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REVIEW

# Advances in Nanoliposomes for the Diagnosis and Treatment of Liver Cancer

Yitong Li[1], Ruihang Zhang[2], Zhen Xu[1], Zhicheng Wang[1]

[1]NHC Key Laboratory of Radiobiology (Jilin University), School of Public Health, Jilin University, Changchun, 130021, Jilin, People's Republic of China; [2]Second Clinical Medical College, Henan University of Traditional Chinese Medicine, Zhengzhou, 450052, Henan, People's Republic of China

Correspondence: Zhicheng Wang, NHC Key Laboratory of Radiobiology (Jilin University), School of Public Health, Jilin University, 1163 Xinmin Street, Changchun, 130021, Jilin, People's Republic of China, Tel +86 13843131059, Fax +86 431185619443, Email zhicheng@jlu.edu.cn

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**Abstract:** The mortality rate of liver cancer is gradually increasing worldwide due to the increasing risk factors such as fatty liver, diabetes, and alcoholic cirrhosis. The diagnostic methods of liver cancer include ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI), among others. The treatment of liver cancer includes surgical resection, transplantation, ablation, and chemoembolization; however, treatment still faces multiple challenges due to its insidious development, high rate of recurrence after surgical resection, and high failure rate of transplantation. The emergence of liposomes has provided new insights into the treatment of liver cancer. Due to their excellent carrier properties and maneuverability, liposomes can be used to perform a variety of functions such as aiding in imaging diagnoses, combinatorial therapies, and integrating disease diagnosis and treatment. In this paper, we further discuss such advantages.

**Keywords:** liver targeting, nanotherapeutics, nanocarriers, multimodal imaging, nanomedicine, diagnostic treatment integration

## Introduction

Liver cancer is the leading cause of cancer death, with an annual increase in incidence.[1] Hepatocellular carcinoma (HCC) represents ~90% of primary liver cancers and constitutes a major global health problem.[2] The risk factors for HCC include chronic hepatitis B and hepatitis C, alcohol addiction, metabolic liver disease (particularly nonalcoholic fatty liver disease) and exposure to dietary toxins, such as aflatoxins and aristolochic acid.[3] The staging of HCC is particularly complicated due to the varying presence of concomitant liver dysfunction. The staging of HCC is generally performed using the Okuda staging system, the Cancer of the Liver Italian Program, the Hong Kong Liver Cancer (HKLC) staging system, and the Barcelona Clinic Liver Cancer (BCLC) staging system, which is the most popular.[4] The current approach to HCC treatment depends on the stage at which the patient is diagnosed.[5] The BCLC staging system recommends that only patients classified as BCLC stage 0 or A be administered surgical resection or liver transplantation. In contrast, patients with BCLC stage B and BCLC stage C are recommended for transarterial chemoembolization (TACE) or transarterial radioembolization (TARE).[6] BCLC stage D, known as the end-stage, is currently treated symptomatically using the best supportive care.[5]

Although several studies have demonstrated the rationality of the BCLC system, the BCLC standard is also controversial. A meta-analysis of 2619 Asian HCC patients showed that surgical resection provided better overall survival than TACE in BCLC stage B patients, without any significant increase in postoperative complications or 30-day mortality.[7]

The Derwent Innovation Platform collects data from a global sample of patents related to HCC treatment technologies, which show future trends in the treatment of HCC. The platform explores novel molecular therapies, localized and visible local therapies, and combinations of immunotherapy with targeted therapies or other conventional treatments.[8]

In addition to surgical resection, liver transplantation, and percutaneous ablation, clinical studies have also recently evaluated liposomes and lipid nanoparticles as therapies for liver cancer. Liposomes are spontaneously-forming and

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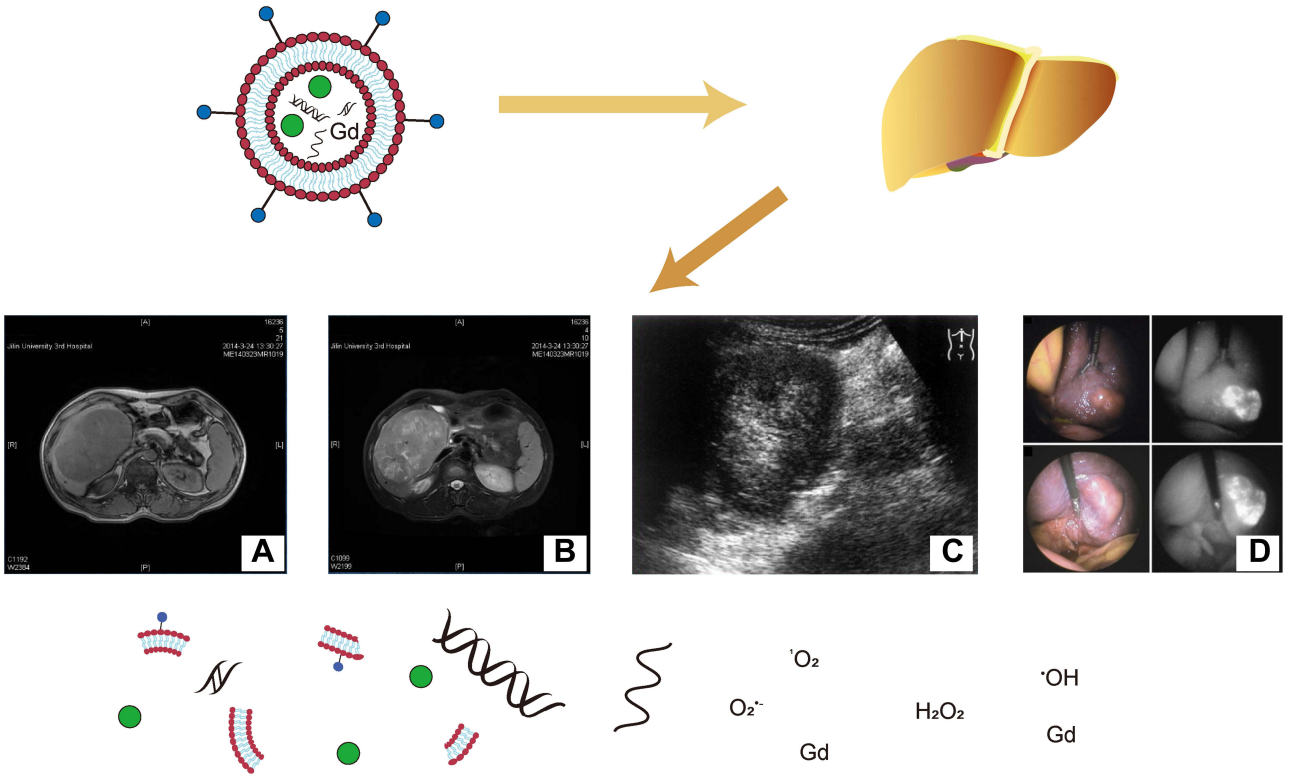
**Graphical Abstract**




