

DOCTORAL THESIS

Engineered Exosomes Via Peptide Post Insertion Encapsulating Vinorelbine as Precision Nanomedicine for Lung Adenocarcinoma

GAURAV, Isha

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**Engineered Exosomes Via Peptide Post Insertion
Encapsulating Vinorelbine as Precision Nanomedicine for
Lung Adenocarcinoma**

GAURAV Isha

A thesis submitted in partial fulfilment of the requirements

for the degree of

Doctor of Philosophy

Principal Supervisor

Prof. YANG Zhijun (Hong Kong Baptist University)

September 2024

DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of PhD at Hong Kong Baptist University and has not been previously included in a thesis or dissertation submitted to this or any institution for a degree, diploma, or other qualifications.

I have read the University's current research ethics guidelines and accept responsibility for the conduct of the procedures in accordance with the University's Research Ethics Committee (REC). I have attempted to identify all the risks related to this research that may arise in conducting this research, obtained the relevant ethical and/or safety approval, and acknowledged my obligations and the rights of the participants.

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ABSTRACT

Cancer is a leading cause of global mortality, with lung cancer being among the most prevalent types. The standard treatments, including surgery, radiation therapy, and chemotherapy, face challenges such as cancer relapse, lack of specificity, and toxicity to healthy cells. Nanotechnology has recently gained attention for potential cancer therapy, particularly in lung cancer, through the development of nanoparticle-based drug delivery systems (NDDS). NDDS, such as polymeric nanoparticles and nanocarriers, offer controlled and sustained drug-release properties, biocompatibility, and promising anticancer effects. They can be tailored to target specific receptors on lung cancer cells, leading to cancer regression. However, challenges like route of administration, size, and immune clearance hinder their clinical use. The use of nanotechnology in lung cancer treatment has shown fundamental advantages, such as localized drug delivery and reduced toxicity to non-cancerous cells. Targeted drug delivery systems, including nanocarriers like liposomes, are being developed to enhance drug efficacy and reduce toxicity. NDDS has demonstrated the ability to address various challenges in cancer treatment, offering advantages such as reduced toxicity and enhanced bioavailability at targeted tumor cells. However, most NDDS, including liposomes and metal-based nanoparticles, are artificially synthesized, leading to drawbacks such as immune response generation and capture by the reticuloendothelial system (RES). The use of NDDS, such as polymeric nanoparticles and nanocarriers, has shown promise in overcoming these challenges. They offer controlled and sustained drug-release properties, biocompatibility, and the ability to target specific receptors on lung cancer cells, leading to cancer regression. However, challenges like route of administration, size, and immune clearance hinder their clinical use. Therefore, we propose to develop a lung cancer targeted DDS by using extracellular vesicles (EVs).

EVs are naturally released nanovesicles from most of the cell types and biofluids such as blood with a size range of 50-5000 nm in diameter, and facilitate intracellular communication, carrying cargoes such as proteins, lipids, and nucleic acids. Interestingly, unlike nanoparticles, EVs can evade the RES without any surface modification, and cause minimum toxicity. Therefore, EVs isolated from human umbilical endothelial cells-derived EVs (HUVEC-EVs) were chosen as a carrier for drug delivery, because HUVEC have shown anti-angiogenic activity in lung cancer mouse model. Further, to achieve the lung cancer specific targeting ability, HUVEC-EVs was engineered to functionalize with GE11 peptide (GE11-HUVEC-EVs) via peptide post-insertion technique, which binds to the epidermal growth factor receptor (EGFR); overexpressed on the surface of lung cancer cells. The GE11-HUVEC-EVs was further loaded with Vinorelbine (GE11-HUVEC-EVs-Vin), followed by their characterization, and evaluation in *in-vitro* and *in-vivo* lung cancer models.

Keywords

Lung cancer, Drug delivery, Extracellular vesicles, Exosomes, HUVEC, GE11 peptide, Vinorelbine, Targeted delivery.

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List of Abbreviations

ABs - Apoptotic Bodies

AD - Alzheimer's Disease

ADIBO - Azadibenzylcyclooctyne

AFM - Atomic Force Microscopy

BBB - Blood-Brain Barrier

BMEVs - Bovine Milk-Derived Extracellular Vesicles

CD63 - Cluster of Differentiation 63

CD81 - Cluster of Differentiation 81

CD9 - Cluster of Differentiation 9

CSF - Cerebrospinal Fluid

CTP - Cardiac Targeting Peptide

Cur - Curcumin

DCEVs - Dendritic Cell-Derived Extracellular Vesicles

DCs - Dendritic Cells

DCs - Dendritic Cells

DDS - Drug Delivery System

DNA - Deoxyribonucleic Acid

Dox - Doxorubicin

DUC - Differential Ultracentrifugation

EDC-NHS - 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-N-hydroxysuccinimide

EGFR - Epidermal Growth Factor Receptor

ELISA - Enzyme-Linked Immunosorbent Assay

EVs - Extracellular Vesicles

ExoTIC - Exosome Total Isolation Chip

FTIR - Fourier Transform Infrared Spectroscopy

GNSTM - Glycosylation Sequence

Hsp70 - Heat Shock Protein 70

IEVs - Immune Cell-Derived Extracellular Vesicles

iRGD - Integrin-Binding Peptide

Lamp2b - Lysosome-Associated Membrane Protein 2b

lncRNA - Long non-coding RNA

MAGE 3 - Melanoma-Associated Antigen 3

MDSCs - Myeloid-Derived Suppressor Cells

MHC - Major Histocompatibility Complex

miR-21i - MicroRNA-21 Inhibitor

miRNA - Micro RNA

mRNA - Messenger RNA

MSCs - Mesenchymal Stem Cells

MVBs - Multivesicular Bodies

MVs - Microvesicles

NDDS - Nano Drug Delivery Systems

NPs - Nanoparticles

NTA - Nanoparticle Tracking Analysis

PEG - Polyethylene Glycol

PEVs - Plant-Derived Extracellular Vesicles

PS - Phosphatidylserine

PTK7 - Protein Tyrosine Kinase 7

RGE - Neuropilin-1-Targeted Peptide

RNA - Ribonucleic Acid

RVG - Rabies Virus Glycoprotein

SELEX - Systematic Evolution of Ligands by Exponential Enrichment

siRNA - Small Interfering RNA

SPIONs - Superparamagnetic Iron Oxide Nanoparticles

SPR - Surface Plasmon Resonance

TDDS - Targeted Drug Delivery System

TEM - Transmission Electron Microscopy

TEVs - Tumor-Derived Extracellular Vesicles

Tsg101 - Tumor Susceptibility Gene 101

XNA - Xeno Nucleic Acid

Research Outputs

Publications

Research Articles

Gaurav I, Thakur A, Zhang K, Thakur S, Xu Z, Kumar G, Jahanathan R, Iyaswamy A, Li M, Ge Z, **Yang Z**^{*}. Peptide Conjugated Vascular Endothelial Exosomes Encapsulating Vinorelbine for Lung Cancer Targeted Therapeutics. (Under revision in *Nanomaterials*) (**Impact Factor:5.3**)

Gaurav I[#], Thakur A^{**}, Kumar G, Long Q, Zhang K, Sidu RK, Thakur S, Sarkar RK, Kumar A, Iyaswamy A^{*}, **Yang Z**^{*}. Delivery of Apoplastic Extracellular Vesicles Encapsulating Green-Synthesized Silver Nanoparticles to Treat Citrus Canker. *Nanomaterials*. **2023** Apr 7;13(8):1306. DOI: [10.3390/biomedicines11072056](https://doi.org/10.3390/biomedicines11072056) (**Impact Factor: 5.3**)

Iyaswamy A^{**}, Thakur A^{**}, Guan X, Krishnamoorthi S, Fung TY, Lu K, **Gaurav I**, **Yang Z**, Su C, Lau KF, Zhang K, NG RCL, Lian Q, Cheung KH, Ye K, Chen HJ, Li M. Fe65 Engineered Neuronal Exosomes Encapsulating Corynoxine-B Ameliorate Cognition and Pathogenesis of Alzheimer's Disease. *Signal Transduction and Targeted Therapy*. 2023. DOI: [10.1038/s41392-023-01657-4](https://doi.org/10.1038/s41392-023-01657-4) (**Impact Factor: 39.2**)

Krishnamoorthi S[#], Iyaswamy A[#], Sreenivasmurthy SG[#], Thakur A[#], Vasudevan K, Kumar G, Guan X, **Gaurav I**, Su C, Zhu Z, Liu J, Kan Y, Jayaraman S, Tong BC, Cheung K, **Yang Z**, Song J, M Li^{*}. PPARα ligand Caudatin improves cognitive functions and mitigates Alzheimer's disease

defects by inducing autophagy in mice models. *Journal of Neuroimmune Pharmacology*. 2023

[DOI: 10.1007/s11481-023-10083-w](https://doi.org/10.1007/s11481-023-10083-w) (Impact Factor: 6.2)

Jingjing S[#], Thakur A^{**}, Pan G, Yan J, **Gaurav I**, Thakur S, Yang Z, Cili A^{*}, Zhang K^{*}. Morus alba derived Kuwanon-A combined with 5-fluorouracil ameliorate Tumor Progression Via Synergistic Activation of GADD153 in Gastric Cancer *MedComm-Oncology*. [DOI: 10.1002/mog2.24](https://doi.org/10.1002/mog2.24)

Review Articles

Gaurav, I., Wang, X., Thakur, A., Iyaswamy, A., Thakur, S., Chen, X., Kumar, G., Li, M. and Yang, Z^{*}, 2021. Peptide Conjugated Nano Delivery Systems for Therapy and Diagnosis of Cancer. *Pharmaceutics*, 13 (9): 1433 (Impact factor: 6.525)

Gaurav, I., Thakur, A., Iyaswamy, A., Wang, X., Chen, X. and Yang, Z^{*}, 2021. Factors Affecting Extracellular Vesicles Based Drug Delivery Systems. *Molecules*, 26(6), p.1544. (Impact factor: 4.927)

Conference Presentations

Proffered Paper Presentation

Gaurav I, Yang Z*. GE11 peptide functionalized exosomes for targeted delivery of vinorelbine to lung cancer. **8th Hong Kong International Oncology Symposium. 6th & 7th Nov 2021**

Conference Poster Presentation

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- **Gaurav I**^{*}, Thakur A, Iyaswamy A, Wang X, Chen X, Yang Z. Factors affecting extracellular vesicles-based drug delivery systems. *Molecules*. 2021 Mar 11;26(6):1544. (DOI: 10.3390/molecules26061544) - ^{*}First author
- **Gaurav I**[^], Wang X, Thakur A, Iyaswamy A, Thakur S, Chen X, Kumar G, Li M, Yang Z. Peptide-conjugated nano delivery systems for therapy and diagnosis of cancer. *Pharmaceutics*. 2021 Sep 9;13(9):1433. (DOI: 10.3390/pharmaceutics13091433)
- [^]First author

CHAPTER 1

INTRODUCTION

A drug delivery system (DDS) consists of various formulations which enable therapeutic substance to reach the desired site of action specifically without going to non-target sites [1]. In nano drug delivery systems (NDDS), different biodegradable and biocompatible materials with size approximately 10-100 nm are utilized as nanocarriers [2][3]. These nanocarriers can be either natural or synthetic polymers, lipids, and metals such as nanoparticles [1][4]. Although NDDS have been used with several drugs including anti-cancer drugs [5][6][7], very few have been approved for use in humans by the Food and Drug Administration [8]. Cytotoxicity and rapid clearance of most of the synthetic NDDS via the mononuclear phagocyte system or reticuloendothelial system have been major bottlenecks in their transition from bench to bedside in clinical setting [9][10]. Several approaches have been employed to modify the nanoparticles (NPs). One example is coating the NPs with polyethylene glycol (PEG); this enhanced circulation time but impeded interaction between the target cells or tissues and the NDDS, thereby interfering with their biodistribution [11][12][13]. Another approach is to look for natural DDS, which could be expected to yield higher therapeutic value owing to their better *in vivo* biocompatibility as compared to the synthetic NDDS [14][15][16]. Extracellular vesicles (EVs) are natural nanovesicles released from most cells and biofluids; they carry various cargo including nucleic acids, proteins, and lipids [17]. EVs have attracted tremendous attention in the context of NDDS due to their ability to facilitate intracellular communication and the transportation of cargo to the target recipient cells [18][19]. Based on their size range and biogenesis, EVs are categorized into

three major types, namely exosomes, microvesicles (MVs), and apoptotic bodies (ABs) [20]. Exosomes are of endocytic origin. They have sizes in the range of 30–100 nm; structurally, exosomes are composed of a lipid bilayer carrying cargoes of different composition including functional proteins, DNA, mRNA, miRNA, and lncRNA (**Figure 1**) [21][22][23].

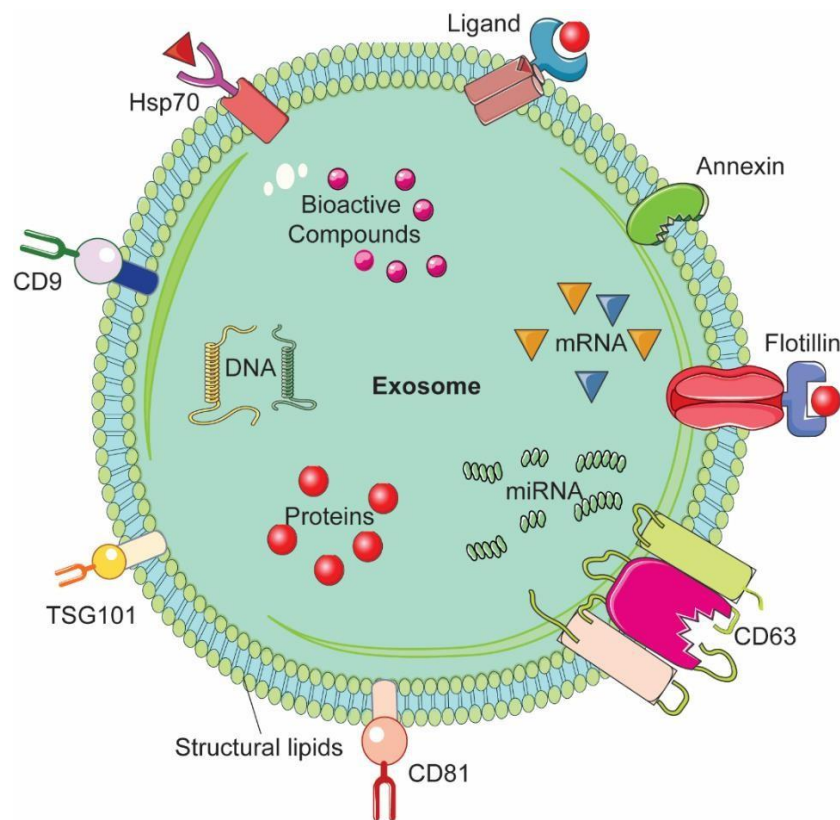


Figure 1. Representative structure of an exosome and its composition. The representative structure of an exosome, a type of extracellular vesicle, embodies a lipid bilayer enclosing various bioactive molecules such as proteins, lipids, and nucleic acids. This intricate composition enables exosomes to serve as potent mediators of intercellular communication, facilitating the transfer of biological information and signaling molecules between cells in diverse physiological and pathological contexts.

Exosomes, a type of extracellular vesicle (EV), are formed through a fascinating cellular process. This begins with the inward budding of the plasma membrane, creating endosome vesicles that develop into multivesicular bodies (MVBs). These MVBs can either fuse with lysosomes for degradation or merge with the plasma membrane, releasing exosomes into the extracellular space. A wide variety of cells produce exosomes, including blood cells, immune cells, epithelial cells, and even cancer cells. These tiny vesicles have been detected in numerous bodily fluids, from blood and urine to cerebrospinal fluid and breast milk. Their presence in such diverse locations underscores their importance in intercellular communication, playing crucial roles in both normal physiological processes and disease states. Exosomes are characterized by specific surface proteins, such as tetraspanins (CD9, CD63, CD81), heat shock proteins, and fusion proteins. These markers distinguish exosomes from other types of EVs, like microvesicles (MVs) and apoptotic bodies (ABs). MVs, another subtype of EVs, are larger than exosomes (100-1000 nm) and form by directly budding off the cell membrane. ABs, on the other hand, are even larger (50-5000 nm) and are released during programmed cell death. The field of EV research is rapidly evolving, with some scientists proposing further subdivisions based on size, markers, and biogenesis. However, for the purposes of this discussion, "EV" refers to the general category unless otherwise specified. This complex world of cellular communication through EVs continues to fascinate researchers, offering potential insights into both normal cellular function and disease processes.

Extracellular vesicles (EVs) have emerged as promising candidates for novel drug delivery systems. Research has demonstrated their ability to transport miRNA and proteins to tumor cells, as well as deliver chemical drugs to inhibit tumor growth. EVs offer advantages over artificial nanocarriers, including enhanced evasion of macrophage phagocytosis and extended drug half-life in circulation. This review builds upon previous summaries, focusing on recent advancements in EV surface modification techniques, the significance of cellular origin, and the critical role of loading efficiency in EV-based targeted drug delivery. We also explore the structural similarities between liposomes and EVs, highlighting their distinct functions in targeted delivery. By harnessing the natural properties of EVs, researchers aim to develop more effective and biocompatible drug delivery systems, potentially revolutionizing therapeutic approaches for various diseases, particularly in cancer treatment.

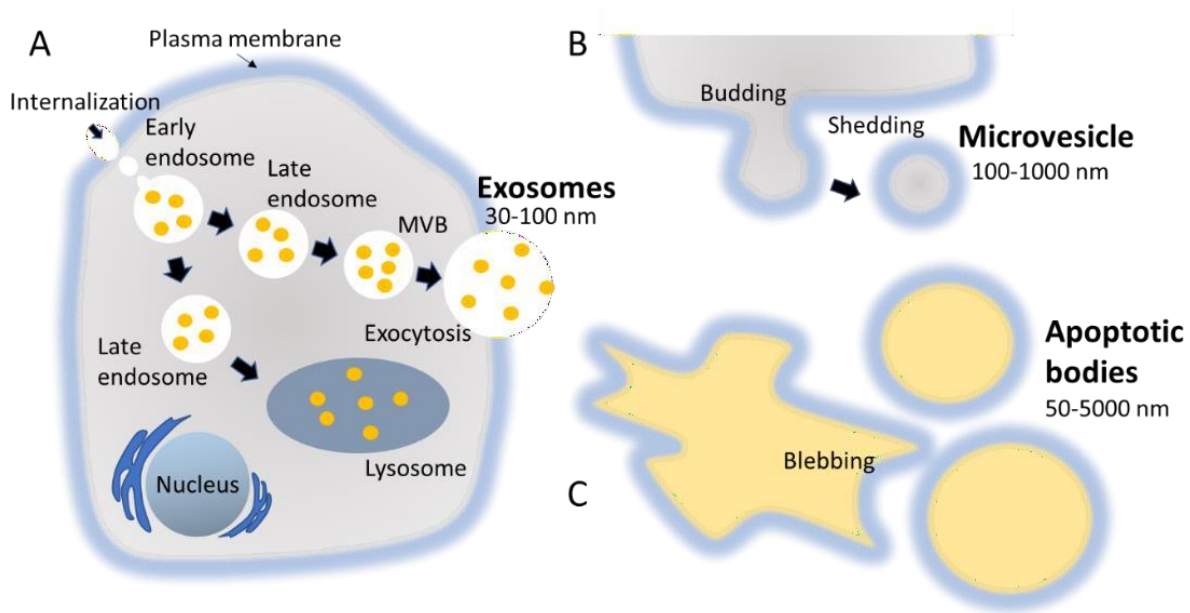


Figure 2. Biogenesis pathways followed by different types of EVs, namely exosomes, MVs, and ABs. Exosomes, microvesicles (MV), and apoptotic bodies (ABs) each follow distinct biogenesis pathways. **(A)** Exosomes originate from the endosomal system, **(B)** MVs bud directly from the plasma membrane, and **(C)** ABs are formed during cell apoptosis. These diverse pathways

reflect the unique cellular processes that give rise to these EVs, each playing specialized roles in intercellular communication and cellular homeostasis.

1.1. Exosomes and liposomes: How similar are they?

Among various synthetic NPs, liposomes share some similarities with exosomes, one of the major types of EVs. **Figure 3** is a representative depiction of exosomes and liposomes in the context of their structure and composition. Exosomes and liposomes are composed of lipid bilayer membrane structure with various dissimilarities. The membrane of exosomes contains various proteins such as tetraspanins, and lipids to enable effective uptake and targeting. Liposomes can be produced via diverse amalgamation of lipids, and their circulation time can be extended by using surface modification with polyethylene glycol. Although liposomes are produced synthetically, their lipid bilayer structure is like that of exosomes. The formulation of liposomes allows the loading of different types of cargo., For example hydrophilic molecules including DNA, RNA, and siRNA can be encapsulated inside the aqueous core of liposomes, and hydrophobic compounds including peptides, proteins, and antibodies can be encapsulated in the lipid bilayer of liposomes [43]. Liposomes can be produced via various methods, such as sonication, membrane extrusion, freeze-thaw, and micro-emulsification [44][45]; however production can be challenging owing to the need for various chemical treatments. In addition, there are multiple steps involved in the modification of the lipid bilayer of the liposomes by using ligands, proteins and other functional elements [46][47]. Surface modification is a crucial factor in achieving targeted DDS. Notably, the limitations due to the tedious and time-consuming steps in the functionalization of the lipid bilayer of liposomes can be overcome via the application of EV-based DDS. Interestingly, exosomes share similar physicochemical characteristics with liposomes; however, one important difference is that exosomes, unlike liposomes, are released naturally, as mentioned earlier.

Importantly, exosomes can efficiently deliver exogenous hydrophilic molecules, and their intrinsic biochemical similarities with that of their originating parent cells [48] enable them to perform better in DDS, as compared to liposomes [49][50][51]. **Table 1** enlists a comparison between exosomes- and liposomes for application in DDS.

Recently, liposome- and exosome-EVs have been proposed as hybrid nanocarriers in advanced DDS. For example, Sato YT *et al.* developed a hybrid exosome via the fusion of the membranes of exosomes with liposomes employing the freeze-thaw technique. Particularly, exosomes expressing specific membrane proteins were isolated from genetically modified cells, then fused with several liposomes. Interestingly, the uptake assay revealed that the interactions between exosomes and the recipient cells can be modified via the altering membrane lipid composition or characteristics of exogenous lipids in hybrid exosomes [52].

Table 1. Comparison between exosomes and liposomes for DDS application.

	Exosomes	Ref.	Liposomes	Ref.
General preparation method	The commonly used methods are traditional ultracentrifugation, density gradient separation, immunomagnetic beads, ultrafiltration, and commercial reagent-based isolation.	[53] [54]	Most of the methods involve four basic steps: (a) Drying down lipids from organic solvent. (b) Dispersing the lipid in aqueous media. (c) Purifying the resultant liposome. (d) Analyzing the final product.	[55]
Yields	Varies depending on isolation method; commercial reagent such as Invitrogen kit could between 200-300 µg exosomal protein amount, whereas immunomagnetic beads-based	[54]	Up to 100% by freeze-drying	[56]

isolation method produces least amount of exosomes.

Loading efficiency	Low (5-50% depending on methods) [16, 57] [58]	High (30-90% depending on methods) [59]
Advantages	High organotropism effect; immune-compatible if isolated from autologous donor cells. [60]	Increased efficacy and therapeutic index of drug; Increased stability via encapsulation; non-toxic, flexible, biocompatible, completely biodegradable, and non-immunogenic for systemic and non-systemic administrations; reduce the toxicity of the encapsulated agent; reduce the exposure of sensitive tissues to toxic drugs; Site avoidance effect; Flexibility to couple with site-specific ligands to achieve active targeting [55]
Disadvantages	Rapid clearance from blood after <i>in vivo</i> administration; Low drug loading efficiency; Unavailability of universally accepted standard manufacturing process. [60]	Low solubility; Short half-life; Sometimes phospholipid undergoes oxidation and hydrolysis-like reaction; Leakage and fusion of encapsulated drug/molecules; Production cost is high; a few stability issue [55]

Clinical progress	<p>Engineered exosomes in [61] preclinical development:</p> <p>Exosome–lipid nanoparticle hybrid loaded with RNA or DNA (Anjarium Bioscience); Cow’s milk exosomes loaded with RNA or DNA (PureTech)</p>	<p>Already available as clinical [62] products, for example Doxil[®], Ambisome[®], DepoDur[™].</p>
	<p>Engineered exosomes in phase-I clinical trials:</p> <p>Cell culture exosomes loaded with KRAS-G12D siRNA (MD Anderson Cancer Center), cell culture exosomes loaded with IL-12 or STRING agonist (Codiak Biosciences)</p>	

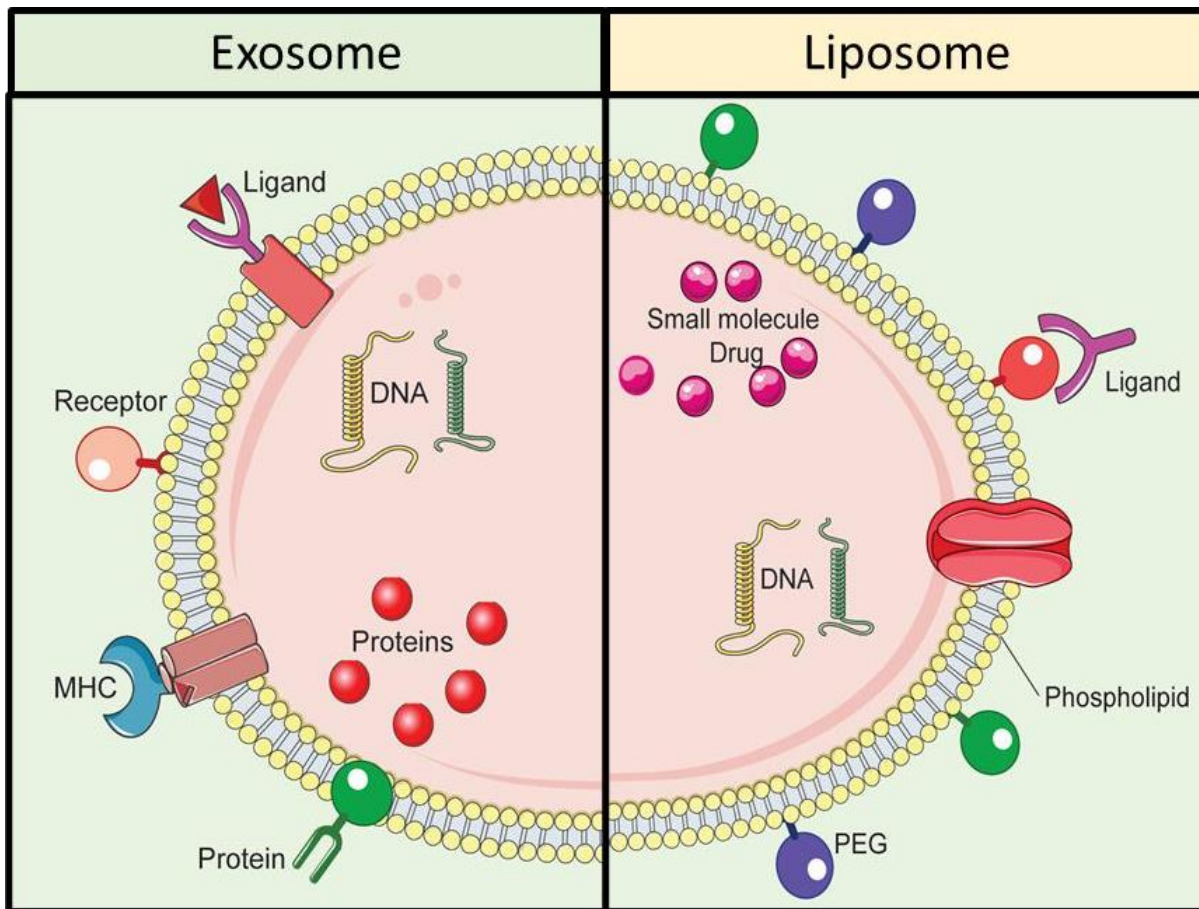


Figure 3. Comparative schematic illustration of exosomes and liposomes. Exosomes, natural extracellular vesicles, and liposomes, synthetic lipid-based nanoparticles, exhibit distinct structural features. Exosomes, derived from cells, encapsulate bioactive cargo within a lipid bilayer. In contrast, liposomes are artificially constructed vesicles with a lipid bilayer enclosing an aqueous core. This comparative schematic highlights the differences in origin and composition between these two types of vesicles.

1.2. How crucial are isolation, yield, and characterization of EVs for delivery purposes?

The isolation of EVs with good quality and high yield from culture-conditioned medium or from biofluids is important for developing EV-based DDS. The isolation of different types of EVs such as exosomes, MVs, and ABs can be carried out based on different parameters including size, density, surface markers, and molecular composition [63]. The qualities and the yield of EVs mostly depend on the type of method employed for their isolation and purification. The most conventional method for isolating EVs is differential ultracentrifugation (DUC), which involves multiple centrifugation steps to eliminate different particles, such as cell debris, at various steps, eventually yielding exosomes in the form of pellets [64]. Although this method is widely used in many labs, there are several limitations and disadvantages. First, it requires heavy instruments, much time, much patience due to the tedious nature of the procedure, and large volumes of sample [52]. Second, there is a probability of loss of and damage to the EVs due to the repeated centrifugation steps.

Various other strategies have been developed including density gradient centrifugation, immunomagnetic bead-based extraction, chromatography, ultrafiltration, and microfluidic device. In addition, various commercial precipitation kits are available such as ExoQuick (from System Biosciences), Total Exosome Isolation Reagent kit (from Thermofisher), and ExoSpin Exosome Purification Kit (Cell Guidance Systems). The advantages and disadvantages of most of these methods have been extensively reviewed elsewhere [53]. Regardless of the numerous available methods, there is no agreement with respect to the best procedure for the isolation of different types of EVs, delivering significant yields with consistent quality [65].

Another important aspect that should be considered while isolating EVs for developing DDS is the yield of EVs. This factor is particularly important in terms of the cost of production. Various advanced methods have been reported recently for isolating EVs with better yield. For example, Liu *et al.* developed an exosome total isolation chip (ExoTIC) for the size-dependent isolation of EVs. This method was found to be simple, convenient to use, and modular; it produced a high yield of EVs with enhanced purity from biofluids. Remarkably, ExoTIC reportedly achieved a yield of EVs approximately 4-1000-fold higher as compared to the conventional techniques including DUC [66]. Two other strategies have been explored for enhancing yield. One is stimulation of donor cells with liposomes. One study revealed that incubation of tumor cells with neutral and cationic-liposomes enhanced the release of EVs, suggesting that, depending on physicochemical properties of liposomes, they can either behave as stimulant or depressant on the release of EVs from tumor cells [67]. The second strategy is the application of stimulating agents, for example in one study, monensin antibiotic has been shown to stimulate the release of EVs in a calcium-dependent manner [68][69].

The source of EVs also plays a crucial role in determining the yield of EVs. For example, mesenchymal stem cells (MSCs) have been reported to be a popular source of EVs for developing into DDS [70]. Importantly, MSCs are easily available and can be produced on a large scale. MSCs release the highest number of EVs as compared to other cell types [71]. In addition, the MSC-derived EVs are reported to show better selective targeting of locations of inflammation and injury. MSC-derived EVs migrate towards a tumor; this propensity can be utilized in developing potential cancer treatments and in regenerative medicine [72]. The origin of EVs has been reported to determine the ability of cells to target and transfer therapeutic cargo to specific recipient cells [73]. In summary, the isolation of EVs can be optimized for higher yield by considering the parameters

of isolation technique and EV source. The precise characterization of EVs or modified EVs is important in any EV-related study. It is critical to be able to establish whether isolated vesicles are indeed EVs, and/or to validate the surface modification or the biochemical constituents of isolated EVs [74][75]. Various methods have been extensively employed by numerous scientists to study different parameters of EVs. These methods include measuring size distribution of EVs via nano tracking analyzer (NTA), determining the morphology and size via transmission electron microscopy (TEM), and examination of exosomal proteins by immunogold-EM, Western blotting, and enzyme linked immunosorbent assay (ELISA) [76]. These methods have already been reviewed in detail elsewhere [77][78]. Recently, several new approaches have been employed by different research groups. One example is the application of surface plasmon resonance (SPR) for the study of surface markers. This method is useful for characterizing the subtypes of EVs based on different proteins on their surface [79][80]. SPR technology has also been utilized for measuring the concentration of EVs in solution; this is useful in the study of EV-based DDS [81]. Despite being an advanced, label-free tool, the SPR method has two serious drawbacks, namely the use of various chemicals in the immobilization of antibody against target antigen to be detected on the surface of EVs, and the need to enhance sensitivity [82]. Another technique, atomic force microscopy (AFM), entails the visualization of the three-dimension surface structure of EVs and the study of their nano-mechanical characteristics [83]. Several other analytical tools are being employed by different research groups to characterize and validate various types of surface of modification of EVs, including Fourier Transform Infrared Spectroscopy (FTIR), and Raman spectroscopy [84]. Several research groups have employed an integrated approach, combining different techniques to achieve better characterization of EVs [85]. Each method, each tool has its

own advantages and disadvantages; the selection of which tool to use mostly depends on the type of study to be done.

1.3. Surface modification of EVs for targeted delivery

Various strategies have been explored for the modification of EVs for targeted delivery such as click chemistry, and genetic engineering. Various targeting molecules including aptamer- and peptide anchorage to the surface of EVs have been employed, which will be described below.

1.3.1. Click chemistry

In this method, an alkyne group is attached to the surface of isolated EVs through 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-N-hydroxysuccinimide (EDC-NHS) condensation reaction. The alkyne group further covalently attaches to the azido group of the targeting structure (on the surface of EVs) in the presence of copper. This approach offers various advantages. First, it is resistant towards fluctuation in temperature and pressure. Second, the reaction of click chemistry can take place in an aqueous buffer as well as organic solvent, without consuming much time. Thirdly, and importantly, the conjugation via click chemistry does not influence the size of EVs nor their uptake by recipient cells [86]. Various groups have employed this approach for the surface modification of EVs. For example, Jia *et al.* loaded superparamagnetic iron oxide nanoparticles (SPIONs) and curcumin (Cur) into EVs and conjugated their membrane with neuropilin-1-targeted peptide (RGERPPR, RGE) via click chemistry to achieve glioma-specific targeting [87]. Liang *et al.* produced azido- modified EVs from MDA-MB-231 cells by changing their glycosylation pathway, and the azido group on EVs further reacted with azadibenzylcyclooctyne (ADIBO) fluorescent dyes via click chemistry, which were useful for *in vivo* tracking [88]. Although the click chemistry method has been widely explored for surface

modification of EVs, its use is limited by the non-specific interaction between the targeting structure and the surface of EVs [89]. In addition, some scientists believe over-modification of proteins on the surface of EVs with alkyne groups may inhibit the function of those exosomal proteins [86].

1.3.2. Genetic engineering

Various proteins are present in the membrane of EVs such as CD9, CD63, CD81, and Lamp, which can be conjugated with target ligands and can facilitate localized delivery of EVs [90]. Another crucial approach is engineering the donor cells producing the EVs by using plasmid vectors. For example, Kim *et al.* performed co-transfection of pcDNA-cardiac targeting peptide (CTP)-Lamp2b in HEK293 cells for producing EVs targeting cardiac cells [91]. Tian *et al.* transfected immature DCs with pEGFP-C1-iRGD-Lamp-2b plasmids for producing iRGD(CRGDKGPDC)-Lamp-2b positive EVs to target breast cancer cells *in vitro* and *in vivo* [92]. Modification of the surface of EVs with peptides has also been explored owing to their small size, high binding affinity, target specificity, low immunogenicity, and low toxicity. Moreover, peptides are conveniently tunable and can be synthesized based on the information about their targeting ligand and screening of library of peptides [93]. Leonard *et al.* conjugated targeting peptides (having glycosylation sequence GNSTM) with EVs to suppress peptide loss and enhance the delivery of EVs to neuroblastoma cells, suggesting that glycosylation does not ablate the interaction between peptide and target [94].

1.4. Targeting molecules-based engineering of EVs

1.4.1. Aptamer-based surface modification of EVs

Aptamers are short, single-stranded DNA, RNA, or xeno nucleic acid (XNA), which can be produced with high specificity and affinity towards desired targets by PCR-based *in vitro* selection; this approach is referred to as systematic evolution of ligands by exponential enrichment (SELEX) [95][96][97]. Aptamers have also been employed in surface modification of exosomes for targeted delivery. For example, Wan *et al.* developed aptamer grafted EVs loaded with paclitaxel for targeting breast cancer *in vivo*. To accomplish this, nucleolin-targeting aptamer AS1411 was attached to cholesterol-polyethylene glycol covalently. The resultant compound was anchored on the membrane of mouse DCs, followed by extrusion of cells by passing them through micro constrictions to get EVs and loading with paclitaxel via sonication method [98]. In another study, Pi *et al.* modified EVs by displaying an aptamer, which could bind to prostate-specific membrane antigen and loaded the modified EVs with survivin siRNA. This aptamer decorated EV loaded with surviving siRNA effectively inhibited the growth of a prostate cancer xenograft. Moreover, the same EV-based delivery system displaying EGFR aptamer could inhibit orthotropic breast cancer [99]. Zou *et al.* developed an aptamer-functionalized exosome for cell type-specific delivery by employing diacyl lipid- sgc8 aptamer conjugates as the targeting ligands. The aptamer sgc8 could specifically recognize membrane-bound protein tyrosine kinase 7 (PTK7), suggesting its applicability as an important theranostic platform [100].

1.4.2. Peptide anchoring on EVs

Anchoring peptide on the surface of EVs is a successful strategy that has been employed by many research groups for achieving targeted delivery. For example, Alvarez-Erviti *et al.* engineered DCs for expressing Lamp2b, an exosomal membrane protein, conjugated with neuron-specific RVG peptide. The exosomes were encapsulated with siRNA via electroporation. The intravenous injection of RVG-targeted exosomes could deliver siRNA specifically to brain cells

including neurons, microglia, and oligodendrocytes, and showed specific gene knockdown [101]. Zhang *et al.* performed conjugation of c(RGDyK) peptide on EVs isolated from mesenchymal stromal cell (MSC), followed by loading with cholesterol-modified miR-210. This delivery system was found to be effective for the treatment of ischemic stroke [102]. Another research led by Zhan *et al.* engineered blood EVs via binding of magnetic molecules and endosomolytic peptides, L17E on the surface of EVs, followed by co-embedding doxorubicin (Dox) and cholesterol-modified miRNA21 inhibitor (miR-21i). Importantly, the surface engineering of blood EVs could enhance the tumor accumulation and increased capacity to escape endosomes, thereby leading to specific and efficient delivery of loaded cargoes to tumor cells *in vitro* and *in vivo* [103].

