



Review article

Liposomes: An emerging carrier for targeting Alzheimer's and Parkinson's diseases[☆]Sureshbabu Ram Kumar Pandian^{a,*}, Kevin Kumar Vijayakumar^b, Sankaranarayanan Murugesan^c, Selvaraj Kunjiappan^a^a Department of Biotechnology, Kalasalingam Academy of Research and Education, Krishnankoil, 626126, Tamilnadu, India^b Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai, Tamilnadu, India^c Medicinal Chemistry Research Laboratory, Department of Pharmacy, Birla Institute of Technology and Science, Pilani Campus, Vidya Vihar, Pilani, 333031, Rajasthan, India

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ABSTRACT

The function of the brain can be affected by various factors that include infection, tumor, and stroke. The major disorders reported with altered brain function are Alzheimer's disease (AD), Parkinson's disease (PD), dementia, brain cancer, seizures, mental disorders, and other movement disorders. The major barrier in treating CNS disease is the blood-brain barrier (BBB), which protects the brain from toxic molecules, and the cerebrospinal fluid (CSF) barrier, which separates blood from CSF. Brain endothelial cells and perivascular elements provide an integrated cellular barrier, the BBB, which hamper the invasion of molecules from the blood to the brain. Even though many drugs are available to treat neurological disorders, it fails to reach the desired site with the required concentration. In this purview, liposomes can carry required concentrations of molecules intracellular by diverse routes such as carrier-mediated transport and receptor-mediated transcytosis. Surface modification of liposomes enables them to deliver drugs to various brain cells, including neurons, astrocytes, oligodendrocytes, and microglia. The research studies supported the role of liposomes in delivering drugs across BBB and in reducing the pathogenesis of AD and PD. The liposomes were surface-functionalized with various molecules to reach the cells intricated with the AD or PD pathogenesis. The targeted and sustained delivery of drugs by liposomes is disturbed due to the antibody formation, renal clearance, accelerated blood clearance, and complement activation-related pseudoallergy (CARPA). Hence, this review will focus on the characteristics, surface functionalization, drug loading, and biodistribution of liposomes respective to AD and PD. In addition, the alternative strategies to overcome immunogenicity are discussed briefly.

1. Introduction

Alzheimer's disease (AD) is commonly known as dementia (*The essential elements of Alzheimer's disease*), whereas Parkinson's disease (PD) is a disorder of neurological movement. The prevalence of both chaoses is ascending worldwide and worsening the burden for older people. They are outcomes of neural dysregulation that severely influence the function of the brain. AD affects 36 million people around the globe, and the prevalence may increase to 115 million by 2050 [1,2]. The progressive aggregates of proteins (β -amyloid (A β) peptides and neurofibrillary tangles (NFTs) are the cause of neurodegeneration and dementia [3, 4, 5]. The spontaneous accumulation of oligomers and amyloid fibrils of A β

leads to synaptic damage and memory deficits in AD [6, 7]. Tau, a phosphoprotein, belongs to the major microtubule-associated protein (MAP) category present in the mature neuron. The microtubule assembly promoting activity of tau is mainly regulated by its degree of phosphorylation. Hyperphosphorylation of tau generally depresses its function. Brain tau is more hyperphosphorylated than the standard level under the pathogenesis of AD. In that state, the tau is polymerized into paired helical filaments ([PHF] conflated with straight filaments (SF), forming neurofibrillary tangles (NFTs). Lowering the level of A β and tau is a therapeutic target of big pharmaceutical companies, and billions of dollars have been exhausted to explore their outcome. However, the redundancy of tau and amyloid protein precursor (APP) is unfortunate

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because their adverse phenotypes are associated with neurodegeneration [8]. Even though more than 30 phase-III trials proceeded with the strategy of lowering the A β level, none showcased cognitive benefit to AD patients or were harmful to the patients' post clearance of amyloid plaques [9, 10].

Drugs that target AD or PD are needed to deliver across the blood-brain barrier (BBB) [11, 12], which prevents 98% of neuro-pharmaceuticals from accessing the brain [13]. BBB contains brain capillary endothelial cells connected through tight junctions, which restricts paracellular transport and prevents the brain from harmful stimuli [14, 15, 16]. The degradative enzymes present in the BBB can destroy the molecules attempting to cross the layer [17, 18]. Non-invasive and bio-compatible treatment strategies are needed to cross BBB and target brain cells, where liposomes are suitable materials due to customized surface functionalization and sustained and targeted delivery [19, 20]. The nanomaterial surface modified with brain targeting ligands with rabies virus glycoprotein peptide (RVG) exhibits promising brain targeting to treat PD, AD, and malignancy [21, 22, 23].

