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The emerging role of lipid nanosystems and nanomicelles in liver diseases

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Abstract. – Nanoparticles (NPs) exhibit remarkable potential in the diagnosis and treatment of various liver ailments, including primary liver cancer or hepatocellular carcinoma (HCC), liver cirrhosis, viral hepatitis, and alcoholic and non-alcoholic liver diseases. High surface area-to-volume ratio with distinct physicochemical and bio-pharmaceutical properties have contributed numerous benefits to NPs, such as high intracellular uptake and efficient drug delivery capabilities stemming from their ability to encapsulate a diverse range of drugs. Lipid-based nanosystems have demonstrated significant potential as reliable and efficient transport vehicles for a variety of actives, including small interfering RNA, targeting the liver, owing to their excellent *in vivo* compatibility, biodegradable nature, and non-toxic properties. Multiple aspects of various lipid-based materials, lipid nanosystems like solid lipid NPs, nanovesicles such as nanoemulsions, liposomes, and nanomicelles for liver-specific active targeting have been comprehensively reviewed. Ongoing and completed clinical trials of lipid nanosystems developed for HCC, hepatic fibrosis, and hepatitis are tabulated. Types of receptors and ligands typically used for active liver targeting in HCC are extensively discussed. The US FDA's recent approval for the use of Onpattro (Patisiran) injection to treat polyneuropathy in adult patients is indicative of the rapid development of lipid nanosystems employed for hepatic targeting. Nanoemulsions loaded with diagnostic imaging agents for multi-modal liver imaging were briefly discussed. Emerging technologies are being developed to integrate desirable properties of nanoparticles (NPs), including high stability, efficient drug loading, opsonization avoidance, active liver targeting, and facilitation of endosomal escape. Clinical translations of many lipid NPs for drug and gene therapy applications targeting different liver diseases are expected in the near future.

Key Words:
Liver targeting, Liver diseases, Solid lipid nanoparticles, Nanoemulsions, Lipsosomes, Nanomicelles, Clinical trials.

Abbreviations

ALD: alcoholic liver disease; ApoB: apolipoprotein B; ApoE: apolipoprotein E; ASGPR: asialoglycoprotein receptor; CLD: chronic liver disease; DLin-MC3-DMA: dilinoleylmethyl-4-dimethylaminobutyrate; DMG: dimyristoyl- sn-glycerol; DOPE: dioleoylphosphatidylethanolamine; DOX: doxorubicin DSPC: dis-tearoyl-sn-glycero-3-phosphocholine; ECM: extracellular matrix; EPR: enhanced permeability and retention; ETO: etoposide; FR: folate receptor; FTLs: folate-targeting liposomes; Gal: galactose; GalNAc: N-acetylgalactosamine; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HSCs: hepatic stellate cells; KCs: Kupffer cells; KSP: kinesin spindle protein; LDL: low density lipoprotein; LDLR: low-density lipoprotein receptors; Lf: Lactoferrin; LNPs: lipid nanoparticles; MEND: multifunctional envelope-type nano device; MPS: mononuclear phagocyte system; NAFLD: non-alcoholic fatty liver diseases; NE: nanoemulsion; NTLs: non-targeting liposomes; PDGFR-β: platelet-derived growth factor receptor-β;

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pDNA: plasmid DNA; PEG: polyethylene glycol; RBP: retinol binding protein; RGD: arginine–glycine–aspartate; RGD: arginyl glycyl aspartic acid; SECs: sinusoidal endothelial cells; SIM: simvastatin; siRNA: small interfering RNA; SLNs: solid-lipid nanoparticles; ssPalm: ss-cleavable and ph-activated lipid-like mate-rial; TPGS-LA conjugate: lactobionic acid-conjugated D—tocopheryl PEG 1000 succinate; VEGF: vascular endothelial growth factor.

Introduction

Chronic liver disease (CLD) is characterized by an ongoing inflammation process that progressively impairs various metabolic, immunological, and endocrine liver functions. This deterioration can lead to architectural distortion of liver tissue, formation of liver nodules, vascular reorganization, and neo-angiogenesis, ultimately resulting in liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). The high prevalence of CLD worldwide, with approximately 1.5 billion cases, combined with limited treatment options, has led to it being recognized as a significant global health issue[1]. It appears most often in patients having various etiological conditions like liver cirrhosis, hepatitis B (HBV) and hepatitis C virus (HCV), alcoholic liver disease (ALD), non-alcoholic fatty liver diseases (NAFLD), non-alcoholic steatohepatitis, adiposity, diabetes mellitus, consuming food adulterated with aflatoxin, hemochromatosis, even though effective treatments are currently available for HCV infection[2]. The most frequent causes reported for CLD are NAFLD (59%), followed by viral hepatitis (38%), and ALD (2%)[3-5]. ALD encompasses a range of disorders that involve the alcoholic fatty liver, with or without hepatitis. On the other hand, NAFLD is linked with metabolic syndromes like obesity, hyperlipidemia, and diabetes mellitus. Some individuals with NAFLD may develop non-alcoholic steatohepatitis, which can result in liver fibrosis. NAFLD is considered the major cause of CLDs and the main indication for liver transplantation[6]. Epidemiologists expect that the mortality rate of liver-related diseases will continue to increase in the forthcoming years[6]. In 2016, cirrhosis was the fifteenth most common cause of morbidity and the eleventh most common cause of death globally. NAFLD has a 24% estimated global prevalence rate[7] while 250 million people are infected with HBV[8] and 71 million people are infected with HCV globally[9]. In several developing nations, chronic infections of hepatitis B, C, and D are the primary causes behind the development of CLD. Hepatitis Bx antigen modifies the expression of genes in viral hepatitis by persistently triggering cytoplasmic signaling pathways and encouraging epigenetic-mediated changes in the regulation of cellular genes. Evidence[10] suggests that the development of HCC from CLD and persistently elevated levels of HBV replication are related. Genetic causes of CLD include Alpha-1 antitrypsin deficiency, hereditary hemochromatosis, and Wilson disease. Though vaccination, screening, and anti-viral treatments have successfully reduced CLD burden in certain regions by targeting hepatitis B and C, however, the global burden of CLD and cirrhosis is significant. The increasing prevalence of injection drug use, alcohol misuse, and metabolic syndrome still pose greater challenges to these advancements[1].

The primary aim of this review article is to comprehensively present emerging roles and positive outcomes of lipid-like materials, lipidic nanosystems such as solid-lipid nanoparticles (SLNs), nanovesicles like liposomes, nanoemulsion (NE), and nanomicelles in targeted drug delivery, gene therapy and diagnostic imaging in the treatment of major liver diseases.

Molecular Pathogenesis of Liver Diseases

Extracellular matrix (ECM) deposition brought on by recurrent and chronic liver injuries resulting from any etiological circumstances is known as hepatic fibrosis. In case of chronic liver injury, activated hepatic stellate cells (HSCs) become proliferative fibrogenic myofibroblasts that express inflammatory receptors like chemokine receptors, *intercellular adhesion molecule-1* (ICAM-1), and other inflammatory mediators. Extracellular signals coming from the ECM, stimuli from damaged parenchymal cells, and infiltrating inflammatory cells all work together to encourage the activation of HSCs. Gene and phenotypic expression in liver cells are altered during the pro-inflammatory or initiation period, rendering them more vulnerable to inflammatory cytokines. This results in the buildup of ECM and the gradual development of fibrosis[11,12]. The accumulation of ECM proteins, particularly collagen type I and III, largely provided by HSCs, distorts the hepatic architecture by forming fibrous scars, and the consequent development of nodules of regenerating hepatocytes defines cirrhosis or advanced liver fibrosis[13]. The persistent deposition

of ECM interferes with several critical liver functions, including detoxification, and can disrupt hepatic blood flow.

Liver injury can result in genetic modifications that lead to changes in hepatocytes, such as dedifferentiation into precursor cells or trans-differentiation into biliary-like cells. These alterations in cell plasticity can contribute to the development of intrahepatic cholangiocarcinoma, indicating abnormal hepatocyte behavior[14,15]. Furthermore, high hepatocyte generation during the progression of liver diseases like cirrhosis and fibrosis can culminate in epigenetic modification and trigger oncogenetic pathways, eventually leading to HCC[16]. A schematic diagram depicting various pathogenic events leading to the development of HCC is shown in Figure 1. The metabolic biomarker α-fetoprotein is considered the most important biomarker to assess HCC[17]. Another metabolic biomarker is des-γ-carboxy-prothrombin, which is an unusual type of prothrombin that is involved the in the induction of the malignant transformation of HCC cells[18]. Glypican-3, Golgi Protein-73 and Squamous Cell Carcinoma Antigen are also metabolic biomarkers that are involved in HCC[19]. The correlation between ABCC1 and the clinicopathological features of HCC has been identified, highlighting ABCC1 as an independent prognostic indicator for HCC[20]. It has been widely accepted that chromosomal instability is associated with clinical and pathological changes in solid tumors, such as HCC.

A recent study[21] has provided evidence suggesting the involvement of FOXD2-AS1 in the malignant progression of HCC by upregulating TWIST1. Various reviews suggest that identifying and characterizing target genes within 1q21 amplicons is significant to understanding the initiation and progression of HCC carcinogenesis.