1.5. How crucial is the origin of exosomes for drug delivery?

There are various sources of EVs which can be employed for developing DDS (**Figure 4**). To achieve the desired effect and to avoid any potential harmful effects, it is necessary to understand the pros and cons of selecting any particular source. For example, the proportion of lipid to surface protein in an EV differs, depending on the source [104]. This proportion can affect certain properties that are crucial for effective delivery, and thus should be considered when selecting a source. It has also been found that the proportion of certain lipids is enhanced in exosomes as compared to the amount of lipids in their donor cells, such as sphingolipid, phosphatidylserine, phosphatidylinositol, and cholesterol. These lipids facilitate in enhancing the rigidity of exosomal membrane [105]. Another factor to consider is the relationship between surface proteins and target cells. Some sources produce EVs with surface proteins that are detrimental to the recipient target cells. A third factor is biocompatibility; and finally yield should also be taken into consideration while making selection of donor source cells for isolation of EVs for developing into EVs-based DDS.

1.5.1. Choice between autologous and heterologous EVs

The choice between autologous and heterologous (also referred as allogenic) EVs for developing into DDS is one of the crucial factors for effective delivery. It has been found that the uptake of autologous EVs and the uptake of heterologous EVs by target recipient cells differ distinctly. As the compositions of EVs have been reported to mimic their parent cells, the selection of heterologous EVs may induce an immune response in the target recipient cells. Therefore, theoretically, autologous EVs may be more suitable for therapeutic purposes [72]. In practice, however, heterologous EVs from MSCs have been found to be safe and reliable for therapeutic purposes. Still, we shouldn't forget about autologous EVs, for example, pathological tissues are generally considered waste; however, if the EVs from these tissues could be isolated and their disease-causing cargo removed, these EVs could be valuable DDS [106]. Lessi *et al.* demonstrated that human primary macrophage-derived EVs could deliver drugs efficiently, [107], suggesting that autologous EVs derived from peripheral blood-derived primary monocytes could be suitable as theranostic agents. The safety profile of these EVs needs to be assessed before developing them into DDS; however, some evidence indicates they are safer than EVs derived from plasma [108].

1.5.2. Tumor-derived EVs

Tumor-derived EVs (TEVs) have been employed by many research groups for drug delivery [109][110]. TEVs have several advantages compared to other delivery carriers. For example, tumor cells release significantly high numbers of EVs [111], suggesting their suitability for studies requiring large amounts of EVs. In addition, TEVs carry MHC class-I molecules and antigens specific to the originating tumor cells. Moreover, TEVs can induce immune response against cancer cells by delivering antigens to DCs [112]. Interestingly, the tetraspanin proteins, common markers for exosomes, have been found to bind with various ligands in a diverse range of tissues,

suggesting their suitability for targeted delivery [113]. TEVs from melanoma patients have been found to increase the release of myeloid-derived suppressor cells (MDSCs), crucial for avoiding immune recognition [114][115]. It is striking that although TEVs have been shown to have potential for targeted delivery, there is also a chance that they can initiate tumor progression due to various of their constituents, such as urokinase plasminogen activator, which can promote cancer cell invasion, and adhesion modulators like vimentin, and annexin A1 [116]. Therefore, again, selection of appropriate source for isolating EVs is a crucial factor for developing successful and effective EV-based targeted DDS.

1.5.3. Immune cell-derived EVs

Another important source from which EVs can be isolated are immune cells. For example cells like macrophages and monocytes have gained attention for EV-based immunotherapy [117][118]. Immune cell derived EVs (IEVs) can evade phagocytosis, a clearance mechanism, which is a major limitation for most of the other types of EVs. Therefore, IEVs possess longer circulation time and better efficacy [119]. Importantly, the DC-derived EVs (DCEVs) seem to have a great potential as various clinical studies have demonstrated their effectiveness on different cancers. DCEVs play a major role as intercellular communicators in adaptive immunity for modulation of immune responses. Therefore, most of the research related to DCEVs are about immunotherapy of cancer leading to clinical advantage [120][121]. Notably, in a Phase-I clinical trial, Escudier *et al.* reported the feasibility and safety of administering DCEVs pulsed with MAGE 3 peptides for immunization in melanoma patients under stage- III/IV [118]. DCEVs have also been found to promote tumor rejection via transporting peptide-MHC complexes from DCs (exposed to an antigen) to other DCs (not exposed to same antigen) [117][122][123].

1.5.4. Biofluid-derived EVs

EVs derived from biofluids such as plasma [124], and ascites [125], have shown potential as delivery carriers. Biofluid-derived EVs have several advantages as delivery carriers. For example, unlike cell culture-derived EVs, plasma-derived EVs are enriched with lyso-phospholipids and do not contain phosphatidylserine (PS). The absence of PS on the surface of plasma-derived EVs prevent their removal from circulation [124][126]. In addition, plasma-derived EVs can cross the blood-brain barrier (BBB), which suggests their applicability for brain delivery [124]. Ascites-derived EVs along with granulocyte–macrophage colony-stimulating factor have been found to be safe and effective for immunotherapy of colorectal cancer [125]. It has also been reported that human peripheral blood-derived EVs loaded with miRNA have potential for treating cardiac diseases [127]. Another study showed that EVs in peripheral blood can be important mediators of lung injury via exosomal shuttling of miR-155 [128]. Blood EVs have been shown to be crucial for targeting brain disease. For example, dopamine-loaded blood EVs can be used as delivery platform in treating Parkinson’s disease and other central nervous system-related disorders [129]. Urine- and saliva-derived EVs have not been much explored for their therapeutic potential as delivery carriers; however, they have been extensively studied for developing biomarker of different diseases including cancer [130][131]. Conclusively, biofluids such as blood and ascites are great sources of EVs for developing novel DDS.

1.5.5. Plant-and bovine milk-derived EVs

Due to safety concerns related to TEVs and IEVs, scientists have explored the applicability of plant-derived EVs (PEVs) as DDS, such as grape-derived EVs [132] or bovine milk-derived EVs (BMEVs) [133]. There are several advantages of using PEVs as DDS, including better safety, consistency of source, scalability for large production, and relative cost effectiveness [132]. Several research groups have isolated EVs from different plants or food sources and showed a

diverse range of applications [134]. For example Ju *et al.* demonstrated that grape- derived EVs are useful in protecting intestinal damage in mice via facilitating growth and differentiation of intestinal stem cells [132]. Subsequently, Wang *et al.* demonstrated that modification of grapefruit derived EVs improved their ability to target tumors and loaded those EVs with doxorubicin and curcumin. Interestingly, those EVs were found to be effective against inflammation *in vivo* [135]. Bovine milk is another important source of EVs for DDS. Mungala *et al.* demonstrated the enhanced activity of various therapeutic cargo loaded BMEVs against lung cancer *in vitro* and *in vivo*. They further showed that modification of BMEVs with folate could enhance tumor-targeting ability as compared to the free drug [133]. Recently, because many clinical trials for the treatment of Alzheimer's disease (AD) using synthetic drugs have failed [136][137][138], scientists are trying to develop targeted delivery using EVs and developing precision medicine-loaded EVs for the treatment of AD [139][140]. Plant-derived traditional medicine have been studied in preclinical models of AD [141][142][143]. Targeted delivery of plant-derived bioactive components using EVs could be more effective for the treatment of AD.

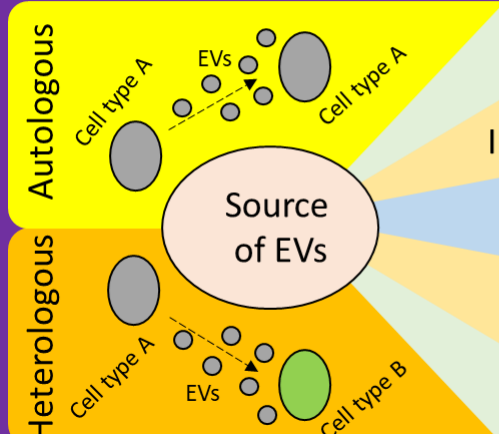
EVs-based Drug Delivery System		Advantages	Disadvantages
 <p>The diagram illustrates the sources and targets of EVs. A central circle labeled 'Source of EVs' is connected to five categories: Autologous (yellow), Heterologous (orange), Tumor (green), Immune cell (yellow), Biofluid (blue), Plant (orange), and Bovine milk (green). Autologous and Heterologous sources show cells (Cell type A and Cell type B) releasing EVs. Tumor, Immune cell, Biofluid, Plant, and Bovine milk are shown as targets for EVs.</p>	Autologous	High number of EVs Induce immune response Bind various ligands	It can initiate tumor progression
	Tumor	Evade phagocytosis Longer circulation time Better efficacy	Tumor rejection may occur
	Immune cell	Removal of blood EVs from circulation is prevented It can cross BBB	Their removal from circulation is prevented It can cross BBB
	Biofluid	Safe; Consistency of source scalability for large - production; Cost effective	Insufficiency to carry high quantity of drug cargo
	Plant	Enhanced activity of various therapeutic cargo	Lack of specificity to recipient cells
	Bovine milk		

Figure 4. Different sources of EVs for developing EV-based DDS, their advantages, and disadvantages. Extracellular vesicles (EVs) sourced from diverse origins like cells, blood, and tissues offer unique advantages for developing EV-based drug delivery systems (DDS). While cell-derived EVs provide natural cargo loading, blood-derived EVs offer systemic delivery potential. However, challenges such as isolation complexity and batch-to-batch variability exist. Understanding these varied sources is crucial for harnessing the full potential of EVs in drug delivery.

1.6. How does loading efficiency play a crucial role in EV-based DDS?

Loading therapeutic cargo into EVs is one of the crucial parts in the process of developing EV-based DDS. The high loading efficiency of EVs ensures better bioavailability of the cargo when it reaches the target site. The major factors that need consideration while loading any cargo onto EVs are how better encapsulation or loading efficiency can be achieved, how the structural integrity of EVs can be maintained, and how the functional properties of the therapeutic cargo can be maintained.

Therapeutic cargo such as proteins, drugs, or small nucleic acids such as miRNA can be loaded in two different ways. First, the therapeutic cargo can be incorporated into donor cells, followed by isolation of EVs; this is referred to as *in vitro* loading [144]. Second, the therapeutic cargo can be loaded after isolation of EVs via various methods including incubation, sonication, electroporation, extrusion, permeabilization, or the freeze-thaw method; this is referred to as *ex vivo* loading (**Figure 5**) [119]. In the simple incubation method, EVs are incubated with drugs, and the drugs enter EVs via diffusion due to the concentration gradient. Incubation is found to be suitable for loading hydrophobic drugs as they interact with lipid layers of EVs' membranes [145][119]. One disadvantage of simple incubation is low loading efficiency. Another method of

loading is incubation of drugs with donor cells, followed by isolation of EVs [144]. **Table 2** summarizes different loading methods with their advantages and disadvantages. The physicochemical properties of the therapeutic cargo partially determine what method is employed for their encapsulation in EVs. For example hydrophobic drugs such as curcumin can be loaded within the inner layers of fatty acid via incubation, whereas hydrophilic molecules including siRNA, miRNA can be loaded by forming transient pores on the membrane of EVs via methods like electroporation [101][146][16].

Recently, membrane permeabilization of EVs has been found to be a promising method for enhancing the loading efficiency of EVs. Saponin has been shown to be particularly effective in enhancing the loading of different cargos in EVs from various sources. Being a surfactant, saponin is able to form a complex with cholesterol in the membranes of cells and create pores, thereby facilitating permeabilization [147]. Haney *et al.* demonstrated that loading efficiency of catalase into exosomes can be enhanced via incubation with saponin, as compared with simple incubation technique. Interestingly, the activity of catalase was not affected by the saponin [119]. Another recent research showed that passing saponin through the microfluidic channels enhances loading of doxorubicin in glioma stem cell-derived exosomes as compared with other conventional methods. The authors reported two different microfluidic channels; one linear. and another sigmoid, which suggests that designing advanced microfluidic channels along with using permeabilizing agent may have a synergistic effect to achieve augmented efficiency of loading cargoes in EVs [16][57]. Fuhrmann *et al.* showed that incubation of a small hydrophilic molecule, porphyrin, with saponin could enhance the loading efficiency as compared with a passive loading technique excluding saponin [148]. Nevertheless, there are some concerns associated with the use of saponin for *in vivo* purposes because of its hemolytic activity [147].

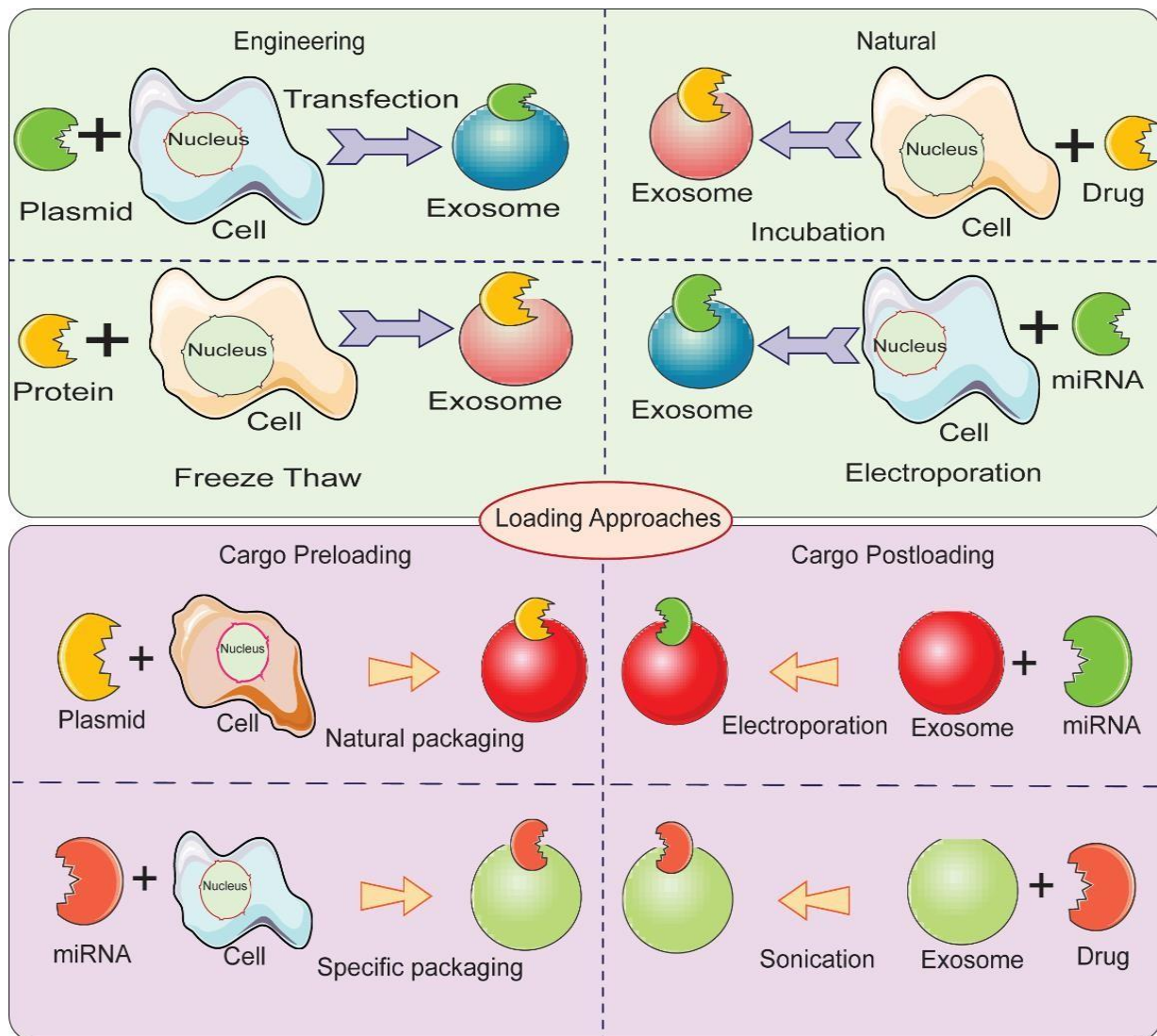


Figure 5. Different methods for loading therapeutic cargoes in EVs for developing EV-based delivery systems.

Table 2. Different techniques for loading cargos in EVs with advantages and disadvantages.

Loading methods	Steps involved	Advantages	Disadvantages	Ref.
Electroporation	Phospholipid bilayer of EVs is disorganized by an electric field, creating pores in the membrane which allow the passage of drug to vesicle.	Loading with large molecules is possible	Disrupts integrity of EVs; Low loading efficiency	[101][149][150][151]
Sonication	Exosomes derived from donor cells are mixed with drug and sonicated through probe sonicator which permits the drug to flow into exosome	Increased loading efficiency; applicable for small RNAs	Potential deformation of membrane eof EVs; Not efficient for hydrophobic drugs.	[60][152]
Extrusion	Exosomes are mixed with drug and loaded into syringe-based lipid extruder and extruded through membrane with 100-400 nm pore size at controlled temperature.	High drug loading efficiency	Potential deformation of membrane.	[60]
Freeze/Thaw Method	Exosome are mixed with drug and incubated, subsequently frozen at -80 °C or in liquid nitrogen and are thawed at room temperature.	Medium loading; Fusion of membranes Possible	Exosomes may aggregate; Low loading efficiency	[60]
Saponin-Assisted Loading	Saponin is incubated with exosomes to generate pores in their membrane by interacting with cholesterol which leads to increased membrane permeability	High drug loading compared to the other methods used in early reports	Generates pores in exosomes; Saponin can cause haemolysis; Toxicity concerns. Saponin concentration control & washing required	[119][147][148]
Dialysis	Exosomes mixed with drug are dialyzed by stirring to obtain drug loaded exosome.	Promotes loading efficiency	Poor cellular uptake. No substantial impact on photodynamic effect	[153]

1.7. Peptide-Conjugated Nano Delivery Systems for Therapy and Diagnosis of Cancer

Despite numerous advancements and breakthroughs in cancer therapy and diagnosis, cancer still ranks among the topmost causes of mortality in every country. In the year 2020, approximately 19.3 million new cases of cancer occurred, and merely cancer accounted for about 10 million deaths around the world [154]. Conventional strategies including chemotherapy are challenged by the low specificity and high toxicity toward cancer cells [155],[156]. There are various biological barriers that hinder the successful delivery of therapeutic agents to the tumor site, for example, the tumor microenvironment (TME), mononuclear phagocytic system, extravasation of nanoparticles (NPs), cellular barriers, and drug efflux transporters [157], as portrayed in Figure 6.

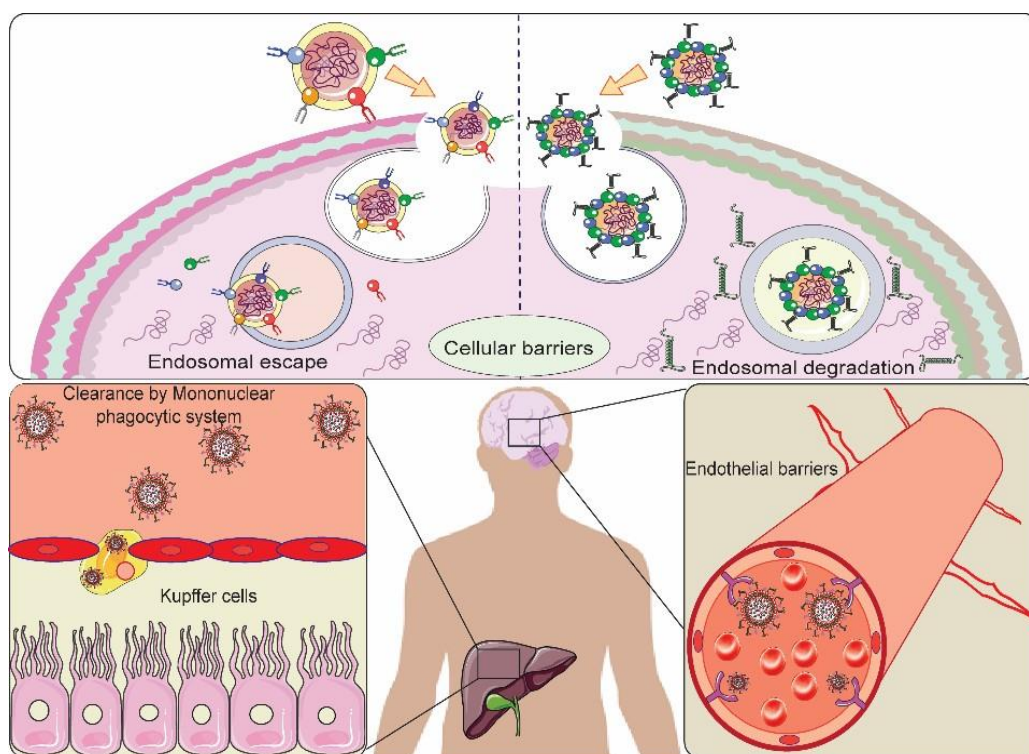


Figure 6. Graphic depiction of major barriers obstructing the nano delivery system (NDS), such as the endosomal–lysosomal system, clearance of NDS via the mononuclear phagocytic system, and endothelial barrier acting in the event of extravasation of NDS in cancer.

1.8. How TME and hypoxia impede drug delivery?

The complex nature of the TME is one of the crucial hindrances to the delivery of therapeutic agents to the tumor site [158]. The TME consists of cellular components such as cancerous and noncancerous stromal cells, blood vessels, lymphatic vessels, and immune cells. In addition, the non-cellular components of TME are composed of cytokines, chemokines, mediators, and growth factors, which are generally affected by the growth of cancer cells [159]. The extracellular matrix (ECM) is another key element of the TME, which differs significantly in terms of composition and framework compared to that under normal tissue. The ECM in the TME is highly abundant, stiffer, and denser, forming another bottleneck to cancer therapy via shielding the cells from anti-cancer drugs. Moreover, the enhanced stiffness of ECM in hypoxic TME has been found to activate the antiapoptotic pathways and contribute toward the development of drug resistance in cancer cells [160].

Therapies such as photodynamic and radiotherapy depend on oxygen, which is restricted by hypoxic TME. When the eruptive growth of the cancer cells occurs, the supply of oxygen and the nutrient is restricted from their neighboring blood vessels [161]. Under hypoxia, the transcriptional factor, hypoxia-inducible factor-1 α (HIF-1 α), induces the metabolic change from oxidative phosphorylation to aerobic glycolysis, which is referred to as the Warburg effect [162]. The proliferation and glycolytic metabolism in the cancer cells enhance the development of excessive reactive oxygen species (ROS), which attack cellular components such as nucleic acid, causing genomic instability and thereby altering the morphology of the cell [162]. Notably, the ability of

ROS to regulate cancer cell survival is found to be cell type specific, for example as observed in MCF-7 and MDA-MB-435 breast cancer cells [163]. In addition to the effect of ROS on cell proliferation, the ROS-mediated activation of extracellular-regulated kinase 1/2 (Erk1/2) are found to play an important role in the augmentation of cell survival, motility, and anchorage-dependent growth of multiple cancers, such as ovarian cancer, breast cancer, melanoma, and leukemia [163]. Such an occurrence, with upregulation of the efflux pump for secreting lactic acid and carbonic acid, leads to benefit the tumor cells as they live longer and succeed in their mission even in extreme condition [164]. Therefore, under hypoxic TME, the delivery of a therapeutic agent to the tumor site is obstructed [165][166]. Contrariwise, ROS has also been applied for therapeutics of cancer by designing strategies to enhance the cellular level of ROS exuberantly in order to include irrevocable damages, leading to the apoptosis of cancer cells. This can be accomplished via chemotherapy or radiotherapy depending on the cancer type. For example, a combinatorial therapy of pancreatic cancer with gemcitabine with trichostatin A, epigallocate-3-gallate (EGCG), capsaicin, and benzyl isothiocyanate (BITC) are found to be working via increasing the intracellular ROS level for triggering ROS [163]. In another research, it was found that gold(III) porphyrin 1a could be a potential anti-cancer lead by acting toward mitochondria, as ROS played a role in gold(III) porphyrin 1a-induced apoptosis [167]. In addition, photodynamic therapy using a synthetic photosensitizer, 5,10,15,20-tetra-sulfo-phenyl-porphyrin (TSPP), is found to enhance the generation of ROS, leading to the decrease in antioxidant capacity in tumor tissue [168]. In addition, palladium porphyrin complexes are also found to generate ROS with higher efficiency. Interestingly, the palladium porphyrin complex showed higher therapeutic activity as compared to free base porphyrin upon irradiation with light [169].

1.9. Mononuclear phagocytic system (MPS) as a barrier to drug delivery

To fetch the desired therapeutic response of the drug, its successful delivery at the tumor site is important, which again relies on the nature of the delivery system and its stability in the blood circulation. Blood carries various proteins including globulin, albumin, and fibrinogen. After entry of the nano delivery system (NDS) in the blood circulation, the blood serum proteins get adsorbed on their surface and form a complex, which is referred to as protein corona [170]. The process of forming protein corona is known as opsonization, which is generally followed by phagocytosis via the macrophage, which is a type of immune cell in MPS [171]. Remarkably, the process of opsonization and phagocytosis by the MPS facilitates the elimination of NDS from the systemic blood circulation.

1.10. How does extravasation react to NDS in the TME?

The presence of the vascular endothelial layer is another hurdle, which is required to be overcome for the successful delivery of NDS at the tumor site. The vascular endothelial layer is composed of a semi-permeable lining of the inner walls of blood vessels. In addition, a proteoglycan layer of glycocalyx controls the permeability of molecules across the blood vessels [172]. The glycocalyx layer has been found to be involved in the enhanced interactions with cationic particles by providing a negative charge to the membrane of endothelial cells [173]. Therefore, the presence of glycocalyx is a limiting factor for the extravasation of NDS in the TME, as it potentially conceals the NDS

[157],[174]. In addition, there are other factors that affect the extravasation of NDS, such as the hydrodynamics of NDS, enhanced permeability, and retention, which favor the nano therapy of cancer [157].

1.11. Cellular barriers as a bane to the nano delivery system

The passage of NDS through the endothelium of the blood vessels into the target site is another obstacle. In general, the NDS cannot traverse through the endothelium; however, in disease conditions, such as cancer, the integrity of the endothelium is compromised owing to the activation of cytokines, and thereby, the endothelial cells' gap is enhanced. Therefore, the NDS can reach the pathological site by traversing through the abnormal endothelial gaps. Unfortunately, after escaping the blood vessels associated with the endothelial barrier, the NDS confronts another hurdle while traversing through the dense interstitial space and extracellular matrix (ECM) to reach the target site. The composition of interstitial space including collagen and an elastic fiber network consisting of proteins and glycosaminoglycan, which form ECM, forms a hydrophilic gel by the interstitial fluid, which fills the interspersed spaces. Even though the ECM and interstitial space render structural integrity to the tissue, under pathological conditions, including cancer, the collagen content is bigger than that in healthy conditions. This suggests that the excessive firmness of ECM is a crucial barrier that could obstruct NDS delivery [175]. Notably, the charged particles have been found to possess enhanced interactions with the membrane, whereas uncharged particles—for example, PEGylated NDS—show lesser interaction due to the steric hindrance. This leads to the accumulation of NDS to form a cluster around the membrane and prevent the entry of successive NDS [157].

1.12. How drug-efflux transporters can pump out therapeutic agents?

Even though the NDS reach the target site after confronting various hurdles, there is a tiny fraction of those that could make it to therapeutic efficacy by exerting intracellular cytotoxicity. Interestingly, various solid tumors possess crucial machinery that facilitates the expulsion of drugs, which is often referred to as drug-efflux transporters. For example, the overexpression of P-glycoprotein (P-gp), a drug-efflux transporter, has been reported to be linked with the efflux of anti-cancer drugs, and also the clinical refractoriness of anti-cancer drugs is associated with P-gp [157]. In addition, there are several other hurdles associated with the obstruction of delivery of NDS to the target site, which have been extensively reviewed elsewhere [176].

Recently, the conjugation of peptides and NDS (CPNDS) has emerged as a versatile technique for multidisciplinary biomedical applications. Compared to antibodies-based targeted NDS, peptide-conjugated NDS offers various advances: for example, most of the therapeutic monoclonal antibodies (TMAs) do not target tumor-specific antigens (TSAs), it requires screening to select monoclonal antibodies for dominant epitopes, the target must be antigenic for conventional monoclonal antibodies, and it also depends on the strain of animals used. However, in case of peptides, the target is not necessarily antigenic, and there is no requirement of prior information about target molecules. In the context of intracellular transport, there is no selection criteria for TMAs, and it is difficult to select during the screening process; however, in case of peptide-based NDS, screening technologies offer a convenient selection of candidates, which could induce endocytosis rapidly. In the context of the conjugation process, only $\approx 50\%$ of the monoclonal antibodies bind to the drug, making it difficult to predict the stoichiometry and drug position. Moreover, the conjugation chemistry is limited to aqueous solutions. On the other hand, in case of

peptide conjugated NDS, the augmented flexibility in conjugation chemistry for coupling to linker and drug allows a wider selection of drugs, including compounds that are insoluble in water. Notably, the significantly lower cost of production and enhanced product reproducibility make peptide-conjugated NDS a preferred choice compared to the antibody-based NDS [177].

The synergistic integration between peptides and NDS allows effective customization of their biological behaviors and facilitates overcoming the inherent limitations of the individual system. Past decades have witnessed the development of several types of CPNDS for various applications including therapeutic drug delivery and diagnostic imaging [178]. This work provides a comprehensive overview of the existing and latest technologies and their application for the development of CPNDS.

1.13. Techniques for Preparing CPNDS

The CPNDS can be prepared by the modification of as-prepared NDS by functionalization with various peptides. In general, the major strategies employed are the chemical conjugation method, ligand exchange method, and chemical reduction method.

1.13.1. Chemical Conjugation Method

In this method, the peptide of choice is attached to the NDS in two steps. First, the NDS is capped by stabilizers (by using either hydrophilic shells or PEG derivatives), which contain active groups that are suitable for binding peptides. Furthermore, peptides can be conjugated on the surface of NDS via a reaction with stabilizers. This method has been found to be suitable for the immobilization of positively charged or neutral peptides on gold nanoparticles (AuNPs) capped with citrate [179][180]. Bartczak et al. demonstrated the conjugation of a positively charged KPQPRPLS peptide (which binds to epidermal growth factor receptor (EGFR)) to carboxy terminated oligoethylene glycol stabilized AuNPs by employing an EDC/sulfo-NHS (1-ethyl-3-(3-

dimethylaminopropyl) carbodiimide hydrochloride/N-hydroxy sulfosuccinimide) coupling technique [181]. In another research study, Fu et al. synthesized manganese-doped iron oxide NPs (MnIO NPs) by the functionalization of monocyclic peptide (MCP, the CXC chemokine receptor 4 (CXCR4) antagonist) [182].

1.13.2. Ligand Exchange Method

“In this method, the existing ligand on the surface of NDS is displaced by the desired peptide ligand. This method is the simplest approach for functionalizing the surface of NDS with peptides [183],[184]. This method has been extensively employed for preparing cysteine (Cys, C)-containing peptides functionalized AuNPs owing to the presence of the thiol group of cycteine, which is capable of forming a strong S-Au covalent bond with the surface of AuNPs [185]–[186]. Lévy et al. demonstrated that the cysteine–alanine–leucine–asparagine–asparagine (CALNN) pentapeptide is capable of converting citrate-capped AuNPs to stable and water-soluble AuNPs equipped with chemical features similar to proteins [186]. It has been shown that the CALNN peptide is mostly captured in the endoplasmic reticulum due to its higher affinity toward the ER signal and its capacity to penetrate the nucleus. Interestingly, the AuNPs modified with CALNN can be functionalized with various biomolecules including nucleic acid and biotin, which is applicable for biomedical application. Later, it was found that the Au-S covalent bond can be degraded by the thiol group, which is often found in the biological system. Notably, the Tang group resolved this limitation by developing a method for preparing peptide-functionalized AuNPs (peptide-Se-AuNPs) via the Au–Se bond in lieu of the Au–S bond by employing a peptide with Se-modified cysteine [187]–[188][189].

1.13.3. Chemical Reduction Method

This method involves three steps: first, the metal ion precursor is premixed with a peptide in a reaction solution. Second, a small amount of reducing agent is added to the reaction solution. Third, the as-prepared peptide-functionalized NPs are purified [190]–[191][192]. Notably, the peptide is responsible for reducing the metal ions and the stabilization of NPs. The presence of amino acid residues in the peptide, for example, tyrosine, aldehyde-functionalized proline, and tryptophan, are capable of reducing the metal ions to the metal via electron transfer [191][193]. Another research study showed that peptides could act as a stabilizing agent; however, other chemicals such as ascorbic acid and sodium borohydride can be used as reducing agents [194]. Corra et al. demonstrated that the HH-dL-dD-NH₂ peptide can be employed as a capping agent to produce palladium NPs (PdNPs), platinum NPs (PtNPs), and AuNPs equipped with high monodispersed and colloidal stability in solution [192].

1.13.4. Peptide Conjugation of NDS for Therapy and Diagnosis of Cancer

A plethora of studies have shown the application of artificial bioactive peptides, and many of those have been commercialized [195][196]. Despite tremendous advancements, most peptides suffer from various limitations including lower binding affinity toward targets, lower selectivity compared to the proteins, susceptibility to digestion by proteases [197], and shorter half-life [198]. Interestingly, the integration of peptides with various non-biological materials such as small molecules, polymers, metals, and hydrogels have shown potential to resolve the inherent limitation of peptides [199][200]. Especially, NDS have shown promising capacity to form conjugates with peptide, which could not only alleviate the peptides' function but also execute abiotic properties, leading to synergistic effects. Therefore, the CPNDS has been considered a promising tool for cancer therapy and diagnosis.

As noted previously, various factors such as hypoxic TME, MPS, cellular barrier, and drug-efflux transporters are major hurdles in nano delivery, and peptide-conjugated NDS have been found to be useful to overcome these scenarios. In the context of hypoxic TME, stimuli-responsive peptide-conjugated nano delivery systems have been developed. For example, pH-responsive insertion peptides possess feasible interactions with the cell membrane at neutral pH, but they can penetrate and form stable transmembrane complexes at acidic pH, which is suitable for targeting hypoxic TME [201]. To overcome the MPS, Tang et al. developed RES-specific blocking systems employing a “don’t-eat-us” approach, where a CD47-derived, enzyme-resistant peptide ligand was designed and placed on a d-self-peptide-labeled liposome (DSL). Interestingly, it facilitated the long-lasting masking of cell membranes, thereby reducing interactions between phagocytes and NDS [202]. Peptide-conjugated NDS have been found to be crucial to overcome the cellular barriers. There are many successful examples of peptide-conjugated particles helping in the targeted delivery of drug to the diseased cells and penetration across physiological barriers. For example, Georgieva et al. showed the conjugation of the G23 peptide to polymersomes for in vivo and in vitro delivery of therapeutic drug across the BBB [203]. Yao et al. reported that pDNA can be delivered across the BBB by conjugating dendrigraft poly-L-lysines (DGL) NP to poly (ethylene glycol) (PEG) and a LIM Kinase 2 derived cell-penetrating peptide (LNP) [204]. Peptide conjugation has been found to be effective in bypassing P-glycoprotein (P-gp), causing drug resistance [205][206].

For a long time, the selective targeted delivery of anti-cancer drugs to the target site has been a major bottleneck in cancer therapy. In the prevailing condition, peptides have shown a great potential for rendering targeted drug delivery selectively, warranting an alleviated performance for treating fatal diseases, including cancer [207][208]. NDS can be engineered via functionalization with specific peptides to achieve the targeted delivery of anti-cancer drugs to the target site (Figure 7).

Table 3 enlists the promising CPNDS based on cancer type, their specific receptor, and the conjugated peptide. There are various receptors, which have been employed as a target for peptide-conjugated NDS for cancer therapy.

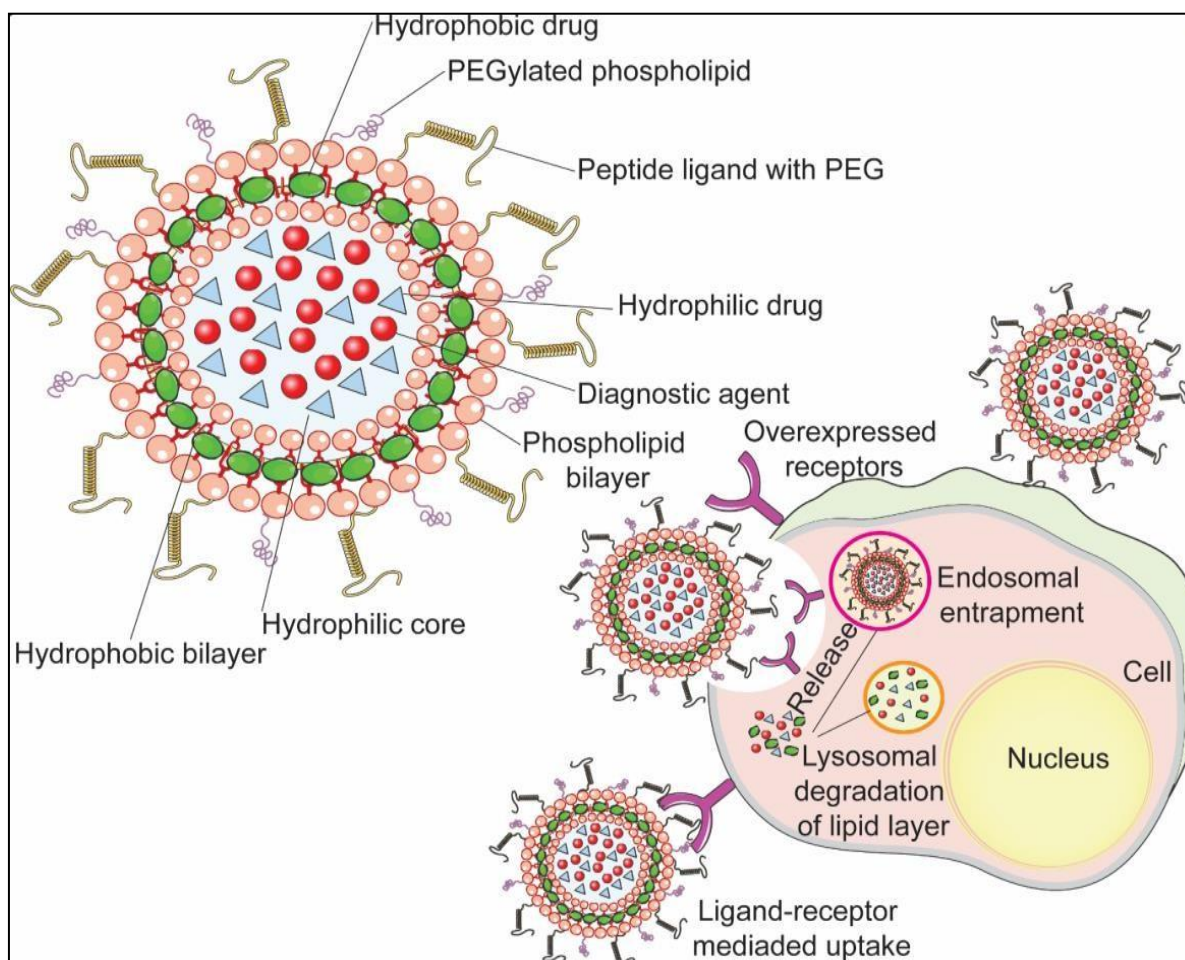


Figure 7. Schematic illustration showing the peptide functionalized liposomal NDS acting on receptors overexpressed on the surface of cancer cells via targeted delivery. The peptide conjugated to the NDS binds specifically to the receptors upregulated on the surface of cancer cells, which is followed by its uptake by the cancer cells through receptor-mediated endocytosis.