spherical fragments consisting of a lipid bilayer and a hydrophilic core.[9] Research on liposome technology has progressed from conventional vesicles (first-generation liposomes) to second-generation liposomes, in which long-circulating liposomes are obtained by modulating the lipid composition, size, and charge of the vesicle.[10] Second-generation liposomes have been designed as tumor-targeting drug delivery vehicles for the precise delivery of active drugs into tumor cells.[11] The unique properties of liposomes, including their biocompatibility, biodegradability, amphiphilic nature, low toxicity, non-ionicity, sustained release, and ability to be actively targeted, have provided many biomedical opportunities, especially for drug delivery.[12] Liposomes can encapsulate drugs to prevent degradation by the immune system, and are highly biocompatible and effectively targeted. As a chemotherapeutic drug delivery system and gene or immunotherapy tools, liposomes enhance the safety of vector systems, the expression of therapeutic proteins, and the silencing of disease-causing genes.

Due to their extensive advantages, liposomes have been studied for drug delivery to tumor tissues via two main targeting approaches: passive targeting and active targeting. The most common active targeting strategy for liposome agents is the selective binding to cancer cell surfaces that express specific receptors.[13] Frequently used ligands for targeting HCC cells include small molecules (eg, folic acid receptor), peptides (eg, the VDAC1-based peptide, R-Tf-D-LP4), proteins (eg, Asialoglycoprotein receptor), and nanoantibodies, among others.[14–16] In addition to nanocarriers, radioisotopes and drugs have also been modified as targeting ligands for molecular imaging and radioimmunotherapy.[17] Liposomes can maintain high concentrations in tumor microenvironments through the enhanced permeability and retention (EPR) effect. In tumorous tissues, the absence of vasculature-supportive tissues initiates the formation of leaky vessels and pores (100 nm to 2 μm in diameter), and the poor lymphatic system offers great opportunity to aggregation of medicine.[18] Many contained drugs can trigger release in multiple forms. Artificial external stimuli such as light and heat therapy can promote the release of drugs from liposomes. Unique features of the tumor microenvironment can also act as endogenous stimuli (pH, redox potential, or unique enzymatic activities), while external stimuli (heat or

light) can also be applied at the target location to trigger the controlled release of the active pharmaceutical ingredient (APIs). In liposome carrier systems, triggered release is generally based on membrane instability due to local defects in the bilayer, thus, resulting in the release of liposome-encapsulated drugs.[19] The drug can be targeted to the lesion with minimal side effects and minimal damage to healthy organs.

For diagnostics, advanced imaging contrast agents increase the differences in signal intensity between diseased and normal tissues. Liposomal multimodal imaging used for drug delivery can also be used as a diagnostic tool to monitor the distribution of drugs in vivo. In terms of therapeutics, liposomes can be modified and adapted for combination therapies, immunotherapies, gene therapies, and other conventional therapies. Thus, such innovations offer opportunities to cure HCC. Despite being an ideal tool for liver cancer treatment, liposome-based therapies also face urgent challenges that must be resolved before their clinical application, such as the stability of carriers and their short residence times in tissues.

The application of liposomes for liver cancer treatment has great potential. In this review, we discuss the current liver cancer treatments and basic information on liposomes. We summarize recent achievements in liposome-based imaging diagnostics and multiple therapies (Chemotherapy, gene therapy and immunotherapy) for HCC. Particular emphasis is placed on the integration of diagnostics and treatment. Finally, we provide perspectives on additional applications of liposome-based technologies.

## Current Status of Liver Cancer Treatment

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and is a malignant tumor that originates from hepatocytes.[20] HCC is the fifth most common cancer in men and the ninth most common cancer in women, with approximately 500,000 and 200,000 new cases per year globally, respectively. HCC also often presents with complex pathogenesis and structural mutations in proto-oncogenes due to the addition of exogenous pathogenic factors such as viruses, excessive alcohol consumption, obesity, and aflatoxins.[21,22] In addition, several epidemiological surveys and experimental studies have shown that the risk of death from HCC was significantly associated with mass size, the number of masses, macrovascular invasion, and intrahepatic metastases; thus, suggesting that hepatocarcinogenesis is influenced by genetic factors.[23–25]

The commonly used staging criteria for HCC include the BCLC staging system and the recently proposed HKLCstaging system. HKLC is derived from studies in Asian cohorts, where advanced HCC is the focus of clinical studies.[2,26,27] In early-stage tumors, ablation is a first-line treatment comparable to hepatic resection due to the lower mortality rate.[28] Ablation and transcatheter chemoembolization are alternative treatments that act locally.[2] HCC, which often occurs in the context of cirrhosis, also suffers from postoperative recurrence, which can be resolved by transplantation. However, recurrence of HCC still occurs in 10–20% of such patients.[29,30]

Non-surgical treatment of HCC (local intra-arterial therapy, systemic multikinase inhibitor therapy, and immunotherapy) has been an emerging field of development in recent years.[31] TACE blocks the blood supply vessels with an embolic agent (usually iodine oil), thus, causing ischemic necrosis of the tumor, with relative preservation of the rest of the liver parenchyma due to the dual blood supply.[2,32] TACE is often contraindicated in cirrhotic decompensation, renal failure, main portal vein obstruction, and extensive tumor burden.[33] Systemic therapy for advanced HCC mainly consists of targeted agents and immune checkpoint inhibitors.[34]

## Liposomes

Nanoparticles are widely fabricated using liposomes, ie lipid nanoparticles (LNPs). Liposomes are typically between 50 and 200 nm and primarily consist of phospholipids and cholesterol. Phospholipids self-assemble into spherical lipid bilayers through their lipophilic tails, and can be used to encapsulate drugs into liposomes. Because liposomes protect the encapsulated cargo from degradation by the immune system, they have the advantage of biocompatibility and enhanced targeting efficiencies.[35] Liposomes can be divided into monolayer vesicles and multilayer vesicles depending on the number of bilayers. In addition, monolayered liposomes are divided into small and large monolayered vesicles.[36]

