 2021). These methods provide crucial information about the function and movement of EVs in real time within an undisturbed in vivo setting. This information is vital for understanding the dynamic interaction between EVs and their targets at the cellular and molecular levels.

4.2 | Optical Imaging

4.2.1 | Fluorescence Imaging

Over the past few years, fluorescence technology has been increasingly used in monitoring and visualizing EVs (Q. Liu et al. 2022b). In fluorescence imaging, EVs are labeled with

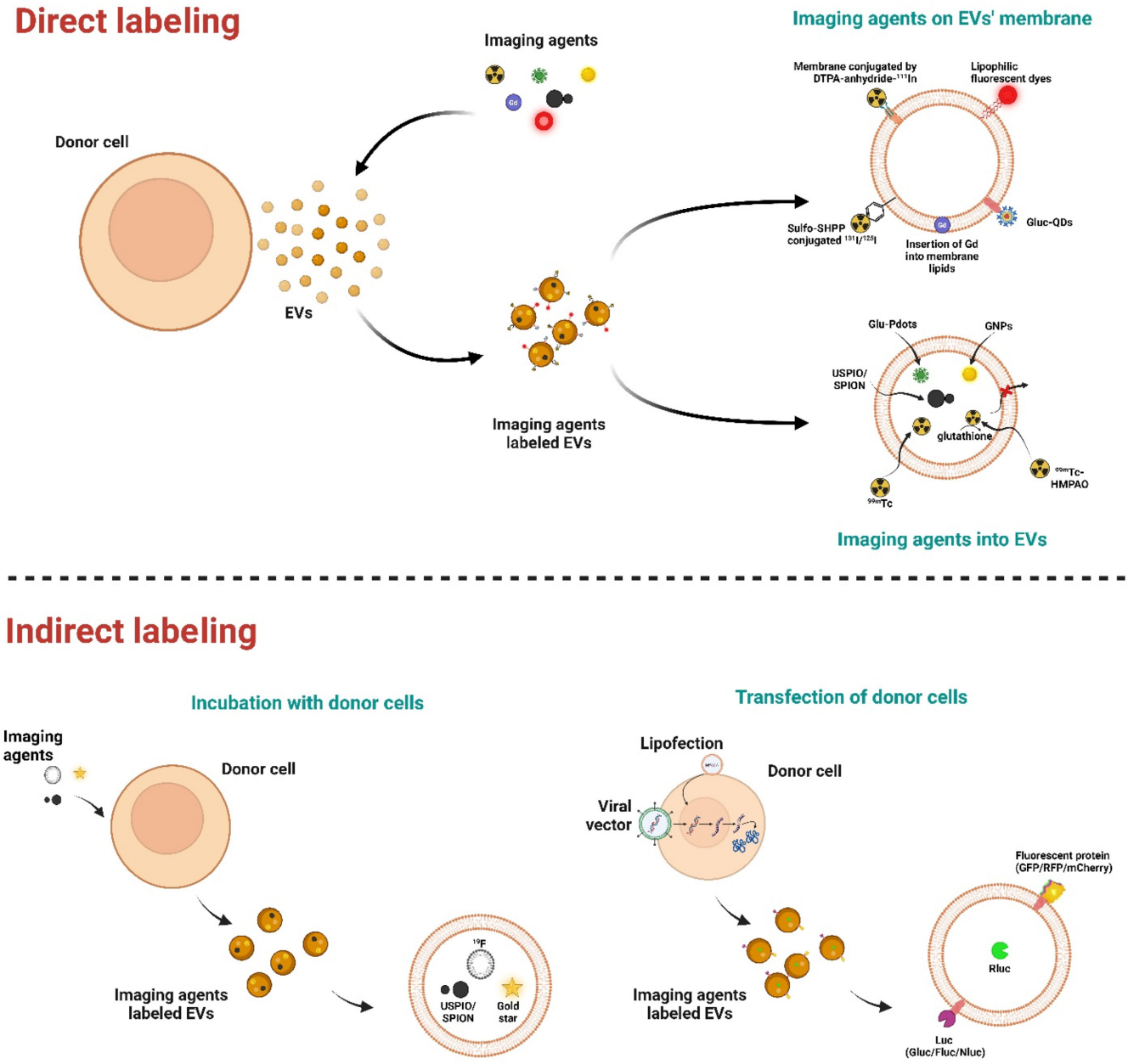

FIGURE 4 | Direct and indirect labeling of EVs. In direct labeling, EV membranes are directly labeled by incorporating agents into the membrane bilayers, attaching agents to the EV surface, or incorporating them onto EVs. These labeling approaches include lipophilic fluorescent dyes (DiR, DiD, Cy7), glucose-coated QDs or Pdots, phospholipids conjugated to gadolinium (Gd), and ^{99m}Tc and ^{111}In. Additionally, GNPs, USPIOs, or SPIONs can be directly loaded into the EV core. Specifically, ^{99m}Tc-HMPAO reacts with glutathione within the vesicle core, facilitating the entrapment of the tracer. Meanwhile, indirect labeling achieved through transduction produces EVs with membranes containing luciferases (Gluc, Rluc, Fluc, and Nluc) or fluorescent proteins (GFP, RFP, and mCherry). Ultrasmall nanoparticles, including USPIOs, GNPs, and gold stars, are also used in these labeling strategies. EVs, extracellular vesicles; QDs, quantum dots; Pdots, polymer dots; GNPs, gold nanoparticles; USPIOs, ultrasmall superparamagnetic iron oxides; SPIONs, superparamagnetic iron oxide nanoparticles; ^{99m}Tc-HMPAO, technetium-99 m hexamethylpropyleneamine oxime; Gluc, Gaussia luciferase; Rluc, Renilla luciferase; Fluc, Firefly luciferase; Nluc, NanoLuc luciferase; GFP, green fluorescent protein; RFP, red fluorescent protein; mCherry, monomeric cherry fluorescent protein. Created with BioRender.com.

fluorescent dyes, proteins, or other substances, allowing them to emit fluorescent signals when exposed to external excitation light. This enables effective tracking and imaging of EVs in living animals.

A study by Grange et al. used NIR dye (DiD) to label EVs and investigate their biodistribution and renal localization in acute kidney injury (AKI). EVs were directly labeled with DiD, or dye-labeled EVs were acquired from mesenchymal stem cells (MSCs) preincubated with DiD (cell-labeled EVs). Fluorescence imaging showed that DiD-labeled EVs accumulated specifically in the kidneys of AKI mice and the liver of healthy mice. Directly labeled EVs produced higher fluorescence and sustained background signals in both AKI and