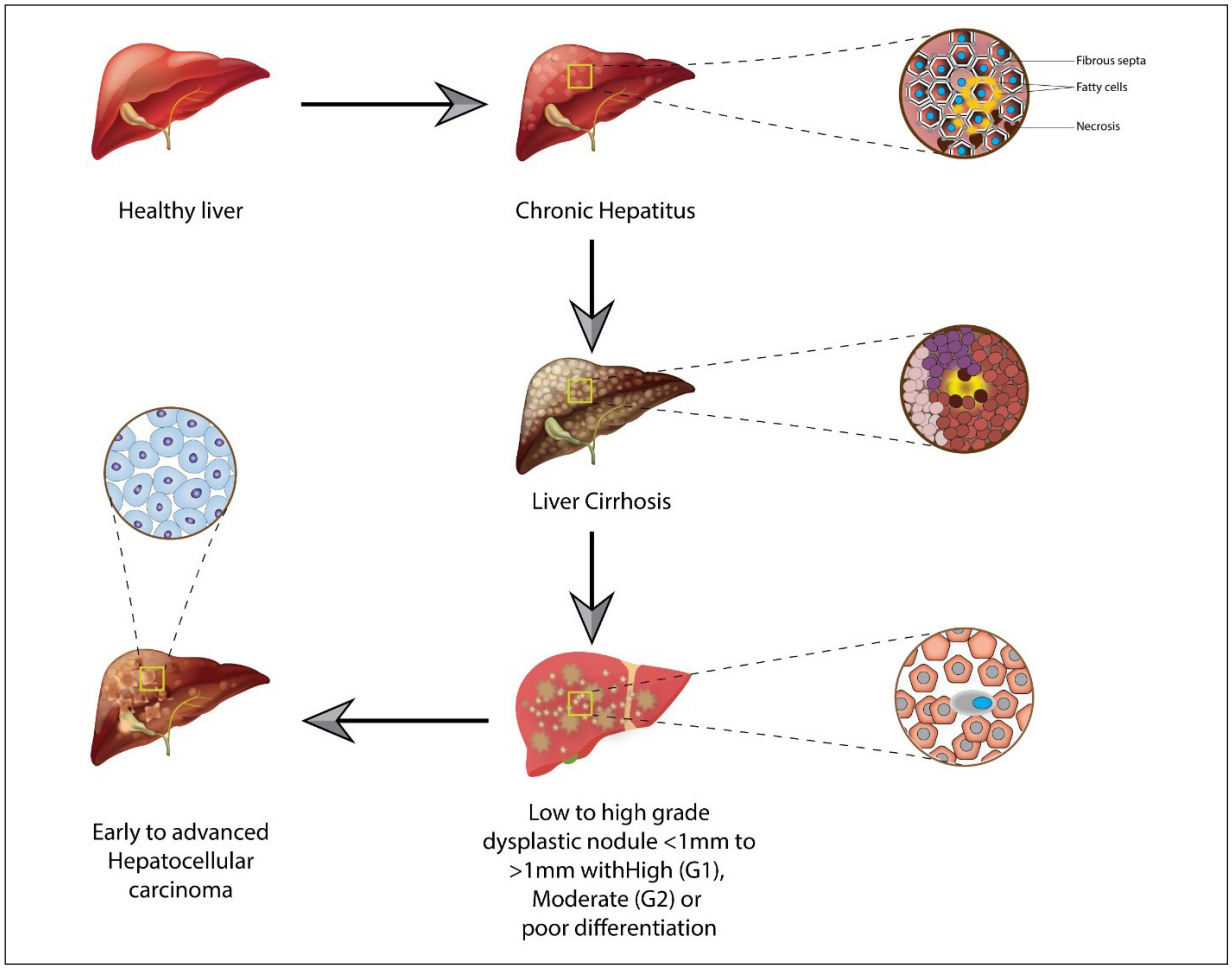

Figure 1. A schematic diagram depicting various pathogenic events during the development of HCC.

Classification of HCC

Hepatitis viruses serve as a major etiological factor for hepatitis, but other factors, including infections, exposure to toxic substances (such as alcohol and certain drugs), and autoimmune disorders, can also lead to this condition. Hepatitis viruses come in five different types: A, B, C, D, and E. Hepatitis A is an RNA virus from the *Picornaviridae* family, while HBV is a DNA virus and is a member of the *Hepadnaviridae* family. Being an RNA virus, HCV belongs to the Flaviviridae family and has a single serotype, at least six major genotypes, and over 80 subtypes of the virus. Hepatitis D is a single species of RNA virus in the Deltavirus genus, and Hepatitis E is a single species of RNA virus in the Hepevirus genus. Automated detection and classification of liver fibrosis stages using 2D contourlet transform and nonlinear features have been proposed. Numerous crucial properties of the contourlet feature transform, including multiresolution, directionality, and asymmetry, which aid in abnormality discrimination. To categorize liver fibrosis into different stages, a kernel discriminant analysis-based feature reduction technique and an analysis of variance-based feature ranking method were combined[22].

The most widely used system employed to classify HCC is Barcelona clinic liver cancer staging, endorsed as a standard system by the European Association for the Study of the Liver, American Gastroenterology Association, American Association for the Study of Liver Diseases, and the European Organization for the Research and Treatment of Cancer[23]. Due to specific molecular signatures in HCC, alternative categorization based on immunologic, metabolic, genomic, and chromosomal characteristics have been attempted[24]. Recently, immunological classification in HCC resulted in grouping under two databases (The Cancer Genome Atlas, Liver Cancer-RIKEN, JP)[25]. There is currently no universally recognized method for evaluating HCC patients due to the disease's extremely complex heterogeneity, despite the fact that a number of staging and scoring systems have been proposed. Therefore, clinicians engaged in treating HCC patients must thoroughly evaluate the features and limitations of the currently available staging system and treatment algorithm.

Basic Concepts of Liver Targeting

When nanoparticles (NPs) are injected intravenously and enter the bloodstream, their non-specific interaction with serum proteins can lead to aggregation or opsonization[26]. The opsonized particles with a size of 7 μm and larger can be cleared *via* alveolar capillaries with a size above 7 μm or/ and eliminated by the macrophages of the reticuloendothelial system that exist in the liver, spleen, and bone marrow[27]. The aggregates with a size above 200 nm get internalized by Kupffer cells (KCs) of the sinusoidal endothelial cells (SECs) *via* phagocytosis. At the same time, non-phagocytic SECs eliminate macromolecules and colloidal particles by receptor-mediated endocytosis[28]. Particles with a negatively charged surface further aid this process, resulting in reduced overall dose and time of circulation. Covalent coupling with polyethylene glycol (PEG), often known as the PEGylation approach, was used to create stealth lipid nanoparticles (LNPs) with long circulation time to protect lipid NPs from plasma protein recognition and untimely reticuloendothelial system(RES) clearance[29-31]. One more physiological barrier is the hepatic sinusoidal fenestrations restricting the access to functional hepatic cells namely HSCs and hepatocytes, through the space of Disse or the perisinusoidal space located between a sinusoid and a hepatocyte. LNPs with a particle size range between 100 nm and 200 nm can be ideal for targeting various liver cells[32].

Passive Liver Targeting

The passive buildup of NPs at a particular tissue site depends upon distinctive pathophysiological or anatomical characteristics besides NP properties[33]. Novel targeting approaches often merge both to facilitate passive liver uptake, followed by ligand-mediated endocytosis for effective and specific treatment. Due to the absence of impermeable basal lamina in the liver cells, most NPs display quick passive liver accumulation after intravenous administration[34]. Passive targeting of a specific liver cell is primarily regulated by the size of the hepatic sinusoidal fenestrae selective to hepatocytes and HSC (< 100 nm) on one side or SECs and KCs (> 100 nm) on the opposite side. Passive targeting approaches generally deliver NPs toward nonparenchymal cells located on the KCs of the sinusoidal endothelium for receptor-mediated endocytosis[28]. Besides KCs, SECs are also responsible for clearing small anionic NPs from circulation utilizing the scavenger receptor stabilin-2[35]. The vascular permeability of NPs into tumors typically occurs due to the gap junctions between tumor cells and

endothelial cells, which are around 400 to 600 nm in diameter[36]. In addition to particle characteristics, the injection route influences liver uptake and intra-liver distribution[37].

Active Liver Targeting

The typical distribution pattern of the delivery system is modified by using exogenous cell-specific ligands, such as an antibody, peptide, carbohydrate, or protein, which identifies and subsequently binds to the cell membrane's corresponding receptor or lipid components[38]. The hepatic cells of non-parenchymal origin consist of SECs, KCs, and HSCs, while hepatocytes control particular liver functions, demonstrating typical morphologies, pathoanatomical attributes, and physiological functions[39]. Hepatocytes are a target for different diseases such as HCC, malaria, and hepatitis virus[40,] while liver fibrosis generally affects HSCs producing an excess amount of ECM as a result of injured KCs, hepatocytes, and SECs[41]. Thus, targeted therapies in these diseases should deliver the actives to these distinct liver cells for maximum therapeutic efficacy and minimum adverse effects. After passive hepatic uptake, the target ligand/moiety attached to the NPs binds to a specific receptor expressed on the liver cell, initiating endocytosis. In the liver, different cell types contain various receptors, possibly suitable for drug targeting. The asialoglycoprotein receptor (ASGPR) is targeted through various moieties for hepatocyte drug delivery[42].

Our research group investigated the possibility of improving the therapeutic efficacy of gemcitabine by encapsulating it within galactosylated chitosan NPs. Hepatic targeting of NPs was observed due to the direct targeting of ASGPRs overexpressed on hepatocytes[43]. Galactosylated chitosan was synthesized by condensing chitosan and lactobionic acid using crosslinking agents namely, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide and N-hydroxy succinimide. Drug-loaded chitosan NPs were formulated (G1-G5) with differing amounts of drug and polymer by ionotropic gelation method. When compared to pure gemcitabine, the optimized batch showed accelerated plasma elimination of gemcitabine (>70% in 30 minutes) in rats induced with HCC, indicating a higher hepatic uptake. Preferential accumulation of the drug in hepatocytes (64% of the given dose) indicated promising liver-targeted delivery and reduced systemic adverse effects (Figure 2). In addition to measuring the blood levels of tumor marker α-fetoprotein,

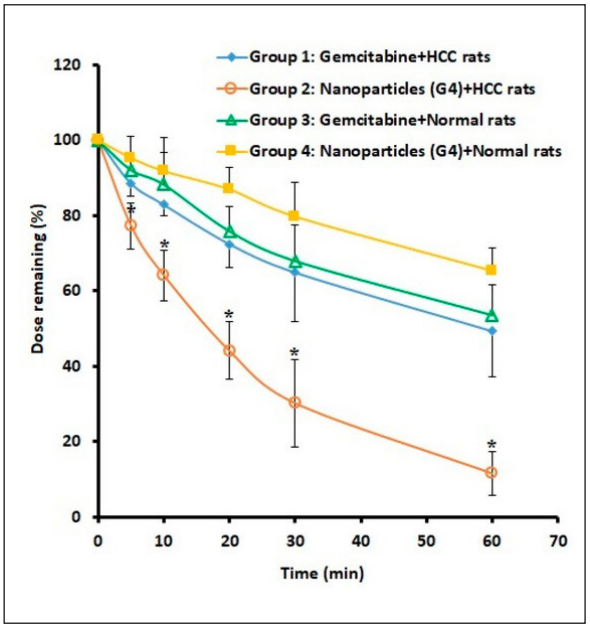

Figure 2. Comparative organ and plasma distribution in HCC-induced rats 60 min after intravenous administration of free gemcitabine and nanoparticles loaded gemcitabine (G4). *Significant difference ($p < 0.0001$) of gemcitabine accumulated in liver tissues of HCC-induced rats (group 2) compared to the amount in liver tissues of other treatment groups (1, 3 and 4) (adapted from[43], published by MDPI, 2019).

histological and biochemical studies were also used to confirm the therapeutic efficacy of G4 in rats. At the end of the experimental investigation carried out in the diethylnitrosamine-induced HCC animal model, the pure gemcitabine-administered and G4-dosed groups demonstrated hepatoprotective effects with minimum cellular injury. Immunohistochemical examination revealed an enhanced level of tumor suppressor gene, *p53*, confirming the anti-HCC efficacy of G4. The study concluded liver targeted delivery and increased *in vivo* performance of gemcitabine by G4, which can be suggested as a potential drug carrier in HCC.

Active targeting methods resulting in endocytosis mediated by the ligand for the transportation of drugs to different types of hepatic cells have been extensively reviewed[44]. Figure 3 illustrates the receptors and their corresponding targeting moiety that have been employed to actively target nanoparticles (NPs) to liver cells.