The mechanisms of liposome-targeted therapy include active targeting and passive targeting. Passive targeting relies on the enhanced permeability and retention (EPR) effect, also known as the high permeability long retention effect,

which is a unique phenomenon in the vascular system of solid tumors that is based on specific anatomical structures and pathophysiology. Examples of EPR include leaky vessels with holes; abundant vascular transmitters such as bradykinin, nitric oxide (NO), carbon monoxide (CO), and vascular endothelial growth factor; and poor lymphatic drainage. Thus, such features lead to nanoparticle deposition in tumor tissues.[37] However, the existence and importance of the EPR effect have recently been hotly debated, and the heterogeneity of EPR between different tumors has called its application into question.[38] Therefore, the effectiveness of nanomedicine can be improved with advances in active targeting strategies. Active targeting of nanomaterials usually targets substances overexpressed in tumors, and can be classified as liver-cell targeting or tumor endothelial vascular targeting, among other types of targeting depending on the site (Table 1 and Figure 1).[39]

# Use of Liposomes

## Liposomes for Diagnostics

HCC usually occurs with chronic liver disease or cirrhosis. In a randomized controlled trial of 18,816 Hepatitis B virus (HBV)-infected patients in China, it was shown that serum alpha-fetoprotein (AFP) testing and abdominal ultrasound (US) at 6-month intervals decreased mortality from HCC by 37%. Thus, early detection of patients at high risk of HCC increases the ability for treatment and improves survival rates.[56] Traditional diagnostic tools for HCC are based on

**Table 1** Active Targeting of HCC Through VCAM-1, GA-R, ASGPR as Well as Others Receptors

| | Target Points | Nanomaterials | References |
|---|---|---|---|
| Microvascular endothelial cells | VCAM-1 | Dexamethasone loaded anti-VCAM-1 SAINT-O-Somes | 40 |
| HCC cells | GA-R | GA and PNAmodified doxorubicin-loaded liposomes (DOX-GA/PNA-Lips) | 41 |
| | | Glycyrrhetinic acid-modified oxaliplatin liposome (GA-OX) | 42 |
| | ASGPR | Galactose Modified Liposomes (Gal-LP) | 43 |
| | | Celastrol-Loaded Galactosylated Liposomes (C-GPL) | 44 |
| | | ASF-lipoplexes | 45 |
| | | Lactobionic acid coupled liposomes (LA-LP) | 46 |
| | | Gal-doxorubicin/vimentin siRNA liposome (Gal-DOX/siRNA-L) | 47 |
| | | Lactoferrin-modified-Stealth liposome (Lf-Stealth-LIPO) | 48 |
| | Pin1 | Liposome-Encapsulated Pin1 Inhibitor (API-LP) | 49 |
| | FA | Folate receptor-targeted liposomes loaded with a diacid metabolite of norcantharidin (DM-NCTD/FA-PEG) | 50 |
| | TfR | Tf-targeted anti-miR-221 liposome (Tf-RL) | 51 |
| | | Transferrin-guided polycarbonate-based polymersomal doxorubicin (Tf-Ps-Dox) | 52 |
| | LDLR | Liposomes loaded with Cholesteryl-succinyl-5-fluorouracil conjugate (5-FUC liposome) | 53 |
| | EGFR | GE11 peptide-functionalized polymersomal doxorubicin (GE11-PS-DOX) | 54 |
| | | Targeted LPD conjugated with anti-EGFR Fab' co-delivering ADR and RRM2 siRNA (ADR-RRM2-TLPD) | 55 |

**Abbreviations**: HCC, hepatocellular carcinoma; VCAM-1, vascular cell adhesion molecule-1; GA-R, glycyrrhetinic acid receptors; ASGPR, asialoglycoprotein receptor; Pin1, peptidyl-prolyl cis/trans isomerase; FA, folic acid; TfR, transferrin receptor; LDLR, low-density lipoprotein receptor; EGFR, epidermal growth factor receptor; SAINT-C18, 1-methyl-4-(cis-9-dioleyl) methyl-pyridinium-chloride; PNA, peanut agglutinin; ASF, asialofetuin; LPD, liposome-polycation-DNA complex; ADR, Adriamycin; RRM2, ribonucleotide reductase M2, siRNA, small interfering RNA.

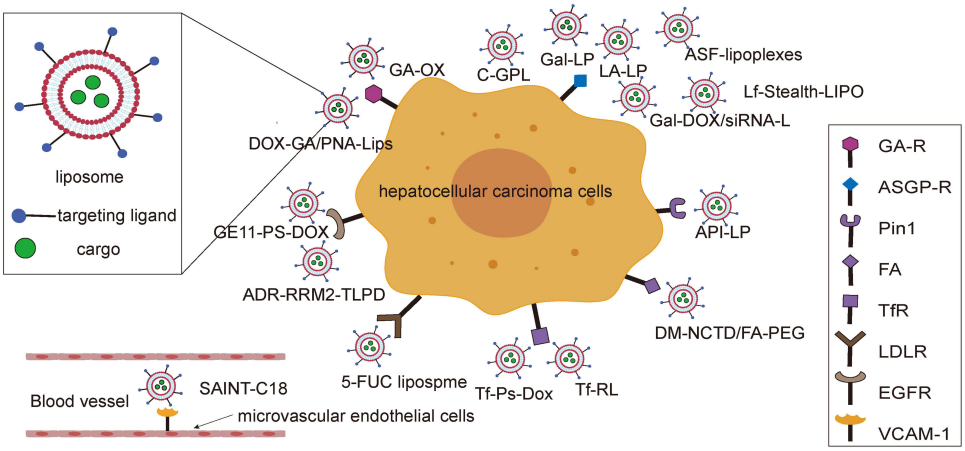



**Figure 1** Liposomes act on tumor microvascular endothelial cells and HCC cells.
**Note**: The liposome targeting ligand recognizes the receptor then undergoes internalization and release of the delivered cargo, thus exerts therapeutic effects.
**Abbreviations**: VCAM-1, vascular cell adhesion molecule-1; GA-R, glycyrrhetinic acid receptors; ASGPR, asialoglycoprotein receptor; Pin1, peptidyl-prolyl cis/trans isomerase; FA, folic acid; TfR, transferrin receptor; LDLR, low-density lipoprotein receptor; EGFR, epidermal growth factor receptor.

cytology or histology, and lesion tissue is obtained by biopsy. With advances in computed tomography (CT) and magnetic resonance imaging (MRI), it is now possible to reliably diagnose HCC without biopsies.[3] The American Association for the Study of Liver Diseases (AASLD) recommends the use of multiphasic CT or multiphasic MRI for the diagnostic evaluation of HCC. According to the Liver Imaging Reporting and Data System (LI-RADS), masses larger than 10 mm on multiphase examinations are assigned category codes reflecting their relative probability of being benign, HCC, or other liver malignancies. For example LI-RADS 1 and LI-RADS 2 are used to indicate definitely and probably benign tumors, respectively.[26] The coding system has played an important role in the early diagnosis of liver cancer.