healthy mice, but cell-labeled EVs displayed greater specificity for the injured kidney (Grange et al. 2014; Haque et al. 2019). Wiklander et al. explored the biodistribution of EVs labeled with an NIR lipophilic dye (DiR) in mice. Intravenously delivered DiR-labeled HEK293T EVs exhibited a dose-dependent tissue uptake, prominently in the liver, spleen, gastrointestinal tract, and lungs. Different administration routes (intraperitoneal, subcutaneous, and intravenous) influenced organ biodistribution of the administered EVs. EVs from various mouse cell sources (C2C12, B16F10, and DC cells) predominantly accumulated in the liver, spleen, and lungs. Additionally, using a rabies virus glycoprotein-targeting moiety in DC-EVs showed a brain-specific distribution (Wiklander et al. 2015). In another study, Wen et al. labeled EVs from highly metastatic murine breast cancer cells (EO771, 4T1, and 67NR) with DiD dye and injected them into healthy mice. These EVs accumulated predominantly in the lungs, highlighting their role in breast cancer metastasis and immunosuppression (Wen et al. 2016). Imai et al. addressed the challenges of low production yield and rapid clearance of EVs via liver macrophages. They produced EVs more efficiently using a hollow-fiber bioreactor. Proteomic analyses identified scavenger receptor class A (SR-A) as a receptor for monocyte/macrophage uptake of EVs. Blocking SR-A reduced liver uptake of DiR-labeled EVs and increased circulating EVs, altering their biodistribution. This increased tumor accumulation of EVs, providing insights for enhancing EV therapeutic effects (Imai et al. 2015). Additionally, Watson et al. employed a hollow-fiber bioreactor for efficient production of EVs. They also identified SR-A as a new receptor involved in the monocyte/macrophage uptake of EVs. They altered the biodistribution of DiR-labeled EVs in mice by blocking SR-A with dextran sulfate or chondroitin. This SR-A blockade reduced liver uptake of EVs by approximately 50% and increased the amount of circulating EVs. This also resulted in higher tumor accumulation of EVs, suggesting significant implications for EV-based therapeutic strategies (Watson et al. 2016). Gangadaran et al. presented a method to enhance the retention of MSC-derived EVs (MSC-EVs) at target sites using a gel matrix. They injected MSC-EV/DiD with and without gel into the mouse thigh muscles. The gel-incorporated MSC-EV/DiD maintained substantial fluorescence signals for up to 48 h, indicating increased retention at the injection site (Gangadaran et al. 2017b). Luminescent materials with aggregation-induced emission characteristics (AIEgens), such as DPA-SCP, provide advantages in biomedical imaging, including excellent biocompatibility, photobleaching resistance, and high signal-to-noise ratio (Wang et al. 2018). Cao et al. developed DPA-SCP for efficient labeling of EVs. In a mouse model of acute liver injury (ALI), AIEgens-labeled EVs accumulated in the liver, peaking between days 1 and 6 in healthy mice and showing significant signals up to day 7 in ALI mice. This method allows for noninvasive, real-time monitoring of EVs in vivo (Cao et al. 2019). In another study, DiR-labeled EVs from 4T1 breast cancer cells were injected into mice. These EVs specifically accumulated in the lungs, liver, and spine, modifying immune cell composition in these organs and suggesting their role in metastasis (Gerwing et al. 2020). Scott et al. utilized a membrane-tethered fluorophore reporter system to fluorescently label cells and their corresponding EVs in the transgenic zebrafish model. They tracked endogenous EVs from various cell types using high-spatiotemporal-resolution light-sheet live imaging allowing for the precise tracking of endothelial and cardiomyocyte-derived EVs in vivo, providing insights into EV dynamics and function in the model system (Scott et al. 2021). The potential of MSC-EVs for targeted drug delivery to thyroid tumors was investigated using the IL-4R-binding peptide (IL4RPep-1). In a Cal-62 tumor model, mice received intravenous DiD-labeled IL4RPep-1-conjugated EVs and a control peptide (NSSSVDK). IL4RPep-1-EVs specifically accumulated in the tumor within 2 h and remained present at 3 and 24 h, unlike NSSSVDK-EVs. This study highlights the potential of IL4RPep-1 for targeted delivery to IL-4R-expressing anaplastic thyroid tumors (Gangadaran et al. 2022). Zhang et al. demonstrated that neutrophil exosomes (N-Ex) could induce apoptosis in tumor cells by delivering cytotoxic proteins. They decorated N-Ex with SPIONs to enhance tumor targeting under an external magnetic field (Figure 5). DiR-labeled SPION-Exs selectively accumulated in the tumor, significantly inhibiting tumor growth and prolonging survival in mice. This approach provides a promising strategy for targeted tumor therapy using magnetic fields to guide EVs (Zhang et al. 2022). These studies collectively illustrate the advancements and applications of fluorescence imaging in tracking and understanding the biodistribution, therapeutic potential, and dynamics of EVs in vivo.

4.2.2 | BLI

BLI is extensively applied in basic research for in vivo visualization. Tagging EVs with luciferase, similar to fluorescent proteins, is performed by genetically fusing luciferase with exosome-associated proteins. This fusion allows real-time tracking of EVs within living organisms by detecting the optical signal emitted when luciferase interacts with its substrate.

Takahashi et al. demonstrated that Gluc-lactadherin could be used to label EV membranes, which were then isolated from transfected B16BL6 murine melanoma cells. In vivo BLI showed rapid distribution of these EVs to various organs, primarily the liver and lungs, with minimal serum luciferase activity 4 h post-injection, indicating quick clearance (Y. Takahashi et al. 2013). They further investigated the role of macrophages in this process by depleting macrophages in mice, causing a significantly reduced clearance of EVs and highlighting the role of macrophages in EV elimination (Imai et al. 2015). Gangadaran et al. developed an in vivo BLI system using Rluc to track EVs from thyroid (CAL-62) and breast cancer (MDA-MB-231) cells. CAL-62 EVs were localized mainly in the lungs, while MDA-MB-231 EVs were located primarily in the liver, demonstrating differential EV distribution (Gangadaran et al. 2017a). Charoenviriyakul et al. used Gluc-labeled EVs from various mouse cell lines, observing predominant liver localization shortly after injection despite differences in EV characteristics (Charoenviriyakul et al. 2017). In another study, Gupta et al. explored bioluminescent labeling of EVs using luciferase enzymes bound to CD63, creating a sensitive system for in vivo EV tracking. EVs were isolated after tetraspanins were fused with NanoLuc or ThermoLuc and transduced into HEK-293T cells. NanoLuc-labeled HEK-293T EVs showed increased luminescence

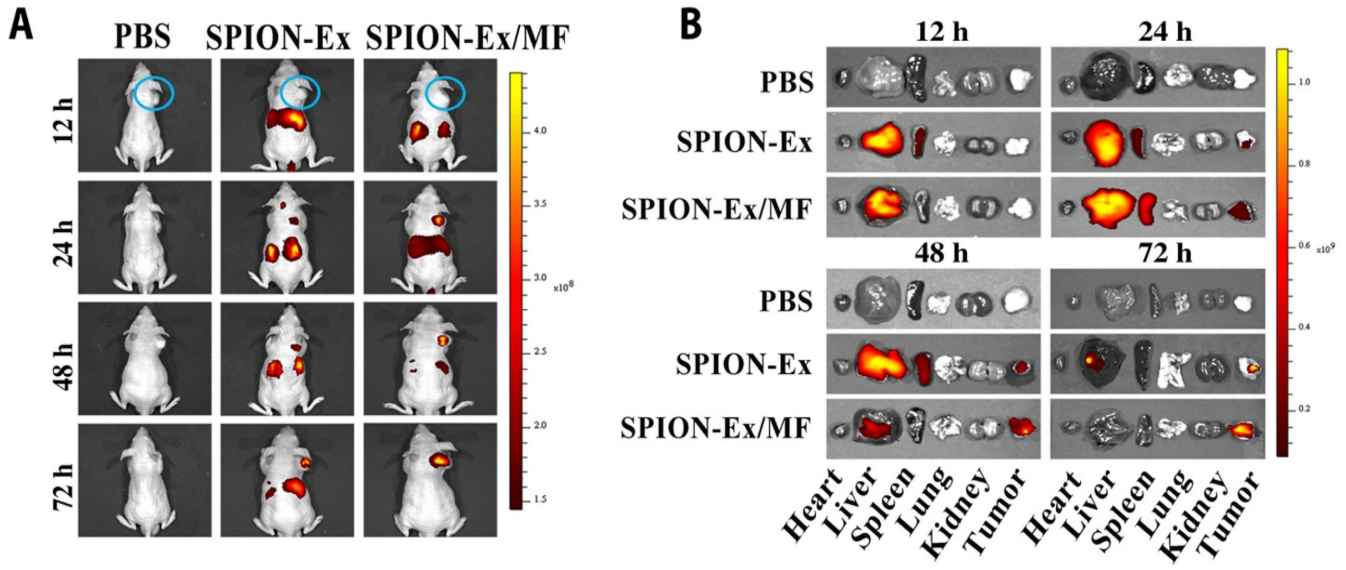

FIGURE 5 | In vivo assessment of SPION-Ex tumor-targeting capability. (A) Images showing the dispersion of DiR-labeled SPION-Ex (5 mg/kg of body weight) in BALB/c nude mice were captured at 12, 24, 48, and 72 h after an intravenous injection under the influence of an external magnetic field. Each group comprised four mice ($n = 4$), and circles indicate tumor sites. (B) Representative images depicting ex vivo fluorescent signals from DiR-labeled SPION-Ex (5 mg/kg of body weight) in major organs (the liver, spleen, lung, heart, and kidney) and tumors were captured at 12, 24, 48, and 72 h after intravenous injection. SPION, superparamagnetic iron oxide nanoparticles; Ex, exosomes from neutrophils; DiR, 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine; MF, external magnetic field.
Source: The cropped figure has been reprinted with permission from Zhang et al. (2022).

efficiency and stability in vitro. BLI of ThermoLuc-labeled EVs demonstrated superior performance due to its NIR emission wavelength. In vivo BLI revealed distinct liver and spleen distribution patterns of ThermoLuc-labeled HEK-293 T EVs 30 min after injection. This technology allows for noninvasive, real-time imaging of EVs, providing insights into their rapid distribution to various internal organs shortly after administration (Gupta et al. 2020; Figure 6).