Subsequently, the payload of the NDS is released by the degradation of the lipid bilayer via the endosomal–lysosomal pathway.

Table 3. Peptides used in conjugation with NDS for targeting cancer-specific receptors.

Type of Cancer	Target Receptor	Peptide	Ref.
Breast cancer	SSTR	Octreotide	[209]
	$\alpha 1\beta 5$ integrin	ATN-161	[210]
	$\alpha v\beta 3$ integrin	Cyclic RGD	[211]
	HER2	KCCYSL	[212]
		AHNP	[213]
Colon cancer	$\alpha v\beta 3$ integrin	Cyclic RGD	[211]
Fibrosarcoma	Aminopeptidase	NGR	[214]
Glioma	SSTR	Octreotide	[215]
	$\alpha v\beta 3$ integrin	Cyclic RGD	[216]
	TFR	T7/TAT	[217]
	Aminopeptidase	NGR	[218]
Lung Cancer	SSTR	Octreotide	[219]
	TFR	T7/TAT	[220]
	LHRH	LHRL	[221]
Melanoma	$\alpha v\beta 3$ integrin	RGD	[222]
	$\alpha v\beta 3$ integrin	Cyclic RGD	[211]
Ovarian cancer	TFR	T7	[223]
	HER2	LTVSPWY	[224]

1.13.4.1. CPNDS Targeting Somatostatin Receptor

Somatostatin receptors (SSTR) are transmembrane GPCRs that have been found to be upregulated in several cancers, including adenocarcinoma and breast cancer [225]–[226]. Notably, the somatostatin peptide in its native form possesses a binding affinity toward SSTR, making them an alluring targeting agent for cancer treatment [227][228]. However, the somatostatin peptide possesses a shorter half-life owing to the enzymatic deterioration. Hence, octreotide was developed, which is an analog of the somatostatin peptide that can endure the enzymatic deterioration [227]. Various research groups employed the octreotide peptide-based functionalized NDS for cancer treatment [209],[219]. Zhang et al. prepared octreotide-PEG-distearoylphosphatidylethanolamine (DSPE), followed by developing octreotide-modified PEGylated liposomes loaded with doxorubicin (DOX), which promoted the delivery of DOX via an intracellular route. Notably, octreotide-functionalized NDS showed enhanced toxicity toward SSTR2-positive cancer cells through endocytosis [219]. Another research by Chang et al. developed octreotide-functionalized PEGylated liposome loaded with cantharidin, which could efficiently induce the cell death of MCF7 breast cancer cells by specifically targeting somatostatin receptors and demonstrated the lowered toxicity as compared to cantharidin alone [209].

The SSTR-based diagnosis of cancer has also been demonstrated; for example, SSTR-based imaging of gastroentero-pancreatic neuroendocrine tumors has been conducted by [111In-DTPA0]-octreotide (Octreoscan), octreotide chelator conjugates, 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA)-d-Phe1-Tyr3-octreotide (DOTATOC), and DOTA-dPhe1-Tyr3-octreotate (DOTATATE), which showed enhanced affinity toward SSTR [229][230]. In another research, ⁸⁹Zr- and gadolinium (Gd)-labeled

PEGylated liposomes functionalized with octreotide, which demonstrated SSTR2-targeting specificity and dual PET/MR imaging features [231].

1.13.4.2. CPNDS Targeting Integrin Receptor

Integrin is a transmembrane heterodimeric protein essential for the regulation of the different biological functions of cancer cells, including cell–cell and cell–ECM interaction [232]. Among various forms of integrins, $\alpha\beta3$, $\alpha\beta5$, and $\alpha5\beta1$ integrins are upregulated in cancer cells and associated with cancer cell phenotypes such as angiogenesis, tumor growth, and metastasis [233], suggesting that peptide-based ligands targeting integrins could be promising therapeutic agents for drug delivery as well as molecular imaging. One of the natural ligands of integrin is glycoproteins, which express themselves on the surface of the cell or protein of the extracellular matrix. Therefore, short peptide sequences that produce integrin-binding motives have gathered huge attention as a potential therapy; however, it was not found to pass the clinical trial successfully. Therefore, the integrin peptide ligand was alternatively used in conjugation with NDS for the specific delivery of drug to the cell, which is overexpressing the integrin receptor [234].

Various Arg–Gly–Asp (RGD)-based CPNDS were also developed as potential anti-cancer therapies and diagnostic probes [235]–[236]. For example, tripeptide RGD was reported as a ligand for $\alpha\beta3$ integrin overexpressed in solid tumors [237]. The RGD-modified PEGylated liposome-encapsulated DOX enhanced drug accumulation in cancer cells by internalization through the integrin receptor-mediated endocytosis pathway and showed antitumor effects [222]. Furthermore, to enhance the targeting efficacy, cyclic RGD-modified PEGylated liposomes were developed; for example, c(RGDfK), c(RGDfC), and RGD10 (DGARYCRGDCFDG) were found to be more stable at neutral pH as compared to

the noncyclic RGD peptide, which enabled them to resist proteolysis [235],[211],[238]. Additionally, they showed high affinity toward $\alpha\beta 3$ integrin in human BcaP-37 breast cancer, HT29 colon cancer, and A375 melanoma cells [211],[239].

In the context of cancer diagnosis, RGD-modified probes have been developed, such as [18F] Galacto-RGD, [18F] Alfatide, [68Ga] NOTA-PRGD2, 99mTcHYNIC-3PEG4-E[c(RGDfK)2], and 64Cu-DOTA-QD-RGD, which allowed the visualization of tumors in vivo [240]. Moreover, [18F] Galacto-RGD did not accumulate in the normal brain, unlike 18F-fluorodeoxyglucose (FDG), when used clinically as a PET tracer, suggesting that the RGD PET tracer can be applied to the imaging of glioma. [18F] Alfatide showed a higher tumor/background ratio in brain metastases compared with before the affinity was optimized [241]. Integrin $\alpha 5\beta 1$ shows potent anti-cancer activity, which is recognized by a non-RGD peptide, ATN-161 (Ac-Pro-His-Ser-Cys-Asn-NH₂) [242]. By coupling the PEGylated DOX liposome and ATN-161 lysine analog, the ATN-161-modified PEGylated DOX liposome was produced. It was reported that the integrin-mediated endocytosis mediates the cellular uptake of the ANT-161-modified liposome. Thereby, the ATN-161-modified PEGylated DOX liposome showed the significant antitumor effect on breast cancer cells and human umbilical vein endothelial cells [210].

1.13.4.3. CPNDS Targeting Transferrin Receptor (TFR)

TFRs are transmembrane glycoproteins receptors that facilitate the iron uptake by interacting with transferrin, an iron-binding protein [243]. Since the TFR is found to be upregulated on the surface of various cancer cells including breast cancer, lung adenocarcinoma, glioma, and chronic lymphocytic leukemia, it became an attractive molecule for cancer therapeutics [244]–[245][246]. Interestingly, the transport of various substances

including anti-cancer drugs across the blood–brain barrier (BBB) is found to be regulated via P-glycoprotein and tight junction [247]. As the expression level of TFR in the BBB is high, the NDS conjugated with TF can cross the BBB through receptor-based endocytosis. Research reported that dual targeting DOX liposomes conjugated with TF and folate yielded anti-cancer effects in C6 glioma cells [248]. Lee et al. developed peptide T7 (HAIYPRH) using a phagedisplay method and showed higher TFR binding activity compared with TF [249].

TFR also represents a unique target for the specific imaging of cancer cells, suggesting its applicability in the diagnosis of cancer progression. Zhang et al. developed a light-up probe TPETH-2T7 by conjugating a red-emissive photosensitizer with aggregation-induced emission (AIE) with peptide HAIYPRH(T7), enabling them to target TFR. The probe alone is non-emissive; however, it yields turn-on fluorescence in the presence of Tfr. In vitro experiments showed that the probe specifically binds to TFR, which is overexpressed on the MDA-MB-231 breast cancer cells. Notably, the image-guided photodynamic cancer ablation is evidence of its cancer therapeutic ability as well [250]. Wang et al. developed self-assembled IR780-loaded transferrin NDS, which are applicable for imaging and targeting, and offered a combined value as photothermal and photodynamic therapy, which is suitable for cancer therapeutics [251]. Another class of transferrin, which is known as lactoferrin, has been found to be highly expressed in the BBB [252], and it possesses better permeability than transferrin [253],[254]. Notably, Miao et al. functionalized lactoferrin to the surface of poly(ethylene glycol)-poly(lactic acid) nanoparticles to facilitate BBB/BBTB and glioma cell dual targeting. Interestingly, tLyP-1, a tumor-homing peptide, which contains a C-end Rule sequence that can facilitate tissue penetration via the neuropilin-1-dependent uptake pathway, was coadministrated with lactoferrin-functionalized NPs to augment its

accumulation and deep penetration into the glioma parenchyma, suggesting its suitability for anti-glioma drug delivery [255].

1.13.4.4. CPNDS Targeting the HER2 Receptor

HER2 is highly expressed in various cancers including breast cancer, gastric cancer, and ovarian cancer [256][257]. Trastuzumab, a recombinant monoclonal antibody, has been found to target specifically HER2 [258]. Additionally, combinatorial therapy with trastuzumab showed a higher anti-cancer therapeutic effect [259]. However, a tedious method for producing recombinant monoclonal antibodies makes it relatively costly. Conversely, the production of peptide-based ligands is cost-efficient and equipped with low antigenicity. Therefore, HER2-specific peptide ligands have gained attention; for example, Karasseva et al. developed KCCYSL peptide using the phage display technique and demonstrated its activities against human breast and prostate cancer cells with HER2 overexpression [260]. In another research, the pH-responsive PEGylated DOX liposome was modified with KCCYSL, which could specifically bind to and internalize in HER2-positive cells, and then pH-tunable vesicles release DOX swiftly and significantly. Notably, this liposome inhibited the tumor growth in a breast cancer mouse model with HER2-positive BT474 breast cancer cells [212].

Another peptide AHNP (FCDGFYACYADVGGG) was created from a heavy-chain CDR3 loop of trastuzumab, which was found to have HER2-specific affinity [261]. In another research study, AHNP-PEG-DSPE was developed with three glycine amino acids, and it was applied to AHNP-modified PEGylated DOX liposomes. Notably, this liposome showed tumor inhibition properties in a breast cancer mouse model bearing HER2-positive TUBO cancer cells [213]. In the context of the diagnostic application, PEGylated chitosan-modified

LTVSPWY (LTVSPWY-PEG-CS) was developed as an MRI imaging probe, which could detect cancer efficiently in vivo [224].

1.13.4.5. CPNDS Targeting Aminopeptidase N

Aminopeptidase N (or CD13) is associated with the growth of various cancers and suggested as a potential target for anti-cancer treatment. Interestingly, tripeptide Asn-Gly-Arg (NGR) is a ligand of aminopeptidase N (APN/CD13), which is found to be overexpressed in cancer cells and also target neoangiogenic blood vessels [262]. APN-targeted NDS have been developed by various groups; for example, after the intravenous injection of the c-Myc siRNA loaded in NGR-modified PEGylated liposomes, they are delivered efficiently to the HT1080 fibrosarcoma cytoplasm. Therefore, the result at the tumor site showed the suppression of c-Myc and evoked cellular apoptosis [54]. Antitumor activity was observed in HT1080 fibrosarcoma cells and HUVECs by the quantitative accumulation of docetaxel, which was loaded in NGR-modified PEG-b-PLA polymeric micelles [103]. When NGR, thermosensitive liposomes, and DOX were conjugated with CPP, it showed an inhibition of tumor growth in HT1080 fibrosarcoma cells [104]. If NGR peptides are conjugated with an imaging agent such as fluorescent dye, QDs, micelles, and liposomes show potential in visualizing the tumor. The glioma-associated vessels in a fluorescent imaging system were clearly shown, and CD31 were specifically recognized when PEGylated CdSe/AnS QDs were modified with an NGR peptide [58].

1.13.4.6. CPNDS Targeting Luteinizing Hormone-Releasing Hormone (LHRH)

Another receptor, LHRH, is overexpressed in different cancers such as breast, colorectal, ovarian, and prostate cancers, and it is a crucial anti-cancer target [263],[264]. Bajusz et al.

developed LHRH-based peptides, SB-05, SB-86, SB-40, and SB-95 as cancer-specific ligands. Interestingly, these ligands showed high affinities toward the membrane receptors of human breast and prostate cancer cells as well as rat pituitary Dunning R-3327 prostate cancer cells [265,266]. AEZS-108 (previously known as AN-152), a hybrid molecule consisting of a synthetic peptide carrier covalently coupled to DOX, was found to facilitate the delivery of DOX specifically to cancer cells expressing LHRH, including in uveal melanoma [267] and prostate cancer [268]. Mingqiang et al. developed cisplatin-loaded LHRH-modified dextran NPs (Dex-SA-CDDP-LHRH), which could specifically target LHRH receptors overexpressed on the surface of 4T1 breast cancer cells [269].

1.13.4.7. CPNDS Targeting Epidermal Growth Factor Receptor (EGFR)

EGFR has been widely reported to be crucial for uncontrolled signal transduction associated with cellular growth [270]. Notably, the GE11 peptide binds specifically to EGFR, which is overexpressed in various cancers including breast cancer, lung cancer, and glioma [271]. Therefore, the GE11 peptide has been conjugated with different NDS; for example, Huang et al. developed GE11 peptide-conjugated liposomes loaded with the photosensitizer indocyanine green (ICG) and chemotherapy drug curcumin (CUR), which could demonstrate EGFR targeting as well as an anti-cancer effect [272]. Han et al. demonstrated that small peptide, AEYLR-conjugated, nano lipid carriers increased the specific cellular uptake in cancer cells with EGFR overexpression [273]. Mayr et al. synthesized platinum (IV) complexes conjugated with an EGFR-targeting peptide, LARLLT; however, it was found to be unsuitable for increasing the specific uptake of small-molecule drugs in cancer cells with overexpressed EGFR [274].

1.13.4.8. CPNDS Targeting Epithelial Cell Adhesion Molecule (EpCAM)

EpCAM (or CD326) is an epithelial cell marker that is frequently and most strongly expressed in tumor-associated antigens. It is expressed in various cancers including squamous cell carcinoma and adenocarcinoma [275]. Ma et al. demonstrated that the peptide SNFYMPL (SNF*) could target EpCAM. Next, they conjugated SNF* with poly(histidine)–PEG/DSPE copolymer micelles. Notably, SNF* labeling substantially enhanced the micelles binding with gastric adenocarcinoma and colon cancer cells and augmented the anti-cancer effects, and it also reduced the in vivo toxicities of the micelles. Therefore, SNF* peptide-based targeting paves the way for EpCAM-targeted cancer therapy as well as diagnosis [276].

1.13.4.9. CPNDS Targeting CD133

CD133 is commonly expressed in cancer stem cells from various cancers including glioma, colon cancer, prostate cancer, and lung cancer [277]. Yan et al. developed CD133 peptide-conjugated photosensitizer, CD133-pyropheophorbide-a (Pyro), which showed a targeted photodynamic effect in colorectal cancer stem cells (CRCSC). Conventional photosensitizers such as (Pyro) lack tumor selectivity, triggering unwanted toxicity to the nearby healthy tissue. Interestingly, CD133-Pyro augmented the targeting capacity of Pyro, and it was found that CD133-Pyro exhibits the targeted delivery ability both in CRCSCs and inhibited tumor growth in a mouse model, suggesting its applicability for the therapy of CRC via CRCSC targeting [278].

1.14. Cell-Penetrating Peptides (CPP)

Cell penetration of the peptide is classified into two categories. (A) On the basis of peptide origin, they are subdivided into three types: chimeric, protein derived, and synthetic. Chimeric CPPs are made of two different peptide motifs. Transportan is said to be chimeric CPP that has been derived from mastoparan and galanin. Examples of protein derived CPPs are TAT and penetratin, which is a natural protein derivative. The synthetic peptides are of the polyarginine family [279]. (B) The second category of CPP classification is based on physiochemical property. Based on physiological property, there are three types of CPP: cationic, amphipathic, and hydrophobic. As a result of its positive charge, many CPPs are cationic. The example of cationic CPP is TAT, the transcriptional activator protein in HIV-1 [280]. The amphipathic CPPs, because of the lysine residue in their structure, are the sequences with a high degree of amphipathicity: for example, Transportan, a 27 amino acid long peptide [281]. In the case of hydrophobic CPP, only the hydrophobic motif or non-polar sequence are present [282].

Regarding the mechanism for the internalization of CPP, for the transportation of CPP across the biological membrane, the exact mechanism is still unclear. However, after going through certain literature, the outcome showed that there may be three possible pathways for CPP internalization into the membrane. The three most effective parameters for the internalization pathway of CPP into the cellular membrane are the peptide concentration, peptide sequence, and lipid component in each membrane [283],[284].

On the basis of peptide concentration, the route for the uptake of different cationic CPPs varies. When the concentration is high, rapid cytosolic uptake is detected, and at the lower concentration of peptide, the mechanism of uptake is dominant [285][286]. The second

influential parameter for the uptake mechanism of CPP is peptide sequence. The local concentration of TAT and penetratin, which are arginine-rich CPPs, in a biomembrane may be enhanced due to the highly positively charged CPPs [287][288]. For the internalization of CPPs, there are three possible mechanisms. (i) The first is direct penetration, which is an energy-independent pathway including various mechanisms including pore formation, a carpet-like model, and a membrane-thinning model [289][290]. (ii) The second mechanism is the endocytosis pathway, in which the transduction approach is energy dependent. In endocytosis, the inward folding of the plasma membrane takes place to carry material from outside of the cell and absorb them. The three different classes of endocytosis are pinocytosis, phagocytosis, and receptor-mediated endocytosis. (iii) The third mechanism is translocation through the formation of a transitory structure. In this, the interaction of CPP takes place with the cellular membrane, which causes the disruption of the lipid bilayer of the membrane following the formation of an inverted structure, the inverted micelles [291].

1.15. Conjugation of Peptides and Extracellular Vesicles (CPEVs) for Cancer Therapy

Extracellular vesicles (EVs) are nanovesicles with a size around 30–1000 nm, which are secreted from most of the cell types and are found in various biofluids including blood and urine [48]–[292][293]. Recently, EVs have emerged as a promising NDS with huge application in cancer therapy as well as diagnosis. A detailed review of the factors reacting with EV-based drug delivery systems has been reported by our group previously [294]. Interestingly, the surface modification of EVs has a great potential to achieve the targeting

ability [89]. There are various methods that could be utilized to modify the surface of EVs to conjugate the ligand, such as physical approaches (sonication, extrusion, and freeze–thaw) that can change the surface properties of EVs via membrane rearrangements and biological approaches (genetically and metabolically engineering cells to express protein or cargo molecules of interest in secreted EVs) [89].

Various groups have demonstrated the applicability of the GE11 peptide for the specific targeting toward the EGFR receptor for different purposes [295][296], including drug delivery [297]–[298]. Importantly, Ohno et al. (2013) showed that the delivery of micro RNA (miRNA) to EGFR-expressing breast cancer cells can be achieved efficiently by EVs. For this, the donor cells were engineered to express the transmembrane domain of the platelet-derived growth factor receptor fused to the GE11 peptide. Notably, the exosome that was injected intravenously could deliver the let-7a miRNA to EGFR-expressing xenograft breast cancer tissue in RAG2(-/-) mice. The result showed that EVs can be employed to target the EGFR expressing cancer tissue with nucleic acid drug for therapeutic purposes [40]. In another research study, Nakase et al. developed a novel drug delivery system based on biofunctional peptide-modified exosomes, which includes arginine-rich cell-penetrating peptide-modified exosomes for the active induction of micropinocytosis and the effective intracellular delivery of therapeutic molecules, a pH-sensitive fusogenic peptide for enhanced cytosolic release of exosomal contents, and a receptor target system using an artificial coiled-coil peptide modified on exosomal membranes [299].

1.16. Hypothesis and Aims

Hypothesis

The GE11 peptide specifically targets the Epidermal Growth Factor Receptor (EGFR), prevalent in many cancers, enhancing drug delivery to tumors and improving therapeutic efficacy with reduced side effects. Endothelial cell derived EVs play a significant role in anticancer therapy by transferring bioactive molecules like RNA, proteins, and lipids to cancer cells. They modulate tumor progression, angiogenesis, and immune responses, potentially enhancing the efficacy of anticancer drugs and contributing to the inhibition of tumor growth and metastasis. Apparently, endothelial cells derived EVs can be engineered by conjugating with GE11 peptide, followed by loading of vinorelbine, an anti-cancer drug, which would be useful for lung cancer therapeutics.

Aims

1. To optimize and characterize a novel GE11-HUVEC-EVs-Vin EDDS targeting the tumor-associated cell surface antigen EGFR.
2. To optimize the feasibility of using GE11-HUVEC-EVs-Vin EDDS in a lung cancer model.
3. To examine whether the therapeutic efficacy of vinorelbine can be improved by delivery via EGFR directed GE11-HUVEC-EVs-Vin EDDS.
4. To validate the distribution of the GE11-HUVEC-EVs-Vin EDDS at cellular levels and in a mouse model of lung cancer.

CHAPTER 2

##Note: The partial content of chapter 2 has been taken from following published paper [3], with due permission from all the contributing authors as well as the publisher: Iyaswamy A[#], Thakur A[#], Guan XJ, Krishnamoorthi S, Fung TY, Lu K, Gaurav I, Yang Z, Su CF, Lau KF, Zhang K..., Chen H*, Li M*. Fe65-engineered neuronal exosomes encapsulating corynoxine-B ameliorate cognition and pathology of Alzheimer's disease. Signal Transduction and Targeted Therapy. 2023 Oct 23;8(1):404.

MATERIALS AND METHODS

2.1. Ethics statement

Animal-related experiments were conducted as per protocols approved by the Committee on the Use of Human and Animal Subjects in Teaching and Research (HASC) at the Hong Kong Baptist University (HKBU) (# REC/21-22/0265).

2.2. Analysis of single cell- and bulk RNA- seq data

Single-cell RNA sequencing dataset GSE127465 [300] was obtained from a public database, the Gene Expression Omnibus (GEO). The dataset features total cells (n = 40,362) in patient lung tumor biopsies (n = 7). The single cell portal <https://singlecell.broadinstitute.org/> (Study# SCP739, accessed on 20th Oct 2024) was utilized for generating of two-dimensional visualization (SPRING plots), and the dot-plot showing the cell- type specific gene expression. Lung cancer specific bulk-RNA seq data were analyzed to obtain expression level of EGFR in pan-cancer (<https://tnmplot.com/analysis/> [301]), and the *EGFR* expression-based survival plots were generated using <https://kmplot.com/analysis/> [302].

2.3. *In silico* analysis for target identification

Lung cancer targets were collected through online databases such as GeneCards (<https://www.genecards.org/>), OMIM (<https://www.omim.org/search/advanced/geneMap>),

Therapeutic Target Database (<https://db.idrblab.net/ttd/>), and PharmMapper (<http://www.lilab-ecust.cn/pharmmapper/results/240204030418.html>), among others. The potential targets of compounds were predicted using SuperPred (<https://sea.bkslab.org/>) and SEA (<https://sea.bkslab.org/>). A total of 74 small molecule targets and 1520 lung cancer targets were collected, with an intersection yielding 24 targets. These targets were imported and analyzed and visualized using Cytoscape_v3.9.0. The network consists of 24 nodes and 110 edges.

2.4. Molecular docking

The 3D atomic coordinates for the crystal structures of Nanodisc-reconstituted human ABCB1 in complex with MRK16 Fab and elacridar (PDB id: 7A6C) were downloaded from the RCSB-Protein Data Bank (PDB). Additionally, the 3D structures of Azithromycin, Clarithromycin, Erythromycin, Elacridar, and Vinorelbine were retrieved from the PubChem database. The structure of ABCB1 was prepared for molecular docking by loading it into UCSF Chimera.

A grid box was generated to encompass the elacridar binding site of ABCB1 and provide ample space for the ligands' rotational and translational movement. The grid box parameters were set as follows: the center grid box coordinates were set to 164.622, 159.652, and 157.799 for X, Y, and Z dimensions, respectively, with a spacing of 0.375 Å and number of points of 84, 96, and 86 points in the X, Y, and Z dimensions.

AutoDock Tools 1.5.6 was utilized to perform molecular docking using Lamarckian genetic algorithm (LGA) search parameters. Subsequently, LigPlot+ (v.1.4.5) was employed to visualize the interaction patterns between the ABCB1-ligand complexes.

2.5. Cell culture

HUVEC and A549 cells were cultured in a standard medium containing Dulbecco's Modified Eagle Medium (DMEM) (Cat# 11965084, Gibco) supplemented with 10% fetal bovine serum (FBS) (Cat# 10500064, Gibco), and 1% penicillin-streptomycin solution. The cultured cells were maintained at 37 °C in an incubator with 5 % CO₂.

2.6. Isolation of HUVEC-EVs and GE11-HUVEC-EVs

EVs, including exosomes, were extracted from the HUVEC cell culture medium using the Total Exosome Isolation (TEI) reagent.[303] The process involved collecting the medium, centrifuging it at 2000 × g for 30 min, mixing the supernatant with TEI reagent, and incubating it at 4°C overnight. Subsequently, the mixture was centrifuged at 10,000 × g for 1 h at 4°C. The resulting pellet, which contained exosomes, was then diluted with 1× phosphate buffer saline (PBS) for analysis.

2.7. Surface engineering of HUVEC to express GE11 peptide (GE11-HUVEC-EVs)

The modification of HUVEC to express GE11 peptide was accomplished using the post-insertion technique, as described previously [304][305][306]. In brief, the GE11 peptide was dissolved in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer for 15 min at 60 °C to form micelles. Then, the HUVEC-exosome suspension was mixed with the above suspension for 2 h at

40 °C. After cooling to room temperature, exosomes were immediately purified by size-exclusion chromatography to get GE11-modified HUVEC EVs (GE11-HUVEC-EVs).

2.8. Analysis of size and concentration of EVs via nano tracking analyzer

The size distribution and concentration of exosomes were assessed using a Malvern Nanoparticle Tracking Analysis (NTA) system. A 405 nm laser beam was employed to analyze 500 ml of exosome solutions in the sample chamber. NTA software (version 2.2, NanoSight NS300) was utilized to analyze the captured videos of EVs in Brownian motion, allowing for the determination of size distribution and concentration of EVs.

2.9. Transmission electron microscopy (TEM)

The size, shape, and morphology of exosomes were characterized using transmission electron microscopy (TEM). A negative staining technique was employed, where a 30 µl drop of the exosome suspension in filtered PBS was placed on carbon-coated electron microscope grids, incubated at room temperature for 10 min, transferred to a drop of Uranylless® solution for 1 min, air dried; the excess stain was blotted away and the grids were observed using TEM machine.

2.10. Immunogold EM analysis

The presence of CD63 protein on exosomes was confirmed using immunogold-EM as described previously.[303] Initially, exosome suspensions, fixed with 2% PFA, were adsorbed to CCEM grids and washed in a solution of 0.05 M filtered glycine in PBS. Subsequently, the grids were incubated with anti-CD63 primary antibody for 24 h at 4 ° C, followed by incubation with

10 nm gold-conjugated donkey anti-rabbit antibody for 1 h at room temperature. After several washes, the grids were post-fixed, excess fluid was blotted, and the samples were dried before imaging using TEM machine.

2.11. Imaging flow cytometry analysis of exosomes

The cells were stained with anti-EGFR-conjugated Alexa 488 antibody, followed by analysis with image flow cytometry as described previously with slight modification [307]. Briefly, the cells were immune-stained with anti-EGFR-conjugated primary antibody for 1 hr at room temperature, followed by evaluation of cell samples with an imaging flow cytometer equipped with a laser. The images were acquired and analyzed using IDEAS software.

2.12. Fourier-transform infrared (FT-IR) microscopy

The exosome samples with and without peptide functionalization were analyzed with FT-IR via the potassium bromide (KBr) technique. Briefly, the exosome samples were diluted (ratio =1:10) in potassium bromide, followed by making pellets using a mini hand-held laboratory hydraulic press. A KBr pellet alone was used as a control. The FTIR instrument was used for obtaining the signals from the pellets of exosome samples with and without peptide functionalization, and the KBr alone.

2.13. Loading of vinorelbine on HUVEC-EXO and GE11-HUVEC-EXO, and their evaluation

Exosomes were loaded with vinorelbine using sonication. Equal amounts of vinorelbine and exosomes were mixed and sonicated using 20% amplitude and 6 cycles of 30 s-on/150 s-off. Subsequently, the solution was incubated at 37 °C for 60 min. To separate excess free drug, size exclusion chromatography with a Sephadex G25 column was employed. The efficiency of drug loading was assessed using a UV-visible spectrophotometer.

2.14. Labelling of EXO

Exosomes were labeled with Exo-Green Exosome Protein Fluorescent Labeling reagent (Cat# EXOG200A-1, System Biosciences). 50 µL of 10x Exo-Green was added to a 500 µL exosome solution in 1XPBS (200 µg protein) and mixed by flicking. The solution was then incubated for 10 min at 37 °C, and the labeling reaction was stopped by adding FBS. After incubation at 4 °C for 30 min, the solution was centrifuged at 14,000 rpm for 3 min to remove excess label. The labeled exosome pellet was then resuspended in PBS for subsequent monitoring.

2.15. Exosome uptake assay

Recipient A549 cells were cultured at a density of 30,000 cells per well on Lab-Tek chamber slides (Thermo Scientific, USA) for 24 h. After 24 h, the cells were washed with PBS and then exposed to a medium supplemented with 250 µg Exo-Green labeled- HUVEC-EXO and GE11-HUVEC-EXO. Subsequently, the recipient cells were washed with PBS, fixed with 4% PFA on ice for 30 min, and then washed again with PBS. The cells were stained, and the slide was covered with a thin layer of Vectashield medium containing DAPI for visualization under a confocal microscope at 40x magnification.

2.16. Cell viability assay

The MTT cell viability assay was conducted on A549 cells (10,000 cells per well) in a 96-well microtiter tissue culture plate. After 48 hours, the cells were treated with HUVEC-EVs, GE11-HUVEC-EVs, Vinorelbine (Vin), HUVEC-EVs-Vin, or GE11-HUVEC-EVs-Vino for 24 h. Following treatment, MTT solution was added to each well, and the cells were incubated for 4 hours at 37°C. Subsequently, DMSO was added to solubilize the formazan product, and the absorbance was measured at 570 nm.

2.17. Migration assay

The migration of the A549 cells (control or with various treatments) was evaluated via the scratch migration assay. It involves the creation of a "scratch" in a cell monolayer, typically using a pipette tip, to simulate a wound. The cells were then incubated, and images were captured at the beginning and at regular intervals during cell migration to close the scratch. The rate of migration was quantified by comparing the images, and the time required to close the wound.

2.18. Apoptosis assay

The level of apoptosis in the A549 cells (control and with various treatments) was evaluated via the Annexin-V staining of cells. The staining process involved incubation of the cells with the Annexin V antibody, which could bind to phosphatidylserine (PS) on the surface of apoptotic cells. The annexin-V-stained cells were examined via fluorescent microscopy.

2.19. Immunocytochemistry

The immunocytochemistry procedure was conducted on A549 cells grown on poly-D-lysine coated-glass cover slips until they reached 70% confluence. The cells were then treated with

Exo-Green-labeled EV samples, washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS. Subsequently, the cells were blocked with 1% BSA in PBS. Rhodamine phalloidin-conjugated primary antibody solution was used, and samples were mounted in a Vectashield medium with DAPI. The samples were examined using a confocal microscope.

2.20. Development of lung cancer mouse model and *in vivo* bioluminescence assay

A xenograft mouse model of lung cancer was developed to study the anti-tumor effects of GE11-HUVEC-EVs-Vin *in vivo*. This model was established using the implantation technique previously described by Onn et al. (2003). Briefly, A549 cell suspension was prepared in Matrigel Matrix (BD Biosciences), which is commonly used as an anchor to prevent tumor cells from spreading at the site of injection. SCID mice (6-8 weeks) were anesthetized and placed in the right lateral decubitus position. 1mL syringes with 30-gauge needles were used to inject A549 cells percutaneously into the left lateral thorax, at the lateral dorsal axillary line, about 1.5 cm above the lower rib line just below the inferior border of the scapula. After tumor cell injection, the labeled EV samples namely HUVEC-EVs, GE11-HUVEC-EVs, HUVEC-EVs-Vino, and GE11-HUVEC-EVs-Vino, and Vinorelbine were injected intravenously (4 µg of exosomes/mouse). The mice were observed until fully recovered. In the 9th week after tumor cell implantation, mice were sacrificed and lung tumors were collected. The tumor formation was confirmed via the H&E staining, and EGFR expression was confirmed in the tumors using immunohistochemistry.

2.21. Hematoxylin-Eosin (H&E) Staining

H&E staining was carried out on xenograft tumors, fixed with 4% paraformaldehyde (PFA), embedded in paraffin, and sectioned into 5 μ m slices using an ultra-thin semiautomatic microtome. The sections were then deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) using standard protocols. H&E-stained sections were examined under a light microscope.

2.22. Immunohistochemistry

The lungs of mice (control and with various treatments) were processed for immunohistochemistry by preparing PFA-fixed, frozen, and cryomatrix-embedded sections. These sections were then subjected to immunostaining analysis using primary antibodies for EGFR and Ki67, along with EXO-Green-labeled exosomes. Following the staining, the sections were imaged, and images were analyzed using Image J analysis (NIH) software. This comprehensive process allowed for the examination of protein expression and exosome localization in specific lung regions.

2.23. Statistical analysis

Differences were statistically evaluated using a one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test. P-value < 0.05 was considered statistically significant.

CHAPTER 3

RESULTS

3.1. *EGFR* gene overexpression is associated with poor survival of lung cancer patients

The *EGFR* gene is important in cancer because it plays a role in many of the key processes that drive cancer development and progression, including cell growth, division, survival, and migration. *EGFR* overexpression can lead to cancer by making cancer cells more resistant to apoptosis (programmed cell death) and by making them more likely to metastasize.[308] Therefore, it is pertinent to examine the correlation of the *EGFR* gene with the overall survival of lung cancer patients. Notably, the pan-cancer analysis using RNA-seq data from the TCGA database showed that *EGFR* is in general overexpressed in most cancers including lung cancer compared to their respective control groups. (**Fig. 8A**). We also found that high expression of *EGFR* is correlated with poor survival of lung cancer patients (**Fig. 8B**). Moreover, the lung cancer stage-based analysis showed that in stage IV, the median survival is far less while the expression of *EGFR* is heightened (**Fig. 9A-D**). Single cell transcriptomic analysis of lung cancer patient data (GEO: GSE127465)[300] revealed cell type-specific distribution of *EGFR* in lung cancer TME (**Fig. 10A-E**). Further, *EGFR* expression was positively correlated with *MYC*, *CD44*, *MET*, and

KRAS (**Fig. 8C-F**). *MYC* is a transcription factor that regulates the expression of many genes involved in cell growth, division, and metabolism. *MYC* overexpression is common in many types of cancer, including lung cancer.[309] *CD44* is a cell surface protein that helps cells to interact with their environment. *CD44* overexpression has been linked to cancer cell stemness, invasion, and metastasis.[310] *MET* is a receptor tyrosine kinase that is activated by hepatocyte growth factor (HGF). *MET* overexpression can lead to cancer cell proliferation, survival, and migration.[311] *KRAS* is a small GTPase that plays a role in cell signaling. *KRAS* mutations are common in many types of cancer, including lung cancer, colon cancer, and pancreatic cancer. The expression of *EGFR* is also linked with major markers, contributing to lung cancer development and progression (**Fig. 8C-F**).

Programmed death-ligand 1 (PD-L1) and programmed death-1 (PD-1) are two proteins that play a key role in lung cancer immune evasion. PD-L1 is expressed on the surface of cancer cells and other cells in the TME. PD-1 is expressed on the surface of T cells, which are a type of white blood cell that plays a key role in the immune response. When PD-L1 interacts with PD-1, it sends a signal to the T cell to stop it from attacking the cancer cell. This allows the cancer cell to evade the immune system and continue to grow and spread. PD-L1 overexpression is common in lung cancer, and it is associated with a poor prognosis. Patients with PD-L1-overexpressing tumors are more likely to have a shorter overall survival and are less likely to respond to immunotherapy.[312] Immunotherapy is a type of cancer treatment that harnesses the body's immune system to fight cancer. Immune checkpoint inhibitors are a type of immunotherapy that blocks the interaction between PD-L1 and PD-1. This allows T cells to recognize and attack cancer cells.[313] Immune checkpoint inhibitors are effective in treating some patients with lung cancer, especially those with PD-L1-overexpressing tumors. Targeting the PD-L1/PD-1 pathway with immunotherapy is a

promising approach to cancer treatment.[312][313] Therefore, we further analyzed the effect of anti-PD-L1 or anti-PD-1 therapy on the *EGFR* via bioinformatics analysis using the TCGA dataset. Interestingly, the expression of *EGFR* was significantly reduced in the responder group towards both anti-PD-L1 and anti-PD-1 therapy (**Fig. 8C-F**). We conclude that *EGFR* is a crucial target for developing targeted therapeutics for cancers including lung cancer.