It has been reported that the manipulation of the adsorbed protein corona on NPs can be utilized to achieve active targeting through en-

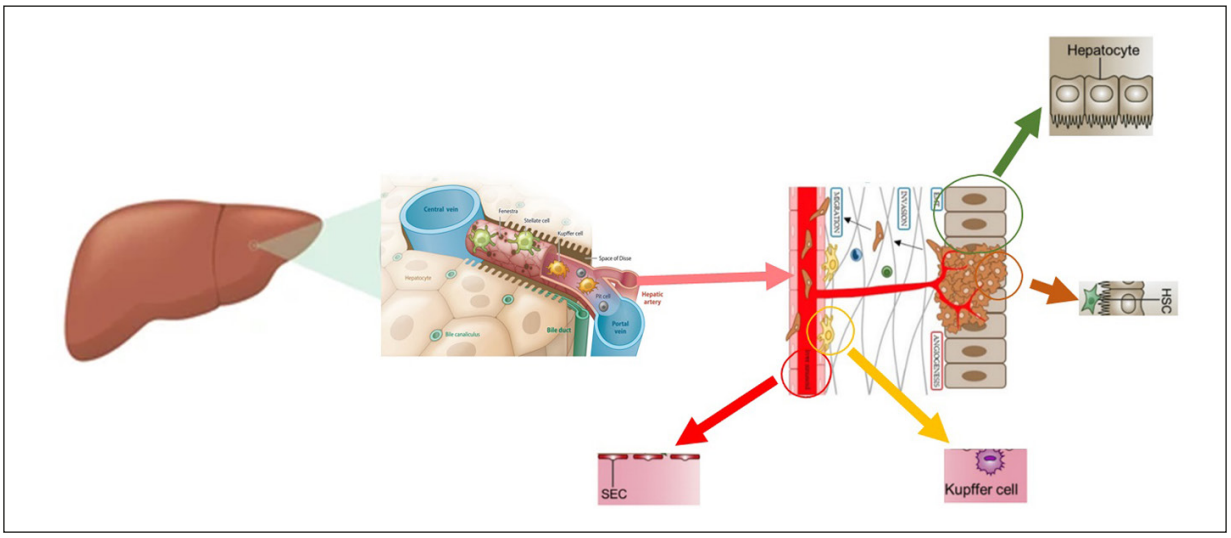

Figure 3. Names of receptors generally exist at specific hepatic cell types meant for active drug targeting.

dogenous systems. This approach aims to target cell-specific receptors in order to enhance the efficiency of drug delivery[45]. For instance, apoprotein-mediated NP-bound drug accumulated extensively in the low-density lipoprotein receptors (LDLR) expressing hepatocytes[46]. The liposomal surface coated with vitamin A showed the selective adsorption of a native retinol-binding protein (RBP). Vitamin-coupled liposomes carrying heat shock protein 47 (HSP47) suggest a novel approach to ameliorate fibrosis in chronic graft-versus-host disease by targeting HSP47^{+} myofibroblasts without initiating immunosuppression[47]. Currently, Nitto Denko and Bristol-Myers Squibb are developing retinol-liposomes to direct small interfering RNA (siRNA) antagonized to HSP47 as a treatment for advanced hepatic fibrosis[48].

Nanoparticles

Nanoparticles (NPs) have multiple advantages due to their high surface-to-volume ratio and distinctive physicochemical properties. These advantages include a high payload capacity, significant intracellular uptake, target-specific delivery, and the ability to incorporate a wide range of drugs[49], making them effective drug transport agents[50]. The controllable shape, size, ability to encapsulate various and multiple constituents, and functional surface characteristics of NPs impart exceptional benefits like improved internalization and penetration, extended circulation time, modifiable drug release, improved drug pharmacokinetics, high contrast, as well as reduced adverse events[51]. Though multiple types of NPs are prepared through different methods, most clinically approved NPs are fabricated using either polymers or lipid materials. In the last few decades, nanoparticulate lipid carriers have acquired tremendous interest as a promising vehicle in various drug and gene delivery applications[52]. Lipid nanosystems offer several benefits as a drug delivery carrier, including customized release, improved intracellular transport, excellent stability, reduced decomposition, scalability, cost-effectiveness, better *in vivo* acceptance, and the ability to be tailored for different administration routes[53,54]. Lipids employed to formulate these NPs have the potential capacity to decrease the undesirable effects typically associated with polymeric nanoformulations[55]. Moreover, lipid nanoparticles have the capability to encapsulate a variety of therapeutic molecules, enhance the bioavailability of poorly water-soluble drugs, and protect them from early elimination.

Functional Role of Nanoparticles in Liver Targeting

The rate of uptake and retention of nanoparticles in cells was observed to be significantly associated with various factors, such as surface charge, size, and ligand properties of the nanoparticles. Passive targeting of a specific liver cell is primarily regulated by the size of the hepatic sinusoidal fenestrae selective to hepatocytes and HSC (<100 nm) on one side or SECs and KCs (>100 nm) on the opposite side. It can be assumed

that NPs with a particle size range between 100 nm and 200 nm can be ideal for targeting various liver cells[32]. Since any systemic administration of NPs will eventually be engulfed by KCs, they might work well as a carrier to reduce or eliminate pro-inflammatory markers and cytokines that occur during an immune response during liver injury[56].

Transient interactions with SECs are believed to be responsible for the extravasation of larger particles (<400 m) into the space of Disse[57]. The distribution of NPs in liver cells was studied using polyaconitylated human serum albumin liposomes as a model. These liposomes were found to accumulate in the liver to a much more than control liposomes, with 80% of the injected polyaconitylated human serum albumin liposomes (17-fold) present in hepatic tissues just 30 min after intravenous injection. Maximum NPs were taken up by liver SECs, while the rest were sequestered by KCs[58].

HSCs residing in the space of Disse are mainly involved with the secretion and maintenance of ECM and therefore, HSC targeting NPs may have implications in liver fibrosis. Furthermore, NPs can be targeted towards specific surface receptors found in HSCs, including but not limited to the mannose 6-phosphate/insulin-like growth factor-II receptor, type VI collagen, integrins, and RBP[59]. However, before interacting with stellate cells, the majority of NPs are expected to be taken up by liver SECs and KCs. Selective interaction of NPs with HSCs can be achieved by cyclic peptides containing the arginine-glycine-aspartate (RGD) motif that can bind to the collagen type VI receptors on HSCs[60]. Scholars[61] have shown that HSCs can sequester human serum albumin conjugated with RGD peptides. A similar trend was shown by RGD-labeled liposomes compared to liposomes without RGD peptide.

The delivery of drug molecules, siRNA, and antibodies for liver targeting and imaging can potentially be carried out using liposomes, SLNs, polymers, and targeting moieties. Moreover, conjugation with target ligands could pave the way for diverse applications, including drug delivery, radiology imaging, and diagnosis. Liposomes are presently being evaluated in clinical trials for the treatment of liver fibrosis and HCC. This is because liposomes have the ability to encapsulate both hydrophobic as well as hydrophilic drugs, showcasing their superior efficacy compared to other nanoparticle varieties. Surface-modified serum albumin-based liposomes of human origin can be considered an efficient transport vehicle for therapeutic activities. These liposomes can be delivered to activated HSCs *via* specific protein binding pathways, offering the potential for liver fibrosis treatment[62] and also in HCC therapy[63]. Exploring siRNA to bring about a gene-silencing effect without inducing immuno-response provides an excellent opportunity to control post-transcriptional gene expression. In this context, lipid-based nanosystems demonstrate sufficient resistance to nuclease degradation, enabling them to deliver the desired effect while avoiding off-target regions and promoting cellular uptake. The lipid-based materials commonly used for the construction of these nanocarriers possess noteworthy tissue compatibility and tolerance characteristics as they are non-toxic and non-immunogenic. Presently lipid NP therapeutics have been extensively researched to treat various liver diseases[64]. Moreover, lipid nanosystems have been considered as most effective transporting carrier for short interfering RNA (siRNA) based formulation as endorsed by the recent clinical approval of Onapattro from the Food and Drug Administration (FDA) in genetic disease of transthyretin-mediated amyloidosis[65]. Protein aggregates like human serum albumin and bovine serum albumin NPs are capable of specifically targeting the liver in the treatment of liver fibrosis as well as HCC[66]. As a nanocarrier, nanomicelles offer numerous advantages, such as enhanced structural stability, reduced toxicity, the ability to encapsulate a high payload of actives, stimuli-responsive drug release, and improved aqueous solubility in water[67]. Nonetheless, the drug loading and long-term stability of these nanoparticles (NPs) are considerably restricted due to the reduced dimensions of the polymeric micelles[68]. Polymeric NPs have been investigated for numerous applications mainly because of their synthetic adaptability to modify their physicochemical and biopharmaceutical properties[69]. Surface-modified polymeric NPs decorated with target ligands for binding with specific receptors overexpressed on hepatic cells are extensively evaluated for liver targeting. A common feature of lipid-based and polymer-type NPs is their tendency to accumulate in regions with high vascular permeation contributed by inflammation, infection, or tumor typically associated with liver diseases[70]. The enhanced permeability and retention effect (EPR) has been shown to improve nano-based drug delivery systems, opening up possibilities for passive targeting of tumors. The defective devel-

opment of the tumor angiogenesis phase to gain extra oxygen and nutrients resulting in the faulty tumor vasculature along with reduced lymphatic drainage is responsible for this unique phenomenon[71]. However, drug concentration in the target site is less than 2-fold higher from nano-delivery compared to critical normal organs. This usually is less than the therapeutic concentration required to cure most cancer although side effects are significantly reduced because of decreased concentration in normal tissues[72].

Solid Lipid Nanoparticles for Liver-Targeted Gene Delivery

Rapid progress in molecular biology and biotechnology fields has led to significant advancement in the field of gene therapeutics. The latest clinical trials indicated the possible application of siRNA as an approach for suppressing specific gene expression through a process called RNAi (RNA interference)[73]. However, siRNA typically encounters challenges related to *in vivo* degradation, as well as inherent difficulties in crossing membrane barriers due to their relatively larger molecular size (13 kDa) and its negative charge[74]. To achieve pharmacological activity, systemically administered oligonucleotides must undergo several steps. These include withstanding degradation of nuclease in the extracellular space, avoiding elimination *via* the kidneys, escaping non-productive binding by specific plasma proteins, and evading clearance by the RES. They must also cross the capillary endothelium in the anticipated target cell(s) within an organ or tissue *via* transcellular or paracellular routes. Additionally, they need to cross the cell membrane, escape uptake by endosomes, avoid degradation by lysosomes or re-export by exocytosis, and finally enter the desired intracellular site of action[75]. These delivery issues can be resolved to a certain extent by chemical and backbone modification to oligonucleotides and nanosized delivery carriers with or without surface functionalization that can significantly decrease rapid elimination[76].

Lipid-based delivery systems have demonstrated their potential as a feasible and efficient delivery vehicle for gene therapy in clinical trials conducted on humans due to their nontoxic and biocompatible characteristics. To deliver siRNA using particle size, drug-to-lipid ratio, lipid-based systems, lipid composition, and the manufacturing process should be adjusted[77]. Advancement of siRNA-LNPs has made excellent progress, confirmed by the recent FDA approval given for Onpattro has made excellent progress in a class that delivers siRNA to treat a rare hereditary disease termed transthyretin amyloidosis (ATTR amyloidosis)[78]. The lipid constituents of lipid NPs have a significant role in determining the size of the particle, surface characteristics, and its efficiency for encapsulation. The pre-eminently effective LNPs that target liver genes using siRNA comprise three lipid categories: a polarizable amino-lipid, such as DLin-MC3-DMA, a cholesterol-like helper lipid-like DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), and a PEG lipid like 1,2-dimyristoyl-sn-glycerol, methoxy PEG, or PEG-DMG[79]. The goals of utilizing ionizable amino-lipids are twofold: first, to achieve high encapsulation efficiencies of nucleic acids at acidic pH by entrapment, and second, to maintain a neutral surface charge at physiological pH (7.4). The desired surface charge is neutral, *in vivo*, since it prevents non-specific adsorption of negatively charged biomolecules and facilitates endosomal escape[80]. At acidic conditions (pH ~5) of the endosomal compartment, the lipids gain a positive charge, allowing interaction with the anionic endosomal membrane, which leads to temporary destabilization and cytosolic release of the nucleic acid[81]. In the best-case scenario, the complete pDNA-vector complex should break out from the endosomes before the maturation stage to lysosomes.