4.3 | ^{1}H-MRI

SPIONs, ranging from 5 to 200 nm, exhibit superparamagnetic properties allowing precise control by magnetic fields, making them valuable in in vivo MR tracking and visualization of EVs (Laurent et al. 2014). Recently, MRI technology has become increasingly used to monitor and visualize EVs in living subjects. Hu et al. used electroporation to load melanoma exosomes with SPIONs for in vivo MR tracking in C57BL/6 mice. After injecting SPIONs and SPION-loaded exosomes into the mice's left foot pads, a T1-weighted MRI revealed significant nodal enhancement and enlargement in 48 h. Mice with SPION-loaded exosomes showed a 110% increase in lymph node size, compared to an 80% increase with free SPIONs. This study demonstrates the potential of in vivo exosome tracking, paving the way for its future diagnostic and therapeutic applications (Hu, Wickline, and Hood 2015). Another study developed a protocol to label adipose stem cell (ASC)-derived exosomes with USPIO for MR visualization. The ASCs were fully labeled with USPIO while maintaining viability, with their exosomes retaining the nanoparticles. In vivo MRI of mice that received USPIO-labeled exosomes showed distinct localized signal reductions, similar to those with unmodified USPIO, confirming high MRI sensitivity. This method enables effective exosome detection by MRI without altering their morphology or physiological properties (Busato et al. 2016).

Magnetic particle imaging (MPI) has been used for cancer detection by visualizing tumors through the accumulation of magnetic nanoparticles in malignant tissues (Huang et al. 2023; Makela et al. 2020; Williams et al. 2023). MPI has been used to track EVs in tumors animal models. For examples, Jung et al. developed SPIO-labeled exosomes to target hypoxic tumor regions and monitored the process with an MRI. In healthy mice, these exosomes predominantly accumulated in the liver. When loaded with olaparib, a PARP inhibitor, and injected into tumors, MRI confirmed the retention of SPIO-labeled exosomes, which exhibited therapeutic efficacy. This study demonstrated a promising strategy for monitoring and treating hypoxic tumors using exosomes (Jung et al. 2018). A recent study Toomajian et al. used MPI to track EV biodistribution by labeling them with SPIO nanoparticles. Results show that iron-labeled EVs accumulate longer in tumors and can be detected in brain metastases, highlighting their potential for targeted drug delivery in metastatic disease (Toomajian et al. 2024).

4.4 | CT

Exosomes can traverse the blood–brain barrier. However, their migration and homing within the brain have not been extensively studied. A recent study has used in vivo neuroimaging with x-ray CT and gold nanoparticles to track intranasally administered MSC-Exos in various brain diseases. They found specific targeting and accumulation in pathological brain regions

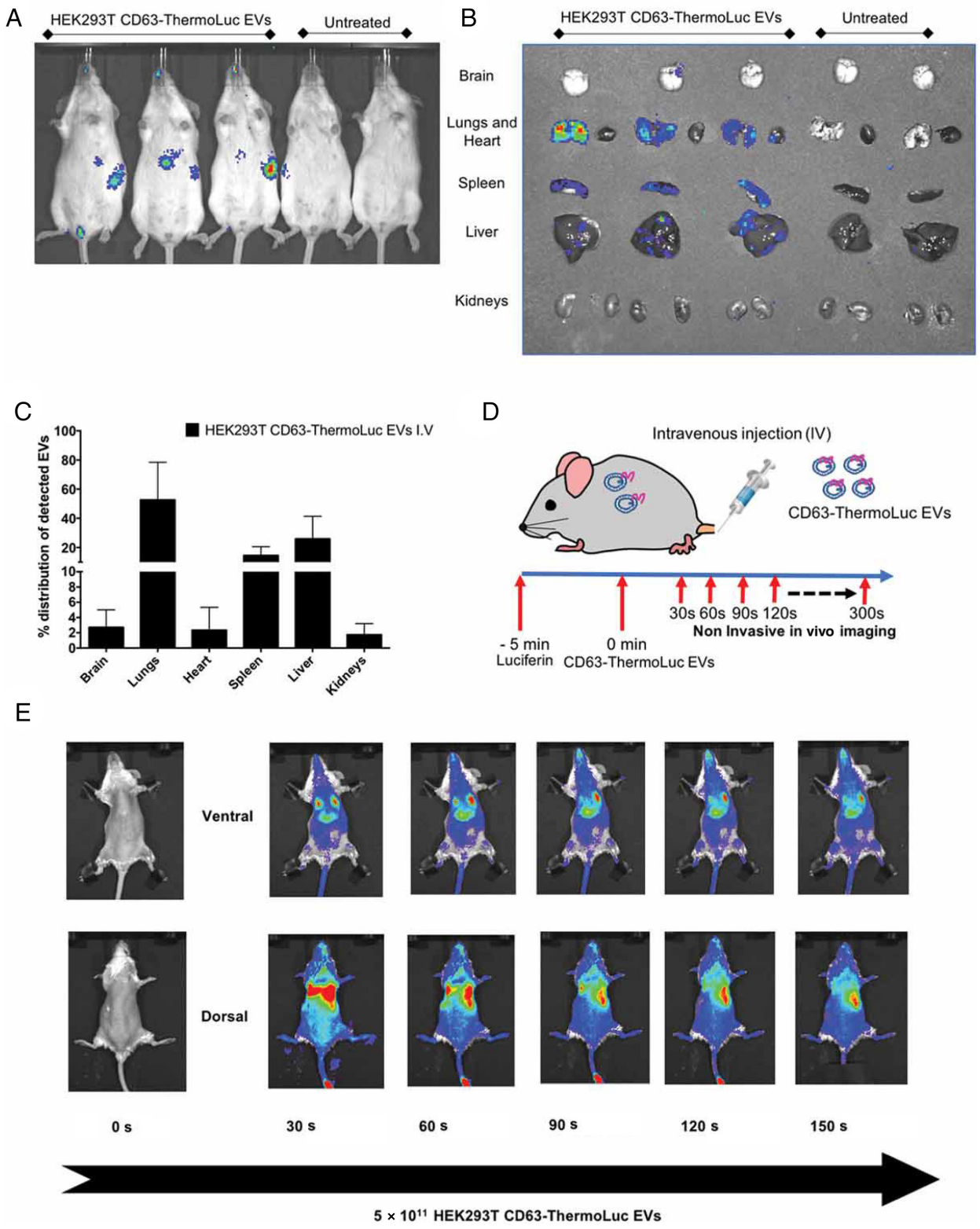

FIGURE 6 | Bioluminescence labeling for real-time in vivo tracking of EVs. (A and B) Noninvasive live animal and ex vivo organ imaging, respectively, in animals 30 min after receiving intravenous 5×10^{11} HEK-293 T cell-derived CD63-ThermoLuc EVs or phosphate-buffered saline ($n = 4$). All animals received an intraperitoneal injection of D-luciferin before imaging. (C) Percentage distribution of detected CD63-ThermoLuc EVs determined through ex vivo quantification. (D) Visualization of EV biodistribution in real time. (E) Noninvasive imaging of mice seconds after the intravenous administration of CD63-ThermoLuc EVs ($n = 2$). EVs, extracellular vesicles.