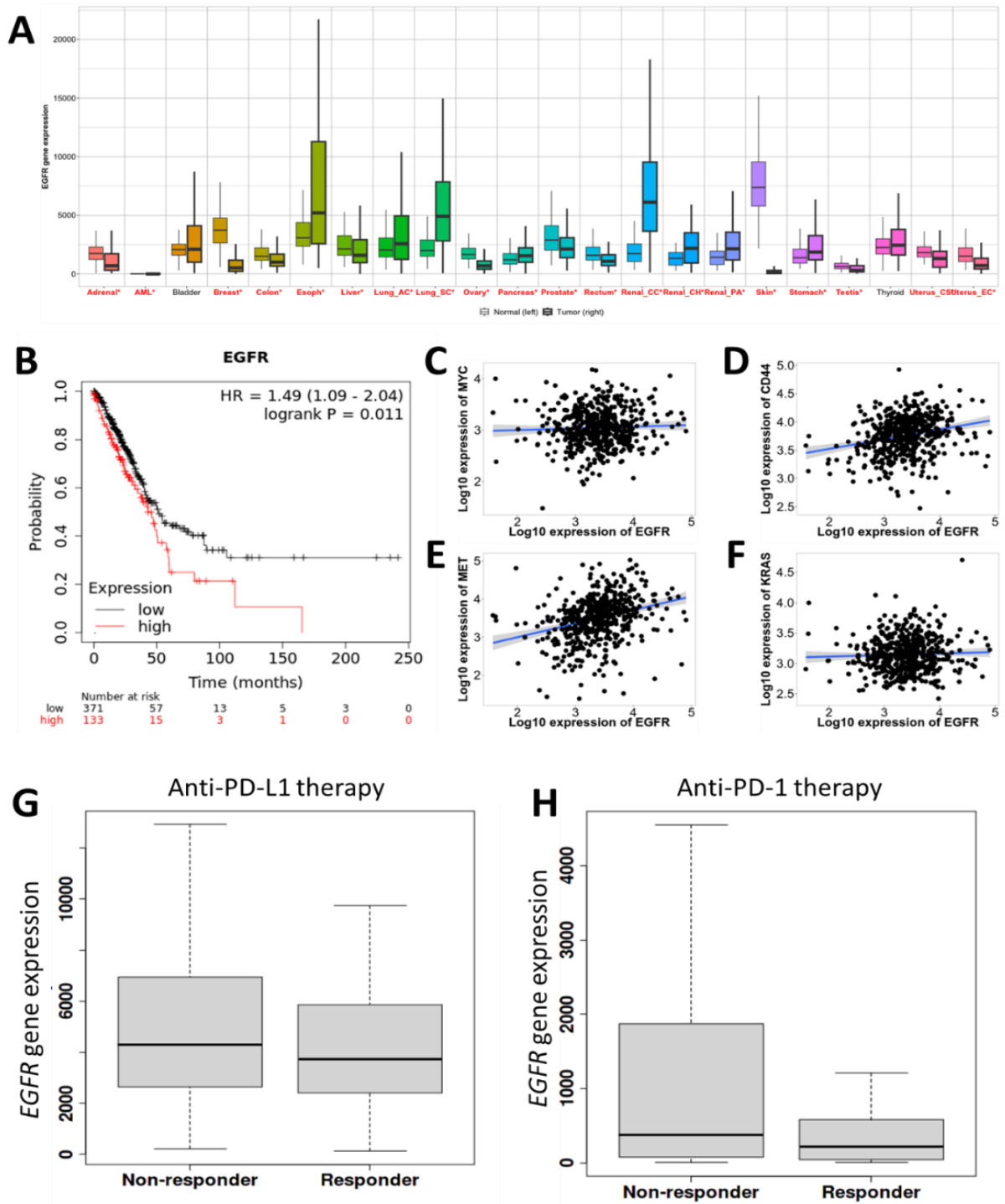


Figure 8. High expression of *EGFR* is correlated with poor survival of lung cancer patients.

(A) Bar graphs showing the expression of *EGFR* in different types of cancer as compared to their respective controls. (B) Survival curve showing the time dependent probability of survival with

EGFR expression in lung cancer patients. **(C-F)** Graphs showing the positive correlation between (C) *EGFR* vs *MYC*, (D) *EGFR* vs *CD44*, (E) *EGFR* vs *MET*, and (F) *EGFR* vs *KRAS*. **(G, H)** Bar graphs showing the expression of *EGFR* in non-responder and responder towards the treatment of (G) anti-PD-L1 therapy, and (H) anti-PD-1 therapy.

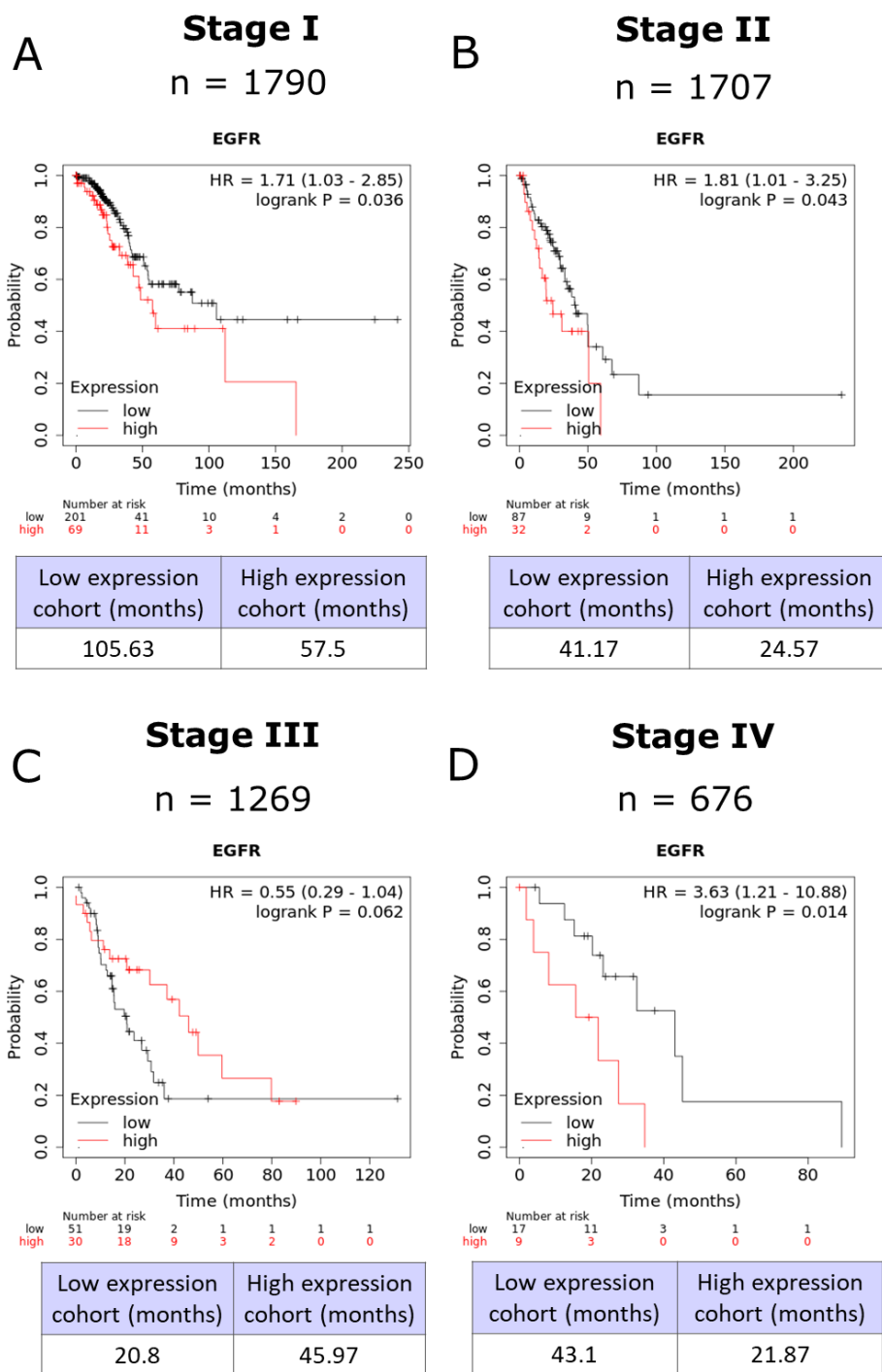


Figure 9. Stage-dependent survival of lung cancer patients with the corresponding expression level of *EGFR*. Survival curves of patients with lung cancer with the expression of *EGFR* in (A) stage I, (B) stage II, (C) stage III, and (D) stage IV.

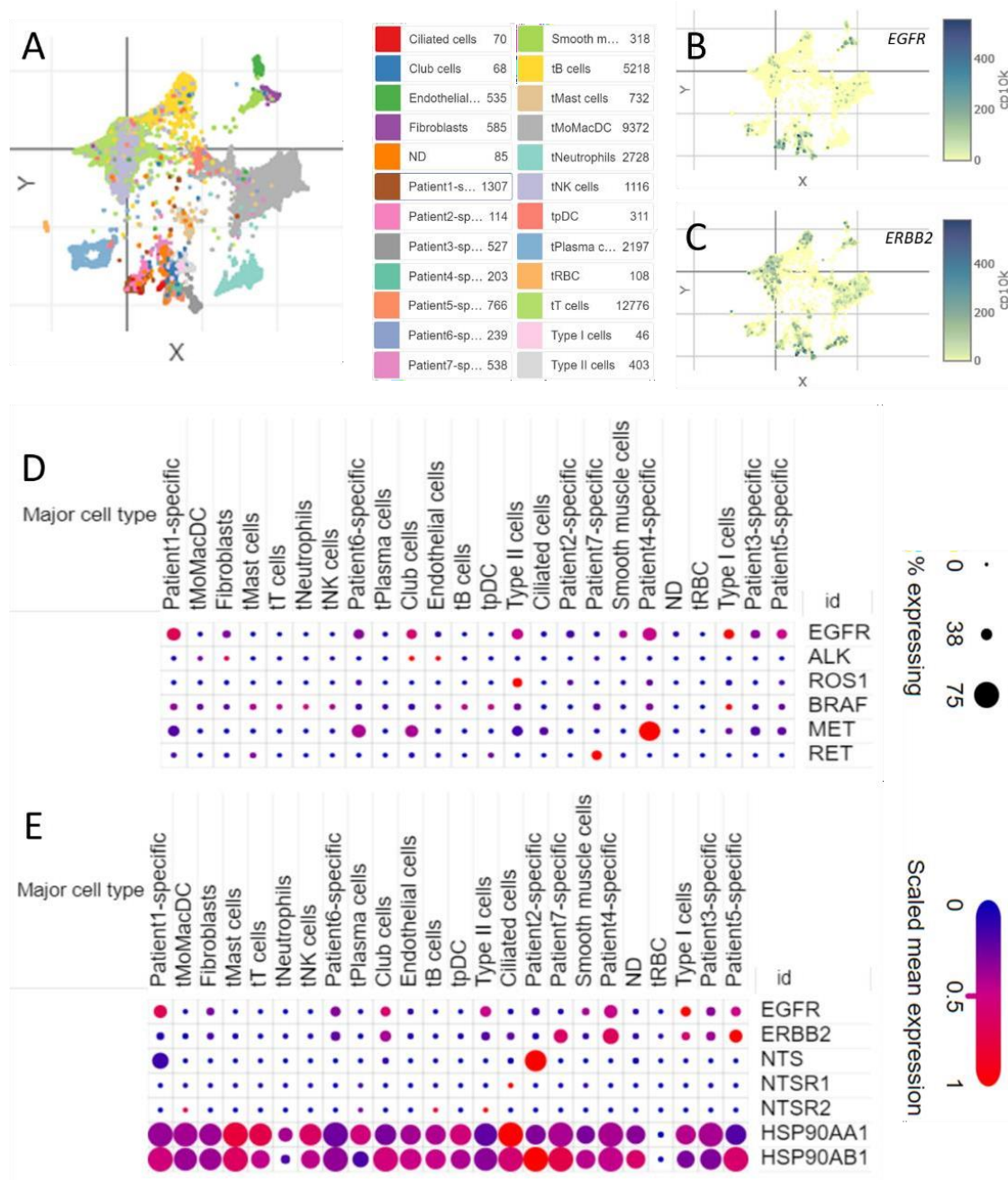


Figure 10. Single-cell RNA-seq analysis of tumor microenvironments (TMEs) in lung cancer patients. (A) UMAP depicting the different cell types in the TME of human non-small cell lung

cancer compared to control group (based on GEO: GSE127465). **(B, C)** UMAPs showing the expressions of (B) *EGFR* and (C) *ERBB2* in the clusters, representing cancer cell types. **(D, E)** Dot plots showing the cell type-specific expression of major cancer markers including *EGFR* and *ERBB2*. The figures are based on the analysis of single cell RNA-seq data publicly available (GSE127465).

Discussion

The correlation between EGFR gene mutation and the overall survival of lung cancer patients is a crucial aspect of cancer research. The EGFR gene plays a pivotal role in cancer development and progression by influencing cell growth, division, survival, and migration. Overexpression of EGFR can lead to cancer by making cancer cells more resistant to apoptosis and more likely to metastasize. Studies have shown that EGFR mutations are associated with specific features, such as female gender, never-smoking history, ethnicity, and adenocarcinoma histology. The presence of EGFR mutations is a predicting factor for the response to EGFR tyrosine kinase inhibitors (TKIs) and is associated with a good prognosis in advanced NSCLC patients. In non-small cell lung cancer (NSCLC), EGFR mutations are more common in females, never smokers, and patients of Asian ethnicity. The most common EGFR mutations are in-frame deletions in exon 19 and missense substitutions in exon 21. The correlation between EGFR mutation status and the incidence of brain metastases in patients with NSCLC has also been investigated. The results suggest that patients with EGFR mutations have a higher incidence of brain metastases, which may be due to the use of EGFR-TKIs that prolong overall survival but increase the risk of brain metastases⁴. Furthermore, the expression of EGFR is positively correlated with MYC, CD44, MET, and KRAS, which are all involved in cell growth, division, survival, and

migration⁵. Overexpression of these genes is common in many types of cancer, including lung cancer, and is associated with a poor prognosis. The expression of EGFR is also linked to major markers contributing to lung cancer development and progression, including programmed death-ligand 1 (PD-L1) and programmed death-1 (PD-1), which play a key role in lung cancer immune evasion. Overexpression of PD-L1 is common in lung cancer and is associated with a poor prognosis, while targeting the PD-L1/PD-1 pathway with immunotherapy is a promising approach to cancer treatment. Conclusively, the EGFR gene is a crucial target for developing targeted therapeutics for cancers, including lung cancer. The correlation between EGFR gene mutation and the overall survival of lung cancer patients is complex and influenced by various factors, including gender, smoking status, tumor markers, and FDG uptake. Further research is needed to fully understand the role of EGFR in lung cancer and to develop effective targeted therapies.

3.2. EGFR protein is significantly over-expressed on the membrane of A549 cells in hypoxic TMEs.

As the over-expression of EGFR on the surface of lung cancer cells has been linked with their malignant behavior[314][315], we anticipated that a ligand targeting EGFR could be used for lung cancer therapeutics if it were conjugated on the surface of EVs. We performed immunocytochemistry (ICC), which helps to reveal the distribution of EGFR on the surface of lung cancer cells by using anti-EGFR primary antibodies. Interestingly, the ICC results showed that EGFR is primarily localized on the surface of A549 lung cancer cells (**Fig. 11A-C**). As

hypoxia, or low oxygen levels, is a common feature of the TME and is closely associated with cell proliferation, angiogenesis, metabolism, and the tumor immune response, it can promote tumor progression, increase its aggressiveness, and enhance metastatic potential—all of which translates to poor prognosis [316]. Therefore, we examined the effect of hypoxia (1% O₂ level) on the protein level of EGFR in A549 cells via image flow cytometry. The level of EGFR was significantly enhanced in the hypoxia exposed A549 cells as compared to the control A549 cells (**Fig. 11D, E**), and normal control cell HEK293T cell (**Fig. 11F**). This result shows that EGFR would be a good target for the delivery of medicine in lung cancer treatment.

Discussion

The use of ligands targeting EGFR, conjugated on the surface of extracellular vesicles (EVs), has been proposed as a potential strategy for lung cancer treatment. Immunocytochemistry (ICC) has been employed to reveal the distribution of EGFR on the surface of lung cancer cells, demonstrating that EGFR is primarily localized on the surface of A549 lung cancer cells. This finding supports the idea that EGFR could be a suitable target for the delivery of medicine in lung cancer treatment. Hypoxia, a common feature of the tumor microenvironment (TME), has been shown to promote tumor progression, increase aggressiveness, and enhance metastatic potential, leading to poor prognosis. The effect of hypoxia on the protein level of EGFR in A549 cells has been examined using image flow cytometry, revealing that the level of EGFR is significantly enhanced in hypoxia-exposed A549 cells compared to control A549 cells. This result further supports the targeting of EGFR for the delivery of medicine in lung cancer treatment. The role of EGFR in lung cancer progression and metastasis has been extensively studied. EGFR-tyrosine kinase inhibitors, such as erlotinib and gefitinib, have been found to be effective in suppressing

the proliferation of malignant lung cells, enhancing apoptosis, and reducing lung cancer metastasis. These inhibitors have been successful in suppressing lung cancer progression in preclinical studies and have been used in second and third-line treatments following chemotherapies. The correlation between EGFR mutation status and the incidence of brain metastases in patients with non-small cell lung cancer (NSCLC) has also been investigated. The results suggest that patients with EGFR mutations have a higher incidence of brain metastases, which may be due to the use of EGFR-tyrosine kinase inhibitors that prolong overall survival but increase the risk of brain metastases. Furthermore, the expression of EGFR is positively correlated with MYC, CD44, MET, and KRAS, which are all involved in cell growth, division, survival, and migration. Overexpression of these genes is common in many types of cancer, including lung cancer, and is associated with a poor prognosis. In conclusion, the targeting of EGFR for the delivery of medicine in lung cancer treatment is a promising approach. The over-expression of EGFR on the surface of lung cancer cells, its enhancement under hypoxic conditions, and its correlation with other genes involved in cell growth and migration make it an attractive target for lung cancer therapeutics. Further research is needed to fully understand the role of EGFR in lung cancer and to develop effective targeted therapies.

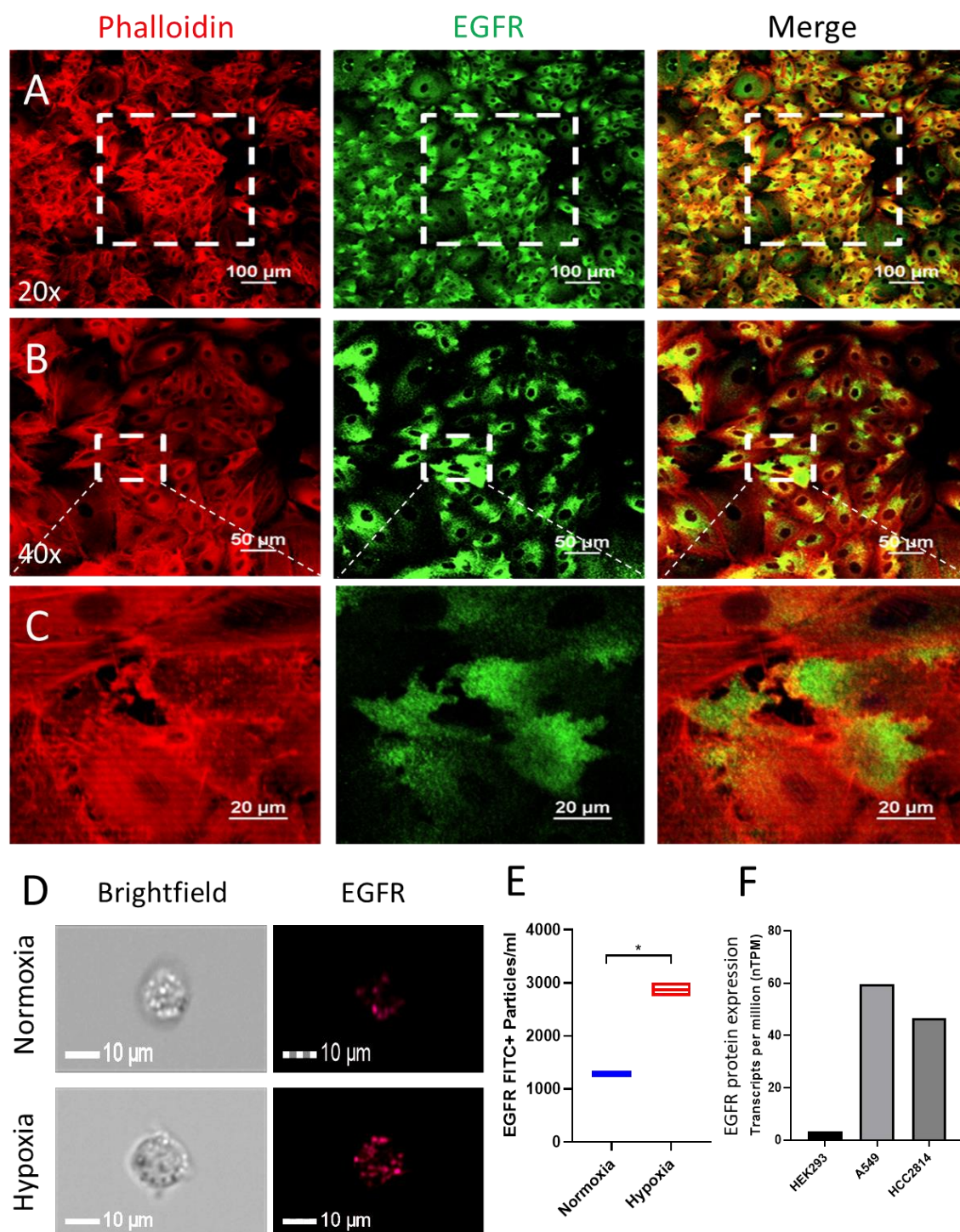


Figure 11. Expression of EGFR on the membranes of lung cancer cells. Immunofluorescence microscopy showing the expression and distribution pattern of EGFR protein (green colour) in A549 lung cancer cells at (A, B) 20x and 40x magnifications, and (C) the corresponding enlarged

images. **(D, E)** Image flow cytometry-based expression of EGFR on the A549 cells under normal and hypoxic conditions, and the corresponding quantitative bar graph. **(F)** Protein expression level of EGFR in normal cell (HEK293), and cancer cells (A549 and HCC2814), as determined via proteomicsatlas.org.

3.3. Endothelial cell derived EVs attenuated the migration of lung cancer cells.

Vascular endothelial cell-derived EVs have been found to inhibit the malignant progression of lung cancer [317], suggesting that endothelial cells could be an apt source of exosomes for developing exosome-based drug delivery tools. HUVEC cells were first cultured in a complete medium, then in an exosome-depleted medium. **Fig. 12A** shows a brightfield image of a typical HUVEC cell, which was utilized for exosome isolation. Subsequently, the HUVEC-EVs were characterized with a nano tracking analyzer and electron microscope. The average diameter of HUVEC-EXO was found to be around 172 nm, and the concentration of released exosomes was 1.73×10^9 particles/ml (**Fig. 12B**). Electron microscopy showed the morphology of the exosomes, and immunogold labeling revealed the expression of CD63, an EV biomarker (**Fig. 12C, D**). This further established that the isolated vesicles were indeed exosomes. The EVs were added to the culture of A549 lung cancer cells at a concentration of 50 $\mu\text{g/ml}$ and incubated at room temperature for 24 hrs. Notably, the HUVEC-EVs were found to inhibit the migration of lung cancer cells (**Fig. 12E**), demonstrating that they were a suitable source of exosomes for lung cancer therapeutics.

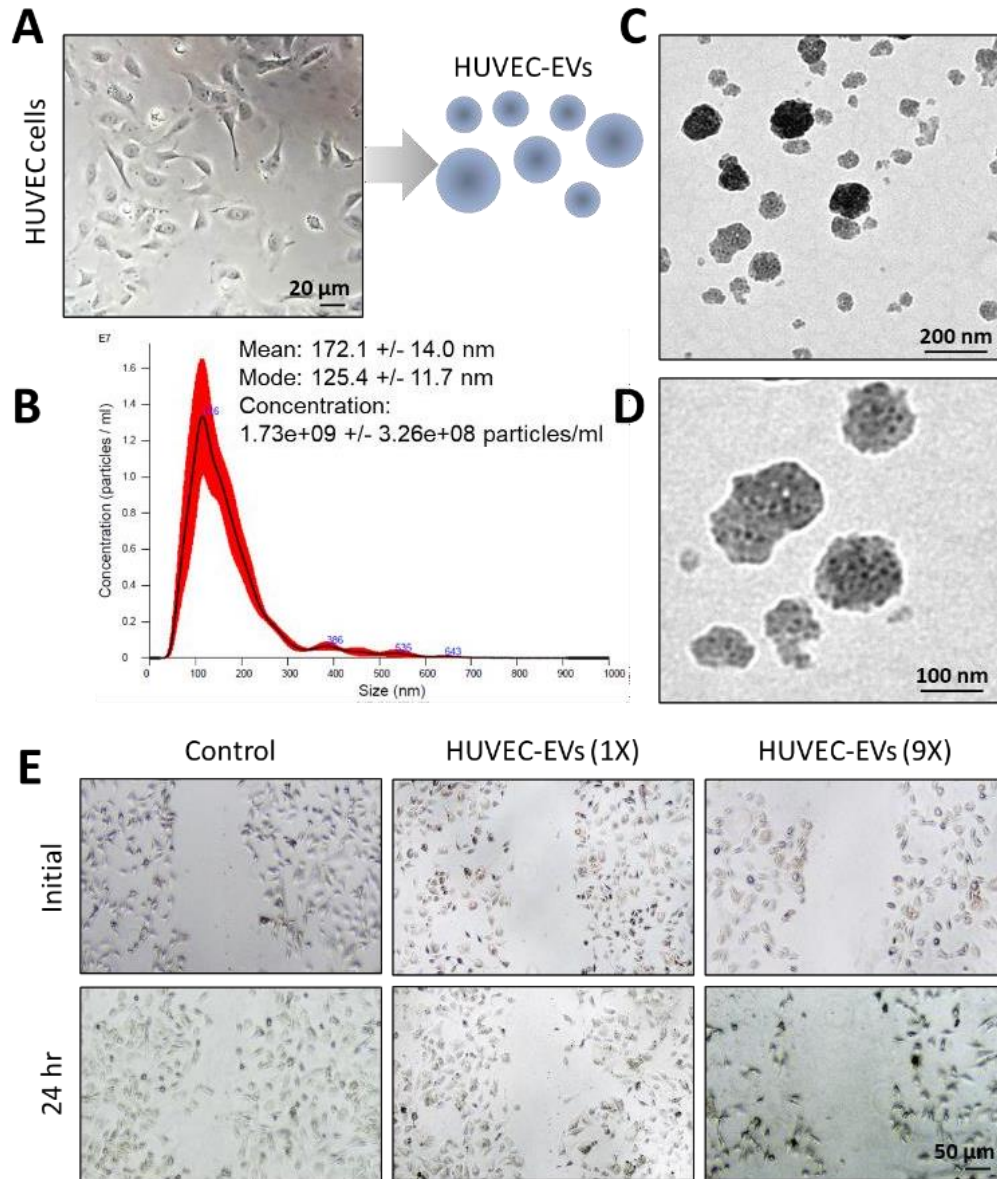


Figure 12. Isolation and characterization of exosomes from endothelial cells. (A) Representative bright field image of HUVEC cells that had been cultured in exosome-depleted medium for 24 hrs., before the isolation of EVs. (B-D) Representative (B) size distribution plot of the HUVEC-EVs; (C, D) immunogold dots showing the expression of CD63 on the HUVEC-EXO. (E) Representative brightfield images showing the migration of A549 cells at the start and 24 hrs. after the addition of HUVEC-EVs at different dilutions (1x and 9x). Scale bars: Fig 10A, 20 nm; Fig. 3C, D, 100 nm; Fig. 3E, 50 nm.

Discussion

The use of vascular endothelial cell-derived EVs as a potential tool for developing exosome-based drug delivery systems for lung cancer treatment has been explored. The inhibition of malignant progression of lung cancer by these EVs suggests that endothelial cells could be a suitable source of exosomes for this purpose. The characterization of HUVEC-EVs, including their average diameter and concentration, as well as their morphology and expression of CD63, an EV biomarker, has been demonstrated. The addition of these EVs to the culture of A549 lung cancer cells has been shown to inhibit the migration of lung cancer cells, indicating their potential as a source of exosomes for lung cancer therapeutics. The role of exosomes in lung cancer metastasis and their clinical applications have been discussed. Exosomes derived from lung cancer cells enhance cell proliferation, angiogenesis, and metastasis, regulate drug resistance, and modulate the immune response³. The clinical application of exosomes in diagnosis, prognosis, drug resistance, and therapeutics has been presented, including their potential as a novel therapeutic strategy for cancer. The use of exosomes as a therapeutic approach for cancer treatment has been reviewed. Exosomes can efficiently elicit an immune response in the tumor microenvironment, and their applications must be substantially monitored in both cancer progression and tumor suppression⁴. The promotion of exosome application and its prominent utility as a therapeutic agent must be regularized in complex intracellular pathways⁴. Furthermore, the role of tumor-derived exosomal miR-3157-3p in promoting angiogenesis and metastasis in non-small cell lung carcinoma (NSCLC) has been investigated. The expression level of miR-3157-3p in circulating exosomes was significantly higher in metastatic NSCLC patients than in non-metastatic NSCLC patients, and it was found to be involved in the formation of pre-metastatic niche formation before tumor metastasis. In conclusion, the use of vascular endothelial cell-derived EVs as a potential

tool for developing exosome-based drug delivery systems for lung cancer treatment is a promising approach. The characterization of these EVs and their ability to inhibit the migration of lung cancer cells demonstrate their potential as a source of exosomes for lung cancer therapeutics. The role of exosomes in lung cancer metastasis and their clinical applications, as well as their use as a therapeutic approach for cancer treatment, have been extensively discussed. Further research is needed to fully understand the role of exosomes in lung cancer and to develop effective targeted therapies.

3.4. Engineering of endothelial cells EVs via GE11 peptide post-insertion and loading of Vinorelbine

As we found that HUVEC-EVs could be useful for inhibiting the malignant phenotype of lung cancer cells, we further questioned if we could tailor the exosomes to achieve the specific targeting of lung cancer cells. As most of the cancer cells including lung cancer cells express high levels of EGFR[315], we engineered the HUVEC-EVs via post-insertion of GE11 peptide, which has been reported to interact with the EGFR receptor.[314]:[318] Briefly, the GE11 peptide was dissolved in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer for 15 min at 60 °C, during which time micelles formed. This was then mixed with HUVEC-derived exosome suspension and incubated for 2 h at 40 °C. Next, after cooling to room temperature, exosomes were immediately purified by size-exclusion chromatography to get GE11-modified HUVEC-EVs (GE11-HUVEC-EVs) (**Fig. 13A**). The engineered exosomes were analyzed by FT-IR to confirm successful insertion of GE11 peptide into the exosomes. **Fig. 13B** shows the FT-IR-based graphs and marked characteristic peaks for the HUVEC-EVs, GE11 peptide, and GE11-HUVEC-EVs.

As evident from the **Figure 13B**, there has been a shift in the IR peak after the GE11 -HUVECs-EVs as compared to the G11 peptide alone (as indicate by the IR peak with wavenumber 1043), potentially revealing successful integration of GE11 into the EV membrane.

This suggests about the successful and efficient functionalization of HUVEC-EVs with GE11 peptide. Size distribution analysis revealed a small shift in size after the conjugation with GE11 peptide. There was a slight change in the value of the zeta potential of HUVEC-EVs after the functionalization with GE11 peptide, but it was not very significant (**Fig. 13C, D**). This suggests that the post-insertion of GE11 peptide into the HUVEC-EVs was accomplished without much impact on the properties of the exosome membranes. We identified ABCB1 as one of the potential targets for lung cancer via *in silico* analysis (**Fig. 14A-D**). *ABCB1* gene, which codes for P-glycoprotein, plays crucial physiological roles, particularly in the protection of cells and organs against toxic compounds. Owing to its ability to recognize and transport a broad range of substrates, this transporter can impart a chemoresistance phenotype to cancer cells. Elacridar has been found to show a potent effect on docetaxel-resistant NSCLC cells [319]. We examined the binding affinity of Vinorelbine towards the ABCB1 protein and compared it with that of elacridar and other relevant drugs. Azithromycin, clarithromycin, and erythromycin were used as additional controls. Notably, it was found that the binding energy for the interaction between Vinorelbine and ABCB1 was comparable to that between elacridar and ABCB1 (**Fig. 15A-E**, and **Table 4**). Therefore, we concluded that Vinorelbine would be an appropriate candidate anti-cancer drug for loading into engineered exosomes. GE11-HUVEC-EVs were loaded with Vinorelbine (GE11-HUVEC-EVs-Vino) via sonication method, and UV-visible spectroscopy analysis showed that the loading efficiency was approximately 32.67 %. The engineering of EVs with peptide can also be affected by their moisture content. **Table 5** lists the moisture content from the available literature. The size distribution of engineered EVs was not remarkably altered, however the concentration of

EVs was decreased (**Fig. 16A-D, Table 6**). We used GE11-HUVEC-EVs-Vino to examine its anti-cancer effect against lung cancer cells *in vitro* and *in vivo*.

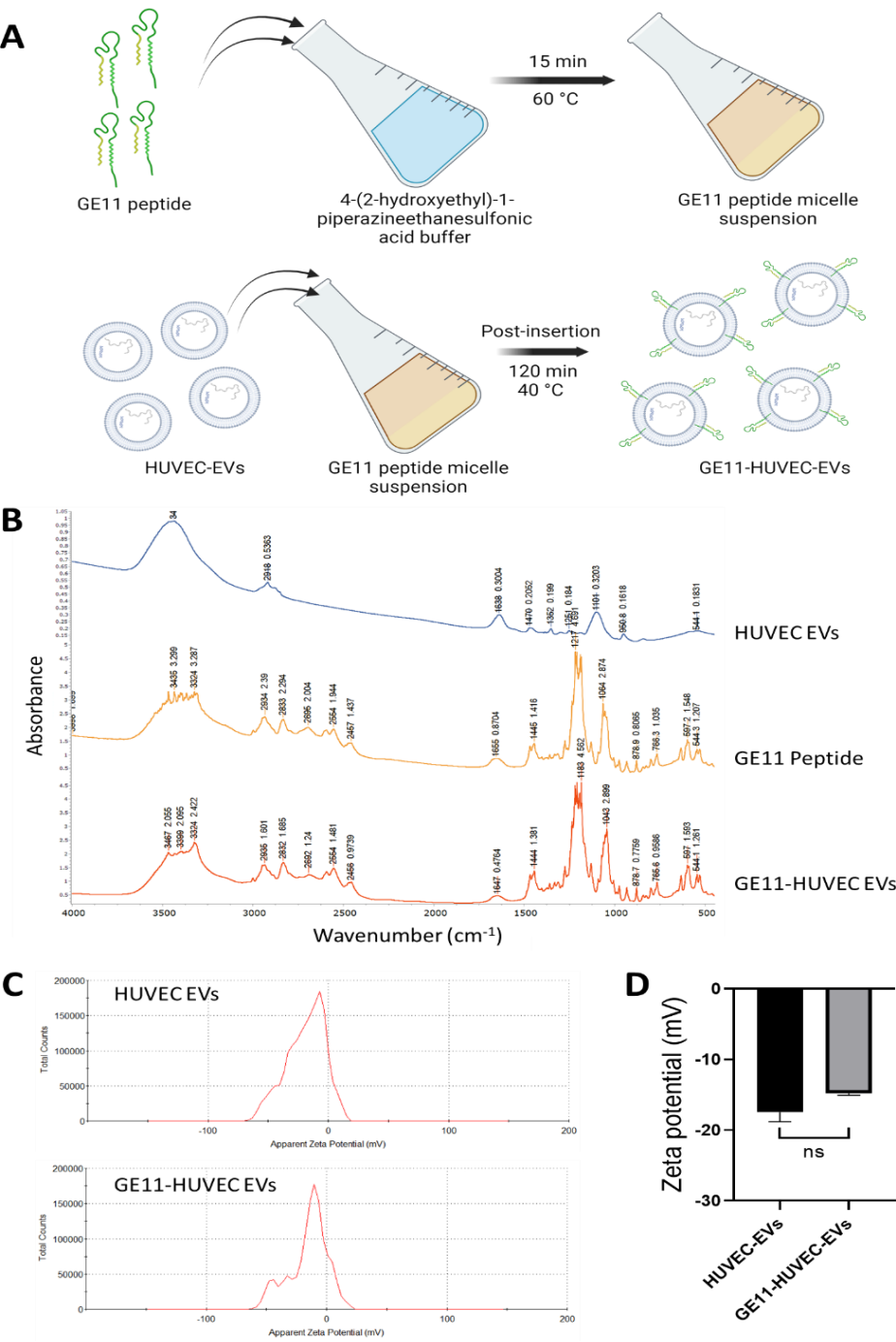


Figure 13. Characterization of engineered exosomes with GE11 peptide via post-insertion t.

(A) A schematic showing the functionalization of HUVEC-EVs with the post-insertion of GE11 peptide. (B) Representative FT-IR graphs with characteristic peaks for HUVEC-EVs, GE11 peptide, and GE11-HUVEC-EVs. (C, D) Zeta potential graphs and the corresponding quantitative bar graph for the exosomes before and after post-insertion of the GE11 peptide. Data are shown as mean \pm S.E.M. (N=2). Statistical analysis was performed using the student's t-test for control HUVEC-EVs vs. GE11-HUVEC-EXO. Significance level at *P<0.05, and **P<0.01; ns = not significant.

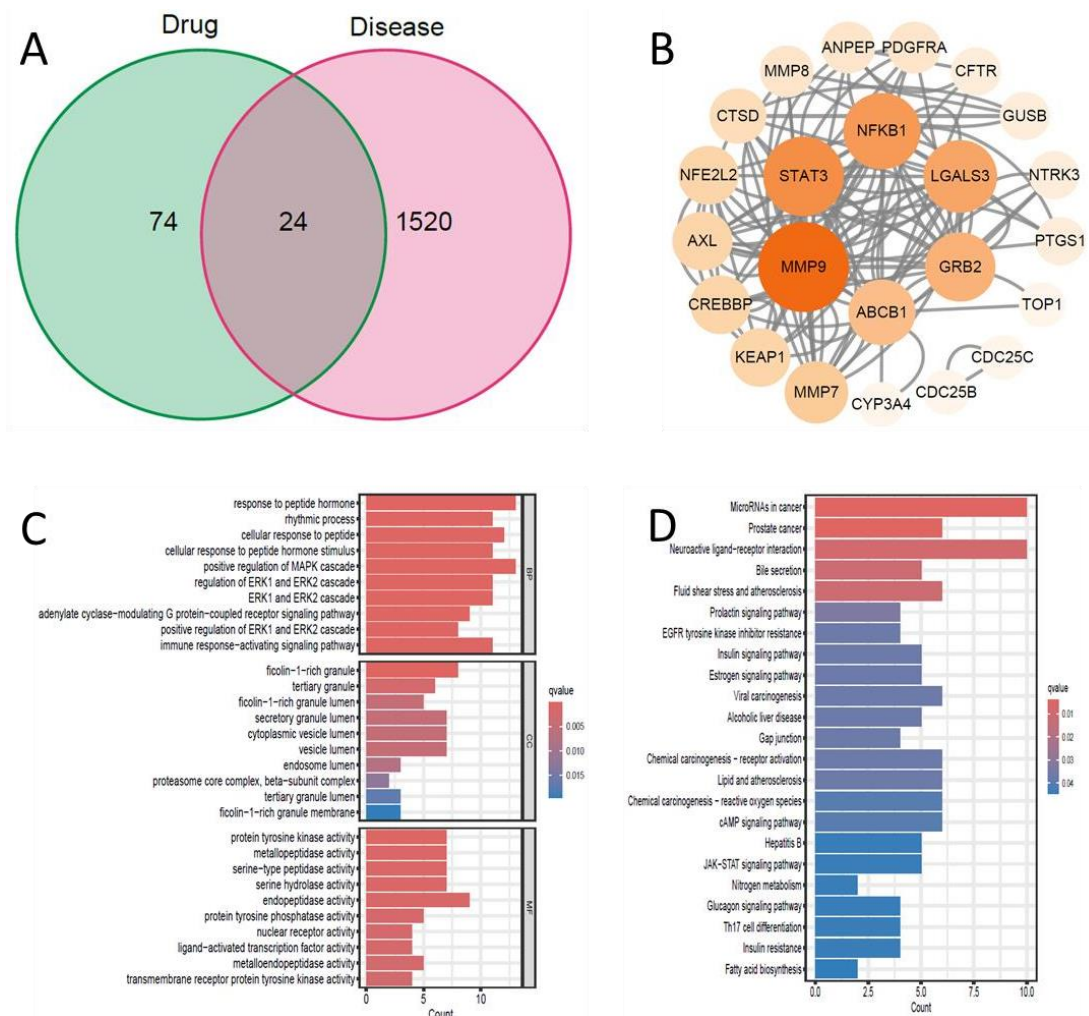


Figure 14. Identification of potential lung cancer targets via *in silico* analysis. (A) The intersection of compound target proteins and lung cancer treatment targets resulted in 44 intersecting targets. (B) These 24 potential targets were imported into the STRING (<https://cn.string-db.org/>) online website to obtain a PPI network, which contains 24 nodes and 110 edges. The depth of color and circle size represent the corresponding size of the degree. (C) GO enrichment analysis was conducted on these 24 potential targets, and the top 10 enrichment

results were displayed. **(D)** KEGG enrichment analysis was performed on these 24 potential targets, and the top 30 enrichment results were displayed.