Liposomes are natural carriers explored for the last three decades for various diseases, including malignancies. The core materials used for liposome fabrication possess the properties such as bio-compatibility, bio-degradability, and low toxicity [24, 25]. Liposomes have recently been recognized as carriers to reach the central nervous system (CNS), though their therapeutic potential was proved in 1961 [26]. As a brain targeting and drug delivery system, liposomes provide several advantages, including biocompatibility, highly stable, enhanced peripheral circulation, and easy to attach ligands on the surface of liposomes for receptor-mediated drug delivery system. PEGylation of liposomes enhances the efficacy of encapsulated molecules by reducing the opsonization and RES clearance *in vivo*. This not only slows drug removal by extending blood circulation and allowing therapeutics to accumulate at the required site, and decreases side effects. The pharmacokinetic properties of liposomes are varied based on their physico-chemical characteristics, and modification in their properties enhances their circulation time up to 24 h in rodents and as high as 45h in humans [27, 28]. The brain targeting mannose modified liposomes were reported during the late 1980s, and their incorporation in the mouse brain and glial cells were proved *in vivo* [29, 30]. Liposomes can encompass both hydrophilic and hydrophobic agents, where hydrophilic can be occupied in the aqueous core, and lipophilic can be incorporated in the hydrophobic region, respectively [31]. Liposome formulations are highly suitable for the medication of various illnesses [32]. Monumental evidence suggests that the nanomaterials are sought-after materials for drug delivery since they are tailored to target the surface receptors and off-load the molecules intracellularly in a controlled manner [33]. The stability of the liposomes can be enhanced by PEGylation or the addition of polyethyleneimine (PEI) with the phospholipid portion. Liposomes possess numerous advantages over viral counterparts, such as high loading capacity, easy scale-up, reproducibility, low immunogenicity, and safe and effective gene therapy [34]. This review deliberates the strategy to reach BBB, the advantage of liposome formulations for brain disorders, and their limitations.

2. Structure of BBB

The BBB is a complex, structural and functional layer located at the interface of blood and brain tissue [18]. The BBB inhibits the passage to the brain through physical (tight junctions) and metabolically by enzymes [35]. BBB is composed of endothelial cells that contribute to the tight junction. Along with a basal lamina sheltered with smooth muscular and neural cells, including astrocytes, neurons, and an extracellular matrix. The barrier function is provided by the tight junctions between endothelial cells, astrocytes, and pericytes [35]. The tight junctions of BBB govern the selective influx of oxygen, iron, nutrients, and cells into the brain parenchyma and the efflux of potentially harmful substances and pathogens from the nervous system [36]. Transmembrane proteins

are a significant part of tight junctions; they bind the cytoskeleton and connect nearby endothelial cells closely, eliminating intercellular spaces [37]. Tight junctions are composed of integral membrane proteins such as claudin family members, i.e., claudin 3, 5, 11, and 12, occludin, and junctional adhesion molecules with many other peripheral proteins to the junction complex [38]. Moreover, astrocyte glial cells provide 90–98% of brain microvasculature and sheaths [39]. Astrocytes contribute to the cellular links between neuronal networks and blood vessels. Pericytes influence BBB integrity in two ways: 1) downregulating trans-endothelial permeability and 2) promoting astrocyte-endothelial cells contacts [40].

3. Liposome transport route across the BBB

The majority of the molecules are prevented from the BBB entry; however, lipophilic compounds (<8 hydrogen bonds and <400 Da) can cross the BBB [41, 42]. Since 98% of active drugs formulated with small molecules fail to cross the BBB, almost all drugs fail to reach the brain in a scheduled medication [43]. Of note, the key issue of rapid clearance is swift phagocytosis mediated through opsonization by the mononuclear phagocyte system (MPS) in combination with renal excretion, unspecific biodistribution, and reduced blood circulation time [44]. Several research works have been performed during the last few decades on developing the brain drug delivery system. Particularly, nano-based carriers focus on enhancing the treatment strategies of neurological diseases by facilitating BBB crossing, accurate delivery of cargo into the brain, site-specific interactions, and systemic release [45]. Intravenous administration of brain targeting therapeutics undergoes peripheral circulation before reaching the brain target [44]. Thus, the main requirements for brain targeting nanoparticles might have prolonged circulation, stability, accumulation at the target site, and ensures longer contact time with the target tissue [46]. Among the variety of nanomaterials, liposomes are the sought-after delivery vehicle for both hydrophobic and hydrophilic molecules into the brain. Liposomes can penetrate the brain through several transport routes [28, 47]. In general, liposomes cross the BBB through carrier-mediated (cationic or PEGylated liposomes) and receptor-mediated endocytosis [48, 49]. The liposome formulations under various phases of clinical trials are represented in Table 1.