When compared to other tested lipids like 1,2-dilinoleyloxy-3-dimethylaminopropane and 1,2-dilinoleoyl-3-dimethylaminopropane, the efficacy of 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane lipid *in vivo* for silencing factor VII was observed to be 2.5 times higher, indicating that the ketal linker had greater potency[82]. Helper lipids are frequently included in LNPs that contribute to their stability as well as accelerate the delivery of activity to the cytosol by disrupting the endosomal membrane[83]. Cone-shaped helper lipids can favor the generation of hexagonal II phases like dioleoylphosphatidylethanolamine (DOPE), which aids in the endosomal release of oligonucleotides. Phosphatidylcholine, a lipid possessing a cylindrical structure, provides superior bilayer stability, which is especially crucial for *in vivo* applications[83]. The most often employed helper lipid in LNP-based siRNA distribution is 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), although the actual mechanism is unknown[84]. The cholesterol component in LNP can influence membrane fluidity, lipid packing, and permeability of the bilayer. Regular

incorporation of cholesterol as a helper enhances intracellular delivery and LNP stability *in vivo*. Including cholesterol in the LNP formulation leads to decreased surface area per lipid due to the condensation effect, tighter lipid packing, and decreased membrane permeability. Based on the lipid exchange effect demonstrated in liposomes, it can be assumed that the inclusion of equimolar concentrations of cholesterol would promote structural integrity in LNPs. There is a lack of information on the structural as well as functional properties of cholesterol in LNP-siRNA mixtures[85]. Site-specific delivery is still hindered by LNPs being sequestered in endosomes. Incorporation of C-24 alkyl phytosterols into LNPs improves gene transfection, and a structure-activity study of cholesterol analogs suggests that alkyl chain length, sterol ring flexibility, and the polarity attributable to the -OH group are all crucial factors in maintaining high transfection efficiency[86]. PEG lipids protect the LNP against opsonization and subsequent uptake by the RES system, besides inhibiting their aggregation in the circulation[87]. Additionally, because the PEG moiety creates a hydrophilic-natured steric barrier on the surface of the particle, PEG lipids prevent the aggregation of particles both during the process of formulation and storage. By virtue of this, the PEG-lipid may lessen particle fusogenicity and adsorption of apolipoprotein E (ApoE), both of which are important for the transfection of LNP to hepatocytes[88].

For effective hepatocyte gene silencing effect *via* subcutaneous delivery, significant advancement took place in the development of LNPs for siRNA-mediated drug delivery[89]. The greater accumulation of PEG2000 carbamate LNPs in the liver indicates that, besides particle size, stable PEG coating is a crucial factor in enabling the nanoparticles to travel from the subcutaneous (SC) injection site to the liver. However, it was suggested that coating on LNPs may also hinder efficient uptake into hepatocytes because this process may require association with ApoE. Although they exhibited desirable systemic blood exposure and liver deposition, small ~30 nm LNPs with a stable PEG2000 carbamate coat were less effective in gene silencing activity. However, PEG2000 carbamate containing intermediate size (45 nm) LNP showed adequate liver accumulation, yet it reduced the level of Factor VII by 80% at 1 mg siRNA per kg of body weight. The addition of N-acetylgalactosamine-conjugated PEG lipid at a concentration of 0.5 mol% in approximately 35 nm PEG2000 carbamate LNPs led to a notable increase in activity. This suggests that the observed decrease in activity in LNPs containing PEG2000 carbamate may be attributed to unproductive cellular uptake. Additionally, it was shown that smaller systems (30 nm) exhibited equal activity to intermediate systems when a N-acetylgalactosamine conjugated PEG lipid of 0.5 mol% was added. The results suggest that lower activity observed with PEG2000 carbamate may be partially attributed to inefficient cellular uptake. This research demonstrates how correctly engineered LNP-siRNA systems may effectively silence hepatocyte genes following SC administration.

Most siRNA-based treatments in clinical trials are based on lipid-based NPs because naked siRNA has weak stability, limited bioavailability, and poor systematic distribution. All of these factors increase the risk of adverse effects. Numerous research findings recommended that more effective and immunogenic lipid components be added to produce safe and stable nucleic acid-lipid nanoparticles (SNALPs). These NPs' smaller particle sizes can speed up circulation time and benefit the agglomeration in solid tumors by enhancing the EPR effect. When administered at a siRNA dosage of about 0.005 mg per kg, SNALPs made with the ionizable lipid called DLin-MC3-DMA (dilinoleylmethyl-4-dimethylaminobutyrate) significantly silenced the hepatic genes in mice (ED_{50}). It was discovered that the pKa and lipid structure of the ionizable lipids are essential for efficiently transporting siRNA to hepatocytes. The ideal ionizable amino-lipids for *in vivo* applications in LNPs should have an apparent pKa ranging from 6.2 to 6.5 as they exhibit a perfect balance between a neutral charge in circulation and a substantial positive charge at endosomal pH[82]. The final composition of DLin-MC3-DMA lipid nanoparticles includes DLin-MC3-DMA, cholesterol, DSPC, and 3-N-[(-methoxy poly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxy-propylamine (DMG-mPEG2000) in a molar ratio of 50:38:10.5:1.5. A fast microfluidic mixing technique created SNALPs containing DLin-KC2-DMA (a cationic ionizable lipid), cholesterol, phospholipid, and a PEG lipid. High siRNA entrapment efficiency and effective particle size control are also features of the microfluidic mixing technique[90]. Large-scale manufacturing is made possible by the microfluidic mixing technique, which effectively regulates particle size with high siRNA

entrapment effectiveness[91]. SiRNA is well protected within the lipid NPs by binding to positively charged lipids in a firmly positioned core. The structural properties of LNPs were analyzed using cryotransmission electron microscopy and small-angle X-ray techniques. The results showed that at pH four, siRNA is sandwiched between the ionizable lipid bilayer structures of DLin-KC2-DMA, while at neutral pH, it exists at the center of LNP[92]. The biocompatible formulation comprises zwitterionic lipids that are modified with PEG and cholesterol, and they do not elicit any immune response.

Several SNALPs developed by Tekmira Pharmaceuticals Corporation (Burnaby, British Columbia, Canada) are now undergoing clinical trials. The development of TKM- apolipoprotein B (ApoB) lipid NPs integrating siRNA to silence ApoB expression in the liver for the treatment of hypercholesterolemia was described[93]. It is worthwhile to note that ApoB is the major lipoprotein that enables the transfer of cholesterol and triglycerides from the circulation into the cells. A specific molar ratio of ingredients including cholesterol, DLin-DMA, DSPC, and PEG-carbamoyl-1,2-dimyristyloxy-propylamine are included in the formulation. The resultant vector is 77-83 nm in size, and siRNA is encapsulated with an efficiency of 92-97%. A phase I study in human volunteers demonstrated a transient 20% decrease in LDL levels and associated ApoB protein. However, one subject experienced flu symptoms, suggesting immune system activation, and the study was terminated.

Successful phase I clinical studies[94] have been conducted on ALN-VSP02, a dual-targeted RNAi drug that targets overexpressed vascular endothelial growth factor (VEGF) and kinesin spindle protein (KSP) in solid tumors such as HCC. The lipid NPs formulation includes DLin-DMA and two different siRNA sequences targeting two distinct genes present in this condition. One of the genes, *VEGF*, is overexpressed in various types of cancer and plays a significant role in lymphangiogenesis and angiogenesis. It is anticipated that VEGF blockade will stop angiogenesis, which suppresses, in turn, the development of tumors. It is worth mentioning that the motor protein, KSP, is overexpressed in multiple kinds of carcinoma cells and is essential for the ideal separation of developing spindle fibers during miosis. The likelihood of complement-mediated rather than cytokine-induced responses to ALNVSP02's infusion-related reactions was higher.

Clinical studies for ALN-PCS, an RNAi therapy that targets PCSK9 to treat severe hypercholesterolemia, are already happening. Low-density lipoprotein cholesterol (LDL-C) is reduced in plasma levels by the PCSK9 production inhibitor ALN-PCS, by overexpression of the LDLR and lowering the extracellular and intracellular levels of PCSK9[95]. The ALN-PCS01 lipid nanoparticles combine the 98N12-5(1) 4HCl lipidoid, which acts as a structural stabilizer, with PEGylated lipid mPEG2000-DMG and cholesterol in a molar ratio of 42:48:10. Preliminary results of phase I clinical trial showed that the extent of PCSK9 silencing was dose-dependent, with the highest dose of 0.4 mg/kg resulting in an 84% decrease in PCSK9 protein and a 50% decrease in low-density lipoprotein (LDL) cholesterol. A 50% decrease in LDL cholesterol was shown when DLin-MC3-DMA was used in place of lipidoid in a mild dosage of 0.25 mg/kg[96]. It was demonstrated that SLNs prepared using a hydrophobic ion pairing approach can incorporate and allow continuous and sustained release of siRNA[97]. An example of this is the extended-release of siRNA in mice for 10 to 13 days, which was accomplished by incorporating the positively charged lipid, DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), within the hydrophobic tristearin core, relying on an ionic interaction between the two[98]. It is worth noting that the siRNA-loaded lipid core NPs' ability to silence genes is increased by having a high cationic lipid/siRNA charge ratio. However, excessive cationic lipid concentrations can be cytotoxic by interacting with negatively charged biomolecules[99].

Lipids-like Materials or Lipidoid Nanoparticles

Achieving secure and effective delivery of RNAi therapeutics remains a crucial obstacle to their successful clinical application. Considerable effort has been made to identify and create carriers capable of facilitating the transport of siRNA across the various biological defenses that safeguard the interior of target cells. A novel category of lipid-based substances known as "lipidoids" has been effectively produced using chemical or combinatorial synthesis techniques for the purpose of delivering RNA therapeutics[100]. NP structures formed from lipid-like materials or "lipidoids," are structurally arranged to improve *in vivo* pharmacokinetics, efficacy, safety, and efficient siRNA delivery. Various investigations were carried out to evaluate the transfec-

tion capability and structure-function activity of degradable lipidoid NPs. In immune cells and hepatocytes, it was revealed that lipidoids facilitate potent gene knockdown upon intravenous administration to mice with low values of siRNA EC50, such as 0.01 mg per kg[101]. Upon establishing an *in vivo* structure-activity relationship, it was discovered that four criteria, one pKa, and three structural criteria, consistently anticipate the ability of lipidoid NPs to facilitate protein silencing *in vivo* for more than 95%. This investigation has also recognized an effective lipidoid NP, 304O13, that did not appear to be toxic. Precisely, lipidoids produced from O13 tails and alkyl-amines containing a minimum of one tertiary amine and a minimum of three substitutional sites had more chance of mediating the potent gene silencing in mice. The three structural function criteria significant for efficient *in vivo* gene silencing effects are identified as possessing an O13 tail, three or more tails, and an alkyl amine containing a minimum of one tertiary amine. Out of the four criteria, the pKa criterion seems to be highly significant in the determination of the *in vivo* efficacy of the lipoidal system. The gene-silencing effect in hepatocytes was assessed using lipopeptide nanoparticles, which exhibited a high degree of effectiveness and selectivity in non-viral siRNA delivery. Similar to LNPs, lipidoids are being considered for the purpose of being covered by serum ApoE and being absorbed by hepatocytes *via* LDL receptors in the liver. Lead material cKK-E12 was exceedingly selective towards hepatic parenchymal cells. It showcased silencing effects that were potent in rats (ED_{50} < 0.01 mg per kg), mice (ED50 ~ 0.002 mg per kg), and non-human primates (above 95% silencing at 0.3 mg per kg)[102]. Toxicological studies[103] revealed that cKK-E12 was tolerated very well in rats at a dose (1 mg per kg) over 100 times that of ED_{50}.