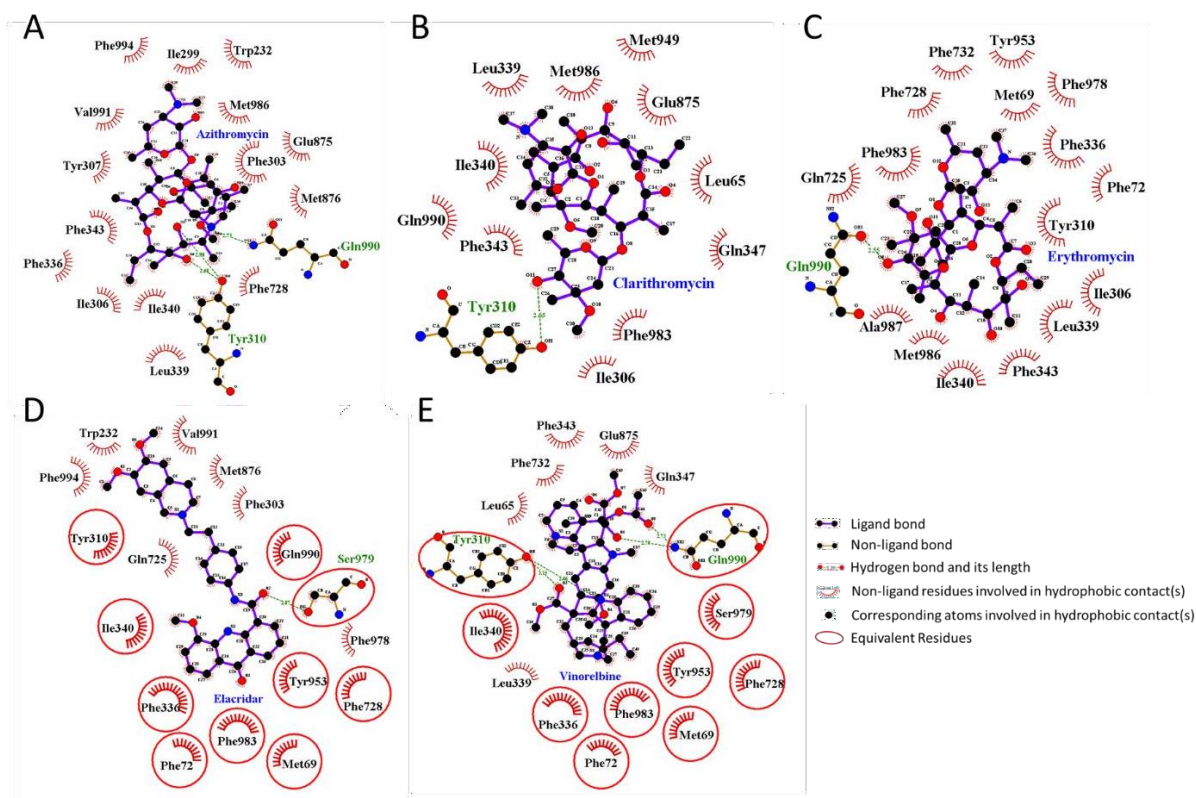


Figure 15. Molecular docking of vinorelbine with ABCB1 protein. Depiction of H-bonding and hydrophobic interaction pattern of control drugs (azithromycin, clarithromycin, erythromycin, and elacridar) and vinorelbine with ABCB1.

Table 4. Docking of different ligands with ABCB1.

Compound name	Binding Energy	Ki	H-bonding with ABCB1
Azithromycin	-9.80 kcal/mol	65.09 nM	Gln990, Tyr310
Clarithromycin	-8.05 kcal/mol	1.25 μ M	Tyr310
Erythromycin	-9.98 kcal/mol	48.71 nM	Gln990
Elacridar	-11.86 kcal/mol	2.03 nM	Ser979
Vinorelbine	-11.31 kcal/mol	5.09 nM	Tyr310, Gln990

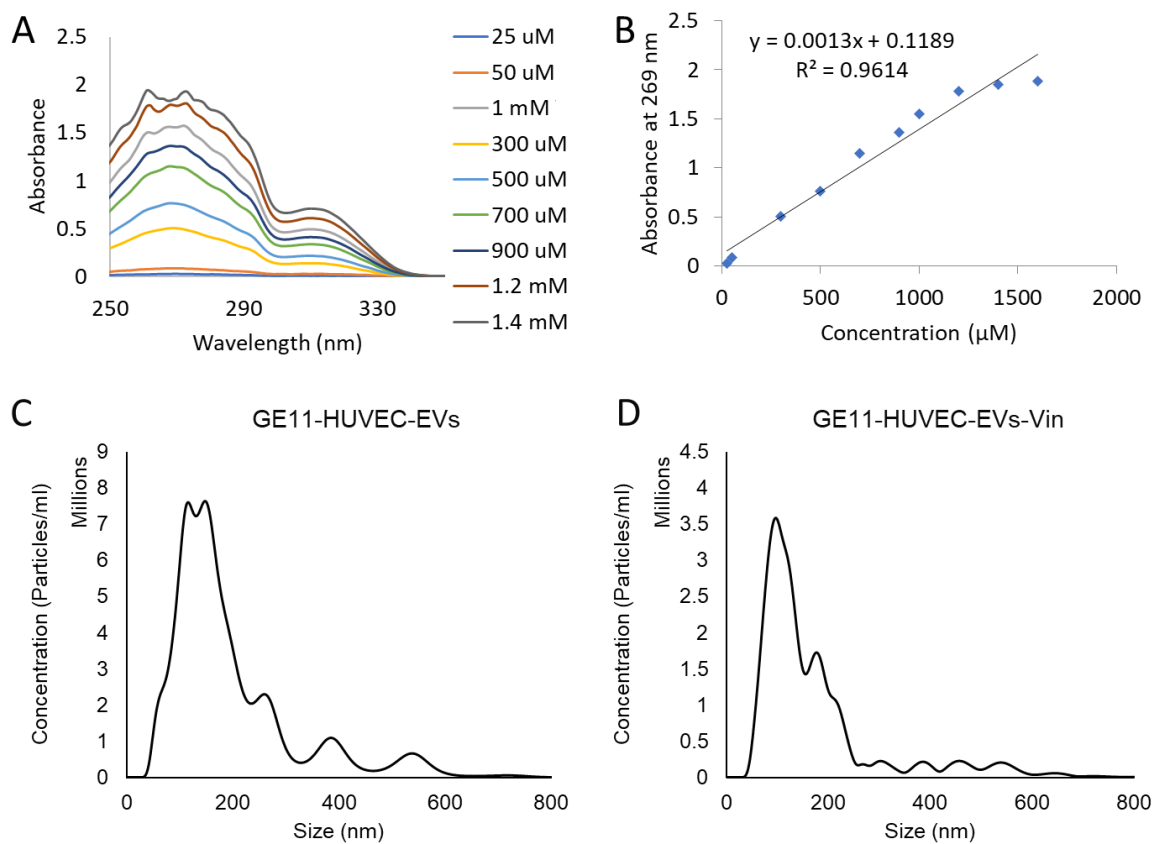


Figure 16. Evaluation of vinorelbine encapsulation in GE11-HUVEC-EVs. (A) UV-visible spectra of difference concentrations of vinorelbine standard solutions. (B) Calibration standard graph showing the linear regression between concentration of vinorelbine standard solutions and the corresponding absorbance at the wavelength of 269 nm (λ_{max}). (C, D) Size distribution of engineered EVs before and after loading with vinorelbine.

Table 5. Moisture content of GE11 peptide and EVs.

Component	Moisture Content	Reference
GE11 peptide	<5%	Zhang et al. [4]
Exosomes	60-70%	Hailing et al. [5] Kar et al. [6]

Table 6. Calculation of efficiency of vinorelbine encapsulation in GE11-HUVEC-EVs.

Components	Absorbance	Conc. (μM)
Initial drug	0.272	200.084615 (A)
Conditioned medium after centrifugation	0.187	134.7 (B)
Drug loading efficiency	$= ((A-B)/A) \times 100$	32.6784194 %

Discussion

The engineering of HUVEC-EVs with the GE11 peptide, which interacts with the EGFR receptor, has been explored as a potential strategy for achieving specific targeting of lung cancer cells. The post-insertion of GE11 peptide into HUVEC-EVs was accomplished without significantly impacting the properties of the exosome membranes, as confirmed by size distribution analysis and zeta potential measurements. The identification of ABCB1 as a potential target for lung cancer via *in silico* analysis and the examination of the binding affinity of Vinorelbine towards the ABCB1 protein have been discussed. The loading of Vinorelbine into engineered exosomes (GE11-HUVEC-EVs-Vino) via sonication method and the analysis of its anti-cancer effect against lung cancer cells *in vitro* and *in vivo* have also been presented. The role of exosomes in cancer therapy, including their potential as a delivery system for anti-cancer drugs, has been reviewed. Exosomes can be engineered to target specific cells or tissues, and their natural ability to cross biological barriers makes them an attractive tool for cancer therapy. The use of exosomes to deliver chemotherapy drugs, such as doxorubicin and paclitaxel, has been shown to enhance their anti-tumor efficacy and reduce their toxicity. The use of nanoparticles, including exosomes, for cancer therapy has been discussed. Nanoparticles can be engineered to target specific cells or tissues, and their small size allows them to penetrate deep into tumors, making them an effective tool for cancer therapy. The use of nanoparticles to deliver chemotherapy drugs, such as doxorubicin and paclitaxel, has been shown to enhance their anti-tumor efficacy and reduce their toxicity. Moreover, the role of ABCB1 in multidrug resistance and its potential as a target for cancer therapy has been extensively studied. ABCB1 is a transmembrane protein that plays a crucial role in the efflux of chemotherapy drugs from cancer cells, leading to multidrug resistance. Inhibitors of ABCB1, such as elacridar, have been shown to enhance the sensitivity of cancer cells to chemotherapy drugs. Apparently, the engineering of HUVEC-EVs with the GE11 peptide and the

loading of Vinorelbine into these engineered exosomes are promising strategies for achieving specific targeting of lung cancer cells and enhancing the anti-cancer effect of chemotherapy drugs. The role of exosomes in cancer therapy, including their potential as a delivery system for anti-cancer drugs, and the use of nanoparticles for cancer therapy have been extensively discussed. The targeting of ABCB1, a key player in multidrug resistance, is a crucial aspect of cancer therapy, and the use of inhibitors of ABCB1, such as elacridar, has been shown to enhance the sensitivity of cancer cells to chemotherapy drugs. Further research is needed to fully understand the potential of engineered exosomes for cancer therapy and to develop effective targeted therapies.

3.5. GE11 peptide-engineered EVs were incorporated into EGFR-expressing lung cancer cells and showed tumoricidal effects *in vitro*

To examine the effectiveness of the GE11-HUVEC-EVs, an uptake assay was performed by incubating A549 cells with PBS, HUVEC-EVs, or GE11-HUVEC-EVs. The EV samples were labeled with EXO-Green, whereas the A549 cells' membrane and nucleus were stained with phalloidin and DAPI, respectively. Interestingly, the confocal analysis revealed that GE11-HUVEC-EVs were significantly taken up by the A549 cells but not the HUVEC-EVs, as evident from the significant co-localization of Exo-Green and phalloidin in A549 cells treated with labeled exosomes (**Fig. 17A-C**). Further, the GE11-HUVEC-EVs loaded with vinorelbine (GE11-HUVEC-EVs-Vino) showed a significant effect on the cell viability as evident from the decline in number of A549 cells (**Fig. 17D**). The enhanced level of Annexin-V, an apoptotic marker, and the decline in the migration ability of the cells treated with GE11-HUVEC-EVs-Vin (**Fig. 17E-H**) also suggest the potential of GE11-HUVEC-EVs as an effective therapeutic for lung cancer.

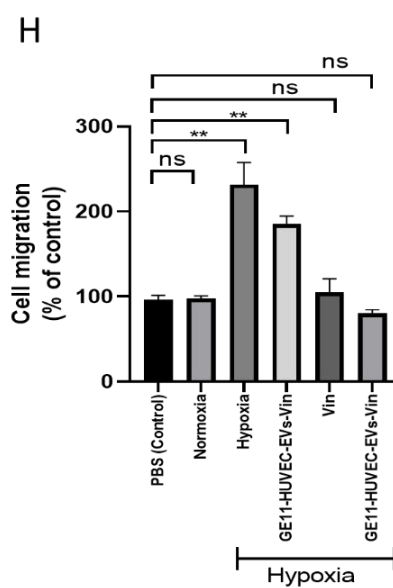
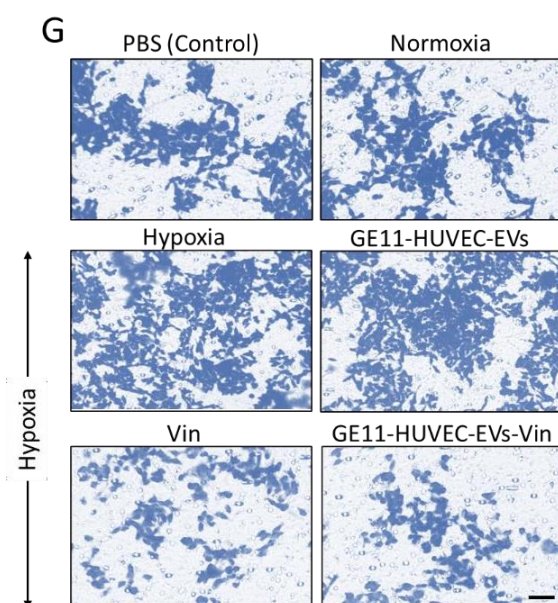
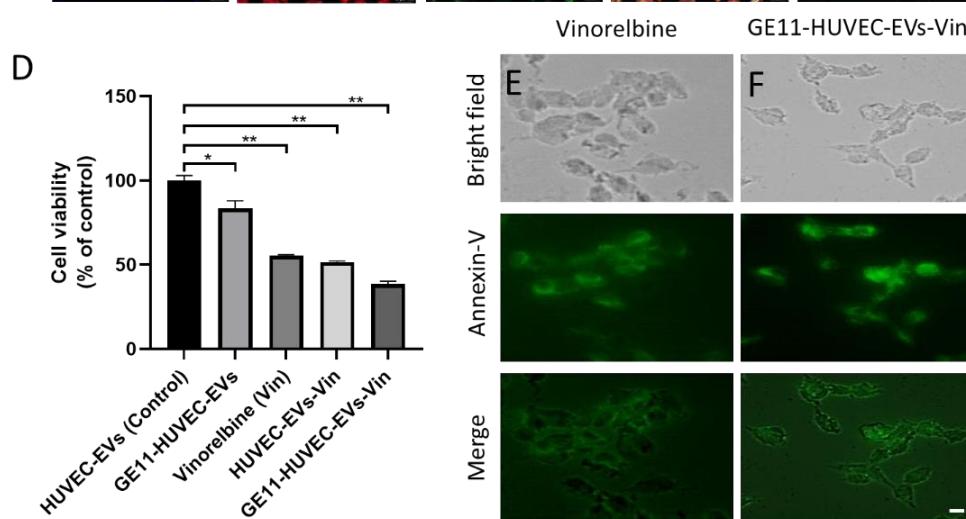
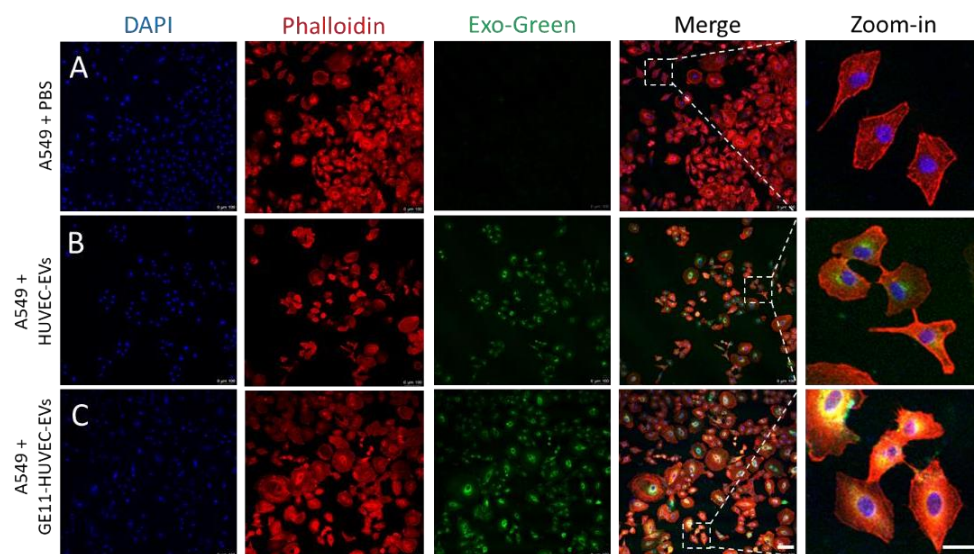


Figure 17. Effect of GE11-HUVEC-EVs-Vin on malignant phenotypes of A549 lung cancer cells. (A-C) GE11 peptide engineered endothelial cells exosomes are efficiently internalized by the lung cancer cells. Representative immunofluorescence images showing the uptake of (A) PBS, (B) HUVEC-EVs, and (C) GE11-HUVEC-EVs by the A549 cells. (Scale bar: 100 nm) (D) GE11-HUVEC-EXO-Vin significantly reduced the cell viability of A549 cells. Representative bar graph showing the effect of different treatment groups: HUVEC-EVs, GE11-HUVEC-EVs, Vinorelbine (Vin), HUVEC-EVs-Vin, and GE11-HUVEC-EVs-Vino on the proliferation of A549 cells, as detected by the MTT cell viability assay. (E, F) Representative immunofluorescence images showing the expression of Annexin-V in A549 cells treated with Vin or HUVEC-EVs-Vin. (Scale bar: 100 nm) (G, H) GE11-HUVEC-EVs significantly reduced the migration of A549 cells. (G) Representative images from the Transwell chamber (Scale bar: 100 nm) and (H) the bar graph showing the effect of different treatment groups: HUVEC-EVs, GE11-HUVEC-EVs, Vinorelbine, HUVEC-EVs-Vin, and GE11-HUVEC-EVs-Vino on the migration ability of A549 cells, as detected by the Transwell migration assay. Data are shown as mean \pm standard error mean (S.E.M.); Significance level: *P<0.05, **P<0.01. The statistical comparison was performed for control vs. HUVEC-EVs; control vs. GE11-HUVEC-EVs; control vs. Vinorelbine, control vs. HUVEC-EVs-Vin, and control vs. GE11-HUVEC-EVs-Vin.

Discussion

The effectiveness of GE11-HUVEC-EVs as a therapeutic for lung cancer has been examined through an uptake assay, where A549 cells were incubated with PBS, HUVEC-EVs, or GE11-HUVEC-EVs. The results showed that GE11-HUVEC-EVs were significantly taken up by the A549 cells, indicating their potential as a targeted delivery system for lung cancer therapy. The

loading of vinorelbine into GE11-HUVEC-EVs (GE11-HUVEC-EVs-Vino) and its effect on cell viability, apoptosis, and migration ability have also been investigated. The results suggest that GE11-HUVEC-EVs-Vino has a significant effect on cell viability, inducing apoptosis and reducing the migration ability of A549 cells, further supporting their potential as an effective therapeutic for lung cancer. The role of exosomes in cancer therapy, including their potential as a delivery system for anti-cancer drugs, has been reviewed. Exosomes can be engineered to target specific cells or tissues, and their natural ability to cross biological barriers makes them an attractive tool for cancer therapy. The use of exosomes to deliver chemotherapy drugs, such as doxorubicin and paclitaxel, has been shown to enhance their anti-tumor efficacy and reduce their toxicity. The use of nanoparticles, including exosomes, for cancer therapy has been discussed. Nanoparticles can be engineered to target specific cells or tissues, and their small size allows them to penetrate deep into tumors, making them an effective tool for cancer therapy. The use of nanoparticles to deliver chemotherapy drugs, such as doxorubicin and paclitaxel, has been shown to enhance their anti-tumor efficacy and reduce their toxicity. Furthermore, the role of ABCB1 in multidrug resistance and its potential as a target for cancer therapy has been extensively studied. ABCB1 is a transmembrane protein that plays a crucial role in the efflux of chemotherapy drugs from cancer cells, leading to multidrug resistance. Inhibitors of ABCB1, such as elacridar, have been shown to enhance the sensitivity of cancer cells to chemotherapy drugs. In conclusion, the use of GE11-HUVEC-EVs as a targeted delivery system for lung cancer therapy is a promising approach. The loading of vinorelbine into these engineered exosomes and their effect on cell viability, apoptosis, and migration ability suggest their potential as an effective therapeutic for lung cancer. The role of exosomes in cancer therapy, including their potential as a delivery system for anti-cancer drugs, and the use of nanoparticles for cancer therapy have been

extensively discussed. The targeting of ABCB1, a key player in multidrug resistance, is a crucial aspect of cancer therapy, and the use of inhibitors of ABCB1, such as elacridar, has been shown to enhance the sensitivity of cancer cells to chemotherapy drugs. Further research is needed to fully understand the potential of engineered exosomes for cancer therapy and to develop effective targeted therapies.

3.6. GE11-HUVEC-EVs-Vin showed a tumoricidal effect in an *in vivo* mouse model of lung cancer.

To validate the *in vitro* results *in vivo*, we developed a xenograft model of lung cancer via injection of A549 cells into severe combined immunodeficiency disease (SCID) mice as described previously [320] and as per the protocol (approval number is REC/21-22/0265) of the Research Ethics Committee at the Hong Kong Baptist University. The H&E staining of lung tissue samples from the xenograft mice showed significant malignant cell proliferation compared to the control mice, which established the formation of the xenograft mice model of lung cancer (**Fig. 18A, B**). Fixed lung tissue sections from the xenograft mice treated with PBS, HUVEC-EVs, GE11-HUVEC-EVs, vinorelbine (Vin), and GE11-HUVEC-EVs-Vin were stained with DAPI, EGFR, and Ki67 (a marker for proliferating cells). Notably, the expression level of EGFR and Ki67 was significantly reduced in the mice treated with GE11-HUVEC-EVs-Vin; the level was comparable with the effect of vinorelbine alone (**Fig. 18C-G**). The H& E staining of tissue samples from different organs of immunodeficient mice treated with engineered exosomes showed that no significant toxicity occurred in brain, heart, lung, liver, spleen, and kidney (**Fig. 19A-F**). This suggests that GE11-HUVEC-EVs-Vin was effective in lung cancer therapeutics in *in vivo*.

Discussion

The validation of *in vitro* results *in vivo* is a crucial step in the development of effective cancer therapeutics. In this context, a xenograft model of lung cancer was developed by injecting A549 cells into severe combined immunodeficiency disease (SCID) mice. The formation of the xenograft mice model of lung cancer was established through H&E staining of lung tissue samples, which showed significant malignant cell proliferation compared to control mice. The treatment of xenograft mice with PBS, HUVEC-EVs, GE11-HUVEC-EVs, vinorelbine (Vin), and GE11-HUVEC-EVs-Vin was evaluated through the staining of fixed lung tissue sections with DAPI, EGFR, and Ki67. The results showed that the expression level of EGFR and Ki67 was significantly reduced in mice treated with GE11-HUVEC-EVs-Vin, comparable to the effect of vinorelbine alone. This suggests that GE11-HUVEC-EVs-Vin was effective in lung cancer therapeutics *in vivo*. The use of xenograft models for studying lung cancer has been extensively discussed. Zebrafish xenograft models have been shown to be a rapid and intuitive model for studying lung cancer, offering advantages over traditional mouse models. The validation of *in vivo* xenograft models has also been demonstrated using multicellular tumor microenvironment systems, which have been shown to predict drug response to EGFR inhibitors in non-small cell lung cancer (NSCLC). The role of EGFR in lung cancer and its targeting for therapy has been extensively studied. EGFR mutations are a common feature of NSCLC, and EGFR tyrosine kinase inhibitors have been developed as a targeted therapy for these mutations. The use of EGFR inhibitors has been shown to be effective in patients with EGFR mutations, highlighting the importance of targeting EGFR in lung cancer therapy. Conclusively, the validation of *in vitro* results *in vivo* is a crucial step in the development of effective cancer therapeutics. The use of xenograft models, including zebrafish and mouse models, has been shown to be effective in

studying lung cancer and validating the efficacy of targeted therapies. The targeting of EGFR in lung cancer therapy has been extensively studied, and EGFR inhibitors have been shown to be effective in patients with EGFR mutations. Further research is needed to fully understand the potential of engineered exosomes for lung cancer therapy and to develop effective targeted therapies.

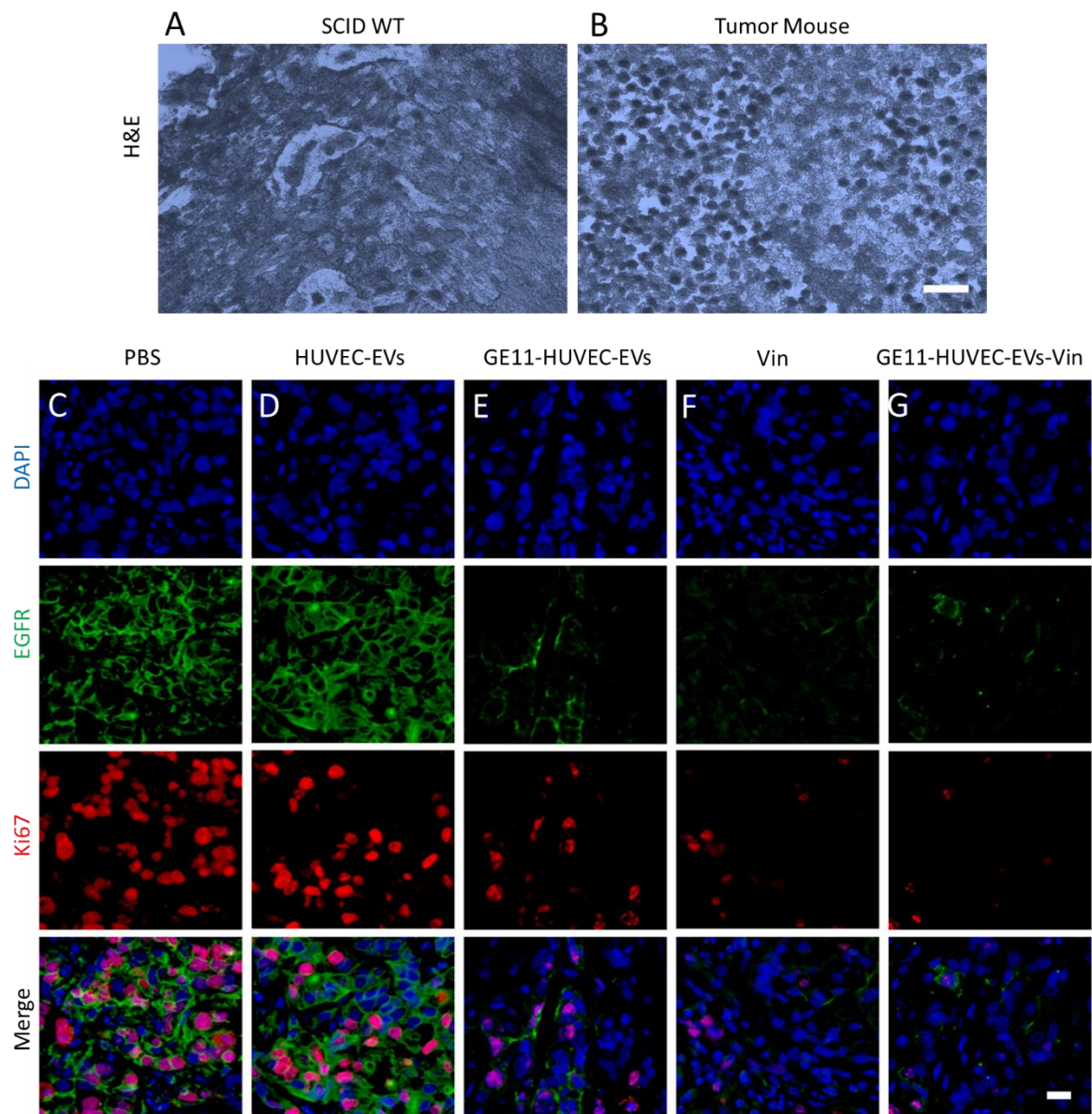


Figure 18. GE11-HUVEC-EVs-Vin significantly reduced the expression of EGFR and Ki67 in the lung tissue of a xenograft mouse model of lung cancer. (A, B) Representative H&E staining of the lung tissue of SCID WT and the lung cancer xenograft mouse model. (Scale bar: 50 μ m) (C-G) Representative immunofluorescence images showing the effect in different treatments: HUVEC-EXO, GE11-HUVEC-EVs, vinorelbine, HUVEC-EVs-Vino, and GE11-HUVEC-EVs-Vino on nuclei (depicted by blue color; DAPI), EGFR (green), and Ki67 (red) in the lung tissue of xenograft mouse model of lung cancer (Scale bar: 50 μ m)

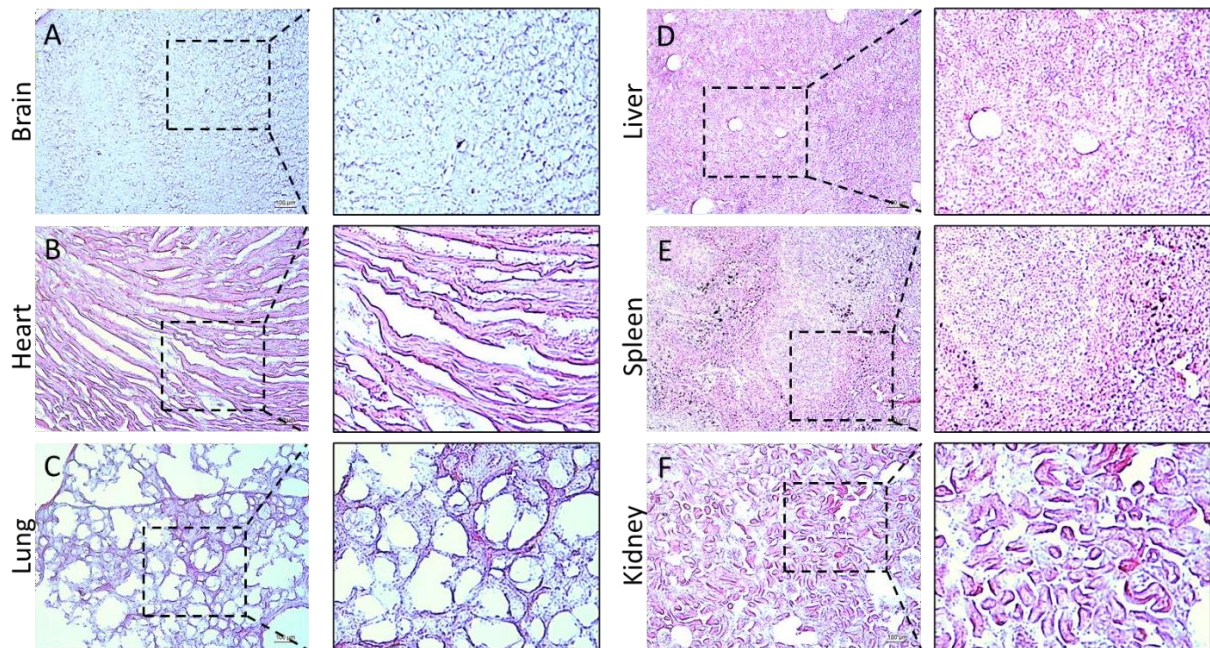


Figure 19. Evaluation of in vivo toxicity of GE11-HUVEC-EVs in major organs of mice. H&E-stained images of major organs of lung cancer xenograft mice: (A) brain, (B) heart, (C) lung, (D) liver, (E) spleen, and (F) kidney.

CHAPTER 4

CONCLUSIONS

Lung cancer, a prevalent form of cancer, presents significant challenges in treatment due to its lack of specificity and potential harm to healthy cells. In addressing these obstacles, NDDS has emerged as a promising solution. While traditional NDDSs have limitations such as immune responses and susceptibility to the reticuloendothelial system (RES), extracellular vesicles (EVs) offer a compelling alternative. These EVs, naturally released from cells, possess the ability to bypass the RES without requiring surface modifications, thereby minimizing toxicity to healthy cells. Consequently, they stand out as a viable candidate for the development of a targeted drug delivery system for lung cancer. In particular, human umbilical endothelial cell (HUVEC)-derived EVs (HUVEC-EVs) have exhibited anti-angiogenic properties in a lung cancer mouse model, leading to their selection as carriers for drug delivery in this study. To enhance their specificity for lung cancer cells, HUVEC-EVs were engineered to display the GE11 peptide (GE11-HUVEC-EVs), which binds to the EGFR overexpressed on the surface of lung cancer cells. Subsequently, these GE11-HUVEC-EVs were loaded with vinorelbine (GE11-HUVEC-EVs-Vin) and meticulously characterized and assessed in both in vitro and in vivo lung cancer models. Further, the study delved into investigating the binding affinity of ABCB1, which encodes P-glycoprotein—a key player in chemoresistance through drug efflux mechanisms. The findings from this research underscored the efficacy of GE11-HUVEC-EVs-Vin in exerting tumoricidal effects against lung cancer cells in both cell cultures and mouse models.

The overexpression of the *EGFR* gene has emerged as a critical factor associated with the diminished survival rates observed in lung cancer patients. This genetic anomaly not only serves as a prognostic indicator but also underscores the complexity of treatment strategies required to combat this aggressive form of cancer effectively. The intricate interplay between genetic predisposition and therapeutic interventions forms the cornerstone of advancements in lung cancer research and treatment modalities. Next, moving beyond the genetic level, the overexpression of the EGFR protein on the membrane of A549 cells within hypoxic tumor microenvironments (TMEs) unveils a microenvironmental influence on cancer progression and treatment response. This heightened expression of EGFR protein signifies a potential target for precision medicine approaches aimed at disrupting signaling pathways crucial for tumor growth and metastasis. Understanding the spatial and temporal dynamics of protein expression within the tumor microenvironment opens new avenues for personalized therapies tailored to individual patient profiles. In the realm of innovative therapeutic strategies, endothelial cell-derived extracellular vesicles (EVs) have emerged as versatile vehicles capable of modulating the behavior of lung cancer cells. By attenuating the migration of these malignant cells, EVs derived from endothelial cells showcase their potential as regulators of tumor progression and metastatic spread. This nuanced interaction between EVs, and cancer cells highlights the intricate crosstalk within the tumor microenvironment, shedding light on novel mechanisms that can be harnessed for therapeutic benefit.

The evolution of drug delivery systems has witnessed a paradigm shift with the engineering of endothelial cell EVs through GE11 peptide post-insertion and subsequent loading of Vinorelbine. This innovative approach not only enhances the specificity of drug delivery to target cancer cells but also minimizes off-target effects on healthy tissues. By leveraging the unique

properties of EVs and molecular targeting strategies, researchers have unlocked a new dimension in precision medicine, where therapeutic payloads can be precisely delivered to their intended cellular destinations.

The integration of GE11 peptide-engineered EVs into EGFR-expressing lung cancer cells marks a pivotal advancement in targeted therapy for lung cancer. Through meticulous in vitro studies, these engineered EVs have demonstrated potent tumoricidal effects, underscoring their efficacy in selectively targeting cancer cells while sparing normal tissues. This targeted approach holds immense promise for enhancing treatment outcomes and reducing adverse effects associated with conventional chemotherapy regimens.

Translating these promising findings from bench to bedside, GE11-HUVEC-EVs loaded with Vinorelbine have exhibited remarkable tumoricidal effects in an in vivo mouse model of lung cancer. This preclinical success not only validates the efficacy of this novel drug delivery system but also paves the way for future clinical trials aimed at evaluating its safety and efficacy in human patients. The ability of GE11-HUVEC-EVs-Vin to elicit a tumoricidal response in a living organism underscores its potential as a transformative therapy for lung cancer, offering new hope for patients battling this devastating disease.

Conclusively, the convergence of genetic insights, microenvironmental influences, innovative drug delivery systems, and targeted therapies has ushered in a new era in lung cancer research and treatment. The journey from understanding genetic aberrations to developing precision therapies tailored to individual patients represents a paradigm shift in oncology. As we navigate this complex landscape of molecular intricacies and therapeutic innovations, one thing remains clear: our relentless pursuit of scientific discovery and clinical translation holds the key to unlocking brighter prospects for lung cancer patients worldwide. ▯

CHAPTER 5

DISCUSSION

EGFR overexpression is a common feature of lung cancer and is associated with poor patient survival. It has been found that EGFR overexpression drives cancer cell proliferation, migration, and invasion, and also confers resistance to conventional therapy. Even our *in-silico* bioinformatics analysis using the TCGA dataset, we found that EGFR is highly expressed on the lung cancer cells, and the high expression of EGFR is correlated with poor survival of lung cancer patients (**Fig. 1A-H, 2A-C**). Therefore, developing effective strategies to target EGFR overexpression is a critical unmet need in lung cancer treatment. Furthermore, in the TME, various stromal cells including endothelial cells support cancer cells' malignant progression [321]. Interestingly, the HUVEC has shown anti-angiogenic activity in lung cancer mouse model [317]. This compelled us to examine the anti-migration ability of the HUVEC-EVs, as the autologous exosomes have been reported to mimic the constituents as well as the phenotypes of their parent cells [16][322]. In a similar line, we isolated exosomes from HUVEC and incubated them with A549 lung cancer cells. It was found that HUVEC-EVs exhibit inhibitory effect on the migration of lung cancer cells (**Fig. 3A-E**), demonstrating their aptness as a source of exosomes for lung cancer therapeutics.