## MRI

MRI has become one of the most powerful diagnostic tools in biomedicine, largely due to its non-invasive nature, sub-millimetre spatial resolution, high anatomical contrast, and excellent soft-tissue differentiation. In many cases, however, gadolinium-based contrast agents (CA) are required to enhance the differences in signals between diseased areas and normal tissue. Liposomes are biocompatible platforms based on nanocarriers. There are two main forms of binding of CA to liposomes: encapsulation of water-soluble reagents of gadolinium chelates in liposome cavities or modification of liposome bilayers with gadolinium chelates.[25] One research group prepared a rare-earth-doped nanoparticle (Gd-REs@Lips) and imaged patients with primary liver cancer (HCC), showing that the nanoparticle used as a $T_2$-weighted imaging contrast agent increased the differences in signal intensities between HCC tissue and the surrounding normal liver tissue on MRI; thus, enabling the accurate detection of liver cancer.[57] Another advantage of gadolinium-based liposomes over conventional gadolinium-based contrast agents is their non-toxicity. Gadolinium deposition has been shown to disrupt intracellular lysosomal function, and cause oxidative damage and fibrous deposition.[58] However, Simeckova et al evaluated gadolinium-based liposome complexes containing 1, 2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid (PE-DTPA) chelated with $Gd^{+3}$, and reverse transcription-polymerase chain reaction (RT-PCR) assays revealed that such complexes did not increase the expression of stress-related genes, including early growth response-1 (EGR1), activating transcription factor 3 (ATF3), growth differentiation factor 15 (GDF15), and fibroblast growth factor 21 (FGF21). The complex also failed to increase the production of inflammatory factors and exhibited good biocompatibility.[59]

CT also requires the use of contrast agents to aid in disease diagnosis. Fouillet et al used iodomelanol-containing liposomes to enhance liver contrast and found that it increased the diagnostic rate to more than 60% higher than the pre-push control values when detecting liver tumors in rats.[60]

### Multimodal Imaging

Multimodal imaging is a novel imaging technique that integrates two or more imaging modalities, and is often used to combine anatomical information with high-resolution anatomical information with molecular-level biological information. It allows simultaneous high spatial resolution, soft tissue contrast and high sensitivity to molecular level biological information. At the same time it overcomes the limitations of individual imaging modalities in the full description of the disease and produces accurate images. For example, PET/CT complements the low resolution of PET and the inability of CT to perform functional imaging.[61]

Radiolabelled liposomes have been used as diagnostic tools to monitor distribution of drugs in vivo and in real time, which optimizes the therapeutic efficacy of liposomal drug delivery. It has been reported that a [$^{111}$In]-liposome platform was used as a drug delivery vehicle to encapsulate a novel $^{18}$F-labeled carboplatin drug derivative ([$^{18}$F]-FCP), which was used as a bimolecular imaging tool. Dual-modality imaging is performed using $^{18}$F positron emission tomography (PET) and $^{111}$In single-photon emission CT (SPECT) when both the vehicle and the drug are labelled. PET/SPECT imaging can greatly enhance the tracking of the movement of encapsulated drugs in vivo.[62]

Guan et al fabricated gold nanorods@liposome core-shell nanoparticles (Au@liposome-ICG) containing indocyanine green (ICG). ICG is the most commonly used near-infrared (NIR) fluorescent dye, however, several factors limit its potential application as a bimodal contrast agent, including its aggregation, rapid clearance, fluorescence burst, and low efficiency in converting laser energy to heat. Gold nanorods (AuNR) are effective photoacoustic probes due to their low toxicity, good biocompatibility, and easy exudation into the tumor region. With Au@liposome-ICG-mediated photoacoustic fluorescence dual-modality imaging, enhanced photoacoustic tomography (PAT) signals can be obtained for preoperative diagnoses, the use of stable fluorescence signals for intraoperative manipulation, and the safe resection of tumors.[63]

High-intensity focused ultrasound (HIFU) uses a high-energy focusing device to focus multiple low-energy ultrasound beams on the target tumor to induce irreversible coagulative necrosis. HIFU requires multiple imaging techniques for synergistic detection. Wang et al constructed a multifunctional bio-targeting booster consisting of *Bifidobacterium longum*, ICG, and perfluorohexane (PFH) co-loaded cationic lipid nanoparticles (CL-ICG-PFH-NPs). The booster used the negative surface charge of *B. longum* to guide the cationic lipid nanoparticles to the tumor area by electrostatic adsorption, and achieved HIFU synergy and multimodality imaging using photoacoustic imaging (PA), fluorescence imaging (FL), and ultrasound imaging (US) in vitro. Thus, the biologically-targeted potentiator not only provided information for the early diagnosis of tumors, but also improved HIFU therapy by enabling multimodal imaging and monitoring during HIFU ablation.[64]

## Liposomes for Therapeutic Usage

### Liposome Applications in Chemotherapy

There are many types of cells in the HCC tumor microenvironment, including HCC cells and other stromal cells, vascular endothelial cells, immune cells, and fibroblasts. Such cells and the extracellular matrices they produce have a great impact on tumor development and the response to antitumor therapy.[65] HCC cells constitute the primary component of HCC and overexpress antigens and receptors, including ASGP-R, GA-R and transferrin receptor (TfR). Such overexpression is a characteristic that distinguishes HCC cells from other cells, and can be used as targets for liposomes used for HCC therapy.[66–68]

There is substantial evidence to suggest that HCC may originate from cancer stem cells (CSCs). CSCs have the ability to self-renew and differentiate, which can initiate tumor formation, promote tumor growth, produce distant metastases, and cause recurrence following treatment. Thus, targeting CSCs may efficiently inhibit hepatocellular carcinoma growth and recurrence at the source.[69] Hepatic macrophages, the largest population of innate immune cells in the liver, consist of Kupffer cells (KCs) from the fetal yolk sac and infiltrated bone marrow-derived monocytes/macrophages, which are important target cells for the treatment of HCC.[70] Hepatic macrophages suppress antitumor immunity, and high levels of macrophage infiltration is predictive of poor prognosis in HCC patients. Thus, targeting KCs may help maintain hepatic antitumor immunity and inhibit tumor growth.[71–73] Chemotherapy is a primary systemic antitumor therapy, and the inability to distinguish between normal and malignant cells is its main drawback as it can lead to adverse systemic

effects. Therefore, chemotherapy is often used for the treatment of HCC with local therapies, such as TACE.[74] Thus, nanoliposome delivery to target chemotherapies has great prospects. Such delivery mechanisms can improve the targeting of treatments and the concentration of chemotherapeutic drugs in tumor tissues, thus, improving the efficacy of such treatments while reducing adverse effects.