Multifunctional Envelope-type Nano Device (MEND) System

The ED_{50} of proconvertin (Factor VII) knockdown in mice after intravenous (IV) administration of second-generation ionizable lipid, YSK13-MEND (pKa,6.45), was found to be more than four times less than that of first-generation ionizable lipid, YSK05-MEND with pKa value of 6.5[104]. In the case of third-generation ionizable lipids, CL4H6 LNPs (pKa, 6.25) ED50 of FVII knockdown in mice was much lower[105]. Another study[106] found that ionizable lipid molecules with higher pKa, such as YSK13-C4 (pKa 6.8) and YSK15-C4 (pKa 7.10), were taken up by liver SECs in greater amounts. These findings suggest that these molecules may be suitable for cell-specific drug delivery in liver-related conditions. Instead of modifying the lipids' chemical structure to achieve the desired pKa value, pH-sensitive cationic lipids, such as YSK12-C4 and YSK05, with pKa values of 8.00 and 6.50, respectively, were mixed at a specific molar ratio to obtain single LNP formulation[107]. The LNPs prepared with a pKa of 7.15 (YSK05/12-LNP) successfully targeted and delivered siRNA to hepatic SECs. When administered at a dose of 0.1 mg per kg targeting the CD31 gene, the optimized YSK05/12-LNP resulted in an mRNA knockdown of almost 60% in SECs. These systematic studies indicated that the ionizable lipids' structural arrangement and properties determine the nanocarrier's nature. Furthermore, it is important for the distribution of LNPs in the liver and their escape from endosomes.

SS-Cleavable and pH-Activated Lipid-like Material (ssPalm)

As a lipid derivative, ssPalm destabilizes the endosomal membrane under lower pH conditions and releases the nucleic acid contents under a reducing acidic environment in the cytoplasm. Under acidic conditions, the positive charge present in the tertiary amines of ssPalm causes cleavage of the disulfide bond, ultimately leading to the disintegration of NPs. The ssPalmE loaded plasmid DNA (pDNA) prepared with vitamin A scaffold demonstrated 15-fold gene transfer ability in comparison to NPs made using a simple acyl chain (myristoyl group)-scaffold (LNP-PalmM)[108]. Imaging studies[109] conducted intracellularly showed that LNPPalmA considerably exhibited endosome-disruptive characteristics. It was concluded that lipid particles delivered to the cytoplasm in particle form *via* the intrinsic nuclear transport system of retinoic acid and subsequently released the enclosed pDNA for efficient gene expression. Similarly, ssPalmE-LNPs having vitamin E as a lipophilic scaffold and tertiary amine structures knocked down hepatocyte-specific markers (factor VII: FVII) more effectively than ssPalmM or ssPalmA[110]. Moreover, an enhanced siRNA transfer efficiency was noticed after ssPalmE developed with tertiary amine attached to the piperidine ring, and the space between tertiary amine and the surface of the particle was increased. After further optimization by fixing the lipid/siRNA ratio, the factor VII

knockdown effect of the improvised ssPalmE-LNP was attained at an ED50 of 0.035 mg per kg, representing a propitious platform used as a hepatocyte-targeting siRNA carrier. HSCs produce extracellular matrices, such as collagen and elastin, leading to the progression of liver fibrosis and liver cirrhosis. An attempt was carried out to deliver siRNA to HSCs using ssPalms containing hydrophobic vitamin A (ssPalmA) or myristic acid (ssPalmM) and E (ssPalmE) as lipophilic scaffolds[111]. When tested in liver fibrosis-induced mice, an ED_{50} of around 0.25 mg siRNA per kg was shown by LNPssPalmA, proving that it is highly efficacious.

Nanoemulsion

Nanocarrier systems, such as nanoemulsion (NE), work as a diagnostic tool or to deliver therapeutic drugs to particular target sites of the body in a controlled way. NEs are submicron (20-200 nanometers) isotropic colloidal dispersion systems that are optically clear or translucent and composed of an oil and aqueous phase stabilized by surfactants and co-surfactants acting as emulsifying agents. These NEs are generally kinetically stable but thermodynamically unstable[112,113]. The oil droplets in IV lipid emulsions are cleared either by metabolism as endogenous chylomicrons or *via* the mononuclear phagocyte system (MPS, such as hepatic KCs and splenic macrophages). Thus, employing NE can increase the biodistribution of drugs in the liver and MPS systems. It was demonstrated that incorporating an antiparasitic drug, primaquine bisphosphate, into NE led to a substantial 2.5-fold increase in liver uptake compared to the drug solution[114]. NE was prepared with phosphatidylcholine, olive oil, lysophosphatidylcholine, cholesteryl oleate, and cholesterol using the method of lipid film hydration, following which vortexing and sonication processes were carried out. The drug was complexed with sodium lauryl sulfate and egg phosphatidic acid, respectively and co-dissolved with the lipid component of the emulsion. In another study, hepatoprotective 2-(allylthio)pyrazine (2-AP)-loaded NE formulation was developed utilizing varying ratios of oil, drug, and lecithin ratio with different co-surfactants[115]. The optimized NE contained water (91%), soybean lecithin (4%), soybean oil (4%), and 2-AP (1%). The *in vivo* investigation demonstrated a significant reduction in clearance and a two-fold increase in drug accumulation in the liver, when compared with drug solution. To escalate the *in vivo* circulation time, imparting hydrophilicity to the NE droplet surface of NE utilizing polyethylene oxide or PEG is often required. However, PEGylation can cause decreased accumulation of the nanocarrier in the liver or mononuclear phagocyte system (MPS). Sorafenib can inhibit the activities of multiple tyrosine and serine kinases, thereby restraining hepatic tumor angiogenesis, cell proliferation, and apoptosis[115]. A NE of sorafenib has been developed using high-energy emulsification with medium-chain triglycerides and lecithin constituting the oil phase – glycerol and polysorbate 80 made up the aqueous phase[116]. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay of the prepared emulsion demonstrated cell viability of the normal cells while killing cancerous cells. The NE-based delivery of sorafenib indicates the practical feasibility of effective and safe parenteral drug administration than conventional tablet formulation since it contains a comparatively less drug and superior therapeutic efficacy.

An oral NE encapsulated with curcumin and 5-fluorouracil demonstrated synergistic efficacy toward hepatic carcinoma and mitigated toxicity[117]. Drug-loaded NE was synthesized using an ultrasonic-assisted emulsification technique. Briefly, NE was prepared by dissolving curcumin, PEG-400, lecithin, medium chain triglyceride in ethyl alcohol to form an oil phase, while the aqueous phase comprised of 5-fluorouracil, Tween 80, and deionized water. The $AUC_{(0-t)}$ of drug-loaded NE was found to be 8.59 times and 8.85 times higher than that of fluorouracil and plain curcumin, respectively. In HepG2 cells, the IC_{50} of prepared NE was 4.6 times and 4.9 times lower than that of fluorouracil and curcumin, respectively. The anti-tumor inhibition rate *in vivo* was significantly enhanced by nano-formulation (49.29%) compared to 4.72% and 24.84% for curcumin and fluorouracil, respectively. The Ki-67 immunohistochemical analysis indicated that CU/FU-LN had a distinct anti-proliferative effect. In L02 cells, the IC_{50} of CU/FU-LN was 1.51 times and 2.60 times higher than that of CU and FU, respectively. Compared to injectable chemotherapeutic agents, orally administered NE-based drug delivery systems most probably would diminish the toxicity and improve efficacy and patient compliance[117]. Antitumor activity of the combinations of anticancer drug, doxorubicin (DOX) and lipid-lowering drug, simvastatin (SIM) in DOX-SIM-solution or NEs (DOX-SIM-NE), was assessed in a Swiss albino mouse model induced with Ehrlich ascites carcinoma[118].

Though an increase in percentage life span was spotted with the DOX-SIM-solution group, DOX-SIM-NE demonstrated decreased side effects on the hematological parameter, such as hemoglobin and lymphocytes as well as hepatocytes of the liver. Furthermore, the entrapment of DOX and SIM into NEs increases the concentration of all biochemical parameters seen in blood compared to the DOX-SIM solution. This investigation proved that encapsulation of drugs within NEs can significantly improve drug delivery efficiency while reducing adverse effects.

Nanoemulsion a Liver Diagnostic Agent

Nanotechnology advancements have revolutionized therapeutics, treatment management, and diagnostic medicine, including intraoperative fluorescence imaging[119]. With the help of fluorescent indocyanine green-directed intraoperative imaging, the fluorescent margin of the carcinomatous tissues can be delineated from the adjacent healthy tissue[120]. However, this novel contrasting agent suffers from limitations such as high background noise, non-specific binding, rapid clearance from the tumor site, degradation, and severe photobleaching issues thus decreasing its detectability or observation time critical for surgical applications[121]. An iodized poppy seed oil called Lipiodol (Andre Guerbet, Aulnay-sous-Bois, France) has been extensively utilized as an embolic material in the transcatheter arterial procedure chemoembolization for treating HCC[122,123]. NE incorporating lipiodol in combination with a fluorescent dye, indocyanine green, has been developed for optical navigation using self-emulsifying nanotechnology[124]. The anti-photobleaching ability and fluorescence stability of NE were significantly improved compared to the indocyanine green probe due to the shielding effect of lipiodol, indicating the high capability of long-standing *in-vivo* imaging during surgery. Complete resection by surgery of the orthotopic HCC-bearing mice was performed after the tumor tissues were precisely defined with a signal-to-noise ratio above 5-fold under the fluorescence guidance of NE. These ultrahigh tumor-to-liver contrast, superior fluorescence performances, and high biosafety indicate the substantial translational potential of NE in enhanced HCC imaging and surgical navigation.

In another study, oil in water NE (60 nm) constituted of the novel, non-toxic contrasting agent,2-3-5-triiodo α-tocopheryl was stabilized by PEGylated and non-ionic emulsifying agent Kolliphor ELP[125]. An extended elimination half-life of 9 h was observed for the NEs, and maximum liver deposition occurred within 24 h. When examined with micro-CT imaging in a mouse orthotopic HCC model, the NEs were observed to be absorbed by the hepatocytes. The visualization of the HCC within the liver could be continuously monitored using NE to check for disease progression for many days. Furthermore, NE could provide image contrast with a resolution similar to MRI. Differentiated HCC cells may not show significant image contrast due to similar uptake of the NEs.