Exosome-based targeted delivery is of paramount importance in cancer therapy. Exosomes, with their small size, biocompatibility, and low immunogenicity, serve as ideal drug carriers. They can deliver various therapeutic agents such as chemotherapeutic drugs, nucleic acids, and proteins to cancer cells, enhancing drug stability and tissue-specific targeted delivery. Tumor-derived

exosomes exhibit remarkable targeting ability against cancer cells, offering a promising approach for effective and personalized cancer treatment. Genetically engineered exosomes loaded with anti-cancer drugs hold potential for more effective and personalized cancer therapies [323][294][324]. Besides, peptide-based functionalization of exosomes has also attracted attention owing to their ability to accomplish targeted delivery [325]. Particularly, GE11; a synthetic peptide that binds to EGFR with high specificity and affinity, has been shown to be effective for targeting cancer cells *in vitro* and *in vivo* [318]. In this study, we for the first time decorated HUVEC-EVs with GE11 peptide via post-insertion technique (**Fig. 4A**). Post-insertion technique offers several advantages for targeted drug delivery in cancer therapy. Unlike genetic modification at the cellular level, post-insertion allows for the introduction of targeting moieties in exosomes after their isolation. This method is relatively simple and avoids gene-related side effects, providing a more controlled and specific approach to functionalizing exosomes. Additionally, it facilitates the induction of functionality in lipid membrane-based nanomaterials without the limitations of direct chemical modification methods [326][327]. Subsequently, the evaluation of exosomes with FT-IR and DLS established their successful engineering with GE11 peptide (**Fig. 4B**), without much impact on their membrane potential (**Fig. 4C**).

Using autologous exosomes for drug delivery to the same origin of parent cells offers significant advantages. These exosomes, derived from the patient's own cells, are well-tolerated and less likely to trigger immune responses. They also reflect the phenotypic state of the parent cells, enhancing their specificity and effectiveness in delivering therapeutic agents to the target cells. This personalized approach holds great potential for improved treatment outcomes in cancer therapy [16][328]. Apparently, as we chose endothelial cells-derived exosomes, it is imperative that HUVEC-EVs would be attracted to the endothelial cells, which is one of the crucial

components of the TME [329]. Further, we examined the uptake of GE11-HUVEC-EVs by the A549 lung cancer cells, and importantly, GE11-HUVEC-EVs was internalized by the A549 cells, relatively better than HUVEC-EVs, suggesting towards the successful targeting ability of GE11 toward the EGFR (**Fig. 5A-C**). Subsequently, the GE11-HUVEC-EVs was found to be effective in showing tumoricidal effect towards the lung cancer cells *in vitro* (**Fig. 5D-H**) as well as *in vivo* (**Fig. 6A-G**). Conclusively, the engineered endothelial cells-derived exosomes with GE11 peptide via post-insertion method, was found to be an effective targeted drug delivery approach for potential lung cancer therapeutics. The stability of HUVEC-EV in the lung cancer microenvironment is quite evident with the green fluorescent protein (GFP) expression of EXO-Green labelled control and engineered HUVEC-EVs, as shown in **Fig. 17A-C**. Moreover, the positive effect of the EVs on the migration, proliferation, and apoptosis of A549 cells, further validated the functional integrity and stability in the lung cancer microenvironment.

Despite the effectiveness of our strategy in *in vitro* and pre-clinical model, several potential challenges do persist. One potential challenge of using engineered exosomes for lung cancer treatment is that exosomes can also be released by tumor cells. Tumor-derived exosomes can promote tumor growth and metastasis [330][331]. Therefore, it is important to develop strategies to distinguish between engineered exosomes and tumor-derived exosomes. Another potential challenge is that engineered exosomes may be degraded by enzymes in the bloodstream or taken up by non-target cells. Therefore, it is important to engineer exosomes to be resistant to degradation and to target them specifically to tumor cells [330]. Moreover, obtaining the appropriate quantity of peptide-functionalized exosomes is difficult due to peptide breakdown by endosomal proteases in the cell during exosome production. To stop peptide degradation, Huang et al. conjugated a targeting peptide with a glycosylation sequence using a genetic engineering technique [94].

In future, this study could also be extended to other types of cancer, via customizing the exosome with different ligands against their respective cancer specific receptors. Further, the source of exosomes can be changed depending on the cell types, which is being targeted.

In the context of clinical translational value, EVs show promise in lung cancer therapy by enhancing drug delivery and stimulating immune responses. Conjugating EVs with the GE11 peptide targets EGFR-expressing tumors, which can enhance the delivery of therapeutic agents to lung cancer cells. This approach potentially reduces side effects and improves treatment efficacy, offering promising clinical translational value in treating lung cancer. Additionally, clinical evaluations are warranted to evaluate the safety and efficacy of GE11 peptideengineered exosomes in patients with lung cancer.

References

- [1] J. Rojo, A. Sousa-Herves, A. Mascaraque, Perspectives of Carbohydrates in Drug Discovery, in: *Compr. Med. Chem. III*, Elsevier: Amsterdam, The Netherlands, 2017: pp. 577–610. <https://doi.org/10.1016/B978-0-12-409547-2.12311-X>.
- [2] S. Bhattacharjee, DLS and zeta potential – What they are and what they are not?, *J. Control. Release*. 235 (2016) 337–351. <https://doi.org/10.1016/j.jconrel.2016.06.017>.
- [3] J.K. Patra, G. Das, L.F. Fraceto, E.V.R. Campos, M. del P. Rodriguez-Torres, L.S. Acosta-Torres, L.A. Diaz-Torres, R. Grillo, M.K. Swamy, S. Sharma, S. Habtemariam, H.-S. Shin, Nano based drug delivery systems: recent developments and future prospects, *J. Nanobiotechnology*. 16 (2018) 71. <https://doi.org/10.1186/s12951-018-0392-8>.
- [4] A. Chowdhury, S. Kunjiappan, T. Panneerselvam, B. Somasundaram, C. Bhattacharjee, Nanotechnology and nanocarrier-based approaches on treatment of degenerative diseases, *Int. Nano Lett.* 7 (2017) 91–122. <https://doi.org/10.1007/s40089-017-0208-0>.
- [5] E. Hinde, K. Thammasiraphop, H.T.T. Duong, J. Yeow, B. Karagoz, C. Boyer, J.J. Gooding, K. Gaus, Pair correlation microscopy reveals the role of nanoparticle shape in intracellular transport and site of drug release, *Nat. Nanotechnol.* 12 (2017) 81–89. <https://doi.org/10.1038/nnano.2016.160>.
- [6] D. Liu, F. Yang, F. Xiong, N. Gu, The Smart Drug Delivery System and Its Clinical Potential, *Theranostics*. 6 (2016) 1306–1323. <https://doi.org/10.7150/thno.14858>.
- [7] A.K. Sharma, A. Gothwal, P. Kesharwani, H. Alsaab, A.K. Iyer, U. Gupta, Dendrimer nanoarchitectures for cancer diagnosis and anticancer drug delivery, *Drug Discov. Today*.

- 22 (2017) 314–326. <https://doi.org/10.1016/j.drudis.2016.09.013>.
- [8] Y. Matsumura, The Drug Discovery by NanoMedicine and its Clinical Experience, *Jpn. J. Clin. Oncol.* 44 (2014) 515–525. <https://doi.org/10.1093/jjco/hyu046>.
- [9] S. Haque, M.R. Whittaker, M.P. McIntosh, C.W. Pouton, L.M. Kaminskas, Disposition and safety of inhaled biodegradable nanomedicines: Opportunities and challenges, *Nanomedicine Nanotechnology, Biol. Med.* 12 (2016) 1703–1724. <https://doi.org/10.1016/j.nano.2016.03.002>.
- [10] X. Luan, K. Sansanaphongpricha, I. Myers, H. Chen, H. Yuan, D. Sun, Engineering exosomes as refined biological nanoplatfroms for drug delivery, *Acta Pharmacol. Sin.* 38 (2017) 754–763. <https://doi.org/10.1038/aps.2017.12>.
- [11] L. Sun, Q. Wu, F. Peng, L. Liu, C. Gong, Strategies of polymeric nanoparticles for enhanced internalization in cancer therapy, *Colloids Surfaces B Biointerfaces.* 135 (2015) 56–72. <https://doi.org/10.1016/j.colsurfb.2015.07.013>.
- [12] T.-H. Chow, Y.-Y. Lin, J.-J. Hwang, H.-E. Wang, Y.-L. Tseng, S.-J. Wang, R.-S. Liu, W.-J. Lin, C.-S. Yang, G. Ting, Improvement of biodistribution and therapeutic index via increase of polyethylene glycol on drug-carrying liposomes in an HT-29/luc xenografted mouse model., *Anticancer Res.* 29 (2009) 2111–20.
- [13] J.S. Suk, Q. Xu, N. Kim, J. Hanes, L.M. Ensign, PEGylation as a strategy for improving nanoparticle-based drug and gene delivery, *Adv. Drug Deliv. Rev.* 99 (2016) 28–51. <https://doi.org/10.1016/j.addr.2015.09.012>.
- [14] P. Vader, E.A. Mol, G. Pasterkamp, R.M. Schiffelers, Extracellular vesicles for drug

- delivery, *Adv. Drug Deliv. Rev.* 106 (2016) 148–156.
<https://doi.org/10.1016/j.addr.2016.02.006>.
- [15] E. V. Batrakova, M.S. Kim, Using exosomes, naturally-equipped nanocarriers, for drug delivery, *J. Control. Release.* 219 (2015) 396–405.
<https://doi.org/10.1016/j.jconrel.2015.07.030>.
- [16] A. Thakur, R.K. Sidu, H. Zou, M.K. Alam, M. Yang, Y. Lee, Inhibition of Glioma Cells' Proliferation by Doxorubicin-Loaded Exosomes via Microfluidics, *Int. J. Nanomedicine*. Volume 15 (2020) 8331–8343. <https://doi.org/10.2147/IJN.S263956>.
- [17] A. Thakur, G. Qiu, S.-P. NG, C.-M.L. Wu, Y. Lee, Detection of membrane antigens of extracellular vesicles by surface plasmon resonance, *J. Lab. Precis. Med.* 2 (2017) 98–98.
<https://doi.org/10.21037/jlpm.2017.12.08>.
- [18] L. Milane, A. Singh, G. Mattheolabakis, M. Suresh, M.M. Amiji, Exosome mediated communication within the tumor microenvironment., *J. Control. Release.* 219 (2015) 278–294. <https://doi.org/10.1016/j.jconrel.2015.06.029>.
- [19] A. Thakur, G. Qiu, C. Xu, X. Han, T. Yang, S.P. NG, K.W.Y. Chan, C.M.L. Wu, Y. Lee, Label-free sensing of exosomal MCT1 and CD147 for tracking metabolic reprogramming and malignant progression in glioma, *Sci. Adv.* 6 (2020) eaaz6119.
<https://doi.org/10.1126/sciadv.aaz6119>.
- [20] A. Thakur, G. Qiu, S.-P. NG, J. Guan, J. Yue, Y. Lee, C.-M.L. Wu, Direct detection of two different tumor-derived extracellular vesicles by SAM-AuNIs LSPR biosensor, *Biosens. Bioelectron.* 94 (2017) 400–407. <https://doi.org/10.1016/j.bios.2017.03.036>.

- [21] L. Mashouri, H. Yousefi, A.R. Aref, A. mohammad Ahadi, F. Molaei, S.K. Alahari, Exosomes: composition, biogenesis, and mechanisms in cancer metastasis and drug resistance, *Mol. Cancer*. 18 (2019) 75. <https://doi.org/10.1186/s12943-019-0991-5>.
- [22] Y. Zhang, Y. Liu, H. Liu, W.H. Tang, Exosomes: biogenesis, biologic function and clinical potential, *Cell Biosci*. 9 (2019) 19. <https://doi.org/10.1186/s13578-019-0282-2>.
- [23] D. Li, J. Liu, B. Guo, C. Liang, L. Dang, C. Lu, X. He, H.Y.-S. Cheung, L. Xu, C. Lu, B. He, B. Liu, A.B. Shaikh, F. Li, L. Wang, Z. Yang, D.W.-T. Au, S. Peng, Z. Zhang, B.-T. Zhang, X. Pan, A. Qian, P. Shang, L. Xiao, B. Jiang, C.K.-C. Wong, J. Xu, Z. Bian, Z. Liang, D. Guo, H. Zhu, W. Tan, A. Lu, G. Zhang, Osteoclast-derived exosomal miR-214-3p inhibits osteoblastic bone formation, *Nat. Commun*. 7 (2016) 10872. <https://doi.org/10.1038/ncomms10872>.
- [24] M. Colombo, G. Raposo, C. Théry, Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles, *Annu. Rev. Cell Dev. Biol*. 30 (2014) 255–289. <https://doi.org/10.1146/annurev-cellbio-101512-122326>.
- [25] B. György, T.G. Szabó, M. Pásztói, Z. Pál, P. Misják, B. Aradi, V. László, É. Pállinger, E. Pap, Á. Kittel, G. Nagy, A. Falus, E.I. Buzás, Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles, *Cell. Mol. Life Sci*. 68 (2011) 2667–2688. <https://doi.org/10.1007/s00018-011-0689-3>.
- [26] A. Isola, S. Chen, Exosomes: The Messengers of Health and Disease, *Curr. Neuropharmacol*. 15 (2016) 157–165. <https://doi.org/10.2174/1570159X14666160825160421>.
- [27] L. Urbanelli, A. Magini, S. Buratta, A. Brozzi, K. Sagini, A. Polchi, B. Tancini, C. Emiliani,

- Signaling Pathways in Exosomes Biogenesis, Secretion and Fate, *Genes (Basel)*. 4 (2013)152–170. <https://doi.org/10.3390/genes4020152>.
- [28] N. Wall, Aspe, Survivin-T34A: molecular mechanism and therapeutic potential, *Onco. Targets. Ther.* (2010) 247. <https://doi.org/10.2147/OTT.S15293>.
- [29] S. Keller, J. Ridinger, A.-K. Rupp, J.W. Janssen, P. Altevogt, Body fluid derived exosomes as a novel template for clinical diagnostics, *J. Transl. Med.* 9 (2011) 86. <https://doi.org/10.1186/1479-5876-9-86>.
- [30] M. Rodriguez-dorantes, S. Romero-Cordoba, O. Peralta-Zaragoza, I. Salido-Guadarrama, A. Hidalgo-Miranda, MicroRNAs transported by exosomes in body fluids as mediators of intercellular communication in cancer, *Onco. Targets. Ther.* (2014) 1327. <https://doi.org/10.2147/OTT.S61562>.
- [31] C. Théry, L. Zitvogel, S. Amigorena, Exosomes: composition, biogenesis and function, *Nat. Rev. Immunol.* 2 (2002) 569–579. <https://doi.org/10.1038/nri855>.
- [32] C. Kahlert, S.A. Melo, A. Protopopov, J. Tang, S. Seth, M. Koch, J. Zhang, J. Weitz, L. Chin, A. Futreal, R. Kalluri, Identification of Double-stranded Genomic DNA Spanning All Chromosomes with Mutated KRAS and p53 DNA in the Serum Exosomes of Patients with Pancreatic Cancer, *J. Biol. Chem.* 289 (2014) 3869–3875. <https://doi.org/10.1074/jbc.C113.532267>.
- [33] M. Battistelli, E. Falcieri, Apoptotic Bodies: Particular Extracellular Vesicles Involved in Intercellular Communication, *Biology (Basel)*. 9 (2020) 21. <https://doi.org/10.3390/biology9010021>.

- [34] R. Kakarla, J. Hur, Y.J. Kim, J. Kim, Y.-J. Chwae, Apoptotic cell-derived exosomes: messages from dying cells, *Exp. Mol. Med.* 52 (2020) 1–6. <https://doi.org/10.1038/s12276-019-0362-8>.
- [35] E. Willms, C. Cabañas, I. Mäger, M.J.A. Wood, P. Vader, Extracellular Vesicle Heterogeneity: Subpopulations, Isolation Techniques, and Diverse Functions in Cancer Progression, *Front. Immunol.* 9 (2018). <https://doi.org/10.3389/fimmu.2018.00738>.
- [36] M.T. Roefs, J.P.G. Sluijter, P. Vader, Extracellular Vesicle-Associated Proteins in Tissue Repair, *Trends Cell Biol.* 30 (2020) 990–1013. <https://doi.org/10.1016/j.tcb.2020.09.009>.
- [37] A. V. Vlassov, S. Magdaleno, R. Setterquist, R. Conrad, Exosomes: Current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials, *Biochim. Biophys. Acta - Gen. Subj.* 1820 (2012) 940–948. <https://doi.org/10.1016/j.bbagen.2012.03.017>.
- [38] M. Tkach, C. Théry, Communication by Extracellular Vesicles: Where We Are and Where We Need to Go, *Cell.* 164 (2016) 1226–1232. <https://doi.org/10.1016/j.cell.2016.01.043>.
- [39] R. Schiffelers, S. Kooijmans, Vader, van Dommelen, Van Solinge, Exosome mimetics: a novel class of drug delivery systems, *Int. J. Nanomedicine.* (2012) 1525. <https://doi.org/10.2147/IJN.S29661>.
- [40] S. Ohno, M. Takanashi, K. Sudo, S. Ueda, A. Ishikawa, N. Matsuyama, K. Fujita, T. Mizutani, T. Ohgi, T. Ochiya, N. Gotoh, M. Kuroda, Systemically Injected Exosomes Targeted to EGFR Deliver Antitumor MicroRNA to Breast Cancer Cells, *Mol. Ther.* 21 (2013) 185–191. <https://doi.org/10.1038/mt.2012.180>.

- [41] S. Rani, A.E. Ryan, M.D. Griffin, T. Ritter, Mesenchymal Stem Cell-derived Extracellular Vesicles: Toward Cell-free Therapeutic Applications, *Mol. Ther.* 23 (2015) 812–823. <https://doi.org/10.1038/mt.2015.44>.
- [42] S.M. van Dommelen, P. Vader, S. Lakhal, S.A.A. Kooijmans, W.W. van Solinge, M.J.A. Wood, R.M. Schiffelers, Microvesicles and exosomes: Opportunities for cell-derived membrane vesicles in drug delivery, *J. Control. Release.* 161 (2012) 635–644. <https://doi.org/10.1016/j.jconrel.2011.11.021>.
- [43] M. Çağdaş, A.D. Sezer, S. Bucak, Liposomes as Potential Drug Carrier Systems for Drug Delivery, in: *Appl. Nanotechnol. Drug Deliv.*, InTech, 2014. <https://doi.org/10.5772/58459>.
- [44] Y. Fan, Q. Zhang, Development of liposomal formulations: From concept to clinical investigations, *Asian J. Pharm. Sci.* 8 (2013) 81–87. <https://doi.org/10.1016/j.ajps.2013.07.010>.
- [45] K.H. Wong, A. Lu, X. Chen, Z. Yang, Natural Ingredient-Based Polymeric Nanoparticles for Cancer Treatment, *Molecules.* 25 (2020) 3620. <https://doi.org/10.3390/molecules25163620>.
- [46] T. Ishida, D.L. Iden, T.M. Allen, A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs, *FEBS Lett.* 460 (1999) 129–133. [https://doi.org/10.1016/S0014-5793\(99\)01320-4](https://doi.org/10.1016/S0014-5793(99)01320-4).
- [47] M. Riaz, M. Riaz, X. Zhang, C. Lin, K. Wong, X. Chen, G. Zhang, A. Lu, Z. Yang, Surface Functionalization and Targeting Strategies of Liposomes in Solid Tumor Therapy: A Review, *Int. J. Mol. Sci.* 19 (2018) 195. <https://doi.org/10.3390/ijms19010195>.

- [48] A. Thakur, A.P. Mishra, B. Panda, K. Sweta, B. Majhi, Detection of Disease-Specific Parent Cells Via Distinct Population of Nano-Vesicles by Machine Learning, *Curr. Pharm. Des.* 26 (2020) 3985–3996. <https://doi.org/10.2174/1381612826666200422091753>.
- [49] S. Keller, M.P. Sanderson, A. Stoeck, P. Altevogt, Exosomes: From biogenesis and secretion to biological function, *Immunol. Lett.* 107 (2006) 102–108. <https://doi.org/10.1016/j.imlet.2006.09.005>.
- [50] S. Stremersch, R.E. Vandenbroucke, E. Van Wonterghem, A. Hendrix, S.C. De Smedt, K. Raemdonck, Comparing exosome-like vesicles with liposomes for the functional cellular delivery of small RNAs, *J. Control. Release.* 232 (2016) 51–61. <https://doi.org/10.1016/j.jconrel.2016.04.005>.
- [51] P. Akuma, O.D. Okagu, C.C. Udenigwe, Naturally Occurring Exosome Vesicles as Potential Delivery Vehicle for Bioactive Compounds, *Front. Sustain. Food Syst.* 3 (2019). <https://doi.org/10.3389/fsufs.2019.00023>.
- [52] Y.T. Sato, K. Umezaki, S. Sawada, S. Mukai, Y. Sasaki, N. Harada, H. Shiku, K. Akiyoshi, Engineering hybrid exosomes by membrane fusion with liposomes, *Sci. Rep.* 6 (2016) 21933. <https://doi.org/10.1038/srep21933>.
- [53] L.-L. Yu, J. Zhu, J.-X. Liu, F. Jiang, W.-K. Ni, L.-S. Qu, R.-Z. Ni, C.-H. Lu, M.-B. Xiao, A Comparison of Traditional and Novel Methods for the Separation of Exosomes from Human Samples, *Biomed Res. Int.* 2018 (2018) 1–9. <https://doi.org/10.1155/2018/3634563>.
- [54] G.K. Patel, M.A. Khan, H. Zubair, S.K. Srivastava, M. Khushman, S. Singh, A.P. Singh, Comparative analysis of exosome isolation methods using culture supernatant for optimum yield, purity and downstream applications, *Sci. Rep.* 9 (2019) 5335.

<https://doi.org/10.1038/s41598-019-41800-2>.

- [55] A. Akbarzadeh, R. Rezaei-Sadabady, S. Davaran, S.W. Joo, N. Zarghami, Y. Hanifepour, M. Samiei, M. Kouhi, K. Nejati-Koshki, Liposome: classification, preparation, and applications, *Nanoscale Res. Lett.* 8 (2013) 102. <https://doi.org/10.1186/1556-276X-8-102>.
- [56] M.; Wiggenghorn, Scale-Up of Liposome Manufacturing: Combining High Pressure Liposome Extrusion with Drying Technologies, Ludwig-Maximilians-University: München, Germany, 2007.
- [57] A. Thakur, H. Zou, M. Yang, Y. Lee, Abstract 3720: Augmented loading efficiency of doxorubicin into glioma-derived exosomes by an integrated microfluidic device, *Cancer Res.* 78 (2018) 3720–3720. <https://doi.org/10.1158/1538-7445.AM2018-3720>.
- [58] M. Sancho-Albero, M. del M. Encabo-Berzosa, M. Beltrán-Visiedo, L. Fernández-Messina, V. Sebastián, F. Sánchez-Madrid, M. Arruebo, J. Santamaría, P. Martín-Duque, Efficient encapsulation of theranostic nanoparticles in cell-derived exosomes: leveraging the exosomal biogenesis pathway to obtain hollow gold nanoparticle-hybrids, *Nanoscale.* 11 (2019) 18825–18836. <https://doi.org/10.1039/C9NR06183E>.
- [59] A. Silva-Weiss, M. Quilaqueo, O. Venegas, M. Ahumada, W. Silva, F. Osorio, B. Giménez, Design of dipalmitoyl lecithin liposomes loaded with quercetin and rutin and their release kinetics from carboxymethyl cellulose edible films, *J. Food Eng.* 224 (2018) 165–173. <https://doi.org/10.1016/j.jfoodeng.2018.01.001>.
- [60] S. Antimisiaris, S. Mourtas, A. Marazioti, Exosomes and Exosome-Inspired Vesicles for Targeted Drug Delivery, *Pharmaceutics.* 10 (2018) 218. <https://doi.org/10.3390/pharmaceutics10040218>.

- [61] M. Cully, Exosome-based candidates move into the clinic, *Nat. Rev. Drug Discov.* 20 (2021) 6–7. <https://doi.org/10.1038/d41573-020-00220-y>.
- [62] U. Bulbake, S. Doppalapudi, N. Kommineni, W. Khan, Liposomal Formulations in Clinical Use: An Updated Review, *Pharmaceutics*. 9 (2017) 12. <https://doi.org/10.3390/pharmaceutics9020012>.
- [63] L. Doyle, M. Wang, Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis, *Cells*. 8 (2019) 727. <https://doi.org/10.3390/cells8070727>.
- [64] M.A. Livshits, E. Khomyakova, E.G. Evtushenko, V.N. Lazarev, N.A. Kulemin, S.E. Semina, E. V. Generozov, V.M. Govorun, Isolation of exosomes by differential centrifugation: Theoretical analysis of a commonly used protocol, *Sci. Rep.* 5 (2015) 17319. <https://doi.org/10.1038/srep17319>.
- [65] T. Soares Martins, J. Catita, I. Martins Rosa, O. A. B. da Cruz e Silva, A.G. Henriques, Exosome isolation from distinct biofluids using precipitation and column-based approaches, *PLoS One*. 13 (2018) e0198820. <https://doi.org/10.1371/journal.pone.0198820>.
- [66] F. Liu, O. Vermesh, V. Mani, T.J. Ge, S.J. Madsen, A. Sabour, E.-C. Hsu, G. Gowrishankar, M. Kanada, J. V. Jokerst, R.G. Sierra, E. Chang, K. Lau, K. Sridhar, A. Bermudez, S.J. Pitteri, T. Stoyanova, R. Sinclair, V.S. Nair, S.S. Gambhir, U. Demirci, The Exosome Total Isolation Chip, *ACS Nano*. 11 (2017) 10712–10723. <https://doi.org/10.1021/acsnano.7b04878>.
- [67] S.E. Emam, H. Ando, A.S. Abu Lila, T. Shimizu, M. Ukawa, K. Okuhira, Y. Ishima, M.A. Mahdy, F.S. Ghazy, T. Ishida, A Novel Strategy to Increase the Yield of Exosomes

- (Extracellular Vesicles) for an Expansion of Basic Research, *Biol. Pharm. Bull.* 41 (2018) 733–742. <https://doi.org/10.1248/bpb.b17-00919>.
- [68] A. Savina, M. Furlán, M. Vidal, M.I. Colombo, Exosome Release Is Regulated by a Calcium-dependent Mechanism in K562 Cells, *J. Biol. Chem.* 278 (2003) 20083–20090. <https://doi.org/10.1074/jbc.M301642200>.
- [69] B.B. Guo, S.A. Bellingham, A.F. Hill, Stimulating the Release of Exosomes Increases the Intercellular Transfer of Prions, *J. Biol. Chem.* 291 (2016) 5128–5137. <https://doi.org/10.1074/jbc.M115.684258>.
- [70] R.C. Lai, R.W.Y. Yeo, K.H. Tan, S.K. Lim, Exosomes for drug delivery — a novel application for the mesenchymal stem cell, *Biotechnol. Adv.* 31 (2013) 543–551. <https://doi.org/10.1016/j.biotechadv.2012.08.008>.
- [71] R.W.Y. Yeo, R.C. Lai, B. Zhang, S.S. Tan, Y. Yin, B.J. Teh, S.K. Lim, Mesenchymal stem cell: An efficient mass producer of exosomes for drug delivery, *Adv. Drug Deliv. Rev.* 65 (2013) 336–341. <https://doi.org/10.1016/j.addr.2012.07.001>.
- [72] J. Ren, W. He, L. Zheng, H. Duan, From structures to functions: insights into exosomes as promising drug delivery vehicles, *Biomater. Sci.* 4 (2016) 910–921. <https://doi.org/10.1039/C5BM00583C>.
- [73] M. Sancho-Albero, N. Navascués, G. Mendoza, V. Sebastián, M. Arruebo, P. Martín-Duque, J. Santamaría, Exosome origin determines cell targeting and the transfer of therapeutic nanoparticles towards target cells, *J. Nanobiotechnology.* 17 (2019) 16. <https://doi.org/10.1186/s12951-018-0437-z>.

- [74] N. Chopra, B. Dutt Arya, N. Jain, P. Yadav, S. Wajid, S.P. Singh, S. Choudhury, Biophysical Characterization and Drug Delivery Potential of Exosomes from Human Wharton's Jelly-Derived Mesenchymal Stem Cells, *ACS Omega*. 4 (2019) 13143–13152. <https://doi.org/10.1021/acsomega.9b01180>.
- [75] Y. Si, S. Kim, E. Zhang, Y. Tang, R. Jaskula-Sztul, J.M. Markert, H. Chen, L. Zhou, X. (Margaret) Liu, Targeted Exosomes for Drug Delivery: Biomanufacturing, Surface Tagging, and Validation, *Biotechnol. J.* 15 (2020) 1900163. <https://doi.org/10.1002/biot.201900163>.
- [76] S. Gurunathan, M.-H. Kang, M. Jeyaraj, M. Qasim, J.-H. Kim, Review of the Isolation, Characterization, Biological Function, and Multifarious Therapeutic Approaches of Exosomes, *Cells*. 8 (2019) 307. <https://doi.org/10.3390/cells8040307>.
- [77] J.M. Carnino, H. Lee, Y. Jin, Isolation and characterization of extracellular vesicles from Broncho-alveolar lavage fluid: a review and comparison of different methods, *Respir. Res.* 20 (2019) 240. <https://doi.org/10.1186/s12931-019-1210-z>.
- [78] Y. Zhang, J. Bi, J. Huang, Y. Tang, S. Du, P. Li, Exosome: A Review of Its Classification, Isolation Techniques, Storage, Diagnostic and Targeted Therapy Applications, *Int. J. Nanomedicine*. Volume 15 (2020) 6917–6934. <https://doi.org/10.2147/IJN.S264498>.
- [79] L. Grasso, R. Wyss, L. Weidenauer, A. Thampi, D. Demurtas, M. Prudent, N. Lion, H. Vogel, Molecular screening of cancer-derived exosomes by surface plasmon resonance spectroscopy, *Anal. Bioanal. Chem.* 407 (2015) 5425–5432. <https://doi.org/10.1007/s00216-015-8711-5>.
- [80] C.Z.J. Lim, Y. Zhang, Y. Chen, H. Zhao, M.C. Stephenson, N.R.Y. Ho, Y. Chen, J. Chung,

- A. Reilhac, T.P. Loh, C.L.H. Chen, H. Shao, Subtyping of circulating exosome-bound amyloid β reflects brain plaque deposition, *Nat. Commun.* 10 (2019) 1144. <https://doi.org/10.1038/s41467-019-09030-2>.
- [81] D.L.M. Rupert, C. Lässer, M. Eldh, S. Block, V.P. Zhdanov, J.O. Lotvall, M. Bally, F. Höök, Determination of Exosome Concentration in Solution Using Surface Plasmon Resonance Spectroscopy, *Anal. Chem.* 86 (2014) 5929–5936. <https://doi.org/10.1021/ac500931f>.
- [82] H. Kholafazad Kordasht, M. Hasanzadeh, Biomedical analysis of exosomes using biosensing methods: recent progress, *Anal. Methods.* 12 (2020) 2795–2811. <https://doi.org/10.1039/D0AY00722F>.
- [83] S. Sharma, H.I. Rasool, V. Palanisamy, C. Mathisen, M. Schmidt, D.T. Wong, J.K. Gimzewski, Structural-Mechanical Characterization of Nanoparticle Exosomes in Human Saliva, Using Correlative AFM, FESEM, and Force Spectroscopy, *ACS Nano.* 4 (2010) 1921–1926. <https://doi.org/10.1021/nn901824n>.
- [84] J. Mihály, R. Deák, I.C. Szigyártó, A. Bóta, T. Beke-Somfai, Z. Varga, Characterization of extracellular vesicles by IR spectroscopy: Fast and simple classification based on amide and C H stretching vibrations, *Biochim. Biophys. Acta - Biomembr.* 1859 (2017) 459–466. <https://doi.org/10.1016/j.bbamem.2016.12.005>.
- [85] J.C. Contreras-Naranjo, H.-J. Wu, V.M. Ugaz, Microfluidics for exosome isolation and analysis: enabling liquid biopsy for personalized medicine, *Lab Chip.* 17 (2017) 3558–3577. <https://doi.org/10.1039/C7LC00592J>.
- [86] T. Smyth, K. Petrova, N.M. Payton, I. Persaud, J.S. Redzic, M.W. Graner, P. Smith-Jones,

- T.J. Anchordoquy, Surface Functionalization of Exosomes Using Click Chemistry, *Bioconjug. Chem.* 25 (2014) 1777–1784. <https://doi.org/10.1021/bc500291r>.
- [87] G. Jia, Y. Han, Y. An, Y. Ding, C. He, X. Wang, Q. Tang, NRP-1 targeted and cargo-loaded exosomes facilitate simultaneous imaging and therapy of glioma in vitro and in vivo, *Biomaterials*. 178 (2018) 302–316. <https://doi.org/10.1016/j.biomaterials.2018.06.029>.
- [88] T.S. Lee, Y. Kim, W. Zhang, I.H. Song, C.-H. Tung, Facile metabolic glycan labeling strategy for exosome tracking, *Biochim. Biophys. Acta - Gen. Subj.* 1862 (2018) 1091–1100. <https://doi.org/10.1016/j.bbagen.2018.02.001>.
- [89] S. Salunkhe, Dheeraj, M. Basak, D. Chitkara, A. Mittal, Surface functionalization of exosomes for target-specific delivery and in vivo imaging & tracking: Strategies and significance, *J. Control. Release.* 326 (2020) 599–614. <https://doi.org/10.1016/j.jconrel.2020.07.042>.
- [90] K.I. Mentkowski, J.D. Snitzer, S. Rusnak, J.K. Lang, Therapeutic Potential of Engineered Extracellular Vesicles, *AAPS J.* 20 (2018) 50. <https://doi.org/10.1208/s12248-018-0211-z>.
- [91] H. Kim, N. Yun, D. Mun, J.-Y. Kang, S.-H. Lee, H. Park, H. Park, B. Joung, Cardiac-specific delivery by cardiac tissue-targeting peptide-expressing exosomes, *Biochem. Biophys. Res. Commun.* 499 (2018) 803–808. <https://doi.org/10.1016/j.bbrc.2018.03.227>.
- [92] Y. Tian, S. Li, J. Song, T. Ji, M. Zhu, G.J. Anderson, J. Wei, G. Nie, A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy, *Biomaterials*. 35 (2014) 2383–2390. <https://doi.org/10.1016/j.biomaterials.2013.11.083>.
- [93] K. Chen, P.S. Conti, Target-specific delivery of peptide-based probes for PET imaging,

- Adv. Drug Deliv. Rev. 62 (2010) 1005–1022. <https://doi.org/10.1016/j.addr.2010.09.004>.
- [94] M.E. Hung, J.N. Leonard, Stabilization of Exosome-targeting Peptides via Engineered Glycosylation, *J. Biol. Chem.* 290 (2015) 8166–8172. <https://doi.org/10.1074/jbc.M114.621383>.
- [95] Y. Zhang, B. Lai, M. Juhas, Recent Advances in Aptamer Discovery and Applications, *Molecules*. 24 (2019) 941. <https://doi.org/10.3390/molecules24050941>.
- [96] A.D. Ellington, J.W. Szostak, In vitro selection of RNA molecules that bind specific ligands, *Nature*. 346 (1990) 818–822. <https://doi.org/10.1038/346818a0>.
- [97] C. Tuerk, L. Gold, Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, *Science* (80-.). 249 (1990) 505–510. <https://doi.org/10.1126/science.2200121>.
- [98] Y. Wan, L. Wang, C. Zhu, Q. Zheng, G. Wang, J. Tong, Y. Fang, Y. Xia, G. Cheng, X. He, S.-Y. Zheng, Aptamer-Conjugated Extracellular Nanovesicles for Targeted Drug Delivery, *Cancer Res.* 78 (2018) 798–808. <https://doi.org/10.1158/0008-5472.CAN-17-2880>.
- [99] F. Pi, D.W. Binzel, T.J. Lee, Z. Li, M. Sun, P. Rychahou, H. Li, F. Haque, S. Wang, C.M. Croce, B. Guo, B.M. Evers, P. Guo, Nanoparticle orientation to control RNA loading and ligand display on extracellular vesicles for cancer regression, *Nat. Nanotechnol.* 13 (2018) 82–89. <https://doi.org/10.1038/s41565-017-0012-z>.
- [100] J. Zou, M. Shi, X. Liu, C. Jin, X. Xing, L. Qiu, W. Tan, Aptamer-Functionalized Exosomes: Elucidating the Cellular Uptake Mechanism and the Potential for Cancer-Targeted Chemotherapy, *Anal. Chem.* 91 (2019) 2425–2430.

<https://doi.org/10.1021/acs.analchem.8b05204>.