Source: The figure has been reprinted with permission from Gupta et al. (2020).

up to 96 h post-administration, suggesting potential therapeutic applications for exosomes in neurologic disorders (Perets et al. 2019).

In another study, Guo et al. demonstrated that intranasally administered MSC-Exos labeled with GNPs can traverse the blood–brain barrier and migrate to the injured spinal cord area. Micro-CT scanning revealed a significant accumulation of GNPs in the spinal cord lesion, with minimal presence in healthy brain tissue. Additionally, MSC-Exos loaded with ExoPTEN effectively reduced PTEN expression in the injured spinal cord, promoting recovery in spinal cord injury models (Guo et al. 2019).

4.5 | ^{19}F-MRI

Current imaging tools limit our understanding of the in vivo behavior of EVs, while chemical labels pose a risk of altering membrane features. To address these problems, Sancho-Albero et al. introduced the first fluorinated EVs incorporating PERFECTA, a molecule with 36 magnetically equivalent ^{19}F atoms, as ^{19}F-MRI probes. PERFECTA-EVs demonstrated a distinct fluorine signal in tumor and bone marrow regions upon intravenous administration into tumor-bearing mice (Figure 7), confirming their tumor-targeting capabilities. This study highlights their potential for visualizing biodistribution and EV delivery throughout the body (Sancho-Albero et al. 2023).

4.6 | Nuclear Imaging

Radionuclide labeling is a valuable tool in EV tracing, offering superior sensitivity and stable imaging performance compared to conventional optical labeling methods. This technique is significantly useful in various applications, including tissue imaging, in vivo studies, and clinical imaging (B.-C. Ahn 2014; Lee et al. 2013). Among the modalities used in preclinical research, nuclear imaging stands out for its exceptional sensitivity, safety, and potential for seamless translation into clinical settings (Pellico, Gawne, and de Rosales 2021).

Hwang et al. developed a radiolabeled macrophage-derived exosome-mimetic nanovesicles (ENVs) with technetium-99 m hexamethylpropyleneamine oxime (^{99m}Tc-HMPAO) and monitored their in vivo distribution in living mice using SPECT/CT.

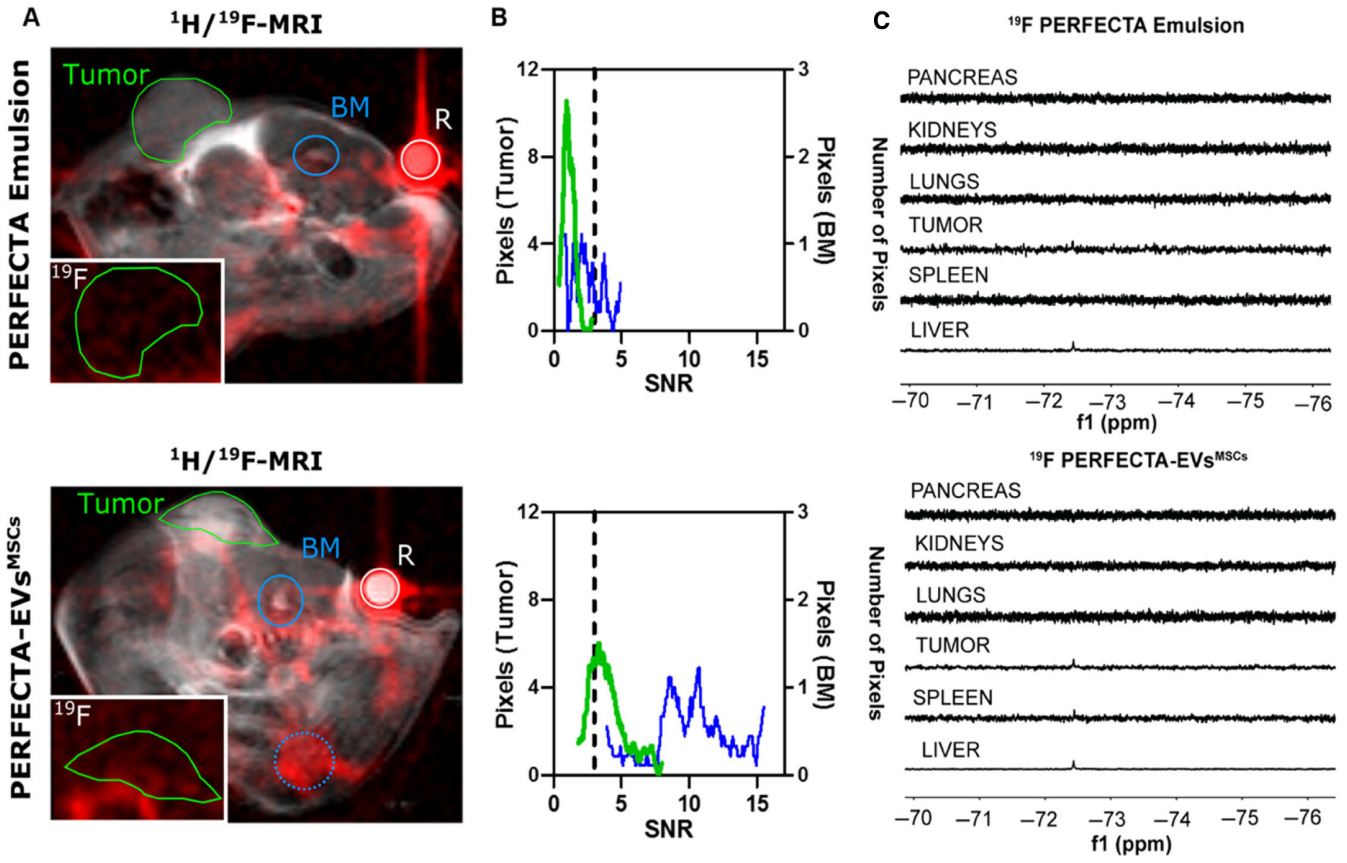

FIGURE 7 | Tumor-homing ability of PERFECTA-EVs^{MSCs}. In vivo ^{19}F-MRI of BALB/c nude mice with HeLa tumors is shown in (A) with merged ^{1}H and ^{19}F-MRI images (red) from representative mice 48 h after the administration of PERFECTA emulsion (top) and PERFECTA-EVs^{MSCs} (bottom). The tumor region is delineated by a green circle, whereas the bone marrow (BM) of the spinal cord (solid) and knee (dotted) are indicated by blue circles. The white circle in each image represents the standard reference (R). The bottom-left white box shows the magnified ^{19}F-MRI image, focusing on the tumor mass. (B) Histograms of ^{19}F signal-to-noise ratio (SNR) distribution in the tumor and bone marrow of the spinal cord for each mouse. The dashed line signifies the SNR limit above which the ^{19}F signal is detectable (SNR > 3). (C) ^{19}F-NMR spectra of harvested organs from a representative mouse treated with PERFECTA emulsion and PERFECTA-EVs^{MSCs}. EVs, extracellular vesicles; MRI, magnetic resonance imaging. *Source:* The figure has been reprinted with permission from Sancho-Albero et al. (2023).