Nanomicelles

They are colloidal drug carriers constituted of amphiphilic molecules that undergo self-assembly in an aqueous environment to create organized supramolecular structures. Nanomicelles have a high potential to solubilize lipophilic molecules in aqueous media and modifiable surface properties effective for controlled drug delivery[126]. Moreover, nanomicelles can encapsulate contrasting agents to improve sensitivity during radiological imaging and diagnosis. Targeted delivery of DOX against HCC utilizing multifunctional micelles was reported[127]. Micelles were developed using pH-sensitive carboxymethyl chitosan chemically conjugated with reactive oxygen species response part phenylboronic acid pinacol ester and covalently linked with active target ligand CD147 monoclonal antibody. The targeting ability of micelles was evaluated in BALB/C nude mice SC injected into the armpit with HepG2 cells (1×10^7). In BALB/C nude mice with HepG2 tumors, the biodistribution studies of micelles loaded with the lipophilic fluorescent dye 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide (Dir) were assessed using the IVIS spectrum system. *In vitro* distribution studies indicated a higher intracellular uptake of specific micelles (DOX-CD147-CMCh-BAPE) compared to another (DOX-CMCh-BAPE). The accumulation in tumor tissue was larger than that of the Dir-CMCh-BAPE group from 3 h to 24 h, according to the stronger fluorescence signal of the CD147-Dir-CMCh-BAPE group.

One of the most effective cytotoxic medications for a variety of cancers, namely lung and testicular cancer, is etoposide (ETO), which blocks type 2 topoisomerase enzyme during cell division[128]. However, the therapeutic response reported with ETO was very low because of numerous resistance mechanisms in carcinomatous

cells[129]. To promote the efficacy of ETO against HCC, lactobionic acid-conjugated D—tocopheryl PEG 1000 succinate (TPGS-LA conjugate) has been characterized as an effective ASGPR-targeted nanomicelle and a possible P-glycoprotein (P-gp) inhibitor[130]. The drug loading capacity and entrapment efficiency of the nanomicelles were 13.06% and 90.99%, respectively. By avoiding the elimination mechanisms, nanosized (~145 mV) positively charged (+15 mV) ETO-loaded TPGS-LA NPs demonstrated targeted delivery to hepatoma cells more effectively. ETO-loaded TPGS-LA NPs were found to increase the cytotoxicity of etoposide in HepG2 cells significantly. *In vivo* data in mice revealed greater internalization and accumulation in HepG2 cells with ASGPR overexpression at the tumor site by the TPGS-LA NPs group. In addition, TPGS-LA NPs also demonstrated a significant inhibitory effect on P-gp compared to non-targeted ETO-loaded TPGS NPs. Following SC administration, the inhibition of tumor growth in HCC-induced mice was increased from 28.3% to 72.89%, and there was a twofold enhancement in apoptosis within the tumor. These findings suggest that ETO can be effectively delivered *via* TPGS-LA NPs for the treatment of HCC. Table I shows the functional role of drug-loaded nanomicelles in HCC along with key components, the preparation method, typical properties, and significant highlights.

Liposomes

Liposomes are sphere-shaped bilayer microscopic vesicles wherein an aqueous core is entirely surrounded by a lipid membrane(s) composed of synthetic or natural phospholipids. Lipophilic drugs are typically intercalated into the phospholipid bilayer membrane, while hydrophilic drugs are entrapped within the aqueous core. Usually, the drug-to-lipid ratio (D/L) obtained by the passive loading technique is relatively low, and the stability of the entrapped drug is minimal because of the weak association between the liposomes and the drug ensuing poor drug retaining property and long-term stability[137]. In active loading, drugs are loaded into the preformed blank liposomes *via* transmembrane gradient followed by interactions with the liposome to lock in the drug. Active loading can usually attain an increased drug-to-lipid ratio with high storage stability compared to the passive process. Though active or remote loading is primarily applicable to amphipathic compounds, methods such as solvent-assisted active loading technology and drug derivatization enable large encapsulation of hydrophobic drugs with high drug-to-lipid ratios[138,139].

Liposomal Liver Targeting

Drug targeting is the ability of the drug to selectively or quantitatively aggregate in the target tissue or organ without relying on the site and method of delivery. Drug targeting involves a synchronized behavior of three essential components: the drug, the targeting moiety, and the suitable drug carrier. The selectivity towards hepatic cells, whether it's fenestrated SECs and KCs on one side (>100 nm) or HSCs and hepatocytes on the other side (100 nm), is influenced by the size of the nanocarrier and the diameter of the hepatic sinusoidal fenestrae, respectively. Deformable nanovesicles larger than 400 nm may sometimes extravasate into the space of Disse of the liver sinusoids through forceful extrusion, which is likely facilitated by brief interactions with SECs[32]. Active drug targeting allows the distribution of drugs at the target tissues at the cellular or subcellular level. Furthermore, it will maintain the optimum drug concentration of the actives for the desired period. It can enhance the drug's activity and specificity, reducing any undesirable toxic effects. An efficient targeting system may include drug, carrier, and targeting group to accomplish site-specific drug delivery. Interactions between a targeting group and a corresponding receptor aid in the targeting of a carrier to a specific cell. Typically, targeting groups are affixed to the drug's or the carrier's surface.

Targeting to Hepatocytes

Parenchymal cells or hepatocytes occupy about 60% of the hepatic cellular mass and 80% of the cytoplasmic mass of the liver. On the other hand, non-parenchymal cells make up just 6.5% of the entire liver volume and hepatocytes make up 40% of the sinusoidal compartment of the hepatic tissue. They are mainly composed of sinusoidal hepatic endothelial cells, HSCs, and KCs[140]. The hepatocytes have multifunctional roles such as lipid and carbohydrate metabolism, detoxification, albumin secretion, production of clotting factors, and complement proteins.

Various investigations revealed hepatocytes to be an active participant in several pathological conditions, including HCC[141], hepatitis B/C[142], liver cirrhosis, liver fibrosis[143], Wilson's Disease, and many other diseases. Delivering drugs to hepatocytes utilizing liposomes attached to various

Table I. Examples of nanomicelles in hepatic carcinoma and their important characteristics.

Drug	Key constituents	Method	Characteristics of nano micelles	Highlights	Ref.
Macrolide brefeldin A (BFA)	TPGS and F127	Solvent exchange method	Spherical morphology Average diameter 43.9 nm The critical micelle concentration wa 0.015 mg/mL.	*In vitro*, M-BFA remarkably reduced the liver carcinoma M-BFA leads to autophagy *via* Akt/mTOR and ERK path-ways. In HepG2 tumor-bearing xenograft mice, ICG fluorescent probe loaded in M-BFA demon-strated long blood circulation time, extensive distribution, and accumulation in the tumor tissue	131
Doxorubicin (DOX)	PEG-pLys-pPhe (Poly(ethylene glycol)-b-poly(L-lysine)-b-poly(L-phenylalanine)	Synthesis *via* ring-opening polymerization reaction followed by dialy-sis method	Redox-responsive cross-links cleaved once internalized cells Dehydroascorbic acid (DHAA) present on the micellar surface for identifying target tumor cells *via* GLUT1 receptors The mean diameter and zeta po-tential of DOX loaded DHAA modified crosslinked was 60.34 nm and 23.7 mV	*In vitro* release study showed only 20% release in 48 h compared to rapid release demonstrated by non-crosslinked micelle DHAA-modified micelle demonstrated high uptake on human hepatocarcinoma cell line Bel-7402 due to passive diffusion DHAA maintained gradual and high tumor-targeting capacity *in vivo* including 3D imaging showed a fraction of micelle confined even at the core of the tumor A fluorescent signal was detected in the mice up to 24 h	132
γ-Fe_2O_3	Poly(ethylene glycol)-b-poly(ε-caprolactone) (PEG-PCL)	Synthesis *via* ring-opening polymerization of ε-caprolactone (CL) and conjugated with cyclic pentapeptide Arg-Gly-Asp (cRGD)	Self-assembled PEG-PCL micelles were loaded with superparamagnetic γ-Fe_2O_3 nanoparticles	Prussian blue staining verified active targeting of micelles with cRGD to tumor integrin αvβ3 re-ceptors of human hepatic vascular endothelial cells. *In vitro* 1.5T MRI showed T2WI and T2 relaxation times were sub-stantially lower in the targeting group than in non-targeting groups Polymer micelles are actively tar-geted to the T3A cells revealed via a clinical 1.5T MRI scanner. The MTT results indicated a significant reduction of γ-Fe_2O_3 cytotoxicity	133

Continued

liver-specific receptor recognition moieties has been investigated[144]. Thus, liposomes prepared with the desired size would allow them to extravasate into the perisinusoidal space or space of Disse of the liver sinusoids The EPR effect in tumor tissues, coupled with reduced fluid drainage into the lymphatic circulation, enables liposomes as large as 400 nm to be retained at the affected sites[145]. In addition, the upregulation of angiogenesis factors like VEGF in tumor tissues typically results in complex vascular architecture and increased vascular permeability[146].

Table I *(Continued)*. Examples of nanomicelles in hepatic carcinoma and their important characteristics.

Drug	Key constituents	Method	Characteristics of nano micelles	Highlights	Ref.
Doxorubicin (DOX)	Poly(ethylene glycol and poly(D, L-lactide) with a targeting ligand, folate attached to the distal ends of the PEG	The multistep chemical reaction procedure	Nanomicelles (< 100 nm) size was influenced by copolymer composition. Folate functionalization of copolymers led to a subtle change in micelle size.	Delivery of DOX from the micelle followed a biphasic release pattern and is pH dependent Folate receptor-mediated cell up-take of DOX-loaded micelles was confirmed using fluorescence and flow cytometry analysis Fo-late-PEG-PDLLA-DOX-micelles demonstrated efficient *in vitro* targeting tropism to the hepatic carcinoma cells	134
Glycyrrhetinic acid (GA)	Stearic acid-modified fenugreek gum (FG-C18).	FG-C18 synthesized through esterification reaction and nano micelles formed by ultrasonication dispersion technique	Particle size nearly 200 nm with a narrow PDI (< 0.3). Particles were negatively charged due to the presence of the hy-droxyl groups of FG-C_{18} The entrapment efficiency value of GA/ FG-C_{18} NMs was nearly 80%, and the drug loading value was 13.34±0.24%,	GA/FG-C_{18} nanomicelles reported high cytotoxicity towards HepG2 cells compared to MCF-7 cells. Pre-treatment with galactose showed increased cell viability to HepG2 cells not MCF-7 cells The liver sections from the nanomicelle group displayed stronger fluorescence at 30 min and faint fluorescence signals after 4 and 8 h indicating increased up-take by overexpressed ASGP-R cells compared with the DiR solution group. In comparison to free GA, the GA/FG-C18 NMs demonstrated higher plasma drug concentration	135
Docetaxel (DTX)	Galactose conjugated with D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS)	Solvent casting method followed by hydration and sonication	CMC, particle size range, PDI, zeta potential of TPGS micelle and Gal-TPGS was 0.02% w/v and 0.025% w/v; 111.40 ± 10.42 and 147.36 ± 10.85 nm, 0.254 ±0.018 and 0.248 ± 0.021; −17.2 ± 0.061 and−15.6 ±0.052, respectively.	Slower elimination rate constant (Kel)(h-1) observed with DTX-TPGS-Gal (0.0261 ±0.006) compared to DTX-TPGS (0.0356 ± 0.007) and DTX (0.1847 ± 0.011) respectively DTX recovered from targeted DTX-TPGS-Gal from liver tissue was significantly ($p < 0.01$) higher (3.845 ± 0.682 µg/g) than non-targeted one (DTX-TPGS), DTX recovered from targeted DTX-TPGS-Gal from liver tissue was significantly ($p < 0.01$) higher (3.845 ± 0.682 µg/g) than non-targeted one (DTX-TPGS)	136

The ASGPRs or lectins are integral membrane proteins demonstrated to have a high expression on the surface of hepatocytes[147]. The ASGPR binds with a broad range of ligands that possess either N-acetylgalactosamine (GalNAc) or terminal galactose (Gal) residues. Under *in-vitro* conditions, ASGPR density has been estimated to be around 1.09 million molecules per mammalian

hepatocyte[148]. Thus, the highly abundant and selective cell surface ASGPR has been extensively evaluated for the targeted delivery of actives to hepatocytes. The uptake through the ASGPR depends on the number of receptors as well as the turnover rate of the receptors. Notably, the receptor-mediated drug delivery technique can get saturated and consequently lead to decreased selectivity for the liver, escalating the prospect of systemic adverse effects.