- [101] L. Alvarez-Erviti, Y. Seow, H. Yin, C. Betts, S. Lakhal, M.J.A. Wood, Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes, *Nat. Biotechnol.* 29 (2011) 341–345. <https://doi.org/10.1038/nbt.1807>.
- [102] H. Zhang, J. Wu, J. Wu, Q. Fan, J. Zhou, J. Wu, S. Liu, J. Zang, J. Ye, M. Xiao, T. Tian, J. Gao, Exosome-mediated targeted delivery of miR-210 for angiogenic therapy after cerebral ischemia in mice, *J. Nanobiotechnology.* 17 (2019) 29. <https://doi.org/10.1186/s12951-019-0461-7>.
- [103] Q. Zhan, K. Yi, H. Qi, S. Li, X. Li, Q. Wang, Y. Wang, C. Liu, M. Qiu, X. Yuan, J. Zhao, X. Hou, C. Kang, Engineering blood exosomes for tumor-targeting efficient gene/chemo combination therapy, *Theranostics.* 10 (2020) 7889–7905. <https://doi.org/10.7150/thno.45028>.
- [104] X. Osteikoetxea, A. Balogh, K. Szabó-Taylor, A. Németh, T.G. Szabó, K. Pálóczi, B. Sódar, Á. Kittel, B. György, É. Pállinger, J. Matkó, E.I. Buzás, Improved Characterization of EV Preparations Based on Protein to Lipid Ratio and Lipid Properties, *PLoS One.* 10 (2015) e0121184. <https://doi.org/10.1371/journal.pone.0121184>.
- [105] M. Frydrychowicz, A. Kolečka-Bednarczyk, M. Madejczyk, S. Yasar, G. Dworacki, Exosomes - Structure, Biogenesis and Biological Role in Non-Small-Cell Lung Cancer, *Scand. J. Immunol.* 81 (2015) 2–10. <https://doi.org/10.1111/sji.12247>.
- [106] C. Federici, F. Petrucci, S. Caimi, A. Cesolini, M. Logozzi, M. Borghi, S. D’Ilio, L. Lugini, N. Violante, T. Azzarito, C. Majorani, D. Brambilla, S. Fais, Exosome Release and Low pH Belong to a Framework of Resistance of Human Melanoma Cells to Cisplatin, *PLoS One.*

- 9 (2014) e88193. <https://doi.org/10.1371/journal.pone.0088193>.
- [107] E. Iessi, M. Logozzi, L. Lugini, T. Azzarito, C. Federici, E.P. Spugnini, D. Mizzoni, R. Di Raimo, D.F. Angelini, L. Battistini, S. Cecchetti, S. Fais, Acridine Orange/exosomes increase the delivery and the effectiveness of Acridine Orange in human melanoma cells: A new prototype for theranostics of tumors, *J. Enzyme Inhib. Med. Chem.* 32 (2017) 648–657. <https://doi.org/10.1080/14756366.2017.1292263>.
- [108] C. Campanella, C. Caruso Bavisotto, M. Logozzi, A. Marino Gammazza, D. Mizzoni, F. Cappello, S. Fais, On the Choice of the Extracellular Vesicles for Therapeutic Purposes, *Int. J. Mol. Sci.* 20 (2019) 236. <https://doi.org/10.3390/ijms20020236>.
- [109] N.M. Mahaweni, M.E.H. Kaijen-Lambers, J. Dekkers, J.G.J.V. Aerts, J.P.J.J. Hegmans, Tumour-derived exosomes as antigen delivery carriers in dendritic cell-based immunotherapy for malignant mesothelioma, *J. Extracell. Vesicles.* 2 (2013) 22492. <https://doi.org/10.3402/jev.v2i0.22492>.
- [110] A.J. Vázquez-Ríos, Á. Molina-Crespo, B.L. Bouzo, R. López-López, G. Moreno-Bueno, M. de la Fuente, Exosome-mimetic nanoplateforms for targeted cancer drug delivery, *J. Nanobiotechnology.* 17 (2019) 85. <https://doi.org/10.1186/s12951-019-0517-8>.
- [111] E. Spugnini, M. Logozzi, R. Di Raimo, D. Mizzoni, S. Fais, A Role of Tumor-Released Exosomes in Paracrine Dissemination and Metastasis, *Int. J. Mol. Sci.* 19 (2018) 3968. <https://doi.org/10.3390/ijms19123968>.
- [112] J. Wolfers, A. Lozier, G. Raposo, A. Regnault, C. Théry, C. Masurier, C. Flament, S. Pouzieux, F. Faure, T. Tursz, E. Angevin, S. Amigorena, L. Zitvogel, Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming, *Nat. Med.*

- 7 (2001) 297–303. <https://doi.org/10.1038/85438>.
- [113] S. Rana, S. Yue, D. Stadel, M. Zöller, Toward tailored exosomes: The exosomal tetraspanin web contributes to target cell selection, *Int. J. Biochem. Cell Biol.* 44 (2012) 1574–1584. <https://doi.org/10.1016/j.biocel.2012.06.018>.
- [114] A.B. Frey, Myeloid suppressor cells regulate the adaptive immune response to cancer, *J. Clin. Invest.* 116 (2006) 2587–2590. <https://doi.org/10.1172/JCI29906>.
- [115] D.D. Taylor, C. Gercel-Taylor, Exosomes/microvesicles: mediators of cancer-associated immunosuppressive microenvironments, *Semin. Immunopathol.* 33 (2011) 441–454. <https://doi.org/10.1007/s00281-010-0234-8>.
- [116] D.A. Harris, S.H. Patel, M. Gucek, A. Hendrix, W. Westbroek, J.W. Taraska, Exosomes Released from Breast Cancer Carcinomas Stimulate Cell Movement, *PLoS One.* 10 (2015) e0117495. <https://doi.org/10.1371/journal.pone.0117495>.
- [117] B.B. Shenoda, S.K. Ajit, Modulation of Immune Responses by Exosomes Derived from Antigen-Presenting Cells, *Clin. Med. Insights Pathol.* 9s1 (2016) CPath.S39925. <https://doi.org/10.4137/CPath.S39925>.
- [118] B. Escudier, T. Dorval, N. Chaput, F. André, M.-P. Caby, S. Novault, C. Flament, C. Leboulaire, C. Borg, S. Amigorena, C. Boccaccio, C. Bonnerot, O. Dhellin, M. Movassagh, S. Piperno, C. Robert, V. Serra, N. Valente, J.-B. Le Pecq, A. Spatz, O. Lantz, T. Tursz, E. Angevin, L. Zitvogel, Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of the first phase I clinical trial., *J. Transl. Med.* 3 (2005) 10. <https://doi.org/10.1186/1479-5876-3-10>.

- [119] M.J. Haney, N.L. Klyachko, Y. Zhao, R. Gupta, E.G. Plotnikova, Z. He, T. Patel, A. Piroyan, M. Sokolsky, A. V. Kabanov, E. V. Batrakova, Exosomes as drug delivery vehicles for Parkinson's disease therapy, *J. Control. Release.* 207 (2015) 18–30. <https://doi.org/10.1016/j.jconrel.2015.03.033>.
- [120] S. Shi, Q. Rao, C. Zhang, X. Zhang, Y. Qin, Z. Niu, Dendritic Cells Pulsed with Exosomes in Combination with PD-1 Antibody Increase the Efficacy of Sorafenib in Hepatocellular Carcinoma Model, *Transl. Oncol.* 11 (2018) 250–258. <https://doi.org/10.1016/j.tranon.2018.01.001>.
- [121] J.M. Pitt, M. Charrier, S. Viaud, F. André, B. Besse, N. Chaput, L. Zitvogel, Dendritic Cell-Derived Exosomes as Immunotherapies in the Fight against Cancer, *J. Immunol.* 193 (2014) 1006–1011. <https://doi.org/10.4049/jimmunol.1400703>.
- [122] C. Théry, L. Duban, E. Segura, P. Véron, O. Lantz, S. Amigorena, Indirect activation of naïve CD4⁺ T cells by dendritic cell-derived exosomes, *Nat. Immunol.* 3 (2002) 1156–1162. <https://doi.org/10.1038/ni854>.
- [123] S. Viaud, M. Terme, C. Flament, J. Taieb, F. André, S. Novault, B. Escudier, C. Robert, S. Caillat-Zucman, T. Tursz, L. Zitvogel, N. Chaput, Dendritic Cell-Derived Exosomes Promote Natural Killer Cell Activation and Proliferation: A Role for NKG2D Ligands and IL-15R α , *PLoS One.* 4 (2009) e4942. <https://doi.org/10.1371/journal.pone.0004942>.
- [124] M. Jakubec, J. Maple-Grødem, S. Akbari, S. Nesse, Ø. Halskau, A.E. Mork-Jansson, Plasma-derived exosome-like vesicles are enriched in lyso-phospholipids and pass the blood-brain barrier, *PLoS One.* 15 (2020) e0232442. <https://doi.org/10.1371/journal.pone.0232442>.

- [125] S. Dai, D. Wei, Z. Wu, X. Zhou, X. Wei, H. Huang, G. Li, Phase I Clinical Trial of Autologous Ascites-derived Exosomes Combined With GM-CSF for Colorectal Cancer, *Mol. Ther.* 16 (2008) 782–790. <https://doi.org/10.1038/mt.2008.1>.
- [126] G. van Niel, G. D’Angelo, G. Raposo, Shedding light on the cell biology of extracellular vesicles, *Nat. Rev. Mol. Cell Biol.* 19 (2018) 213–228. <https://doi.org/10.1038/nrm.2017.125>.
- [127] J. Kang, H. Park, H. Kim, D. Mun, H. Park, N. Yun, B. Joung, Human peripheral blood-derived exosomes for microRNA delivery, *Int. J. Mol. Med.* (2019). <https://doi.org/10.3892/ijmm.2019.4150>.
- [128] K. Jiang, J. Yang, S. Guo, G. Zhao, H. Wu, G. Deng, Peripheral Circulating Exosome-Mediated Delivery of miR-155 as a Novel Mechanism for Acute Lung Inflammation, *Mol. Ther.* 27 (2019) 1758–1771. <https://doi.org/10.1016/j.ymthe.2019.07.003>.
- [129] M. Qu, Q. Lin, L. Huang, Y. Fu, L. Wang, S. He, Y. Fu, S. Yang, Z. Zhang, L. Zhang, X. Sun, Dopamine-loaded blood exosomes targeted to brain for better treatment of Parkinson’s disease, *J. Control. Release.* 287 (2018) 156–166. <https://doi.org/10.1016/j.jconrel.2018.08.035>.
- [130] S. Nair, K.D. Tang, L. Kenny, C. Punyadeera, Salivary exosomes as potential biomarkers in cancer, *Oral Oncol.* 84 (2018) 31–40. <https://doi.org/10.1016/j.oraloncology.2018.07.001>.
- [131] A.R. Huebner, P. Somparn, T. Benjachat, A. Leelahavanichkul, Y. Avihingsanon, R.A. Fenton, T. Pisitkun, Exosomes in Urine Biomarker Discovery, in: *Urin. Proteomics Kidney Dis. Biomark. Discov.* Springer Dordrecht, Netherlands, 2015: pp. 43–58.

https://doi.org/10.1007/978-94-017-9523-4_5.

- [132] S. Ju, J. Mu, T. Dokland, X. Zhuang, Q. Wang, H. Jiang, X. Xiang, Z.-B. Deng, B. Wang, L. Zhang, M. Roth, R. Welti, J. Mobley, Y. Jun, D. Miller, H.-G. Zhang, Grape Exosome-like Nanoparticles Induce Intestinal Stem Cells and Protect Mice From DSS-Induced Colitis, *Mol. Ther.* 21 (2013) 1345–1357. <https://doi.org/10.1038/mt.2013.64>.
- [133] R. Munagala, F. Aqil, J. Jeyabalan, R.C. Gupta, Bovine milk-derived exosomes for drug delivery, *Cancer Lett.* 371 (2016) 48–61. <https://doi.org/10.1016/j.canlet.2015.10.020>.
- [134] H.A. Dad, T.-W. Gu, A.-Q. Zhu, L.-Q. Huang, L.-H. Peng, Plant Exosome-like Nanovesicles: Emerging Therapeutics and Drug Delivery Nanoplatfroms, *Mol. Ther.* 29 (2021) 13–31. <https://doi.org/10.1016/j.ymthe.2020.11.030>.
- [135] Q. Wang, Y. Ren, J. Mu, N.K. Egilmez, X. Zhuang, Z. Deng, L. Zhang, J. Yan, D. Miller, H.-G. Zhang, Grapefruit-Derived Nanovectors Use an Activated Leukocyte Trafficking Pathway to Deliver Therapeutic Agents to Inflammatory Tumor Sites, *Cancer Res.* 75 (2015) 2520–2529. <https://doi.org/10.1158/0008-5472.CAN-14-3095>.
- [136] L.-K. Huang, S.-P. Chao, C.-J. Hu, Clinical trials of new drugs for Alzheimer disease, *J. Biomed. Sci.* 27 (2020) 18. <https://doi.org/10.1186/s12929-019-0609-7>.
- [137] A.E. Oxford, E.S. Stewart, T.T. Rohn, Clinical Trials in Alzheimer’s Disease: A Hurdle in the Path of Remedy, *Int. J. Alzheimers. Dis.* 2020 (2020) 1–13. <https://doi.org/10.1155/2020/5380346>.
- [138] P.S. Aisen, Failure After Failure. What Next in AD Drug Development?, *J Prev Alzheimers Dis.* 6 (2019). <https://doi.org/https://doi.org/10.14283/jpad.2019.23>.

- [139] Q. Yin, X. Ji, R. Lv, J.-J. Pei, Y. Du, C. Shen, X. Hou, Targetting Exosomes as a New Biomarker and Therapeutic Approach for Alzheimer's Disease, *Clin. Interv. Aging*. Volume 15 (2020) 195–205. <https://doi.org/10.2147/CIA.S240400>.
- [140] M. Losurdo, M. Pedrazzoli, C. D'Agostino, C.A. Elia, F. Massenzio, E. Lonati, M. Mauri, L. Rizzi, L. Molteni, E. Bresciani, E. Dander, G. D'Amico, A. Bulbarelli, A. Torsello, M. Matteoli, M. Buffelli, S. Coco, Intranasal delivery of mesenchymal stem cell-derived extracellular vesicles exerts immunomodulatory and neuroprotective effects in a 3xTg model of Alzheimer's disease, *Stem Cells Transl. Med.* 9 (2020) 1068–1084. <https://doi.org/10.1002/sctm.19-0327>.
- [141] A. Iyaswamy, S.K. Krishnamoorthi, Y.W. Liu, J.X. Song, A.K. Kammala, S.G. Sreenivasmurthy, S. Malampati, B.C.K. Tong, K. Selvarasu, K.H. Cheung, J.H. Lu, J.Q. Tan, C.Y. Huang, S.S.K. Durairajan, M. Li, Yuan-Hu Zhi Tong Prescription Mitigates Tau Pathology and Alleviates Memory Deficiency in the Preclinical Models of Alzheimer's Disease, *Front. Pharmacol.* 11 (2020). <https://doi.org/10.3389/fphar.2020.584770>.
- [142] A. Iyaswamy, S.K. Krishnamoorthi, J.-X. Song, C.-B. Yang, V. Kaliyamoorthy, H. Zhang, S.G. Sreenivasmurthy, S. Malampati, Z.-Y. Wang, Z. Zhu, B.C.-K. Tong, K.-H. Cheung, J.-H. Lu, S.S.K. Durairajan, M. Li, NeuroDefend, a novel Chinese medicine, attenuates amyloid- β and tau pathology in experimental Alzheimer's disease models, *J. Food Drug Anal.* 28 (2020) 132–146. <https://doi.org/10.1016/j.jfda.2019.09.004>.
- [143] S.S.K. Durairajan, A. Iyaswamy, S.G. Shetty, A.K. Kammella, S. Malampati, W. Shang, C. Yang, J. Song, S. Chung, J. Huang, K. Ilango, Q.-B. Han, M. Li, A modified formulation of Huanglian-Jie-Du-Tang reduces memory impairments and β -amyloid plaques in a triple

- transgenic mouse model of Alzheimer's disease, *Sci. Rep.* 7 (2017) 6238. <https://doi.org/10.1038/s41598-017-06217-9>.
- [144] L. Pascucci, V. Coccè, A. Bonomi, D. Ami, P. Ceccarelli, E. Ciusani, L. Viganò, A. Locatelli, F. Sisto, S.M. Doglia, E. Parati, M.E. Bernardo, M. Muraca, G. Alessandri, G. Bondiolotti, A. Pessina, Paclitaxel is incorporated by mesenchymal stromal cells and released in exosomes that inhibit in vitro tumor growth: A new approach for drug delivery, *J. Control. Release.* 192 (2014) 262–270. <https://doi.org/10.1016/j.jconrel.2014.07.042>.
- [145] D. Sun, X. Zhuang, X. Xiang, Y. Liu, S. Zhang, C. Liu, S. Barnes, W. Grizzle, D. Miller, H.-G. Zhang, A Novel Nanoparticle Drug Delivery System: The Anti-inflammatory Activity of Curcumin Is Enhanced When Encapsulated in Exosomes, *Mol. Ther.* 18 (2010) 1606–1614. <https://doi.org/10.1038/mt.2010.105>.
- [146] X. Zhuang, X. Xiang, W. Grizzle, D. Sun, S. Zhang, R.C. Axtell, S. Ju, J. Mu, L. Zhang, L. Steinman, D. Miller, H.-G. Zhang, Treatment of Brain Inflammatory Diseases by Delivering Exosome Encapsulated Anti-inflammatory Drugs From the Nasal Region to the Brain, *Mol. Ther.* 19 (2011) 1769–1779. <https://doi.org/10.1038/mt.2011.164>.
- [147] I. Podolak, A. Galanty, D. Sobolewska, Saponins as cytotoxic agents: a review, *Phytochem. Rev.* 9 (2010) 425–474. <https://doi.org/10.1007/s11101-010-9183-z>.
- [148] G. Fuhrmann, A. Serio, M. Mazo, R. Nair, M.M. Stevens, Active loading into extracellular vesicles significantly improves the cellular uptake and photodynamic effect of porphyrins, *J. Control. Release.* 205 (2015) 35–44. <https://doi.org/10.1016/j.jconrel.2014.11.029>.
- [149] J. Wahlgren, T.D.L. Karlson, M. Brisslert, F. Vaziri Sani, E. Telemo, P. Sunnerhagen, H. Valadi, Plasma exosomes can deliver exogenous short interfering RNA to monocytes and

- lymphocytes, *Nucleic Acids Res.* 40 (2012) e130–e130. <https://doi.org/10.1093/nar/gks463>.
- [150] T.A. Shtam, R.A. Kovalev, E. Varfolomeeva, E.M. Makarov, Y. V Kil, M. V Filatov, Exosomes are natural carriers of exogenous siRNA to human cells in vitro, *Cell Commun. Signal.* 11 (2013) 88. <https://doi.org/10.1186/1478-811X-11-88>.
- [151] S.A.A. Kooijmans, S. Stremersch, K. Braeckmans, S.C. de Smedt, A. Hendrix, M.J.A. Wood, R.M. Schiffelers, K. Raemdonck, P. Vader, Electroporation-induced siRNA precipitation obscures the efficiency of siRNA loading into extracellular vesicles, *J. Control. Release.* 172 (2013) 229–238. <https://doi.org/10.1016/j.jconrel.2013.08.014>.
- [152] M.S. Kim, M.J. Haney, Y. Zhao, V. Mahajan, I. Deygen, N.L. Klyachko, E. Inskoe, A. Piroyan, M. Sokolsky, O. Okolie, S.D. Hingtgen, A. V. Kabanov, E. V. Batrakova, Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells, *Nanomedicine Nanotechnology, Biol. Med.* 12 (2016) 655–664. <https://doi.org/10.1016/j.nano.2015.10.012>.
- [153] S. Fu, Y. Wang, X. Xia, J.C. Zheng, Exosome engineering: Current progress in cargo loading and targeted delivery, *NanoImpact.* 20 (2020) 100261. <https://doi.org/10.1016/j.impact.2020.100261>.
- [154] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries, *CA. Cancer J. Clin.* 71 (2021) 209–249. <https://doi.org/10.3322/caac.21660>.
- [155] C. Pucci, C. Martinelli, G. Ciofani, Innovative approaches for cancer treatment: current perspectives and new challenges, *Ecancermedicalsecience.* 13 (2019).

- <https://doi.org/10.3332/ecancer.2019.961>.
- [156] A. Thakur, A. Roy, A. Ghosh, M. Chhabra, S. Banerjee, Abiraterone acetate in the treatment of prostate cancer, *Biomed. Pharmacother.* 101 (2018) 211–218. <https://doi.org/10.1016/j.biopha.2018.02.067>.
- [157] S.K. Sriraman, B. Aryasomayajula, V.P. Torchilin, Barriers to drug delivery in solid tumors, *Tissue Barriers.* 2 (2014) e29528. <https://doi.org/10.4161/tisb.29528>.
- [158] Y. Zhou, X. Chen, J. Cao, H. Gao, Overcoming the biological barriers in the tumor microenvironment for improving drug delivery and efficacy, *J. Mater. Chem. B.* 8 (2020) 6765–6781. <https://doi.org/10.1039/D0TB00649A>.
- [159] J.L. Chitty, E.C. Filipe, M.C. Lucas, D. Herrmann, T.R. Cox, P. Timpson, Recent advances in understanding the complexities of metastasis, *F1000Research.* 7 (2018) 1169. <https://doi.org/10.12688/f1000research.15064.2>.
- [160] E. Henke, R. Nandigama, S. Ergün, Extracellular Matrix in the Tumor Microenvironment and Its Impact on Cancer Therapy, *Front. Mol. Biosci.* 6 (2020). <https://doi.org/10.3389/fmolb.2019.00160>.
- [161] D. Hanahan, R.A. Weinberg, Hallmarks of Cancer: The Next Generation, *Cell.* 144 (2011) 646–674. <https://doi.org/10.1016/j.cell.2011.02.013>.
- [162] M.V. Gwangwa, A.M. Joubert, M.H. Visagie, Crosstalk between the Warburg effect, redox regulation and autophagy induction in tumourigenesis, *Cell. Mol. Biol. Lett.* 23 (2018) 20. <https://doi.org/10.1186/s11658-018-0088-y>.
- [163] G.-Y. Liou, P. Storz, Reactive oxygen species in cancer, *Free Radic. Res.* 44 (2010) 479–

496. <https://doi.org/10.3109/10715761003667554>.
- [164] C. Roma-Rodrigues, I. Pombo, L. Raposo, P. Pedrosa, A.R. Fernandes, P. V. Baptista, Nanotheranostics Targeting the Tumor Microenvironment, *Front. Bioeng. Biotechnol.* 7 (2019). <https://doi.org/10.3389/fbioe.2019.00197>.
- [165] A.E.M. Dirkx, M.G.A. oude Egbrink, K. Castermans, D.W.J. Schaft, V.L.J.L. Thijssen, R.P.M. Dings, L. Kwee, K.H. Mayo, J. Wagstaff, J.C.A.B. Steege, A.W. Griffioen, Anti-angiogenesis therapy can overcome endothelial cell anergy and promote leukocyte-endothelium interactions and infiltration in tumors, *FASEB J.* 20 (2006) 621–630. <https://doi.org/10.1096/fj.05-4493com>.
- [166] D. Klein, The Tumor Vascular Endothelium as Decision Maker in Cancer Therapy, *Front. Oncol.* 8 (2018). <https://doi.org/10.3389/fonc.2018.00367>.
- [167] Y. Wang, Q.-Y. He, R.W.-Y. Sun, C.-M. Che, J.-F. Chiu, Gold(III) Porphyrin 1a Induced Apoptosis by Mitochondrial Death Pathways Related to Reactive Oxygen Species, *Cancer Res.* 65 (2005) 11553–11564. <https://doi.org/10.1158/0008-5472.CAN-05-2867>.
- [168] S. Clichici, A. Filip, D. Daicoviciu, R. Ion, T. Mocan, C. Tatomir, L. Rogojan, D. Olteanu, A. Muresan, The dynamics of reactive oxygen species in photodynamic therapy with tetra sulfophenyl-porphyrin, *Acta Physiol. Hung.* 97 (2010) 41–51. <https://doi.org/10.1556/APhysiol.97.2010.1.5>.
- [169] J. Deng, H. Li, M. Yang, F. Wu, Palladium porphyrin complexes for photodynamic cancer therapy: effect of porphyrin units and metal, *Photochem. Photobiol. Sci.* 19 (2020) 905–912. <https://doi.org/10.1039/C9PP00363K>.

- [170] A.E. Nel, L. Mädler, D. Velegol, T. Xia, E.M. V. Hoek, P. Somasundaran, F. Klaessig, V. Castranova, M. Thompson, Understanding biophysicochemical interactions at the nano–bio interface, *Nat. Mater.* 8 (2009) 543–557. <https://doi.org/10.1038/nmat2442>.
- [171] A. Chow, B.D. Brown, M. Merad, Studying the mononuclear phagocyte system in the molecular age, *Nat. Rev. Immunol.* 11 (2011) 788–798. <https://doi.org/10.1038/nri3087>.
- [172] M. Rehm, S. Zahler, M. Lötsch, U. Welsch, P. Conzen, M. Jacob, B.F. Becker, Endothelial Glycocalyx as an Additional Barrier Determining Extravasation of 6% Hydroxyethyl Starch or 5% Albumin Solutions in the Coronary Vascular Bed, *Anesthesiology*. 100 (2004) 1211–1223. <https://doi.org/10.1097/00000542-200405000-00025>.
- [173] R.O. Dull, R. Dinavahi, L. Schwartz, D.E. Humphries, D. Berry, R. Sasisekharan, J.G.N. Garcia, Lung endothelial heparan sulfates mediate cationic peptide-induced barrier dysfunction: a new role for the glycocalyx, *Am. J. Physiol. Cell. Mol. Physiol.* 285 (2003) L986–L995. <https://doi.org/10.1152/ajplung.00022.2003>.
- [174] N. Kutuzov, H. Flyvbjerg, M. Lauritzen, Contributions of the glycocalyx, endothelium, and extravascular compartment to the blood–brain barrier, *Proc. Natl. Acad. Sci.* 115 (2018) E9429–E9438. <https://doi.org/10.1073/pnas.1802155115>.
- [175] S. Barua, S. Mitragotri, Challenges associated with penetration of nanoparticles across cell and tissue barriers: A review of current status and future prospects, *Nano Today*. 9 (2014) 223–243. <https://doi.org/10.1016/j.nantod.2014.04.008>.
- [176] J. Jia, Z. Wang, T. Yue, G. Su, C. Teng, B. Yan, Crossing Biological Barriers by Engineered Nanoparticles, *Chem. Res. Toxicol.* 33 (2020) 1055–1060. <https://doi.org/10.1021/acs.chemrestox.9b00483>.

- [177] M.A. Firer, G. Gellerman, Targeted drug delivery for cancer therapy: the other side of antibodies, *J. Hematol. Oncol.* 5 (2012) 70. <https://doi.org/10.1186/1756-8722-5-70>.
- [178] W. Jeong, J. Bu, L.J. Kubiawicz, S.S. Chen, Y. Kim, S. Hong, Peptide–nanoparticle conjugates: a next generation of diagnostic and therapeutic platforms?, *Nano Converg.* 5 (2018) 38. <https://doi.org/10.1186/s40580-018-0170-1>.
- [179] P. Baptista, A. Kanaras, A. Heuer-Jungemann, C. Roma-Rodrigues, A. Fernandes, Peptide-coated gold nanoparticles for modulation of angiogenesis in vivo, *Int. J. Nanomedicine.* (2016) 2633. <https://doi.org/10.2147/IJN.S108661>.
- [180] X. Li, L. Liu, Y. Fu, H. Chen, M.M.A. Abualrejal, H. Zhang, Z. Wang, H. Zhang, Peptide-enhanced tumor accumulation of upconversion nanoparticles for sensitive upconversion luminescence/magnetic resonance dual-mode bioimaging of colorectal tumors, *Acta Biomater.* 104 (2020) 167–175. <https://doi.org/10.1016/j.actbio.2020.01.003>.
- [181] D. Bartczak, A.G. Kanaras, Preparation of Peptide-Functionalized Gold Nanoparticles Using One Pot EDC/Sulfo-NHS Coupling, *Langmuir.* 27 (2011) 10119–10123. <https://doi.org/10.1021/la2022177>.
- [182] Y. Fu, X. Li, H. Chen, Z. Wang, W. Yang, H. Zhang, CXC Chemokine Receptor 4 Antagonist Functionalized Renal Clearable Manganese-Doped Iron Oxide Nanoparticles for Active-Tumor-Targeting Magnetic Resonance Imaging-Guided Bio-Photothermal Therapy, *ACS Appl. Bio Mater.* 2 (2019) 3613–3621. <https://doi.org/10.1021/acsabm.9b00475>.
- [183] W. Wang, C.F. Anderson, Z. Wang, W. Wu, H. Cui, C.-J. Liu, Peptide-templated noble metal catalysts: syntheses and applications, *Chem. Sci.* 8 (2017) 3310–3324.

<https://doi.org/10.1039/C7SC00069C>.

- [184] Y. Li, Z. Tang, P.N. Prasad, M.R. Knecht, M.T. Swihart, Peptide-mediated synthesis of gold nanoparticles: effects of peptide sequence and nature of binding on physicochemical properties, *Nanoscale*. 6 (2014) 3165–3172. <https://doi.org/10.1039/C3NR06201E>.
- [185] Ž. Krpetić, P. Nativio, F. Porta, M. Brust, A Multidentate Peptide for Stabilization and Facile Bioconjugation of Gold Nanoparticles, *Bioconjug. Chem.* 20 (2009) 619–624. <https://doi.org/10.1021/bc8003028>.
- [186] R. Lévy, N.T.K. Thanh, R.C. Doty, I. Hussain, R.J. Nichols, D.J. Schiffrin, M. Brust, D.G. Fernig, Rational and Combinatorial Design of Peptide Capping Ligands for Gold Nanoparticles, *J. Am. Chem. Soc.* 126 (2004) 10076–10084. <https://doi.org/10.1021/ja0487269>.
- [187] B. Hu, F. Kong, X. Gao, L. Jiang, X. Li, W. Gao, K. Xu, B. Tang, Avoiding Thiol Compound Interference: A Nanoplatform Based on High-Fidelity Au-Se Bonds for Biological Applications, *Angew. Chemie Int. Ed.* 57 (2018) 5306–5309. <https://doi.org/10.1002/anie.201712921>.
- [188] W. Pan, X. Liu, X. Wan, J. Li, Y. Li, F. Lu, N. Li, B. Tang, Rapid Preparation of Au–Se–Peptide Nanoprobe Based on a Freezing Method for Bioimaging, *Anal. Chem.* 91 (2019) 15982–15987. <https://doi.org/10.1021/acs.analchem.9b04616>.
- [189] M. Luan, M. Shi, W. Pan, N. Li, B. Tang, A gold–selenium-bonded nanoprobe for real-time in situ imaging of the upstream and downstream relationship between uPA and MMP-9 in cancer cells, *Chem. Commun.* 55 (2019) 5817–5820. <https://doi.org/10.1039/C9CC01454C>.

- [190] S. Si, T.K. Mandal, Tryptophan-Based Peptides to Synthesize Gold and Silver Nanoparticles: A Mechanistic and Kinetic Study, *Chem. - A Eur. J.* 13 (2007) 3160–3168. <https://doi.org/10.1002/chem.200601492>.
- [191] G. Upert, F. Bouillère, H. Wennemers, Oligoprolines as Scaffolds for the Formation of Silver Nanoparticles in Defined Sizes: Correlating Molecular and Nanoscopic Dimensions, *Angew. Chemie Int. Ed.* 51 (2012) 4231–4234. <https://doi.org/10.1002/anie.201107183>.
- [192] S. Corra, U. Lewandowska, E.M. Benetti, H. Wennemers, Size-Controlled Formation of Noble-Metal Nanoparticles in Aqueous Solution with a Thiol-Free Tripeptide, *Angew. Chemie Int. Ed.* 55 (2016) 8542–8545. <https://doi.org/10.1002/anie.201510337>.
- [193] P. Graf, A. Manton, A. Foelske, A. Shkilnyy, A. Mašić, A.F. Thünemann, A. Taubert, Peptide-Coated Silver Nanoparticles: Synthesis, Surface Chemistry, and pH-Triggered, Reversible Assembly into Particle Assemblies, *Chem. - A Eur. J.* 15 (2009) 5831–5844. <https://doi.org/10.1002/chem.200802329>.
- [194] S. Papst, M.A. Brimble, R.D. Tilley, D.E. Williams, One-Pot Synthesis of Functionalized Noble Metal Nanoparticles Using a Rationally Designed Phosphopeptide, *Part. Part. Syst. Charact.* 31 (2014) 971–975. <https://doi.org/10.1002/ppsc.201300381>.
- [195] A. Henninot, J.C. Collins, J.M. Nuss, The Current State of Peptide Drug Discovery: Back to the Future?, *J. Med. Chem.* 61 (2018) 1382–1414. <https://doi.org/10.1021/acs.jmedchem.7b00318>.
- [196] J.L. Lau, M.K. Dunn, Therapeutic peptides: Historical perspectives, current development trends, and future directions, *Bioorg. Med. Chem.* 26 (2018) 2700–2707. <https://doi.org/10.1016/j.bmc.2017.06.052>.

- [197] M.T. Weinstock, J.N. Francis, J.S. Redman, M.S. Kay, Protease-resistant peptide design-empowering nature's fragile warriors against HIV, *Biopolymers*. 98 (2012) 431–442. <https://doi.org/10.1002/bip.22073>.
- [198] J. Talmadge, Pharmacodynamic aspects of peptide administration biological response modifiers, *Adv. Drug Deliv. Rev.* 33 (1998) 241–252. [https://doi.org/10.1016/S0169-409X\(98\)00032-5](https://doi.org/10.1016/S0169-409X(98)00032-5).
- [199] J.Y. Shu, B. Panganiban, T. Xu, Peptide-Polymer Conjugates: From Fundamental Science to Application, *Annu. Rev. Phys. Chem.* 64 (2013) 631–657. <https://doi.org/10.1146/annurev-physchem-040412-110108>.
- [200] Y. Xiao, L.A. Reis, N. Feric, E.J. Knee, J. Gu, S. Cao, C. Laschinger, C. Londono, J. Antolovich, A.P. McGuigan, M. Radisic, Diabetic wound regeneration using peptide-modified hydrogels to target re-epithelialization, *Proc. Natl. Acad. Sci.* 113 (2016) E5792–E5801. <https://doi.org/10.1073/pnas.1612277113>.
- [201] J. Du, L.A. Lane, S. Nie, Stimuli-responsive nanoparticles for targeting the tumor microenvironment, *J. Control. Release.* 219 (2015) 205–214. <https://doi.org/10.1016/j.jconrel.2015.08.050>.
- [202] Y. Tang, X. Wang, J. Li, Y. Nie, G. Liao, Y. Yu, C. Li, Overcoming the Reticuloendothelial System Barrier to Drug Delivery with a “Don't-Eat-Us” Strategy, *ACS Nano*. 13 (2019) 13015–13026. <https://doi.org/10.1021/acsnano.9b05679>.
- [203] J. V. Georgieva, R.P. Brinkhuis, K. Stojanov, C.A.G.M. Weijers, H. Zuilhof, F.P.J.T. Rutjes, D. Hoekstra, J.C.M. van Hest, I.S. Zuhorn, Peptide-Mediated Blood-Brain Barrier Transport of Polymersomes, *Angew. Chemie Int. Ed.* 51 (2012) 8339–8342.

<https://doi.org/10.1002/anie.201202001>.

- [204] H. Yao, K. Wang, Y. Wang, S. Wang, J. Li, J. Lou, L. Ye, X. Yan, W. Lu, R. Huang, Enhanced blood–brain barrier penetration and glioma therapy mediated by a new peptide modified gene delivery system, *Biomaterials*. 37 (2015) 345–352. <https://doi.org/10.1016/j.biomaterials.2014.10.034>.
- [205] P. Ma, R.J. Mumper, Anthracycline nano-delivery systems to overcome multiple drug resistance: A comprehensive review, *Nano Today*. 8 (2013) 313–331. <https://doi.org/10.1016/j.nantod.2013.04.006>.
- [206] V. Guillemard, H. Uri Saragovi, Prodrug chemotherapeutics bypass p-glycoprotein resistance and kill tumors in vivo with high efficacy and target-dependent selectivity, *Oncogene*. 23 (2004) 3613–3621. <https://doi.org/10.1038/sj.onc.1207463>.
- [207] D.A. Modi, S. Sunoqrot, J. Bugno, D.D. Lantvit, S. Hong, J.E. Burdette, Targeting of follicle stimulating hormone peptide-conjugated dendrimers to ovarian cancer cells, *Nanoscale*. 6 (2014) 2812–2820. <https://doi.org/10.1039/C3NR05042D>.
- [208] X. Jiang, J. Bugno, C. Hu, Y. Yang, T. Herold, J. Qi, P. Chen, S. Gurbuxani, S. Arnovitz, B. Ulrich, H. Weng, Y. Wang, H. Huang, S. Li, J. Strong, M.B. Neilly, R.A. Larson, M.M. Le Beau, S.K. Bohlander, J. Jin, Z. Li, J.E. Bradner, S. Hong, J. Chen, Targeted Treatment of FLT3 -Overexpressing Acute Myeloid Leukemia with MiR-150 Nanoparticles Guided By Conjugated FLT3 Ligand Peptides, *Blood*. 126 (2015) 3784–3784. <https://doi.org/10.1182/blood.V126.23.3784.3784>.
- [209] C.-C. Chang, D.-Z. Liu, S.-Y. Lin, H.-J. Liang, W.-C. Hou, W.-J. Huang, C.-H. Chang, F.-M. Ho, Y.-C. Liang, Liposome encapsulation reduces cantharidin toxicity, *Food Chem.*

- Toxicol. 46 (2008) 3116–3121. <https://doi.org/10.1016/j.fct.2008.06.084>.
- [210] W. Dai, T. Yang, X. Wang, J. Wang, X. Zhang, Q. Zhang, PHSCNK-Modified and doxorubicin-loaded liposomes as a dual targeting system to integrin-overexpressing tumor neovasculature and tumor cells, *J. Drug Target.* 18 (2010) 254–263. <https://doi.org/10.3109/10611860903353354>.
- [211] X. Liu, L. Ruan, W. Mao, J.-Q. Wang, Y. Shen, M. Sui, Preparation of RGD-modified Long Circulating Liposome Loading Matrine, and its in vitro Anti-cancer Effects, *Int. J. Med. Sci.* (2010) 197–208. <https://doi.org/10.7150/ijms.7.197>.
- [212] A. Bandekar, C. Zhu, A. Gomez, M.Z. Menzenski, M. Sempkowski, S. Sofou, Masking and Triggered Unmasking of Targeting Ligands on Liposomal Chemotherapy Selectively Suppress Tumor Growth in Vivo, *Mol. Pharm.* 10 (2013) 152–160. <https://doi.org/10.1021/mp3002717>.
- [213] M. Zahmatkeshan, F. Gheybi, S.M. Rezayat, M.R. Jaafari, Improved drug delivery and therapeutic efficacy of PEGylated liposomal doxorubicin by targeting anti-HER2 peptide in murine breast tumor model, *Eur. J. Pharm. Sci.* 86 (2016) 125–135. <https://doi.org/10.1016/j.ejps.2016.03.009>.
- [214] Y. Chen, J.J. Wu, L. Huang, Nanoparticles Targeted With NGR Motif Deliver c-myc siRNA and Doxorubicin for Anticancer Therapy, *Mol. Ther.* 18 (2010) 828–834. <https://doi.org/10.1038/mt.2009.291>.
- [215] J. Hou, Y. Diao, W. Li, Z. Yang, L. Zhang, Z. Chen, Y. Wu, RGD peptide conjugation results in enhanced antitumor activity of PD0325901 against glioblastoma by both tumor-targeting delivery and combination therapy, *Int. J. Pharm.* 505 (2016) 329–340.

<https://doi.org/10.1016/j.ijpharm.2016.04.017>.