They observed a high uptake of ^{99m}Tc-HMPAO-ENVs in the liver and no uptake in the brain, providing valuable insights into the in vivo behavior of exosomes (Hwang et al. 2015). An alternative approach involves manipulating donor cells through genetic engineering to target nonnative binding groups introduced in EVs. Morishita et al. genetically engineered donor cells to radiolabel melanoma B16BL6 cell-derived exosomes with iodine-125 (^{125}I) using the streptavidin (SAV)-biotin system. The engineered exosomes were injected intravenously into mice and tracked using a gamma counter, depicting their primary accumulation in the liver, followed by the lungs. While effective for radionuclide conjugation without interfering with native active sites, this approach may not be universally applicable to all EV types (Morishita et al. 2015). A novel method was developed for outer-membrane labeling of red blood cell (RBC)-derived EVs using an organometallic technetium-99 m–tricarbonyl complex. This complex has a high affinity to amino acids such as histidine, methionine, and cysteine on EV membranes, enabling straightforward surface labeling. SPECT/CT imaging in a mouse model 1 h after an intravenous administration of labeled EVs revealed significant accumulation in the liver and spleen, validating the reliability and potential application of this radiolabeling procedure for in vivo EV tracking by SPECT imaging. (Varga et al. 2016). Gangadaran et al. created exosome mimetics (EMs) from RBCs and labeled them with technetium-99 m (^{99m}Tc) for in vivo imaging. The labeled RBC-EMs showed nearly 100% radiochemical purity (RCP) up to 2 h, reducing to 97% at 3 h. Upon intravenous administration in mice, in vivo gamma camera imaging revealed higher uptake of ^{99m}Tc-labeled RBC-EMs in the liver and spleen, with no thyroid uptake, compared to free ^{99m}Tc in 1 and 3 h after administration. This study demonstrates a promising approach for in vivo imaging and monitoring of RBC-EM-based drug delivery (Gangadaran et al. 2018; Figure 8). One study investigated ^{99m}Tc-labeled HER2-targeted exosomes for in vivo tumor imaging. These exosomes, derived from genetically engineered cells, exhibited specific binding to HER2 receptors, as demonstrated in cell studies. In tumor-bearing mice, gamma camera imaging confirmed the accumulation of the exosomes in the tumor, suggesting a specific targeting mechanism through interaction with HER2 receptors (Molavipordanjani et al. 2020). Hong et al. demonstrated a reliable method for tracking EVs using radioiodine. EVs from thyroid cancer cells (Cal62) and natural killer cells (NK92-MI) were labeled with radioiodine (I-131 and I-125). In vivo gamma camera imaging after intravenous injection of Cal62-labeled EVs revealed that they accumulated primarily in the liver, spleen, and lungs. These studies highlight the valuable role of nuclear imaging in in vivo tracking EVs in living subjects (Hong et al. 2021).

4.7 | Others

Gold-based nanomaterials, such as gold nanostars, show promise for tumor thermoradiotherapy due to their high atomic numbers and strong absorbance in the NIR-II window. However, they often lack active tumor-targeting ability, penetration efficiency, and stability. A novel solution to these challenges is using tumor cell-derived stellate plasmonic exosomes (TDSP-Exos) that exhibit enhanced accumulation in deep tumor tissues and improved performance in photoacoustic imaging and NIR-II thermoradiotherapy. In a study, TDSP-Exos demonstrated pronounced accumulation in tumor tissues 1–5 min post-injection compared to bare gold nanostars, highlighting their potential as carriers for targeted tumor therapies (Zhu et al. 2020). Another advancement is the use of glucose-coated semiconductor polymer dots (Pdots-Glu), providing efficient labeling and long-term tracking of MSC-Exos without altering their properties. In a liver-resected mouse model, Pdot-labeled MSC-Exos accumulated in the residual liver tissue from 3 h to 7 days post-injection, showing potential for their application in liver regenerative medicine (Ma et al. 2022). Additionally, a study introduced glucose-conjugated quantum dots (QDs-Glu) for noninvasive NIR fluorescence imaging to target and track EVs in a rat model of sciatic nerve injury. QDs-Glu-labeled EVs migrated from uninjured to injured nerve sites, with increased fluorescent signals from 4 to 28 days post-injection. Real-time NIR-II imaging correlated fluorescence intensity with functional recovery, demonstrating the efficacy of EV therapy in peripheral nerve regeneration.

4.8 | Hybrid Multimodal Imaging

Integrating multiple imaging modalities (i.e., multimodality imaging) offers a more effective solution for overcoming the disadvantages of individual techniques. Lai et al. designed a sensitive membrane-bound EV reporter system called EV-GlucB for in vivo multimodal imaging of EVs. This reporter system included Gluc bound to a biotin acceptor domain. In the presence of a biotin ligase in the mammalian system, Gluc is metabolically biotinylated, enabling strong bioluminescent tracking of EVs when incubated with the Gluc substrate coelenterazine. Additionally, the surface biotin enables the labeling and detection of cells using fluorescently tagged streptavidin through fluorescence imaging. Thus, the reporter not only facilitated EV detection but also helped determine the distribution and dynamics of injected EVs (Lai et al. 2014).

In another study, Shaikh et al. simultaneously utilized CT, fluorescence, and MRI to improve early and precise diagnosis of cancer. Specifically, the authors performed in situ biosynthesis of iridium and iron oxide nanoclusters (IrO_2 and Fe_3O_4 NCs) in tumor cells, serving as a versatile probe for both luminescent and MR tracking of tumor cells. Given that NCs form only in cancer cells, the method not only served as an excellent probe for multimodal imaging of tumor-associated EVs but also a potential biomarker for cancer diagnosis (Shaikh et al. 2018).

Table 1 provides a comprehensive overview of the research endeavors focused on in vivo visualization and monitoring of EVs and the limitation of clinical application of FLI, BLI, and photoacoustic imaging.

5 | Implications of in Vivo Imaging in Understanding EV Biology and Related Physiological Events

The in vivo imaging of EVs plays multiple roles. Beyond monitoring the effectiveness and biodistribution of injected EVs