## Targeting Hepatocytes

Multiple cellular hepatoma targets are described in Table 1. It is well documented that the asialoglycoprotein receptor (ASGPR) is highly expressed in well-differentiated HCC cells and that it specifically recognizes glycoproteins, especially D-galactose or N-acetylgalactosamine (galactosamine). Li et al constructed novel ASGPR-targeting poly (polyethylene glycol paclitaxel) (PTX) nanoliposomes that were loaded with PTX with Tn-modified nanoliposomes (Tn-Lipo-PTX), and GalNAc (Tn antigen) as a ligand to target the paclitaxel to tumors. That vehicle improved drug efficacy and tumor site accumulation, while reducing drug toxicity.[75] Zhang et al constructed a Lupeol-loaded liposome system (Gal-lupeol-L system) using lactoferrin as the target ligand to deliver Lupeol to ASGPR-expressing HCC cells. In vivo and in vitro experiments revealed the good tumor-suppressive effects of that liposome system.[76] Additionally, Wang et al modified adriamycin (DOX) liposomes using ester bond-linked shearable polyethylene glycol lipids and galactosyl lipids. The polyethylene glycol-lipid-modified liposomes have been shown to prolong the circulation time and reduce uptake by the mononuclear phagocyte system. Thus, exhibiting a higher safety profile than free drugs.[77]

HBV virus infection is a risk factor for HCC and contains a highly specific amino acid sequence (HBVpreS1) in its envelope protein that imparts extreme affinity for the liver.[78] It was later shown that the specific target of HBV on the hepatocyte membrane is the sodium-taurocholate co-transporting polypeptide (NTCP/SLC10A1).[79] A myristoylated peptide, Myrcludex B, was developed and bound with high affinity and specificity to the NTCP on hepatocyte membranes, preventing the binding of viral particles to their target cells.[80] Zhan was the first to prepare HBV preS1-derived lipopeptide-functionalized liposomes targeting hepatocytes, and showed that HBVpreS/2-48myr conjugated to PEGylated liposomes (HBVP-Lip) could specifically deliver loaded drugs to hepatocyte. Thus, those findings opened new possibilities for liver-specific drug delivery systems, gene delivery systems, and bioimaging systems.[81] However, there are limits to the size of the nanoformulations with entry to the liver being limited when the diameter is higher than the average diameter of the endothelial window of the hepatic sinusoids in healthy humans (approximately 100 nm).[82] To further optimize, Witzigmann et al prepared hepatotropic liposome particles to specifically target NTCP, and found using gamma scintigraphy and fluorescence microscopy that active NTCP could mediate the endocytosis of hepatocytes.[83]

## Targeting Other Cells

Based on the dual action of liposomes and magnetic thermotherapy, thermosensitive magnetoliposomes (TMs) render tumor treatment more effective and safer. An anti-$CD90^+$ Ab-modified TM loaded with 17-allylamino-17-demethoxygeldanamycin (17-AAG) (CD90@17-AAG/TMs) was reported to effectively target and kill $CD90^+$ liver cancer stem cells (LCSC) and reduce the chance of HCC recurrence.[84] KCs account for 35% of the non-parenchymal cells of the liver and 80–90% of the monocyte-macrophage system.[85] They also secrete and synthesize a variety of bioactive substances.[86] The activation of KCs and the overexpression of inflammatory factors such as tumor necrosis factor-α (TNF-α) are the main initiators of pro-inflammatory/sustained imbalance.

Zoledronate (ZOD) stops the progression of HCC, and Zhao et al used ZOD to prepare liposome-encapsulated zoledronate for intravenous administration and selectively induced the apoptosis of rat KCs.[87] Animal experiments have shown that zoledronic acid liposomes help to concentrate ZOD in the target area, while reducing it in others, which reduces toxicity.[88]

Many chemotherapeutic agents such as arsenic trioxide (ATO) are toxic to cells, which has limited their applications. Jin et al prepared liposome-encapsulated arsenic-manganese complexes, denoted as LP@MnAsx, which enhanced therapeutic efficacy through pH-sensitive drug release, prolonged circulation time, and improved tumor accumulation. Manganese ($Mn^{2+}$) ions were recently found to be effective $T_1$ contrast agents.[89] Arsenite ($AsO_3^{3-}$), the ionic state of ATO, forms precipitates with $Mn^{2+}$ in a neutral environment, while in an acidic environment, the compound can dissociate, releasing $Mn^{2+}$ ions and arsenite. In turn, $Mn^{2+}$ enables the real-time MRI detection of arsenite release in

acidic tumor microenvironments, and therefore, a versatile drug delivery system was developed that was sensitive to microenvironmental pH value.[90] Additionally, $Mn^{2+}$ can also cause further damage to tumors by generating reactive oxygen species (ROS) through a Fenton-like reaction.[91]

## Liposome Applications for Gene Therapy

Liposome applications for gene therapy can provide small interfering ribonucleic acid (siRNA), messenger RNA (mRNA), deoxyribonucleic acid (DNA), or gene editing complexes that provide a new approach to the treatment of liver cancer by silencing disease-causing genes, expressing therapeutic proteins, or correcting genetic defects (Figure 2).[92]

RNA interference (RNAi) is a means of regulating gene expression by siRNAs and microRNAs (miRNAs).[93] siRNAs bind to the RNA-induced silencing complex (RISC), and once they enter the cytoplasm, they induce gene silencing by directing the specific cleavage of sequences of fully complementary paired mRNAs. Comparatively, miRNAs mediate the inhibition of translation and the termination of transcription of incompletely complementary mRNAs.[94] miRNAs may also mediate mRNA degradation in cytoplasmic compartments, known as processing bodies (P-bodies), thus preventing protein synthesis.[95] Primate synthesized siRNAs are unable to cross biological membranes by passive diffusion due to their high molecular weight and polyanionic nature; therefore, they require drug delivery strategies. Lipid nanoparticles containing siRNAs can silence genes, and thus, are precise therapeutic tools.[96] Wanatabe's team attempted to silence the gene expression of hepatitis C virus (HCV) in vivo using novel cationic liposome-encapsulated siRNA molecules. They used enzyme-linked immunosorbent assays (ELISA) to measure the expression of the HCV core protein in mouse liver and found that siRNA/lactosylated cationic liposome 5 (CL-LA5) complexes (siRNA/CL-LA5) specifically inhibited HCV protein expression in a dose-dependent manner. Interestingly, siRNA/CL-LA5 did not induce a highly biocompatible interferon (IFN) response in the liver, as conventional siRNAs did.[97]