4. Synthesis of liposomes

Many types of liposomes were synthesized; some of them are more suitable for brain targeting and delivery vehicle. The most basic types of liposomes are multilamellar vesicles, small unilamellar vesicles, large unilamellar vesicles, and multivesicular vesicles, as presented in Figure 1. The most suitable method of liposome formulation is to obtain efficient drug entrapment, loading a variety of drugs, prolonged circulation, highly stable, uniform particle size, and distribution [50]. The most common techniques for all types of liposome synthesis include hydration of the lipid, followed by particle size reduction and removal of the non-encapsulated liposomes [51]. Generally, the preparation of liposomes is followed by two types, namely, passive loading mechanical dispersion and active loading methods. Commonly used methods for the preparation of liposomes are thin-film hydration, pro-liposome method, solvent dispersion or injection (ethanol and ether injection), sonication (probe tip and bath sonication) method, micro emulsification, microfluidics, membrane extrusion, freeze-thawed method, reverse phase evaporation method, detergent removal, dehydration-rehydration, and calcium-induced fusion method [52]. Most liposome formulation procedures require additional post-processing activities to increase liposome homogeneity in terms of size and uniformity, such as freeze-thaw cycles, extrusion, and sonication.

Further, the drug is encapsulated in the passive loading approach by injecting an aqueous phase of a water-soluble drug or an organic phase of a lipid-soluble drug before or during liposome production [53]. The

Table 1. Represents the liposomal formulations are under various phases of clinical trials.

S. No	Formulation	Drug	Target	Phase	Reference
1.	Liposome	Cytarabine	Solid Tumor Neoplastic Meningitis	I	NCT00854867
2.			Brain and Central Nervous System Tumors, Leukemia Lymphoma	I	NCT00003073
3.		Doxorubicin	Brain Metastasis from Breast Cancer	II	NCT00465673
4.			Primary Brain Lymphoma	II	NCT01848652
5.			Brain Tumor, Bone Cancer, Kidney Tumor, Childhood Liver Cancer	I	NCT00019630
6.		2B3-101 (Glutathione pegylated liposomal doxorubicin hydrochloride)	Brain Metastases, Lung Cancer, Breast Cancer, Melanoma, Malignant Glioma	I & II	NCT01386580
7.		Methotrexate, cytarabine	Central Nervous System Metastases, Leptomeningeal Metastases	II	NCT00992602
8.		Marqibo	Sarcoma, Neuroblastoma Wilms Tumor, Leukemia Lymphoma, Brain Tumors	I	NCT01222780
9.		ITV DepoCyt & Temozolomide	Glioblastoma Multiforme Glioma, Astrocytoma Brain Tumor	I & II	NCT01044966
10.		Irinotecan	Diffuse Intrinsic Pontine Glioma	I	NCT03086616
11.			Solid Tumors, ER/PR Positive Breast Cancer, Triple Negative Breast Cancer, Metastatic Breast Cancer with Active Brain Metastasis	I	NCT01770353
12.		Rhenium	Glioma	I & II	NCT01906385
13.		Talineuren	Parkinson Disease	I	NCT04976127
14.		Total tumor mRNA	Adult Glioblastoma	I	NCT04573140
15.		Bupivacaine	Craniofacial Pain, Migraine Cluster Headache, Trigeminal Autonomic Cephalgia, Sphenopalatine Ganglion Neuralgia, Paroxysmal Hemicrania	II	NCT04930887

passive loading method can achieve high drug encapsulation for lipid-soluble drugs and an enhanced affinity towards the lipid membrane. Alongside, ions or drugs can be loaded in the external or internal aqueous phases by creating diffusion gradients [54]. The thin-film hydration method of liposome preparation is one of the most common and simple procedures by dissolving the phospholipids in the organic solvents (dichloromethane, chloroform, ethanol, and chloroform-methanol mixture). This approach includes removing the organic solvent from a round-bottom flask to create a thin lipid film. Heterogeneous liposomes are formed when the dispersion medium is added and agitated. Finally, the uniformity of liposomes is formed following extrusion through polycarbonate membranes. The main disadvantages are low encapsulation, difficulties in scaling up, and heterogeneous size distribution A [55]. Reema Narayan et al. prepared risperidone loaded liposomes through the thin film hydration method for brain targeting [56]. Sonication is a widely used method for preparing liposomes [57]. In the sonication method, multilamellar vesicles sonicated either with a bath type- or probe tip sonicator under potential for significant tissue penetration. Multilamellar vesicles are broken up by the produced pressure in the larger sample, resulting in smaller vesicles that might be either unilamellar or multilamellar in composition. The size of vesicles is determined by the amount of time that lipid solutions are sonicated, with the minimum radius for phospholipid vesicles being in the range of 10.25 ± 0.55 nm, regardless of the length of the phospholipid hydrocarbon chain [58].