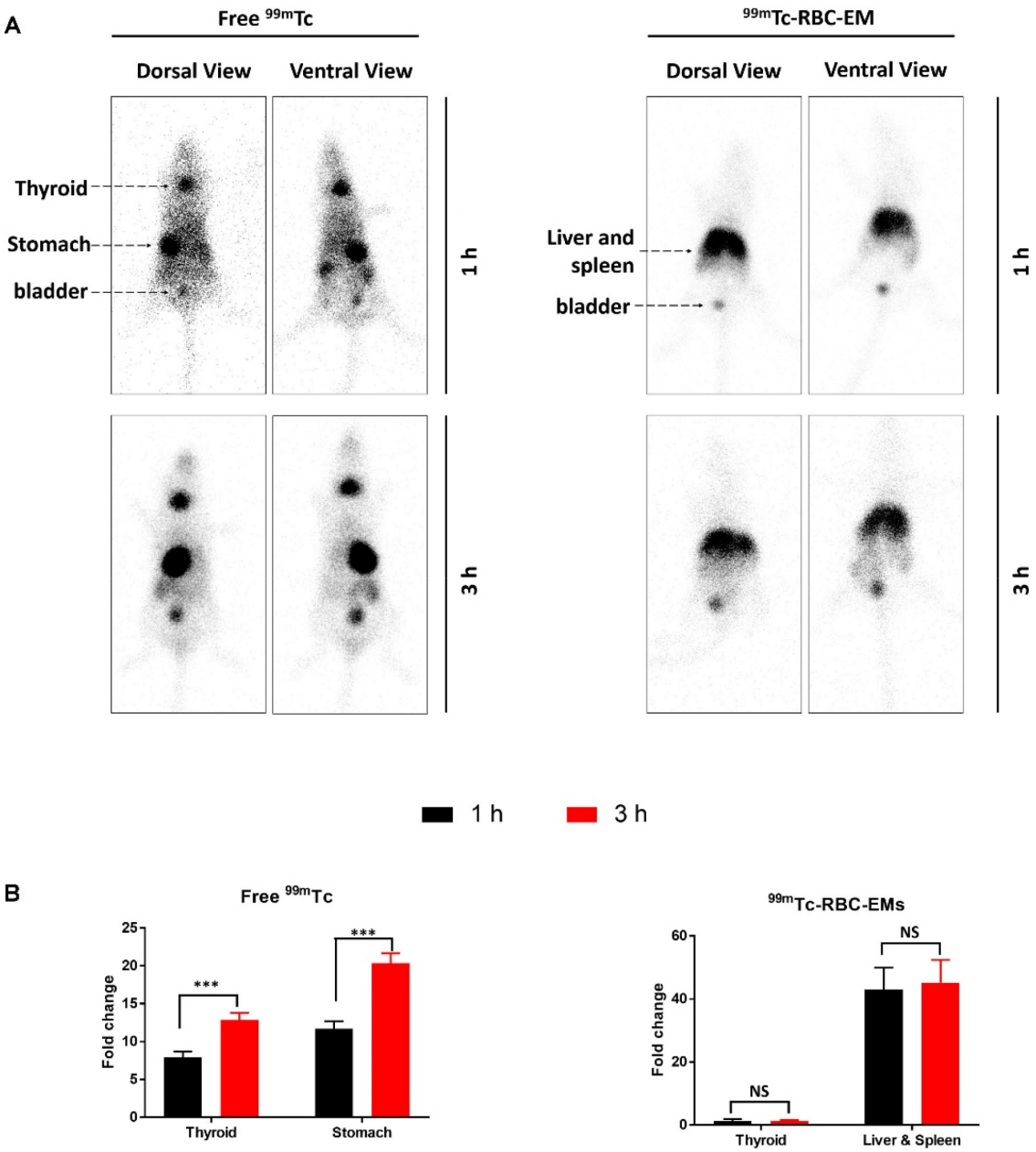

FIGURE 8 | In vivo gamma camera images of free ^{99m}Tc and ^{99m}Tc-RBC-EMs injected into mice. (A) Gamma camera images were acquired at 1 and 3 h post-intravenous injection of free ^{99m}Tc and ^{99m}Tc-RBC-EMs in C57BL/6 mice ($n = 4$). (B) Quantification of ^{99m}Tc and ^{99m}Tc-RBC-EMs in various organs (thyroid, stomach, liver, and spleen) of injected mice, with results depicted in a bar graph. The values are expressed as mean ± standard deviation, with ***$p < 0.001$ (Student's *t*-test). EMs, exosome mimetics; ^{99m}Tc, technetium-99 m; RBC, red blood cell.
Source: The figure has been reprinted with permission from Gangadaran et al. (2018).

carrying specific cargoes, it allows for observing EV biogenesis, secretion, uptake, and function in physiological conditions and related intercellular communication. While mouse models are more relevant to humans, models of lower organisms are frequently used to understand EV-related pathophysiological processes. This choice stems from the limitations of live imaging in complex organisms, such as mice, because of tissue complexity. Additionally, existing technologies may lack the required resolution to capture smaller EVs, and the site of EV accumulation may not always correlate with the site of action. Consequently, lower organisms, such as *Drosophila melanogaster* and vertebrate zebrafish models, with a lower complexity and simpler morphology are widely used to elucidate EV biology. Table 2 summarizes in vivo imaging methods and markers used to study different aspects of EV biology and their function in various model organisms.

6 | Clinical Translatability of Imaging of EVs

In vivo imaging of EVs may enhance their potential for translation into clinical applications. Since, there are currently no clinical trials available. Additional data to accumulate more reference basis for clinical application. Two widely used clinical imaging tools, MRI, and nuclear imaging, may be used for imaging EVs in clinical settings. Table 3 summarizes the clinical

TABLE 1 | In vivo imaging of EVs.

Imaging techniques	Imaging modalities	Imaging instrument	Imaging agent	EVs type	Size	Source of EVs	Animal	Route	Duration	Target	Clinical application	References
Optical imaging	FLI	IVIS	DiD	EVs	145 ± 57 nm	MSCs	CD1 nude mice	IV	15 min, 5 and 25 h	Biodistribution/ injured kidney	Limited#	(Grange et al. 2014)
	FLI	IVIS	DiR	EVs	100 nm	HEK293T, C2C12, B16F10, and DCs	NMRI or C57BL/6	IV, IP, and SC	5, 30 min, 1, 3, 24, and 48 h	Biodistribution/ brain	Limited#	(Wiklander et al. 2015)
	FLI	IVIS	DiD	Exosomes	EO771 (86 nm) 4T1 cells (80 nm)	EO771, 4T1, and 67NR	C57BL/6 or BALB/c mice	IV	4, 24, and 48 h	Biodistribution	Limited#	(Wen et al. 2016)
	FLI	IVIS	Syto RNASelect Green	EVs	40–200 nm	HEK293	FVB or Balb/c mice	IV	1, 2, 4, 8, and 24 h	Biodistribution/ tumor	Limited#	(Watson et al. 2016)
	FLI	IVIS	DiD	EVs	134.8 ± 42.7 nm	MSCs	C57BL/6 mice	IM	12, 24, 36, and 48 h	Ischemic Hindlimb	Limited#	(Gangadaran et al. 2017b)
	FLI	IVIS	AIE (DPA-SCP)	EVs	120 nm	MSCs	FVB mice	IV	1 h; 1–3, 6, and 12 days	Biodistribution/ injured kidney	Limited#	(Cao et al. 2019)
	FLI	IVIS	DiR	EVs	97.3 ± 36.2 nm	4T1	BALB/c mice	IV	30 min, 2 and 24 h	Biodistribution	Limited#	(Gerwing et al. 2020)
	FLI	ZEISS Lightsheet Z.1 system	mCherry	EVs	23–808 nm	Endothelial cells and cardiomyocyte	Zebrafish	Endogenous	—	Biodistribution	Limited#	(Scott et al. 2021)
	FLI	IVIS	DiD	EVs	162.5 ± 8.7 nm	MSCs	Nude Mice	IV	1, 2, 3, and 24 h	Tumor	Limited#	(Gangadaran et al. 2022)
	FLI	IVIS	DiR	EVs	125 ± 10 nm	Neutrophils	BALB/c mice	IV	12, 24, 48, and 72 h	Tumor	Limited#	(Zhang et al. 2022)
	FLI	Pearl Trilogy imaging system	DiR	Exo	171 ± 42 nm (DLS) 66 ± 2 nm (NTA)	UC-MSCs	Nude mice	IV	0.5, 1, 2, 3, 6, 12, 24, and 48 h	Tumor	Limited#	(Abello et al. 2019)
	FLI	IVIS	mCherry	EVs	126–154 nm	Expi293F cells	BALB/c mice	IV	24 h	Biodistribution	Limited#	(Lazaro-Ibanez et al. 2021)
	FLI	IVIS	DiR	EVs	126–154 nm	Expi293F cells	BALB/c mice	IV	24 h	Biodistribution	Limited#	(Lazaro-Ibanez et al. 2021)
	BLI	IVIS	Gluc	Exosome	66 ± 11 nm	B16BL6	BALB/c mice	IV	10, 30, 60, and 120 min	Biodistribution	Limited*	(Y. Takahashi et al. 2013)

(Continues)