- [216] W. Cai, K. Chen, Z.-B. Li, S.S. Gambhir, X. Chen, Dual-Function Probe for PET and Near-Infrared Fluorescence Imaging of Tumor Vasculature, *J. Nucl. Med.* 48 (2007) 1862–1870. <https://doi.org/10.2967/jnumed.107.043216>.
- [217] Y. Qin, H. Chen, Q. Zhang, X. Wang, W. Yuan, R. Kuai, J. Tang, L. Zhang, Z. Zhang, Q. Zhang, J. Liu, Q. He, Liposome formulated with TAT-modified cholesterol for improving brain delivery and therapeutic efficacy on brain glioma in animals, *Int. J. Pharm.* 420 (2011) 304–312. <https://doi.org/10.1016/j.ijpharm.2011.09.008>.
- [218] N. Huang, S. Cheng, X. Zhang, Q. Tian, J. Pi, J. Tang, Q. Huang, F. Wang, J. Chen, Z. Xie, Z. Xu, W. Chen, H. Zheng, Y. Cheng, Efficacy of NGR peptide-modified PEGylated quantum dots for crossing the blood–brain barrier and targeted fluorescence imaging of glioma and tumor vasculature, *Nanomedicine Nanotechnology, Biol. Med.* 13 (2017) 83–93. <https://doi.org/10.1016/j.nano.2016.08.029>.
- [219] J. Zhang, W. Jin, X. Wang, J. Wang, X. Zhang, Q. Zhang, A Novel Octreotide Modified Lipid Vesicle Improved the Anticancer Efficacy of Doxorubicin in Somatostatin Receptor 2 Positive Tumor Models, *Mol. Pharm.* 7 (2010) 1159–1168. <https://doi.org/10.1021/mp1000235>.
- [220] R.-H. Wang, H.-M. Cao, Z.-J. Tian, B. Jin, Q. Wang, H. Ma, J. Wu, Efficacy of dual-functional liposomes containing paclitaxel for treatment of lung cancer, *Oncol. Rep.* 38 (2017) 3285–3285. <https://doi.org/10.3892/or.2017.6006>.
- [221] M. Saad, O.B. Garbuzenko, E. Ber, P. Chandna, J.J. Khandare, V.P. Pozharov, T. Minko, Receptor targeted polymers, dendrimers, liposomes: Which nanocarrier is the most efficient

- for tumor-specific treatment and imaging?, *J. Control. Release.* 130 (2008) 107–114.
<https://doi.org/10.1016/j.jconrel.2008.05.024>.
- [222] X.-B. Xiong, Y. Huang, W.-L. LU, X. Zhang, H. Zhang, T. Nagai, Q. Zhang, Intracellular delivery of doxorubicin with RGD-modified sterically stabilized liposomes for an improved antitumor efficacy: In vitro and in vivo, *J. Pharm. Sci.* 94 (2005) 1782–1793.
<https://doi.org/10.1002/jps.20397>.
- [223] H. Wu, L. Yao, J. Mei, F. Li, Development of synthetic of peptide-functionalized liposome for enhanced targeted ovarian carcinoma therapy., *Int. J. Clin. Exp. Pathol.* 8 (2015) 207–16.
- [224] Y.-Z. Du, Jie, Cai, Wang, Ying, Yu, M. Zhang, Actively-targeted LTVSPWY peptide-modified magnetic nanoparticles for tumor imaging, *Int. J. Nanomedicine.* (2012) 3981.
<https://doi.org/10.2147/IJN.S33593>.
- [225] Y. He, X. Yuan, P. Lei, S. Wu, W. Xing, X. Lan, H. Zhu, T. Huang, G. Wang, R. An, Y. Zhang, G. Shen, The antiproliferative effects of somatostatin receptor subtype 2 in breast cancer cells, *Acta Pharmacol. Sin.* 30 (2009) 1053–1059.
<https://doi.org/10.1038/aps.2009.59>.
- [226] C. Susini, L. Buscail, Rationale for the use of somatostatin analogs as antitumor agents, *Ann. Oncol.* 17 (2006) 1733–1742. <https://doi.org/10.1093/annonc/mdl105>.
- [227] Y.C. Patel, Somatostatin and Its Receptor Family, *Front. Neuroendocrinol.* 20 (1999) 157–198. <https://doi.org/10.1006/frne.1999.0183>.
- [228] T. Florio, G. Schettini, Multiple intracellular effectors modulate physiological functions of

- the cloned somatostatin receptors, *J. Mol. Endocrinol.* 17 (1996) 89–100. <https://doi.org/10.1677/jme.0.0170089>.
- [229] I.M. Modlin, S.F. Moss, K. Oberg, R. Padbury, R.J. Hicks, B.I. Gustafsson, N.A. Wright, M. Kidd, Gastrointestinal neuroendocrine (carcinoid) tumours: current diagnosis and management, *Med. J. Aust.* 193 (2010) 46–52. <https://doi.org/10.5694/j.1326-5377.2010.tb03742.x>.
- [230] D.J. Kwekkeboom, B.L. Kam, M. van Essen, J.J.M. Teunissen, C.H.J. van Eijck, R. Valkema, M. de Jong, W.W. de Herder, E.P. Krenning, Somatostatin receptor-based imaging and therapy of gastroenteropancreatic neuroendocrine tumors, *Endocr. Relat. Cancer.* 17 (2010) R53–R73. <https://doi.org/10.1677/ERC-09-0078>.
- [231] D.S. Abou, D.L.J. Thorek, N.N. Ramos, M.W.H. Pinkse, H.T. Wolterbeek, S.D. Carlin, B.J. Beattie, J.S. Lewis, ⁸⁹Zr-Labeled Paramagnetic Octreotide-Liposomes for PET-MR Imaging of Cancer, *Pharm. Res.* 30 (2013) 878–888. <https://doi.org/10.1007/s11095-012-0929-8>.
- [232] C. Su, J. Li, L. Zhang, H. Wang, F. Wang, Y. Tao, Y. Wang, Q. Guo, J. Li, Y. Liu, Y. Yan, J. Zhang, The Biological Functions and Clinical Applications of Integrins in Cancers, *Front. Pharmacol.* 11 (2020). <https://doi.org/10.3389/fphar.2020.579068>.
- [233] G. SERINI, D. VALDEMBRI, F. BUSSOLINO, Integrins and angiogenesis: A sticky business, *Exp. Cell Res.* 312 (2006) 651–658. <https://doi.org/10.1016/j.yexcr.2005.10.020>.
- [234] J. Zhao, F. Santino, D. Giacomini, L. Gentilucci, Integrin-Targeting Peptides for the Design of Functional Cell-Responsive Biomaterials, *Biomedicines.* 8 (2020) 307. <https://doi.org/10.3390/biomedicines8090307>.

- [235] F. Danhier, A. Le Breton, V. Préat, RGD-Based Strategies To Target Alpha(v) Beta(3) Integrin in Cancer Therapy and Diagnosis, *Mol. Pharm.* 9 (2012) 2961–2973. <https://doi.org/10.1021/mp3002733>.
- [236] D. Arosio, C. Casagrande, L. Manzoni, Integrin-Mediated Drug Delivery in Cancer and Cardiovascular Diseases with Peptide-Functionalized Nanoparticles, *Curr. Med. Chem.* 19 (2012) 3128–3151. <https://doi.org/10.2174/092986712800784748>.
- [237] K. Chen, X. Chen, Integrin Targeted Delivery of Chemotherapeutics, *Theranostics*. 1 (2011) 189–200. <https://doi.org/10.7150/thno/v01p0189>.
- [238] P. Holig, M. Bach, T. Volkel, T. Nahde, S. Hoffmann, R. Muller, R.E. Kontermann, Novel RGD lipopeptides for the targeting of liposomes to integrin-expressing endothelial and melanoma cells, *Protein Eng. Des. Sel.* 17 (2004) 433–441. <https://doi.org/10.1093/protein/gzh055>.
- [239] S.J. Bogdanowich-Knipp, S. Chakrabarti, T.J. Siahaan, T.D. Williams, R.K. Dillman, Solution stability of linear vs. cyclic RGD peptides, *J. Pept. Res.* 53 (1999) 530–541. <https://doi.org/10.1034/j.1399-3011.1999.00052.x>.
- [240] R. Haubner, W.A. Weber, A.J. Beer, E. Vabuliene, D. Reim, M. Sarbia, K.-F. Becker, M. Goebel, R. Hein, H.-J. Wester, H. Kessler, M. Schwaiger, Noninvasive Visualization of the Activated $\alpha v \beta 3$ Integrin in Cancer Patients by Positron Emission Tomography and [18F]Galacto-RGD, *PLoS Med.* 2 (2005) e70. <https://doi.org/10.1371/journal.pmed.0020070>.
- [241] Y.-C. Wei, Y. Gao, J. Zhang, Z. Fu, J. Zheng, N. Liu, X. Hu, W. Hou, J. Yu, S. Yuan, Stereotactic Comparison Study of 18F-Alfatide and 18F-FDG PET Imaging in an LLC

- Tumor-Bearing C57BL/6 Mouse Model, *Sci. Rep.* 6 (2016) 28757.
<https://doi.org/10.1038/srep28757>.
- [242] J. Ren, S. Xu, D. Guo, J. Zhang, S. Liu, Increased expression of $\alpha 5\beta 1$ -integrin is a prognostic marker for patients with gastric cancer, *Clin. Transl. Oncol.* 16 (2014) 668–674.
<https://doi.org/10.1007/s12094-013-1133-y>.
- [243] L.M. Neckers, J.B. Trepel, Transferrin Receptor Expression and the Control of Cell Growth, *Cancer Invest.* 4 (1986) 461–470. <https://doi.org/10.3109/07357908609017524>.
- [244] T.R. Daniels, E. Bernabeu, J.A. Rodríguez, S. Patel, M. Kozman, D.A. Chiappetta, E. Holler, J.Y. Ljubimova, G. Helguera, M.L. Penichet, The transferrin receptor and the targeted delivery of therapeutic agents against cancer, *Biochim. Biophys. Acta - Gen. Subj.* 1820 (2012) 291–317. <https://doi.org/10.1016/j.bbagen.2011.07.016>.
- [245] R. Prior, G. Reifenberger, W. Wechsler, Transferrin receptor expression in tumours of the human nervous system: relation to tumour type, grading and tumour growth fraction, *Virchows Arch. A Pathol. Anat. Histopathol.* 416 (1990) 491–496.
<https://doi.org/10.1007/BF01600299>.
- [246] G.J. Seymour, M.D. Walsh, M.F. Lavin, G. Strutton, R.A. Gardiner, Transferrin receptor expression by human bladder transitional cell carcinomas, *Urol. Res.* 15 (1987).
<https://doi.org/10.1007/BF00265663>.
- [247] C. Rousselle, P. Clair, J.-M. Lefauconnier, M. Kaczorek, J.-M. Scherrmann, J. Temsamani, New Advances in the Transport of Doxorubicin through the Blood-Brain Barrier by a Peptide Vector-Mediated Strategy, *Mol. Pharmacol.* 57 (2000) 679–686.
<https://doi.org/10.1124/mol.57.4.679>.

- [248] J.-Q. Gao, Q. Lv, L.-M. Li, X.-J. Tang, F.-Z. Li, Y.-L. Hu, M. Han, Glioma targeting and blood–brain barrier penetration by dual-targeting doxorubicin liposomes, *Biomaterials*. 34 (2013) 5628–5639. <https://doi.org/10.1016/j.biomaterials.2013.03.097>.
- [249] J.H. Lee, J.A. Engler, J.F. Collawn, B.A. Moore, Receptor mediated uptake of peptides that bind the human transferrin receptor, *Eur. J. Biochem.* 268 (2001) 2004–2012. <https://doi.org/10.1046/j.1432-1327.2001.02073.x>.
- [250] R. Zhang, G. Feng, C.-J. Zhang, X. Cai, X. Cheng, B. Liu, Real-Time Specific Light-Up Sensing of Transferrin Receptor: Image-Guided Photodynamic Ablation of Cancer Cells through Controlled Cytochrome Disintegration, *Anal. Chem.* 88 (2016) 4841–4848. <https://doi.org/10.1021/acs.analchem.6b00524>.
- [251] K. Wang, Y. Zhang, J. Wang, A. Yuan, M. Sun, J. Wu, Y. Hu, Self-assembled IR780-loaded transferrin nanoparticles as an imaging, targeting and PDT/PTT agent for cancer therapy, *Sci. Rep.* 6 (2016) 27421. <https://doi.org/10.1038/srep27421>.
- [252] R. Huang, W. Ke, Y. Qu, J. Zhu, Y. Pei, C. Jiang, Characterization of lactoferrin receptor in brain endothelial capillary cells and mouse brain, *J. Biomed. Sci.* 14 (2007) 121–128. <https://doi.org/10.1007/s11373-006-9121-7>.
- [253] J. Lalani, Y. Raichandani, R. Mathur, M. Lalan, K. Chutani, A.K. Mishra, A. Misra, Comparative Receptor Based Brain Delivery of Tramadol-Loaded Poly(lactic-co-glycolic acid) Nanoparticles, *J. Biomed. Nanotechnol.* 8 (2012) 918–927. <https://doi.org/10.1166/jbn.2012.1462>.
- [254] J. Lalani, M. Rathi, M. Lalan, A. Misra, Protein functionalized tramadol-loaded PLGA nanoparticles: preparation, optimization, stability and pharmacodynamic studies, *Drug Dev.*


- Ind. Pharm. 39 (2013) 854–864. <https://doi.org/10.3109/03639045.2012.684390>.
- [255] D. Miao, M. Jiang, Z. Liu, G. Gu, Q. Hu, T. Kang, Q. Song, L. Yao, W. Li, X. Gao, M. Sun, J. Chen, Co-administration of Dual-Targeting Nanoparticles with Penetration Enhancement Peptide for Antiglioblastoma Therapy, *Mol. Pharm.* 11 (2014) 90–101. <https://doi.org/10.1021/mp400189j>.
- [256] W. Tai, R. Mahato, K. Cheng, The role of HER2 in cancer therapy and targeted drug delivery, *J. Control. Release.* 146 (2010) 264–275. <https://doi.org/10.1016/j.jconrel.2010.04.009>.
- [257] Y. Yarden, Biology of HER2 and Its Importance in Breast Cancer, *Oncology.* 61 (2001) 1–13. <https://doi.org/10.1159/000055396>.
- [258] Z.-L. Zhu, J. Zhang, M.-L. Chen, K. Li, Efficacy and Safety of Trastuzumab Added to Standard Treatments for HER2-positive Metastatic Breast Cancer Patients, *Asian Pacific J. Cancer Prev.* 14 (2013) 7111–7116. <https://doi.org/10.7314/APJCP.2013.14.12.7111>.
- [259] Y.-J. Bang, E. Van Cutsem, A. Feyereislova, H.C. Chung, L. Shen, A. Sawaki, F. Lordick, A. Ohtsu, Y. Omuro, T. Satoh, G. Aprile, E. Kulikov, J. Hill, M. Lehle, J. Rüschhoff, Y.-K. Kang, Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial, *Lancet.* 376 (2010) 687–697. [https://doi.org/10.1016/S0140-6736\(10\)61121-X](https://doi.org/10.1016/S0140-6736(10)61121-X).
- [260] N.G. Karasseva, V. V Glinsky, N.X. Chen, R. Komatireddy, T.P. Quinn, Identification and characterization of peptides that bind human ErbB-2 selected from a bacteriophage display library., *J. Protein Chem.* 21 (2002) 287–96. <https://doi.org/10.1023/a:1019749504418>.

- [261] B.-W. Park, H.-T. Zhang, C. Wu, A. Berezov, X. Zhang, R. Dua, Q. Wang, G. Kao, D.M. O'Rourke, M.I. Greene, R. Murali, Rationally designed anti-HER2/neu peptide mimetic disables P185HER2/neu tyrosine kinases in vitro and in vivo, *Nat. Biotechnol.* 18 (2000) 194–198. <https://doi.org/10.1038/72651>.
- [262] H. Lei, P. Cao, G. Miao, Z. Lin, Z. Diao, Expression and Functional Characterization of Tumor-Targeted Fusion Protein Composed of NGR Peptide and 15-kDa Actin Fragment, *Appl. Biochem. Biotechnol.* 162 (2010) 988–995. <https://doi.org/10.1007/s12010-009-8901-8>.
- [263] S.S. Dharap, Y. Wang, P. Chandna, J.J. Khandare, B. Qiu, S. Gunaseelan, P.J. Sinko, S. Stein, A. Farmanfarmanian, T. Minko, Tumor-specific targeting of an anticancer drug delivery system by LHRH peptide, *Proc. Natl. Acad. Sci.* 102 (2005) 12962–12967. <https://doi.org/10.1073/pnas.0504274102>.
- [264] J.J. Khandare, P. Chandna, Y. Wang, V.P. Pozharov, T. Minko, Novel Polymeric Prodrug with Multivalent Components for Cancer Therapy, *J. Pharmacol. Exp. Ther.* 317 (2006) 929–937. <https://doi.org/10.1124/jpet.105.098855>.
- [265] S. Bajusz, T. Janaky, V.J. Csernus, L. Bokser, M. Fekete, G. Srkalovic, T.W. Redding, A. V. Schally, Highly potent metallopeptide analogues of luteinizing hormone-releasing hormone., *Proc. Natl. Acad. Sci.* 86 (1989) 6313–6317. <https://doi.org/10.1073/pnas.86.16.6313>.
- [266] S. Bajusz, T. Janaky, V.J. Csernus, L. Bokser, M. Fekete, G. Srkalovic, T.W. Redding, A. V. Schally, Highly potent analogues of luteinizing hormone-releasing hormone containing D-phenylalanine nitrogen mustard in position 6., *Proc. Natl. Acad. Sci.* 86 (1989) 6318–

6322. <https://doi.org/10.1073/pnas.86.16.6318>.
- [267] K. Fodor, N. Dobos, A. Schally, Z. Steiber, G. Olah, E. Sipos, L. Szekvolgyi, G. Halmos, The targeted LHRH analog AEZS-108 alters expression of genes related to angiogenesis and development of metastasis in uveal melanoma, *Oncotarget*. 11 (2020) 175–187. <https://doi.org/10.18632/oncotarget.27431>.
- [268] S. V. Liu, D.D. Tsao-Wei, S. Xiong, S. Groshen, T.B. Dorff, D.I. Quinn, Y.-C. Tai, J. Engel, D. Hawes, A. V. Schally, J.K. Pinski, Phase I, Dose-Escalation Study of the Targeted Cytotoxic LHRH Analog AEZS-108 in Patients with Castration- and Taxane-Resistant Prostate Cancer, *Clin. Cancer Res.* 20 (2014) 6277–6283. <https://doi.org/10.1158/1078-0432.CCR-14-0489>.
- [269] M. Li, Z. Tang, Y. Zhang, S. Lv, Q. Li, X. Chen, Targeted delivery of cisplatin by LHRH-peptide conjugated dextran nanoparticles suppresses breast cancer growth and metastasis, *Acta Biomater.* 18 (2015) 132–143. <https://doi.org/10.1016/j.actbio.2015.02.022>.
- [270] H. Zhang, A. Berezov, Q. Wang, G. Zhang, J. Drebin, R. Murali, M.I. Greene, ErbB receptors: from oncogenes to targeted cancer therapies, *J. Clin. Invest.* 117 (2007) 2051–2058. <https://doi.org/10.1172/JCI32278>.
- [271] H. Hossein-Nejad-Ariani, E. Althagafi, K. Kaur, Small Peptide Ligands for Targeting EGFR in Triple Negative Breast Cancer Cells, *Sci. Rep.* 9 (2019) 2723. <https://doi.org/10.1038/s41598-019-38574-y>.
- [272] X. Huang, L. Chen, Y. Zhang, S. Zhou, H.-H. Cai, T. Li, H. Jin, J. Cai, H. Zhou, J. Pi, GE11 Peptide Conjugated Liposomes for EGFR-Targeted and Chemophotothermal Combined Anticancer Therapy, *Bioinorg. Chem. Appl.* 2021 (2021) 1–15.

<https://doi.org/10.1155/2021/5534870>.

- [273] W.-S. Pan, C. Han, L. Yue, L. Tai, Li Zhou, X. Li, G. Xing, X. Yang, Sun, A novel small peptide as an epidermal growth factor receptor targeting ligand for nanodelivery in vitro, *Int. J. Nanomedicine*. (2013) 1541. <https://doi.org/10.2147/IJN.S43627>.
- [274] J. Mayr, S. Hager, B. Koblmüller, M.H.M. Klose, K. Holste, B. Fischer, K. Pelivan, W. Berger, P. Heffeter, C.R. Kowol, B.K. Keppler, EGFR-targeting peptide-coupled platinum(IV) complexes, *JBIC J. Biol. Inorg. Chem.* 22 (2017) 591–603. <https://doi.org/10.1007/s00775-017-1450-7>.
- [275] P.T. Went, A. Lugli, S. Meier, M. Bendi, M. Mirlacher, G. Sauter, S. Dirnhofer, Frequent EpCam protein expression in human carcinomas, *Hum. Pathol.* 35 (2004) 122–128. <https://doi.org/10.1016/j.humpath.2003.08.026>.
- [276] X. Ma, X. Kang, L. He, J. Zhou, J. Zhou, M.B. Sturm, D.G. Beer, R. Kuick, D.J. Nancarrow, H.D. Appelman, Z. Pang, W. Li, C. Zhang, W. Zhang, Y. Zhang, T.D. Wang, M. Li, Identification of Tumor Specific Peptide as EpCAM Ligand and Its Potential Diagnostic and Therapeutic Clinical Application, *Mol. Pharm.* 16 (2019) 2199–2213. <https://doi.org/10.1021/acs.molpharmaceut.9b00185>.
- [277] P.M. Glumac, A.M. LeBeau, The role of CD133 in cancer: a concise review, *Clin. Transl. Med.* 7 (2018). <https://doi.org/10.1186/s40169-018-0198-1>.
- [278] S. Yan, D. Tang, Z. Hong, J. Wang, H. Yao, L. Lu, H. Yi, S. Fu, C. Zheng, G. He, H. Zou, X. Hou, Q. He, L. Xiong, Q. Li, X. Deng, CD133 peptide-conjugated pyropheophorbide-a as a novel photosensitizer for targeted photodynamic therapy in colorectal cancer stem cells, *Biomater. Sci.* 9 (2021) 2020–2031. <https://doi.org/10.1039/D0BM01874K>.

- [279] C. Bechara, S. Sagan, Cell-penetrating peptides: 20 years later, where do we stand?, *FEBS Lett.* 587 (2013) 1693–1702. <https://doi.org/10.1016/j.febslet.2013.04.031>.
- [280] H. Kim, M. Kitamatsu, T. Ohtsuki, Enhanced intracellular peptide delivery by multivalent cell-penetrating peptide with bio-reducible linkage, *Bioorg. Med. Chem. Lett.* 28 (2018) 378–381. <https://doi.org/10.1016/j.bmcl.2017.12.035>.
- [281] Y.-W. Huang, H.-J. Lee, Cell-penetrating peptides for medical theranostics and targeted drug delivery, in: *Pept. Appl. Biomed. Biotechnol. Bioeng.*, Elsevier, 2018: pp. 359–370. <https://doi.org/10.1016/B978-0-08-100736-5.00013-2>.
- [282] F. Milletti, Cell-penetrating peptides: classes, origin, and current landscape, *Drug Discov. Today.* 17 (2012) 850–860. <https://doi.org/10.1016/j.drudis.2012.03.002>.
- [283] G. Guidotti, L. Brambilla, D. Rossi, Cell-Penetrating Peptides: From Basic Research to Clinics, *Trends Pharmacol. Sci.* 38 (2017) 406–424. <https://doi.org/10.1016/j.tips.2017.01.003>.
- [284] P. Lundberg,  L. Langel, A brief introduction to cell-penetrating peptides, *J. Mol. Recognit.* 16 (2003) 227–233. <https://doi.org/10.1002/jmr.630>.
- [285] K. Padari, K. Koppel, A. Lorents, M. Hällbrink, M. Mano, M.C. Pedrosa de Lima, M. Pooga, S4 13 -PV Cell-Penetrating Peptide Forms Nanoparticle-Like Structures to Gain Entry Into Cells, *Bioconjug. Chem.* 21 (2010) 774–783. <https://doi.org/10.1021/bc900577e>.
- [286] F. Duchardt, I.R. Ruttekolk, W.P.R. Verdurmen, H. Lortat-Jacob, J. Bürck, H. Hufnagel, R. Fischer, M. van den Heuvel, D.W.P.M. Löwik, G.W. Vuister, A. Ulrich, M. de Waard, R. Brock, A Cell-penetrating Peptide Derived from Human Lactoferrin with Conformation-

- dependent Uptake Efficiency, *J. Biol. Chem.* 284 (2009) 36099–36108. <https://doi.org/10.1074/jbc.M109.036426>.
- [287] E. Bellet-Amalric, D. Blaudez, B. Desbat, F. Graner, F. Gauthier, A. Renault, Interaction of the third helix of Antennapedia homeodomain and a phospholipid monolayer, studied by ellipsometry and PM-IRRAS at the air–water interface, *Biochim. Biophys. Acta - Biomembr.* 1467 (2000) 131–143. [https://doi.org/10.1016/S0005-2736\(00\)00218-2](https://doi.org/10.1016/S0005-2736(00)00218-2).
- [288] A. Ziegler, X. Li Blatter, A. Seelig, J. Seelig, Protein Transduction Domains of HIV-1 and SIV TAT Interact with Charged Lipid Vesicles. Binding Mechanism and Thermodynamic Analysis †, *Biochemistry.* 42 (2003) 9185–9194. <https://doi.org/10.1021/bi0346805>.
- [289] M.-T. Lee, W.-C. Hung, F.-Y. Chen, H.W. Huang, Many-Body Effect of Antimicrobial Peptides: On the Correlation Between Lipid's Spontaneous Curvature and Pore Formation, *Biophys. J.* 89 (2005) 4006–4016. <https://doi.org/10.1529/biophysj.105.068080>.
- [290] Y. Pouny, D. Rapaport, A. Mor, P. Nicolas, Y. Shai, Interaction of antimicrobial dermaseptin and its fluorescently labeled analogs with phospholipid membranes, *Biochemistry.* 31 (1992) 12416–12423. <https://doi.org/10.1021/bi00164a017>.
- [291] G. Ter-Avetisyan, G. Tünnemann, D. Nowak, M. Nitschke, A. Herrmann, M. Drab, M.C. Cardoso, Cell Entry of Arginine-rich Peptides Is Independent of Endocytosis, *J. Biol. Chem.* 284 (2009) 3370–3378. <https://doi.org/10.1074/jbc.M805550200>.
- [292] A. Thakur, A.P. Mishra, B. Panda, D.C.S. Rodríguez, I. Gaurav, B. Majhi, Application of Artificial Intelligence in Pharmaceutical and Biomedical Studies, *Curr. Pharm. Des.* 26 (2020) 3569–3578. <https://doi.org/10.2174/1381612826666200515131245>.

- [293] A. Thakur, R.K. Sidu, I. Gaurav, K. Sweta, P. Chakraborty, S. Thakur, Modified biopolymer-based systems for drug delivery to the brain, in: *Tailor-Made Funct. Biopolym. Syst.*, Elsevier, 2021: pp. 571–611. <https://doi.org/10.1016/B978-0-12-821437-4.00016-5>.
- [294] I. Gaurav, A. Thakur, A. Iyaswamy, X. Wang, X. Chen, Z. Yang, Factors Affecting Extracellular Vesicles Based Drug Delivery Systems, *Molecules*. 26 (2021) 1544. <https://doi.org/10.3390/molecules26061544>.
- [295] H. Jiao, X. Zhao, J. Han, J. Zhang, J. Wang, Synthesis of a novel ^{99m}Tc labeled GE11 peptide for EGFR SPECT imaging, *Int. J. Radiat. Biol.* (2020) 1–9. <https://doi.org/10.1080/09553002.2020.1811419>.
- [296] R.S. Agnes, A.-M. Broome, J. Wang, A. Verma, K. Lavik, J.P. Basilion, An Optical Probe for Noninvasive Molecular Imaging of Orthotopic Brain Tumors Overexpressing Epidermal Growth Factor Receptor, *Mol. Cancer Ther.* 11 (2012) 2202–2211. <https://doi.org/10.1158/1535-7163.MCT-12-0211>.
- [297] W.-W. Xu, D. Liu, Y. Cao, X. Wang, GE11 peptide-conjugated nanoliposomes to enhance the combinational therapeutic efficacy of docetaxel and siRNA in laryngeal cancers, *Int. J. Nanomedicine*. Volume 12 (2017) 6461–6470. <https://doi.org/10.2147/IJN.S129946>.
- [298] N. Rahmanian, S.J. Hosseinimehr, A. Khalaj, Z. Noaparast, S.M. Abedi, O. Sabzevari, ^{99m}Tc-radiolabeled GE11-modified peptide for ovarian tumor targeting, *DARU J. Pharm. Sci.* 25 (2017) 13. <https://doi.org/10.1186/s40199-017-0179-8>.
- [299] I. Nakase, Biofunctional Peptide-Modified Extracellular Vesicles Enable Effective Intracellular Delivery via the Induction of Macropinocytosis, *Processes*. 9 (2021) 224. <https://doi.org/10.3390/pr9020224>.

- [300] R. Zilionis, C. Engblom, C. Pfirschke, V. Savova, D. Zemmour, H.D. Saaticioglu, I. Krishnan, G. Maroni, C. V. Meyerovitz, C.M. Kerwin, S. Choi, W.G. Richards, A. De Rienzo, D.G. Tenen, R. Bueno, E. Levantini, M.J. Pittet, A.M. Klein, Single-Cell Transcriptomics of Human and Mouse Lung Cancers Reveals Conserved Myeloid Populations across Individuals and Species, *Immunity*. 50 (2019) 1317-1334.e10. <https://doi.org/10.1016/j.immuni.2019.03.009>.
- [301] Á. Bartha, B. Györfy, TNMplot.com: A Web Tool for the Comparison of Gene Expression in Normal, Tumor and Metastatic Tissues, *Int. J. Mol. Sci.* 22 (2021) 2622. <https://doi.org/10.3390/ijms22052622>.
- [302] B. Györfy, Discovery and ranking of the most robust prognostic biomarkers in serous ovarian cancer., *GeroScience*. 45 (2023) 1889–1898. <https://doi.org/10.1007/s11357-023-00742-4>.
- [303] A. Thakur, G. Qiu, C. Xu, X. Han, T. Yang, S.P. NG, K.W.Y. Chan, C.M.L. Wu, Y. Lee, Label-free sensing of exosomal MCT1 and CD147 for tracking metabolic reprogramming and malignant progression in glioma, *Sci. Adv.* 6 (2020). <https://doi.org/10.1126/sciadv.aaz6119>.
- [304] S.A.A. Kooijmans, L.A.L. Fliervoet, R. van der Meel, M.H.A.M. Fens, H.F.G. Heijnen, P.M.P. van Bergen en Henegouwen, P. Vader, R.M. Schiffelers, PEGylated and targeted extracellular vesicles display enhanced cell specificity and circulation time, *J. Control. Release*. 224 (2016) 77–85. <https://doi.org/10.1016/j.jconrel.2016.01.009>.
- [305] Q. Zhu, X. Ling, Y. Yang, J. Zhang, Q. Li, X. Niu, G. Hu, B. Chen, H. Li, Y. Wang, Z. Deng, Embryonic Stem Cells-Derived Exosomes Endowed with Targeting Properties as

- Chemotherapeutics Delivery Vehicles for Glioblastoma Therapy, *Adv. Sci.* 6 (2019) 1801899. <https://doi.org/10.1002/advs.201801899>.
- [306] J.L. Hood, Post isolation modification of exosomes for nanomedicine applications, *Nanomedicine*. 11 (2016) 1745–1756. <https://doi.org/10.2217/nnm-2016-0102>.
- [307] Y. Park, I.S. Abihssira-García, S. Thalmann, G.F. Wiegertjes, D.R. Barreda, P.A. Olsvik, V. Kiron, Imaging Flow Cytometry Protocols for Examining Phagocytosis of Microplastics and Bioparticles by Immune Cells of Aquatic Animals, *Front. Immunol.* 11 (2020). <https://doi.org/10.3389/fimmu.2020.00203>.
- [308] M.L. Uribe, I. Marrocco, Y. Yarden, EGFR in Cancer: Signaling Mechanisms, Drugs, and Acquired Resistance, *Cancers* (Basel). 13 (2021) 2748. <https://doi.org/10.3390/cancers13112748>.
- [309] C.D. Little, M.M. Nau, D.N. Carney, A.F. Gazdar, J.D. Minna, Amplification and expression of the c-myc oncogene in human lung cancer cell lines, *Nature*. 306 (1983) 194–196. <https://doi.org/10.1038/306194a0>.
- [310] A. Thakur, C. Xu, W.K. Li, G. Qiu, B. He, S.-P. Ng, C.-M.L. Wu, Y. Lee, *In vivo* liquid biopsy for glioblastoma malignancy by the AFM and LSPR based sensing of exosomal CD44 and CD133 in a mouse model, *Biosens. Bioelectron.* 191 (2021) 113476. <https://doi.org/10.1016/j.bios.2021.113476>.
- [311] K. Matsumoto, M. Umitsu, D.M. De Silva, A. Roy, D.P. Bottaro, Hepatocyte growth factor/ $\langle \text{scp} \rangle \text{MET} \langle \text{scp} \rangle$ in cancer progression and biomarker discovery, *Cancer Sci.* 108 (2017) 296–307. <https://doi.org/10.1111/cas.13156>.

- [312] J. Yu, X. Wang, F. Teng, L. Kong, PD-L1 expression in human cancers and its association with clinical outcomes, *Onco. Targets. Ther.* Volume 9 (2016) 5023–5039. <https://doi.org/10.2147/OTT.S105862>.
- [313] F.K. Dermani, P. Samadi, G. Rahmani, A.K. Kohlan, R. Najafi, PD-1/PD-L1 immune checkpoint: Potential target for cancer therapy, *J. Cell. Physiol.* 234 (2019) 1313–1325. <https://doi.org/10.1002/jcp.27172>.
- [314] G. V. Scagliotti, The Biology of Epidermal Growth Factor Receptor in Lung Cancer, *Clin. Cancer Res.* 10 (2004) 4227S–4232S. <https://doi.org/10.1158/1078-0432.CCR-040007>.
- [315] P. Seshacharyulu, M.P. Ponnusamy, D. Haridas, M. Jain, A.K. Ganti, S.K. Batra, Targeting the EGFR signaling pathway in cancer therapy, *Expert Opin. Ther. Targets.* 16 (2012) 15–31. <https://doi.org/10.1517/14728222.2011.648617>.
- [316] Y. Li, L. Zhao, X.-F. Li, Hypoxia and the Tumor Microenvironment, *Technol. Cancer Res. Treat.* 20 (2021) 153303382110363. <https://doi.org/10.1177/15330338211036304>.
- [317] K. Tao, J. Liu, J. Liang, X. Xu, L. Xu, W. Mao, Vascular endothelial cell-derived exosomal miR-30a-5p inhibits lung adenocarcinoma malignant progression by targeting CCNE2, *Carcinogenesis.* 42 (2021) 1056–1067. <https://doi.org/10.1093/carcin/bgab051>.
- [318] I. Genta, E. Chiesa, B. Colzani, T. Modena, B. Conti, R. Dorati, GE11 Peptide as an Active Targeting Agent in Antitumor Therapy: A Minireview., *Pharmaceutics.* 10 (2017) 2. <https://doi.org/10.3390/pharmaceutics10010002>.
- [319] H. Chen, K. Shien, K. Suzawa, K. Tsukuda, S. Tomida, H. Sato, H. Torigoe, M. Watanabe, K. Namba, H. Yamamoto, J. Soh, H. Asano, S. Miyoshi, S. Toyooka, Elacridar, a third-

- generation ABCB1 inhibitor, overcomes resistance to docetaxel in non-small cell lung cancer, *Oncol. Lett.* 14 (2017) 4349–4354. <https://doi.org/10.3892/ol.2017.6678>.
- [320] M.K. Riaz, X. Zhang, K.H. Wong, H. Chen, Q. Liu, X. Chen, G. Zhang, A. Lu, Z. Yang, Pulmonary delivery of transferrin receptors targeting peptide surface-functionalized liposomes augments the chemotherapeutic effect of quercetin in lung cancer therapy, *Int. J. Nanomedicine*. Volume 14 (2019) 2879–2902. <https://doi.org/10.2147/IJN.S192219>.
- [321] D. Yang, P. Guo, T. He, C.A. Powell, Role of endothelial cells in tumor microenvironment, *Clin. Transl. Med.* 11 (2021). <https://doi.org/10.1002/ctm2.450>.
- [322] Q. Hu, H. Su, J. Li, C. Lyon, W. Tang, M. Wan, T.Y. Hu, Clinical applications of exosome membrane proteins, *Precis. Clin. Med.* 3 (2020) 54–66. <https://doi.org/10.1093/pcmedi/pbaa007>.
- [323] A. Thakur, A. Johnson, E. Jacobs, K. Zhang, J. Chen, Z. Wei, Q. Lian, H.J. Chen, Energy Sources for Exosome Communication in a Cancer Microenvironment, *Cancers (Basel)*. 14 (2022) 1698. <https://doi.org/10.3390/cancers14071698>.
- [324] A. Thakur, X. Ke, Y.-W. Chen, P. Motallebnejad, K. Zhang, Q. Lian, H.J. Chen, The mini player with diverse functions: extracellular vesicles in cell biology, disease, and therapeutics, *Protein Cell*. 13 (2022) 631–654. <https://doi.org/10.1007/s13238-021-00863-6>.
- [325] I. Gaurav, X. Wang, A. Thakur, A. Iyaswamy, S. Thakur, X. Chen, G. Kumar, M. Li, Z. Yang, Peptide-Conjugated Nano Delivery Systems for Therapy and Diagnosis of Cancer, *Pharmaceutics*. 13 (2021) 1433. <https://doi.org/10.3390/pharmaceutics13091433>.

- [326] O.G. de Jong, S.A.A. Kooijmans, D.E. Murphy, L. Jiang, M.J.W. Evers, J.P.G. Sluijter, P. Vader, R.M. Schiffelers, Drug Delivery with Extracellular Vesicles: From Imagination to Innovation, *Acc. Chem. Res.* 52 (2019) 1761–1770. <https://doi.org/10.1021/acs.accounts.9b00109>.
- [327] H. Jang, H. Kim, E.H. Kim, G. Han, Y. Jang, Y. Kim, J.W. Lee, S.C. Shin, E.E. Kim, S.H. Kim, Y. Yang, Post-insertion technique to introduce targeting moieties in milk exosomes for targeted drug delivery, *Biomater. Res.* 27 (2023) 124. <https://doi.org/10.1186/s40824-023-00456-w>.
- [328] A. Thakur, D.C. Parra, P. Motallebnejad, M. Brocchi, H.J. Chen, Exosomes: Small vesicles with big roles in cancer, vaccine development, and therapeutics, *Bioact. Mater.* 10 (2022) 281–294. <https://doi.org/10.1016/j.bioactmat.2021.08.029>.
- [329] G. Lorusso, C. Rüegg, The tumor microenvironment and its contribution to tumor evolution toward metastasis, *Histochem. Cell Biol.* 130 (2008) 1091–1103. <https://doi.org/10.1007/s00418-008-0530-8>.
- [330] R. Raguraman, D. Bhavsar, D. Kim, X. Ren, V. Sikavitsas, A. Munshi, R. Ramesh, Tumor-targeted exosomes for delivery of anticancer drugs, *Cancer Lett.* 558 (2023) 216093. <https://doi.org/10.1016/j.canlet.2023.216093>.
- [331] H. Zheng, Y. Zhan, S. Liu, J. Lu, J. Luo, J. Feng, S. Fan, The roles of tumor-derived exosomes in non-small cell lung cancer and their clinical implications, *J. Exp. Clin. Cancer Res.* 37 (2018) 226. <https://doi.org/10.1186/s13046-018-0901-5>.

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