As a derivative of Gal, GalNAc demonstrates a higher affinity towards the ASGPR with subsequent uptake *via* clathrin-mediated endocytosis[149]. Despite several setbacks, a lot of investigation based on Gal and GalNAc-mediated liver targeting has been explored[150,151]. It has been proposed that siRNA can be delivered to hepatocytes using liposomes encapsulated in 1,2-dioleoyl-sn-glycerol-3-phosphatidyl-N-(1-deoxylactito-1-yl) ethanolamine (GDOPE), a novel Gal -modified lipid[152]. The targeting ability of the produced liposomes was assessed using the gene silencing activity of liposome: siRNA complexes in human hepatoma cell lines. The most potent action was shown by the liposomal formulation (LIC-G5), which was made with a positively charged lipid: GDOPE in a ratio of 3:5 by weight. When administered to the mice, the intravenous injection of LIC-G5 coupled with 3H-labelled siRNA led to the build-up of radioactivity in the liver. LIC-G5 can be considered a propitious delivery system candidate for liver-targeted siRNA treatment despite increasing cell toxicity at high dosages. In the last few decades, cationic liposomes have gained a lot of acceptance and popularity for siRNA delivery. However, intracellular barriers such as cytosolic siRNA delivery and endosomal escape continue to challenge researchers, including cytotoxicity contributed by the cationic lipids. To identify the factors that impact siRNA efficacy and cytotoxicity, different liposomal formulations were formulated using various cationic lipids (DC-Cholesterol and DOTAP) and changing the ratio of co-lipids (DOPE and cholesterol). It was found that the concentration of cationic lipid, the nitrogen/phosphate ratio, and the nature of the cationic lipid contributed to the cytotoxicity. Moreover, it was concluded from the study that many parameters had to be optimized to prompt a systematic intracytoplasmic release of the siRNA[153].

A hepatocyte-specific targeting strategy was investigated using asialofetuin, a synthetic modification of GalNAc covalently coupled to pegylated liposomes[154]. It was found to be piled up in the hepatocytes, whereas the KCs had taken up the non-decorated liposomes. From the data, it was concluded that the use of PEGylated asialofetuin-conjugated liposomes is an optimal strategy to keep away from the reticuloendothelial system and was found to precisely target hepatocytes *via* the ASGPR, both *in vitro* and *in vivo*. The ethanol injection method was used to create diterpenoid bioactive oridonin (ORI) loaded liposomes with Gal on the surface (NOH-ORI-LP)[155]. *In vitro* studies demonstrated nano-sized (173 ± 12 nm) particles with negative surface potential (-31.5 ± 1.6 mV), and high entrapment competency ($94.1 \pm 1.2\%$). The mean residence time of NOH-ORI-LP in the plasma was approximately 5.56 times more than that of the solution. In contrast to the ORI solution, NOH-ORI-LP distributed about 4.28 times more drugs into the liver.

The unique binding of Lactoferrin (Lf) with ASGPR makes it a prospective target ligand for the HCC cells[156]. The Lf-modified, PEGylated, and DOX-loaded liposome (Lf-PLS) were made by covalent coupling with lactoferrin to the surface of liposomes *via* the carboxyl functional groups of the PEG-lipid DSPE-PEG_{2000}-COOH and the free amino groups of Lactoferrin. The Lf-PLS system demonstrated a noticeably better antitumor activity when compared with free DOX and PEGylated liposome, according to *in vivo* antitumor studies on male BALB/c nude mice with HepG2 xenografts[157].

Retinol-Binding Protein Receptor

The ability to store the most significant quantity of vitamin A as retinyl palmitate in lipid droplets is a crucial characteristic of the quiescent HSCs. Due to the ability to be taken up by the HSC, RBP and vitamin A have been commonly utilized as ligands for HSC-specific delivery[158]. It was proved that albumin's domain III fused to RBP was effectively taken up by the HSCs under both *in vitro* and *in vivo* conditions. Another study[159] demonstrated that NPs modified with retinol and encasing an oligonucleotide could bind to RBP receptors in the bloodstream and be selectively internalized by HSCs *via* the RBP receptor. The NPs effectively inhibited the production of type I collagen in the liver, and reversed liver fibrosis induced by CCl_4 and bile duct ligation in mice[159]. A therapeutic efficacy evaluation was conducted in liver fibrosis utilizing gp46 siRNA encapsulated in liposomes coupled with vitamin A through

an *in vivo* study. The functionalized liposome obstructed collagen secretion in the HSCs, significantly silenced gp46, and overturned CCl4 and bile duct ligation-induced hepatic fibrosis in rats. The investigation demonstrated the therapeutic capacity of siRNAs in an animal model[160].

Type VI Collagen Receptor

The type VI collagen receptor is a crucial matrix protein responsible for cell adhesion. It plays a significant role in continuing the interaction between the extracellular matrix molecules and the cells, thereby regulating the matrix's homeostasis[161]. Its distribution is primarily observed in the portal region, hepatocytes, endothelial cell membranes, and HSCs. During hepatic fibrogenesis, the upregulation of the type VI collagen receptor expression is predominantly observed on activated HSCs. It has been investigated for the targeted delivery of antifibrotic agents specifically to HSCs[162]. An *in vitro* study was carried out on human serum albumin conjugated on cyclic RGD peptide C*GRGDSPC*. Results from biodistribution studies in rats revealed internalization and high accumulation of activated HSCs after liver fibrosis induced in the rats[60]. CGRGDSPK, which binds to activated HSCs, was employed as a target ligand for the type VI collagen receptor, owing to its similarity to the cyclic RGD peptide. The coupling of interferon-a1b-loaded liposomes with the RGD peptide resulted in a 10-fold rise in accumulation of activated HSCs, and hence, an escalation in antifibrotic activity was observed in rats with liver fibrosis induced by bile duct ligation[61].

Folate Receptor

The cell surface-bound folate receptor (FR) is a glycosylphosphatidylinositol-anchored glycoprotein. In neutral pH, it binds strongly with folic acid molecules and initiates cell uptake *via* endocytosis. Folate is overexpressed in various types of cancer tissues. Hence, these receptors can be considered a delivery target in the treatment of HCC by incorporating the conjugation of folic acid onto the nanocarrier surfaces[163]. *In vivo* studies[164,165], FR-targeting in tumor models has indicated differing therapeutic benefits, from encouraging to disappointing. Another study[166] used computed tomography imaging and positron emission tomography to examine the tumor-accumulating capacity of folate-targeting liposomes (FTLs) and non targeting liposomes (NTLs) in an FR overexpressing xenograft model. The high-affinity copper chelator (DOTA) was entrapped in NTLs. FTLs were produced by post-inserting 0.5 mol% DSPE-PEG5k-folate into NTLs that contained DOTA. The study concluded that targeting FRs did not improve the total liposome delivery to solid KB xenografts as established by PET. The outcomes of this research indicate that, regardless of active cancer cell receptor targeting, the circulating properties of the liposomal formulations and the EPR effect regulate the liposome levels that may be obtained. It also emphasizes the need for liposomes to have long-circulating characteristics to benefit from the accumulation of EPR-dependent tumors, which may be lost by functionalization. According to the study, liposome levels that may be acquired, regardless of the active targeting of tumor cell receptors, are determined by the EPR effect as well as the circulation characteristics of liposomal formulations. To comprehend the targeting and therapeutic potential of FTLs and their mechanism of action, studies in other animal models with a folate metabolism that is more similar to human folate metabolism may be helpful. Though several types of research have been carried out to deliver the functionalized nanocarriers to FR-expressing tumors, particularly to hepatocytes and KCs, surface modifications must be examined appropriately as this may drastically affect biodistribution and circulation[167].

Targeting Hepatic Stellate Cells

An essential component in the microenvironment of the HCC tumor is the HSCs[168]. According to ongoing research, activated HSCs may interact with paracrine crosstalk within the tumor site and ECM proteins to initiate hepatocarcinogenesis. The manifestation of liver fibrogenesis is activated HSCs, which cause infiltration to HCC stroma and promote HCC development. Furthermore, the activation of quiescent HSCs in the liver is a significant phenomenon observed during cirrhosis, hepatic fibrosis, and HCC[11]. Promoter hypermethylation in tumors leads to the suppression of the tumor suppressor gene PTEN, which is a significant inhibitor of HSC activation[169]. The interplay between activated HSCs, the hepatic extracellular matrix (ECM), and HCC tumor cells remains poorly understood. Several cell surface receptors, including the insulin-like growth factor-2 receptor, mannose-6-phosphate receptor, RBP receptor, and Platelet-Derived Growth Factor Receptor-β (PDGFR-β), have been identified as potential targets for directing therapies toward HSCs[170].

Platelet-Derived Growth Factor Receptor- Beta

Activated HSCs have an abnormally high level of PDGFR-β, therefore numerous PDGFR-β targeted delivery systems for anti-fibrotic drugs have been investigated[171]. The selective binding of the cyclic peptide to PDGFR-β on activated HSCs is suggested by the predominant presence of CSRNLIDC Peptide conjugated to human serum albumin in HSCs. The fusion of the CSRNLIDC peptide with an antibody fragment was found to redirect the adenovirus towards activated HSCs while abolishing the virus' natural tropism for KCs and hepatocytes.