TABLE 1 | Continued

Imaging techniques	Imaging modalities	Imaging instrument	Imaging agent	EVs type	Size	Source of EVs	Animal	Route	Duration	Target	Clinical application	References
	BLI	IVIS	Gluc	Exosome	—	B16BL6	BALB/c mice	IV	10, 30, 60, and 240 min	Biodistribution after the depletion of macrophages	Limited*	(Imai et al. 2015)
	BLI	IVIS	Rluc	EVs	CAL-62 (107.7 nm) & MDA-MB-231 (101.8 nm)	Cancer cells (CAL-62 & MDA-MB-231)	Nude Mice	IV	10 and 30 min; 1, 2, 3, 6, 9, and 12 days	Biodistribution	Limited*	(Gangadaran et al. 2017a)
	BLI	IVIS	Gluc	Exosome	100 nm	B16BL6, C2C12, NIH3T3, MAEC, and RAW264.7	BALB/c mice	IV	5 min	Biodistribution	Limited*	(Charoenviriyakul et al. 2017)
	BLI	IVIS	NanoLuc or ThermoLuc	EVs	—	HEK-293T	BALB/c mice	IV	30 min	Biodistribution	Limited*	(Gupta et al. 2020)
	BLI	IVIS	Nluc	EVs	126–154 nm	Expi293F cells	BALB/c mice	IV	1, 4, and 24 h	Biodistribution	Limited*	(Lazaro-Ibanez et al. 2021)
Magnetic resonance imaging/ magnetic particle imaging	MRI	MRI	SPIONs	Exosome	100 nm	Melanoma	C57BL/6 mice	Foot injection	1 and 48 h	Lymph nodes	Yes	(Hu, Wickline, and Hood 2015)
	MRI	MRI	USPIO	Exosome	30–100 nm	Stem cells	C57BL/6 mice	IM	24 and 72 h	Muscle	Yes	(Busato et al. 2016)
	MPI	MPI	USPIO	Exosome	30–200 nm	MDA-MB-231	Nude mice	IV and IT	1 h	Biodistribution/ Tumor	Yes	(Jung et al. 2018)
	MPI	MPI	SPIO	EVs	98 nm	4T1	BALB/c mice	IV	24 and 48 h, and 7 day	Tumor	Yes	(Toomajian et al. 2024)
	microCT	microCT	Gold nanoparticle	Exosome	—	MSCs	C57BL/6, 5xFAD mice & BTBR mice	Intranasal	1, 24, and 96 h	Brain (healthy, stroke, Parkinson's disease, Alzheimer's disease, and autism)	Yes	(Perets et al. 2019)
	microCT	microCT	Gold nanoparticle	Exosome	111 ± 64 nm	MSCs	Rats	Intranasal	24 h	Brain (healthy and spinal cord injury)	Yes	(Guo et al. 2019)
	MRI	MRI	^{19}F	EVs	170–220 nm	MSCs	BALB-nu mice	IV	48 h	Tumor	Yes	(Sancho-Albero et al. 2023)

(Continues)

TABLE 1 | Continued

Imaging techniques	Imaging modalities	Imaging instrument	Imaging agent	EVs type	Size	Source of EVs	Animal	Route	Duration	Target	Clinical application	References
	MRI	MRI	Gadolinium	Exo	171 ± 42 nm (DLS) 66 ± 2 nm (NTA)	MSCs	Nude mice	IV	30 and 90 min	Tumor	Yes	(Abello et al. 2019)
Nuclear imaging	NI	SPECT/CT	^{99}mTc-HMPAO	ENVs	218 ± 8.2 nm	Macrophage	BALB/c mice	IV	0.5, 3, and 5 h	Biodistribution	Yes	(Hwang et al. 2015)
	NI	Gamma counter	^{125}I	Exosome	70 ± 3 nm	B16BL6	BALB/c mice	IV	1, 5, 10, and 30 min; 1 and 4 h	Biodistribution	Yes	(Morishita et al. 2015)
	NI	SPECT/CT	^{99m}Tc	Exosome	80–300 nm	RBCs	BALB/c mice	IV	1 h	Biodistribution	Yes	(Varga et al. 2016)
	NI	Gamma camera	^{99m}Tc	EMs	209.1 ± 19.8 nm	RBCs	C57BL/6 mice	IV	1 and 3 h	Biodistribution	Yes	(Gangadaran et al. 2018)
	NI	Gamma camera	^{99m}Tc	Exosome	76.53 nm	HEK293T	BALB/c mice	IV	1, 2, and 4 h	Biodistribution/ tumor	Yes	(Molavipordanjani et al. 2020)
	NI	Gamma camera	^{131}I	EVs	255.7 ± 18.6 nm	Thyroid Cancer	Nude mice	IV	1, 3, 5, and 24 h	Biodistribution	Yes	(Hong et al. 2021)
	NI	SPECT/CT	^{111}In	EVs	126–154 nm	Expi293F cells	BALB/c mice	IV	0.5, 4, and 24 h	Biodistribution	Yes	(Lazaro-Ibanez et al. 2021)
Other imaging	Photoacoustic imaging	Photoacoustic imaging system	Gold Star	Exosome	96 ± 6 nm	CT26 cancer cells	BALC/c mice	IV	1, 3, and 5 min	Tumor	Limited@	(Zhu et al. 2020)
	FLI	NIR-II small animal imaging system	Polymer Dots	Exosomes	100 nm	MSCs	Balb/c mice	IV	3 h; 1, 2, 3, and 7 days	Injured liver	Limited#	(Ma et al. 2022)
	FLI	NIR-II small animal imaging system	Quantum dot	EVs	166.5 nm	Skin-derived precursors-stem cells	Nude mice	Subcutaneous	0, 7, 14, 21, and 28 days	Nerve injury	Limited#	(Wang et al. 2023)

Note: Limited# (FLI): limited tissue penetration, low image quality, autofluorescence, photobleaching, and limited resolution; Limited* (BLI): limited tissue penetration, low spatial penetration, and requirement of substrate administration; Limited@ (photoacoustic imaging): limited tissue penetration, low image quality, and limited resolution.

Abbreviations: ^{111}In, indium-111; ^{125}I, iodine-125; ^{131}I, iodine-131; ^{99m}Tc-HMPAO, technetium-99 m hexamethylpropyleneamine oxime; BLI, bioluminescence imaging; CD, cluster of differentiation; DiD, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine; DiR, 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine; EMs, extracellular microvesicles; ENVs, extracellular nanovesicles; EVs, extracellular vesicles; FLI, fluorescence imaging; gamma counter, gamma radiation counter; h, hours; IM, intramuscular; IP, intraperitoneal; IT, intrathecal; IV, intravenous; IVIS, in vivo imaging system; microCT, micro-computed tomography; min, minutes; MRI, magnetic resonance imaging; MSCs, mesenchymal stem cells; NI, nuclear imaging; NIR, near-infrared; NIR-II, near-infrared II; RBCs, red blood cells; SC, subcutaneous; SPECT/CT, single photon emission computed tomography/computed tomography; SPIONs, superparamagnetic iron oxide nanoparticles; USPIO, ultrasmall superparamagnetic iron oxide.

TABLE 2 | In vivo imaging of endogenous EVs and their function in a healthy state and disease.

EV biology	Key finding	Imaging technique used	EV marker used	Model organism	Reference
Release	Rab11/35/Syx1A/Rop-dependent exosomal secretion for lipid film assembly crucial for airway gas filling of respiratory networks	Fluorescence imaging	CD63	*Drosophila melanogaster*	(Tsarouhas et al. 2023)
Release	EVs released from the male spermathecal secretory cells after mating initiate egg-laying in females and enhance fertility	Fluorescence imaging	CD63	*Drosophila melanogaster*	(Sanchez-Lopez et al. 2022)
Biogenesis and intercellular communication	Epithelial microvilli-EVs contribute to long-range hedgehog morphogenetic signaling	Fluorescence imaging	Prominin-like (PromL)	*Drosophila melanogaster*	(Hurbain et al. 2022)
Biogenesis and release	Stac-EVs are involved in promoting anastomosis in an Arl3- and Rab27/35-dependent manner	Fluorescence imaging	Staccato (Stac; UNC13D) CD63	*Drosophila melanogaster*	(Camelo et al. 2022)
Biogenesis	Synergistic functions of polycystins LOV-1 and PKD-2 for the biogenesis of ciliary EVs	High-resolution fluorescence imaging	LOV-1 PKD-2	*Caenorhabditis elegans*	(Walsh et al. 2022)
Cargo enrichment, biogenesis, and release	A kinesis-2 motor-dependent and independent release of EV subpopulations occurs via sensory cilia	Fluorescence imaging	CLHM-1 PKD-2	*Caenorhabditis elegans*	(Clupper et al. 2022)
Intercellular communication and release	Environmentally released PKD-2^+ EVs are required for animal communication and mating behavior	Optical imaging	LOV-1 PKD-2	*Caenorhabditis elegans*	(Wang et al. 2014)
Biogenesis, release, uptake, and intercellular (organ) communication	Development of a $CD63^+$ zebrafish model as an in vivo system to study EVs at greater spatiotemporal resolution Syntenin-dependent release of EVs in circulation, which are phagocytosed by macrophages and endothelial cells	Fluorescence imaging	CD63	*Danio rerio*	(Verweij et al. 2019)
Biogenesis and intercellular communication	Tspan4- and 7-dependent migrasome formation and release regulates organ morphogenesis	Fluorescence imaging	Tspan4 Tspan7	*Danio rerio*	(Jiang et al. 2019)
Intercellular communication	EVs derived from tumor cells induce malignancy and metastatic behavior in non-malignant cells	Intravital fluorescence imaging	Different dyes and Cre^+ reporters	*Mus musculus*	(Zomer et al. 2015)

Abbreviations: *Caenorhabditis elegans*, nematode worm; CD, cluster of differentiation; CLHM-1, cilia and flagella high-mobility group protein 1; *Danio rerio*, zebrafish; *Drosophila melanogaster*, fruit fly; EV, extracellular vesicle; LOV-1, lobe of Venus 1; *Mus musculus*, house mouse (common laboratory mouse); PKD-2, polycystic kidney disease 2; Rab11, Ras-related protein Rab-11; Rop, Rho-related GTPase from plants; Stac, Staccato (UNC13D); Syx1A, syntaxin-1A; Tspan4, tetraspanin-4; Tspan7, tetraspanin-7.