The disadvantages of thin-film hydration include sonicator interaction with liposomes and the potential of high-temperature exposure, resulting in phospholipid or drug breakdown and dead encapsulation. The reverse-phase evaporation process produces inverted micelles or water-in-oil emulsions and is a widely used preparative option. The water phase carries the medicine, and the organic phase comprises lipids that form the liposome bilayer. The lipid combination is dissolved in solvents, which are then evaporated. After evaporation, the formed lipid film is redissolved in an organic phase. The organic solvent can be gently evaporated under reduced pressure, resulting in the conversion of the dispersion into a viscous gel and then an aqueous suspension containing the liposomes in the end. The procedure of freezing and thawing

liposomal preparations increases the confined volume. This kind of synthesis is significantly suppressed by increasing the phospholipid concentration and the medium's ionic strength. Encapsulation efficiencies ranging from 20% to 30% were achieved [59]. The synthesis of liposomes can be achieved under various conditions with a microfluidics injection made up of a syringe pump. The process of microfluidization is reproducible and yields liposomes with good aqueous phase encapsulation [60, 61]. The most recent and widely applied liposomal formulations have been developed for brain targeting and efficient drug delivery across the BBB, such as cationic liposomes, specifically targeted liposomes, and long-circulating liposomes.

The extrusion method is one of the most extensively utilized methods for producing monodisperse unilamellar liposomes and controlling their sizes. A lipid suspension is typically pushed through a polycarbonate membrane with a well-defined pore size to form vesicles with a diameter close to the pore size of the membrane used in their preparation. This method eliminates the requirement to remove organic solvents or detergents from the final preparations (a common difficulty with other methods). It may be used on a wide range of lipid species and mixes. The most notable advantage of extrusion is that the resulting mean vesicle size and size dispersion are very repeatable from batch to batch due to the physical processing method.

Microfluidics is a multipurpose tool to create liposomes or a technique for tuning the controlled release of a drug encapsulated within a liposome. Microfluidics can provide rapid and tunable mixing, a homogenous reaction environment and a high-throughput experimental platform. Microfluidics fine control of flow and mixing conditions has been used to change particle size and improve the homogeneity of particle size distributions [62, 63]. Liposomes production through microfluidics techniques comprises electroformation and hydration, extrusion, pulsed jetting, double emulsion templating, ice droplet hydration, transient membrane ejection, droplet emulsion transfer and hydrodynamic focusing [64]. Recently, a microfluidic approach based on the hydrodynamic pinch-off process for manufacturing monodisperse, cell-sized (5–20 μm) unilamellar liposomes with good encapsulation efficiency has also been proposed. Microfluidics has a lot of advantages when it comes to overcoming the problems with liposome bulk preparation.