Therefore, using this approach would prevent KCs and hepatocytes from non-specific uptake of antifibrotic genes and delivering them to activated HSCs[172]. According to a study, polypeptide pPB-modified stable SNALPs (pPB-SNALPs) were used to deliver specifically high transfection efficiency siRNAs against HSP47 to the liver for hepatic fibrosis targeted therapy[173]. *In vivo* and *in vitro* imaging studies indicated enhanced uptake of pPB-SNALP by LX-2 cells besides demonstrating an augmented inhibitory effect on Thioacetamide-induced hepatic fibrosis mice with high gp46 mRNA expression. *In vitro*, the antifibrotic medication oxymatrine decreased HSC cell viability and induced apoptosis[174]. The targeting of HSCs by oxymatrine was enhanced by RGD, resulting in lower serum alkaline phosphatase levels (272.51 ± 19.55 U/L *vs.* 344.47 ± 27.52 U/L, $p < 0.05$), reduced collagen deposition (0.26% ± 0.09% *vs.* 2.36% ± 0.09%, $p < 0.05$), improved liver damage, and downregulation of genes associated with fibrosis, including procollagen type I, MMP-2, and TIMP-1 ($p < 0.05$).

Lipid-Based Nanoparticles in Clinical Trials

The US FDA approved Onpattro (Patisiran) infusion in 2018 for the treatment of adult patients with polyneuropathy caused by hereditary transthyretin-mediated amyloidosis (hATTR)[65,175]. Onpattro encapsulates the siRNA into a lipid NP carrier to actively transport the medication to hepatocytes and subsequently alter or stop the synthesis of disease-causing proteins by selectively interacting with apolipoproteins. Advancement in the field of siRNA would enable clinicians to treat disease by either halting or reversing a condition rather than only being capable of managing or treating its symptoms. A study[176] with an open-label design was conducted to evaluate the pharmacokinetics, safety, and effectiveness of Patisiran-LNP in patients with hATTR amyloidosis who experienced disease progression after receiving an orthotopic liver transplant. Moreover, the safety and efficacy of the subcutaneous injection form, Vutrisiran (ALN-TTRSC02) are currently undergoing phase 3 clinical trials (NCT03759379) to check the safety and efficacy against the reference comparator patisiran during the treatment period. The phase I clinical trials of the open-label, multicenter, extension study of ALN-VSP02 in cancer patients who showed a therapeutic response to ALN-VSP02 therapy have been completed. In addition, ALN-VSP02 dose escalation studies were undertaken in patients with advanced solid tumors with liver damage to assess the pharmacokinetics, pharmacodynamics, safety, and tolerability of intravenous ALN-VSP02. Table II provides a summary of the clinical trials, both ongoing and completed, that have utilized various actives for liver targeting in HCC.

Future Perspectives and Challenges

Multiple intracellular and extracellular barriers need to be conquered for effective targeted drug and gene delivery *via* LNPs. NPs must maintain sufficient stability while in circulation and possess excellent target-binding ability before arriving at the desired site of action. LNPs are designed to penetrate through hepatic SECs to combine with liver-specific cells, such as HSCs, KCs, and hepatocytes. It is expected to be internalized by the targeted liver cells to release the loaded actives efficiently. To attain NP stability and to avoid untimely release of actives, drugs, and geneses must be firmly associated with lipids. SLNs prepared with ionizable lipids have been successfully developed to encapsulate genes in a novel targeted RNA-based FDA-approved product, "Onpattro"[77].

Liposomes formulated with transmembrane ionic and pH gradient techniques demonstrated efficient drug loading using active loading methods[177]. Moreover, the inclusion of cholesterol in the lipid formulation can impart fluidity and rigidity to the lipid membrane resulting in improved stability and drug loading[178]. To avoid premature systematic clearance through RES, many lipid NPs are surface-modified with PEGylation. However, one unpredictable response is the fast clearance of PEGylated nanocarriers upon repeated administration, named the accelerated blood clearance phenomenon[179].

Table II. Completed and ongoing clinical trials of lipid-based nanosystems designed for targeting HCC, hepatic fibrosis, and hepatitis.

Identifier	Clinical use	Mode of delivery (technology)	Target cell and receptor	Payload	Trial phase	Trial identifier (Trial starting year)	Trail status
ALN-TTR02	TTR-mediated	Solid Lipid nanoparticles amyloidosis	TTR	TTR siRNA	1 2 3 4	NCT01559077 (2012) NCT02053454 (2014) NCT01617967 (2012) NCT01961921 (2013) NCT01960348 (2013) NCT02510261 (2015) NCT03862807 (2019) NCT03997383 (2019) NCT03759379 (2019) NCT02939820 (2016)	Completed Completed Completed Completed Completed Completed Completed Active Active Approved for marketing
Thermo-Dox®	Hepatocellular carcinoma	Lysolipid thermally sensitive liposome + RFA	Release doxorubicin upon heat exposure	Doxorubicin	1 3	NCT00441376 (2007) NCT02112656 (2014) NCT00617981 (2008)	Completed Completed Completed
ND-L02-s0201 BMS-986263	Hepatic fibrosis	siRNA encapsulated Li-posome	HSP47 inhibitor	Hsp47 siRNA	1 1 1 2	NCT01858935 (2013) NCT02227459 (2014) NCT03142165 (2017) NCT03420768 (2018)	Completed Completed Completed Completed
MTL-CEBPA	Advanced Hepatocellular carcinoma	Smarticles - saRNA encapsulated liposome	CCAAT/ enhancerbinding protein alpha (CEBPα) inducer	CEBPα saRN -tumor suppressor gene	1 1 1 2	NCT02716012 (2016) NCT04105335 (2019) NCT05097911 (2021) NCT04710641 (2021)	Active, Not yet recruiting Active, Not yet recruiting Recruiting Recruiting

Continued

Table II *(continued)*. Completed and ongoing clinical trials of lipid-based nanosystems designed for targeting HCC, hepatic fibrosis, and hepatitis.

Identifier	Clinical use	Mode of delivery (technology)	Target cell and receptor	Payload	Trial phase	Trial identifier (Trial starting year)	Trail status
TKM-080301	Hepatocellular Carcinoma	Solid Lipid nanoparticles	PLK1	PLK1 siRNA	1 2	NCT01262235 (2010) NCT01437007 (2011) NCT02191878 (2014)	Completed Completed Completed
DCR-MYC	Hepatocellular Carcinoma Solid Tumors Multiple Myeloma Non-Hodgkin's Lymphoma	EnCore™ lipid nanoparticle	MYC	MYC siRNA	1 2	NCT02314052 (2014) NCT02110563 (2015)	Terminated (Sponsor decision) Terminated (Sponsor decision)
ARB-001467	Chronic hepatitis B virus (HBV) infection	Solid Lipid nanoparticles	HBV proteins	HBV siRNA	2	NCT02631096 (2015)	Completed
PLM60	Advanced Hepatocellular Carcinoma	PEGylated Liposomal mitoxantrone IV Infusion	Topoisomerase II inhibitor	Mitoxantrone HCl	1	NCT04331743 (2020)	Not yet recruiting
MRX34	Hepatocellular Carcinoma	MicroRNA miR-RX34 Liposomal Injection	Mimic miRNA	miR-34a - tumor suppressor gene	1	NCT01829971 (2013)	Terminated (Five immune-related serious ADRs)
ALN-VSP02	Hepatocellular carcinoma Solid tumors	Solid Lipid nanoparticles	KIF11 and VEGF	KSP/VEGF siRNA	1	NCT00882180 (2009) NCT01158079 (2009)	Completed Completed

To effectively reach the space of Disse, nanoparticles (NPs) need to possess a particle size smaller than 100 nm, enabling them to pass through the endothelial fenestrae. Additionally, for targeted delivery to sinusoidal endothelial cells (SECs) and Kupffer cells (KCs), larger-sized lipid nanoparticles (LNPs) should be employed. Active targeting is typically obtained *via* ligand-receptor binding or ligand-receptor binding or serum protein adsorption. Overcoming the cytosolic and endosomal membrane barriers would allow intracellular transport of actives to deliver the cargo at the site of action. Internalization is typically achieved through many physiological mechanisms, including phagocytosis, pinocytosis, and clathrin-mediated endocytosis. Liposomes made from pH-dependent ionizable cationic lipid or fusogenic lipid, such as DOPE, have been observed to aid in the escape of cargoes from endosomes prior to their release into the cytosol. The functional task of ionizable lipids in endosomal escape is evident after the successful development of Onpattro and Smarticles[77].

The multiple-ligand approaches to target the KCs, have been recently reported for more precise targeting[180]. Patients underwent genomic and proteomic studies to determine the degree of expression and the efficacy of the receptors for the cell-specific targeted administration. The delivery of contrast agents for MRI and CT of diagnosed medical conditions has been assessed utilizing LNPs[181]. LNPs for the codelivery of drugs and contrast media are beneficial in the medical field due to advantages such as improved diagnosis, site-specific delivery of drugs, and reduced toxic effects on normal tissues. Active and passive targeting effects could be merged for efficient cell-specific hepatic drug delivery. Combined liver targeting strategies have been achieved through the development of lactosyl-norcantharidin N-trimethyl chitosan nanoparticles (Lac-NCTD-35 TMC-NPs). In this system, passive targeting was accomplished by controlling the particle size (~120 nm) and active drug targeting through ASGP-R recognition[182]. Paramagnetic MnO and Gd_2O_3 NP-based T1 contrast demonstrated significant advantages over clinically approved T1 contrast agents. In recent years, there has been tremendous interest in the field of nanomaterials as liver contrasting agents. Nano-sized T1 contrast agent, MnO, and T2 contrast agent, Fe_3O_4 integrated into a single hybrid nanocrystal proved that the dual contrast media approach had higher accuracy for liver MRI[183]. NP-based long-circulating contrast agents such as PEGylated Gd2O3:Yb3+ and Er3+nanorods for multi-modality MRI and X-ray CT liver imaging have been described[184].

LNPs prepared using traditional methods are tedious and time-consuming, producing inconsistent results and challenging to scale up. Recently, advanced microfluidic benchtop systems are currently being developed for speedy and consistent LNP manufacture with easy pressure-driven solutions for the encapsulation of API, cells, protein, and DNA[185]. Those methods have allowed researchers to go beyond lipid ingredients' limitations and size differences frequently associated with conventional manufacturing methods. Even though liposomes can load hydrophilic and hydrophobic drugs, various drug encapsulation methods have not yet demonstrated promising entrapment. Transmembrane gradient and solvent-assisted active loading technology demonstrated the potential ability to embed low water-soluble drugs into liposomes efficiently[77,186,187].

Conclusions

The LNPs decorated with ligands are still being utilized for liver targeting to deliver therapeutics, such as siRNA, mRNA, and pDNA. Furthermore, endogenous systems like apolipoproteins and LDLR are also investigated as newer approaches to cell-specific liver targeting. Novel functionalization strategies are emerging to increase active drug loading, improve drug stability, avoid opsonization, allow endosomal escape, and direct cell-specific targeting *via* lipid NPs. Onpattro demonstrates all desirable properties as expected from NPs and results in clinical application after successful FDA approval. This paved the way for many new possibilities of LNPs for drug and gene therapy applications in the near future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' Contribution

Conceptualization, S.J., A.B.N., B.G.; literature review, S.J., A.B.N., S.H.S.B., R.K.A., K.S., T.B., B.G., J.S., M.A.M.; writing–original draft preparation, S.J., S.H.S.B, R.K.A., K.S., T.B., J.S.; writing–review and edit-ing, S.J., A.B.N., B.G., J.S., M.A.M. All authors have read and agreed to the published version of the manu-script.

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Ethics Approval

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