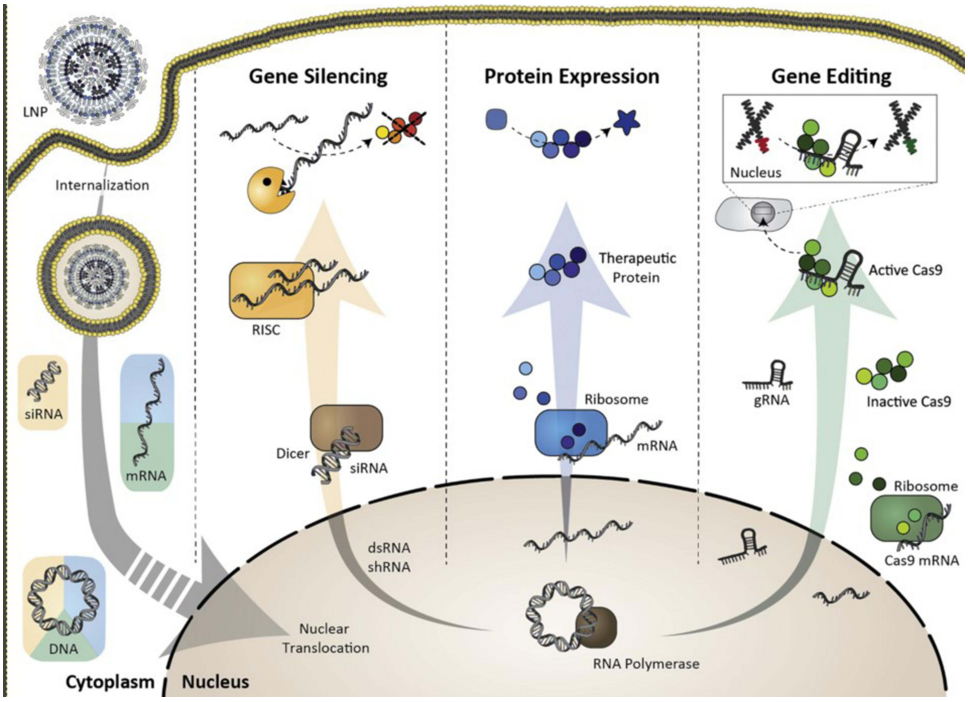



**Figure 2** Mechanism of liposome combined gene therapy.
**Notes**: Encapsulating siRNA, mRNA, or DNA in liposomes. siRNA binds to certain enzymes and proteins in the cell to form an RNA-induced silencing complex (RISC) that binds to the target mRNA, cutting it off and degrading it. mRNA enters the cell and expresses proteins. cas9/tracrRNA/rNcrRNA targets DNA to cause double-stranded breaks (DSB) in DNA, resulting in gene editing. Reprinted from *Advanced Drug Delivery Reviews*, 159, Witzigmann D, Kulkarni JA, Leung J, Chen S, Cullis PR, van der Meel R. Lipid nanoparticle technology for therapeutic gene regulation in the liver, 344-363, Copyright 2022, with permission from Elsevier.[92]
**Abbreviations**: siRNA, small interfering RNA; mRNA, message RNA; cas9, clustered regularly interspaced short palindromic repeat-associated nuclease 9; crRNA, CRISPR RNA; tracrRNA, trans-acting crRNA.

Liver fibrosis genes also have an important role in hepatocarcinogenesis. The encapsulation of a procollagen α1 (I) (Col1a1) siRNA duplex (siCol1a1) into cationic C12-200 LNP (LNP-siCol1a1) has been reported, and the siCol1a1 was validated to have silencing efficacy against fibrosis genes, with a 90% dose-dependence. Thus, those data reflect the importance of anti-fibrotic therapy in inhibiting the progression of liver disease.[93] Specific enhancement of gene expression through the targeted delivery of DNA can also be used for the treatment of HCC. Cationic lipid complexes consist of cationic lipids and neutral lipids or cholesterol. Negatively charged DNA is compressed and forms complexes with positively charged lipids, making it easier to transduce in rapidly-dividing cells.[98] Wang's team proposed a strategy to modify liposomes with the TfR, and delivering the acetylcholinesterase (AChE) gene by targeting the TfR on the surface of HCC cells for gene therapy.[99] Compared to DNA-based therapies, mRNA has a greater potential success of targeted delivery and effect of gene. Because it does not require access to the nucleus. Unfortunately, however, mRNA is capable of inducing an immune response, which can be avoided by mRNA-based liposomes.[100]

Miao et al introduced alkyne and ester groups into the lipid tail, where the alkyne enhanced endosomal escape and systemic tolerance, and the ester promoted cellular uptake of the drug. Such a synergistic formulation was efficacious for gene delivery, with approximately 10-fold higher protein expression compared to unmodified lipids.[101] In some experiments, the protein was infused directly into the tumor, thus, providing unexpected results. Kringle 1-5 (K1-5) is an excellent gene delivery candidate due to its physiological production, non-immunogenic nature, avoidance of transduction to other tumor cells, and strong and specific anti-angiogenic activity.[91,102] Torimura et al prepared liposome-K1-5 cDNA complexes using K1-5-containing Cos-1 cells as the substrate and intravenously injected them into transplanted tumor-bearing mice, showing the inhibitory effect of liposome-K1-5 cDNA complexes on angiogenesis in tumor tissue.[102–106]

### Liposome Applications for Immunotherapy

The immune microenvironment of HCC has suppressive tumor-associated macrophages (HCC-TAM) and liver sinusoidal endothelial cells (LSEC) functions as antigen-presenting cells that regulate the immune effects of the liver. In the physiological state, HCC-TAM and LSEC prevent immune responses by suppressing $CD4^+$ and $CD8^+$ T lymphocytes. Similar to LSEC, KCs and HDCs secrete suppressive cytokines and induce an increase in Treg cells.[107] Furthermore, the HCC microenvironment is characterized by the high expression of immune checkpoint molecules. The combination of LipC6 bound $CD8^+$ cells and eliminated the immune response leads to a significant decrease in the activity of the effectors of anti-tumor immune responses, which results in tumor immune evasion. In light of these findings, improving the tumor microenvironment is key for the treatment of HCC.[108] Li et al have developed a liposome containing C6-ceramide (LipC6) and showed that it not only binds to the $CD8^+$ T cell-mediated immune response to eliminate liver tumors in situ, but also promoted the polarization of TAMs to the M1 phenotype.[109]