TABLE 3 | Clinical translatability of imaging of EVs.

Imaging modalities	Applications of EVs	Strengths	Limitations	Reference
MRI	• Drug delivery • Diagnosis	• Excellent tissue penetration • High resolution due to superior soft tissue contrast • No ionizing radiation exposure • Long-term imaging capability	• Susceptible to artifacts due to motion or metallic implants • Risk of contrast agents • Less effective for calcified tissues like bone • Less sensitivity • Long imaging time • High cost • Limited availability	(Aerts et al. 2014; García-Figueiras et al. 2019; Grover et al. 2015; Zhang et al. 2010)
NI	• Drug delivery • Diagnosis	• High sensitivity • Whole body imaging • Relatively high spatial resolution • High tissue penetration	• Ionizing radiation exposure • Complex radiotracer development • Limited time imaging due to half-life of radionuclides • Long imaging time • Limited availability	(Aerts et al. 2014; García-Figueiras et al. 2019; Israel et al. 2019; Schwenck et al. 2023; Sudarshan et al. 2021; Trotter et al. 2023; Vaquero and Kinahan 2015)

Abbreviations: MRI, magnetic resonance imaging; NI, nuclear imaging.

translatability of imaging modalities for EVs, detailing their applications, strengths, and limitations.

7 | Challenges and Perspectives of in Vivo Imaging of EVs

In vivo imaging of EVs and NVs can provide invaluable insights into their behavior, biodistribution, and functional roles within living organisms. However, the field has its significant challenges. Researchers often rely on using contrast agents or fluorescent markers to visualize EVs (Gangadaran et al. 2018; Verweij et al. 2021; Zhu et al. 2016). Although essential for detection, these markers can alter the properties of EVs, potentially influencing their behavior, biodistribution, and biological functions. Fluorescent probes and fluorescence imaging often face limitations such as diminishing concentration and signal attenuation in tissues over time, making them unsuitable for deep tissues and prolonged EV monitoring.

Although fluorescent proteins generate robust signals, their effectiveness diminishes with tissue penetration, limiting their use to labeling tissues with up to 2-mm depth in animals (Kobayashi et al. 2011). Consequently, this strategy is more valuable for in vitro or ex vivo cell sorting than in vivo deep tissue imaging. EVs labeled with fluorescent dye generally accumulate in the liver possibly because the size of EVs increases after labeling (Dehghani et al. 2020). EVs are rapidly cleared by the reticuloendothelial system, including the liver and spleen, limiting their availability for imaging over extended periods and in various organs. Conventional optical imaging techniques have limited tissue penetration capabilities (Dang et al. 2019; X. Liang et al. 2022). Simultaneously using two or multiple reporter genes (Gluc, Rluc, and Fluc) could overcome the constraints of using a solitary fusion protein with a reporter, enabling the labeling of EV-producing cells, promoting uniform monitoring levels, and achieving simultaneous monitoring of multiple components (Zomer et al. 2015). Compared to traditional membrane dye methods, genetically engineered labeling techniques offer superior specificity, flexibility, minimal cell disruption, and increased stability (Imai et al. 2015). Directly tracing clinical sample-derived EVs is challenging, but the remarkable flexibility and stability of genetic labeling approaches hold promise for investigating EV biogenesis, in vivo transport, cellular uptake, distribution, and clearance. Imaging techniques (fluorescence, bioluminescence, radioactive, and MR imaging) and contrast labeling methods significantly influence the sensitivity and accuracy of detecting and tracking EVs. Therefore, the advantages and disadvantages of different imaging techniques need to be carefully considered before embarking on in vivo biodistribution studies (Gangadaran et al. 2017a; Lazaro-Ibanez et al. 2021). In vivo imaging of EVs presents challenges due to limitations in existing techniques (e.g., MRI and CT) that have restricted sensitivity and rely on surrounding tissue signals (Cassidy and Radda 2005; Kim, Lee, and Hyeon 2017; C. Liu et al. 2022a). Most MRI contrasts loaded onto EVs via electroporation may further influence their integrity, complicating accurate biodistribution assessments (Gangadaran and Ahn 2020; Herrmann, Wood, and Fuhrmann 2021). Nuclear imaging has superior sensitivity and resolution but raises concerns about radiation exposure (Chin et al. 2003; Gangadaran et al. 2018; Hong et al. 2021). Moreover, accurately quantifying the number of EVs in each tissue or organ using imaging techniques is challenging.

Despite these challenges, in vivo imaging of EVs holds significant promise and exciting perspectives. Researchers are actively exploring avenues to enhance EV visualization with improved resolution and sensitivity while minimizing interference with EV function. Sophisticated animal models, including genetically modified mice, facilitate real-time tracking of EVs, providing valuable insights into their behavior and interactions in living organisms. The potential clinical applications of EV imaging,

such as disease detection and therapeutic response monitoring, highlight its significance in revolutionizing personalized medicine. Continued research efforts are crucial for overcoming existing challenges and fully realizing the potential of EV imaging in biomedical research and clinical practice.

8 | Conclusion

The exploration of cutting-edge technologies for in vivo visualization of EVs has advanced our understanding of these elusive cell-derived nanoparticles. These advancements hold immense promise for uncovering their crucial roles in intercellular pathophysiologic communication, disease initiation, disease progression, and therapeutic interventions. We can now observe the previously unseen dynamics of EVs within living organisms through innovative imaging and tracking techniques, illuminating their intricate behaviors and functions.

The rapid evolution of this field ensures that we stand on the brink of discovering more secrets and therapeutic opportunities hidden within these tiny vesicles. As we continue to explore EVs, the potential for groundbreaking discoveries in diagnostics and therapy becomes increasingly apparent. The pursuit of in vivo visualization technologies for EVs represents a pivotal step toward unlocking their full potential in biology and medicine, paving the way for transformative advancements in healthcare.

Author Contributions

Prakash Gangadaran: conceptualization (lead), data curation (lead), formal analysis (lead), funding acquisition (equal), investigation (lead), writing – original draft (lead), writing – review and editing (equal). **Fatima Khan:** conceptualization (equal), data curation (equal), formal analysis (equal), investigation (equal), resources (equal), writing – review and editing (equal). **Ramya Lakshmi Rajendran:** conceptualization (lead), data curation (lead), formal analysis (lead), funding acquisition (equal), investigation (equal), writing – original draft (lead), writing – review and editing (equal). **Akanksha Onkar:** data curation (supporting), investigation (supporting), resources (supporting), writing – review and editing (supporting). **Anshika Goenka:** data curation (supporting), investigation (supporting), resources (supporting), writing – review and editing (supporting). **Byeong-Cheol Ahn:** conceptualization (lead), data curation (equal), formal analysis (equal), funding acquisition (supporting), investigation (lead), methodology (lead), project administration (lead), resources (lead), software (equal), supervision (lead), validation (lead), visualization (lead), writing – original draft (supporting), writing – review and editing (lead).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Related Wires Articles

Advances in imaging strategies for in vivo tracking of exosomes

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