Immunoliposomes are monoclonal antibodies or antibody fragments modified on the surface of liposomes that can actively target tumor tissue for specific immunotherapeutic purposes. During that process, liposomes act as adjuvants in addition to being the antigen carriers. Iwama et al encapsulated glypican-3 (GPC3)-derived cytotoxic T lymphocyte (CTL) peptides in liposomes (pGPC3-liposome), and investigated their antitumor potential. GPC3 behaved as a TAA in HCC tissue, exhibiting high expression. Thus, the results showed that pGPC3-liposomes effectively stimulated CTL in vivo and that liposomes were essential for the induction of CTL.[110]

## The Integration of Diagnosis and Treatment

### US

Ultrasound imaging (US) is widely used for diagnostic imaging due to its physical properties, low-cost, safety, and non-invasiveness. Additionally, US can induce multiple biological effects such as thermal, mechanical, and chemical effects, which can be used for therapeutic purposes (Figure 3).[111]

### US-Guided Thermal Effects

US energy is converted into heat energy and absorbed by the body, thus, raising the tissue temperature in the body to 40°C-45°C. Different tissues have different sensitivities to temperature, with 43°C typically as the cut-off. Temperatures

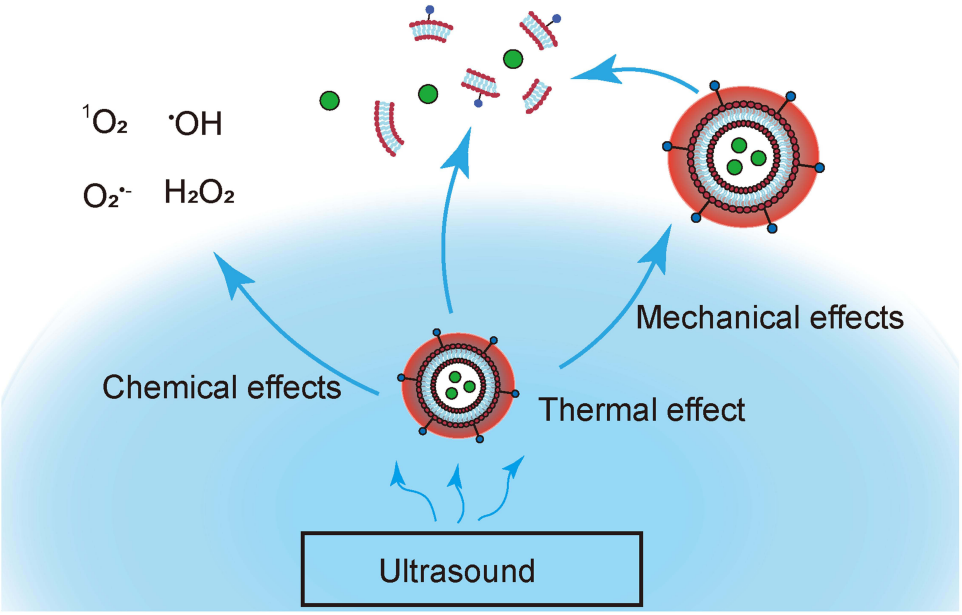



**Figure 3** Ultrasound induces thermal, mechanical and chemical effects.
**Note**: It is worth emphasizing that these effects are not independent, but occur simultaneously and interact with each other.

below 43°C are considered mild hyperthermia, which can cause vasodilation, increase blood flow, and increase the permeability of vessel walls. Thermosensitive liposomes (TSLs) encapsulate hydrophilic drugs in a core surrounded by a lipid bilayer, and they respond to thermal signals and cause changes in the fluidity of the phospholipid bilayer. As the temperature increases, TSLs deform and eventually rupture, contributing to drug release at the site of the lesion.[112] Some researchers used chemotherapy and thermotherapy in combination, where the loaded chemotherapeutic drug and the photothermal agent were released into the HCC tumor area in a controlled way, thus, enhancing the synergistic effect for the treatment.[103–105,113] However, TSLs have some limitations in their practical application, such as causing damage to normal tissues and reduced drug release. To overcome such limitations, recent studies have focused on the structural design of liposomes. For example, TSLs were developed as a novel drug-controlled release system–ThermoDox®–with a relatively short half-life. To take advantage of the short half-life for cycling, thermotherapy is usually administered immediately before or after drug delivery, thus, overcoming the dependence of passive targeting of liposomes to solid tumor sites and ensuring free drug penetration through the tumor mesenchyme.[114,115] Another thermal application of US —thermal ablation of tumors—is achieve by HIFU and occurs at temperatures above 43°C. Such high-temperature processes are classified as intense thermotherapy and lead to faster protein denaturation and necrosis.[116] Thus, liposomes can enhance the therapeutic intensity. In a study by Feng et al, liposomes containing ammonium bicarbonate [Lip-ABC] produced bubbles after HIFU, which not only enhanced the quality of US imaging, but also further enhanced the ablation of tumors by vaporizing the liposomes into microbubbles through acoustic droplets.[117] Liposomes can also increase the precision of treatment. Zhou et al developed adriamycin/indocyanine green (DOX/ICG)-loaded liposomes (DILPs) and combined them with radiofrequency ablation for the treatment of HCC. ICG is a contrast agent for multispectral photoacoustic tomography (MSOT) and facilitates the detection of lesions as small as 2.5 mm; a level of precision that is difficult to achieve by conventional imaging.[118]

### Ultrasound-Guided Mechanical Effects

Mechanical effects mainly include the cavitation effect and the sonoporation effect.[119] The sonoporation effect refers to the formation of pores in the cell membrane from US irradiation, which allow the transfer and accumulation of molecules and nanoparticles into the cell; thus, enhancing the delivery of liposomal drugs.[120] The cavitation effect is classified into stable cavitation (SC) and inertial cavitation (IC) according to the way the US-induced bubbles burst.[121] At low acoustic

pressures, bubbles usually exhibit SC,[122] while at high air pressure, bubbles collapse instantaneously, thus, leading to mechanical damage called IC.[123] In addition, the cavitation effect produces ROS through the thermal dissociation of water.[124] This is referred to as sonodynamic therapy in clinical applications; a combination of low-intensity US and chemotherapeutic agents, which has been explored as a promising alternative for cancer treatment.[125]

Zhao et al prepared multifunctional US molecular probes, hyaluronic acid-mediated cell-penetrating peptide-modified 10-hydroxycamptothecin-loaded phase-transformation lipid nanoparticles (HA/CPPs-10-HCPT-NPs), which actively target the CD44 receptor and accumulate in HCC cells. After low-intensity focused ultrasound (LIFU) irradiation of the lesion, the nanoparticles transform into gaseous microbubbles to enhance US imaging at the cellular level. In turn, US triggers targeted microbubble rupture (UTMD) for the synergistic physicochemical treatment of tumors.[126] Similarly, Klibanov et al studied microbubble complexes coated with liposomes, where US was used to induce microbubble ruptures and trigger liposomes for drug delivery.[127]

### US-Guided Chemical Effects

The process of US-guided chemical effects is closely linked to the cavitation effect, where vacuoles are formed after US waves pass through the blood-rich liver, and vacuole rupture generates large amounts of ROS that kill the diseased tissue.[128]

Echo-liposomes containing gas act as acoustic sensitizers to overcome the hypoxic tumor microenvironment. They are activated to produce ROS, including singlet oxygen ($^1O_2$), hydroxyl radicals ($^{\bullet}OH$), superoxide anions ($O_2^{\bullet-}$), and hydrogen peroxide ($H_2O_2$).[119,129] Lin et al described a 2, 2'-azobis [2-(2-imidazolin-2-yl) propane] dihydrochloride (AIPH)-loaded liposome (Lip-AIPH) that instantaneously generated bubbles and produced large amounts of ROS.[130] It was also used as an ultrasound contrast agent because the air interface produced very high contrast between the circulation and the surrounding tissues.[131]

## Near-Infrared (NIR)

NIR light imaging has been widely discussed in recent years. ICG is a fluorescent dye that absorbs and emits green light in NIR light and can identify and characterize tumors and metastatic lymph nodes. Thus, NIR fluorescence can be used to monitor cancer tissue resection during surgery.[132] NIR light causes the least damage to the human body because its wavelength is in the biologically harmless range (650–950 nm).[133]

Experts have proposed photodynamic therapy (PDT) and photothermal therapy (PTT) as new options for tumor ablation. PDT can directly kill tumor cells, because during the therapy, ROS cause the apoptosis of tumor cells, while the immune response can also kill tumor cells.[134] Kaneko et al evaluated the effectiveness of PDT using ICG and NIR, and found that the dye was directly absorbed by the HuH-7 tumor cell line.[135] Liposomes can convert light energy into thermal energy,[136] and with the aid of NIR-induced photothermal therapy, tumor ablation was achieved by heat stress and the thermal-induced release of therapeutic molecules.[137] Peng et al prepared liposomes responsive to NIR to co-deliver DOX and the molecular targeting agent, sorafenib (SF). The disintegration of liposome structures under NIR light resulted in rapid drug release at the tumor site, which greatly enhanced the synergistic chemotherapeutic effect.[138] Mu et al designed liposomes targeting Glypican-3 (GPC3) containing SF and IR780 iodide (IR780) (GSI-Lip) to perform NIR fluorescence imaging, and found that this system responded to photothermal therapy to improve the accuracy of HCC diagnoses.[139]

## MRI

In recent years, liposomes have become an integrated platform for MRI imaging and therapy due to their excellent carrier properties.[140] Platform can be combined with metallic elements to achieve therapeutic implications using their unique properties. For example, $Fe_3O_4$ is commonly used as a $T_2$ contrast agent for MRI due to its unique superparamagnetic properties.[141] Recent studies have shown that $Fe_3O_4$ exhibits photothermal conversion under NIR irradiation.[142] In Shen's study, multifunctional magnetic nanoparticle-loaded thermosensitive liposomes ($Fe_3O_4$-TSL) were developed for NIR-triggered release and combined with the photothermal-chemical treatment of tumors. After intravenous injection, $Fe_3O_4$-TSL was enriched in tumors over time and exhibited significant MRI and photothermal effects.

Gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA) is also a widely used contrast agent for MRI.[143] Xiao et al prepared SF and Gd co-loaded liposomes (SF/Gd-liposomes) to improve the aqueous solubility of SF and the accurate monitoring of its distribution.[144] It was shown that SF/Gd-liposomes facilitated the MRI-guided visualization of liposome delivery and in vivo biodistribution with longer visualization times and higher signal enhancements in tumor tissues.[145]

## Opportunities and Challenges for Future Applications of Liposomes

Liposomes are ideal tools for the application of HCC therapy. They are easy to prepare, highly biocompatible, adjustable in size, and capable of encapsulating highly toxic chemotherapeutic drugs. They can also respond to stimuli such as high temperatures, pH value, and US to release drugs into the diseased tissue in a targeted manner.[146,147] Given these advantages, why are liposomes still limited to basic research and have not been widely used for clinical applications? Liposomes are prone to degradation through hydrolysis and oxidation, which is due to their inherent instability.[12] Therefore, adjusting the structure of liposomes, controlling the drug loading and drug release rate, overcoming the rapid clearance of liposomes, and increasing the residence time of liposomes in tissues will accelerate their clinical applicability.[148] To this end, this paper makes the following recommendations for the future use of liposomal drugs.

(1) Active targeting of ligands to control the release of drugs. There are many studies on highly expressed ligands for HCC cell membranes, however, few studies have targeted HCC vascular endothelium, or the organelle. Integrin-modified liposomes have been reported to modulate the production of breast cancer.[149] Through reading much documents, we found studies in HCC are still lacking but potential.

(2) Triggered release. There are two main trigger types, including remote triggers (eg, heat, US, and light), and local triggers (eg, enzymes and pH changes) at the disease site or in organelles.[150] For remote trigger release, we must further consider the issue of metastatic foci, and a whole-body Positron Emission Tomography-Computed Tomography (PET/CT) scan is required to determine the location of the lesion and improve the efficiency of treatment before operating.[151]

(3) Combination therapies. Combination therapies with different mechanisms can be used for the development of liposomes. In addition to traditional drug therapies, immunotherapies and gene therapies also have broad applicability, but how to combine them will be a primary area of focus in the field of nanomedicine in the future.

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## Author Contributions

Yitong Li, Ruihang Zhang, Zhen Xu and Zhicheng Wang designed the research, Yitong Li, Ruihang Zhang and Zhen Xu wrote the manuscript, Zhicheng Wang completed the revising. All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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