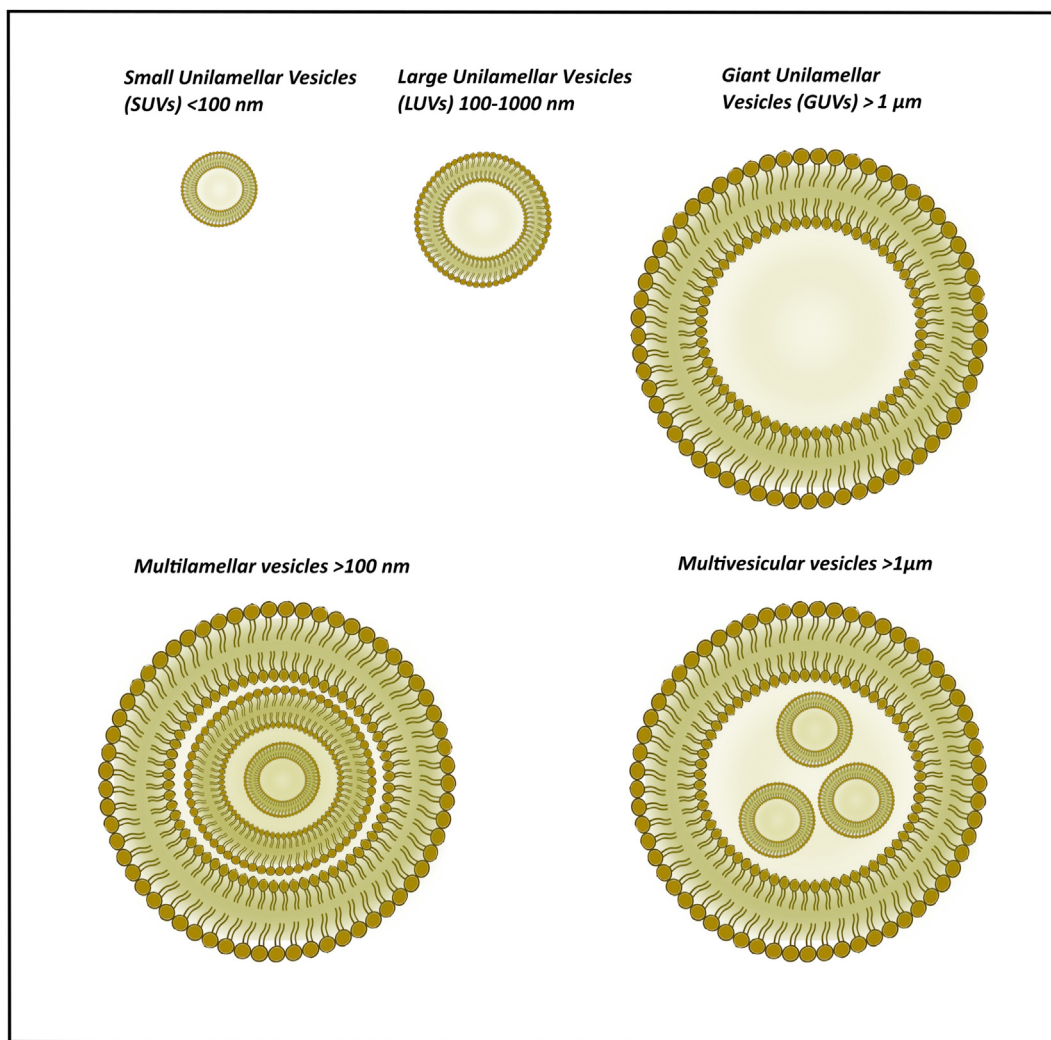


Figure 1. Illustrates the various types of liposomes synthesized for the delivery hydrophilic and -phobic molecules at selected target site.

Microfluidics, with its laminar flow conditions and diffusive mass transfer, enables the synthesis of liposomes with precise size and lamellarity control [65]. In addition, unlike bulk approaches, microfluidics allows for *in situ* monitoring of the liposome formation process, continuous manufacturing, and scaling up via microreactor parallelization.

5. Carrier-mediated transport into the brain

Cationic lipids in liposomes expedite the electrostatic interactions with polyanions, such as DNA and RNA, which exhibit well-ordered condensation of nucleic acids [66]. A variety of mono or multivalent cationic lipids is currently used for gene transfer [67], such as polyethyleneimine, polylysine, dioctadecylamidoglycylspermin (DOGS), dioleoylpropyl trimethylammonium chloride (DOTMA), dimyristooypropyl dimethyl hydroxyethyl ammonium bromide (DMRI), dioleoyloxy-3-(trimethylammonium)-propane (DOTAP). For efficient transfection efficacy, the neutral lipid (Dioleoyl phosphatidylethanolamine (DOPE)) is mostly used as a helper lipid for forming liposomes from these cationic lipids. Cationic liposomes have been used to understand the plasmid-mediated transfection of murine brain cells. For instance, cationic liposomes formulated from the cytokeratin, 3β -(*N*-(*N*', *N*'-dimethylaminomethane)-carbamoyl)-cholesterol (DC-Chol) and DOPE are efficient at transfecting the neuronally derived ND7 cell lines [68].

Moreover, DC-Chol has been applied successfully outside the CNS in various tissues and recently experimented with clinical trials for cystic

fibrosis gene therapy [68]. The efficient binding and diffusion of plasma proteins into the liposome is based on the DOPE or lipid bilayer [69]. The addition of polyethylene glycol (PEG) with liposomes makes a protective layer above the surface of liposomes. It protects the cargo from the binding of plasma proteins, inhibiting the opsonization process and subsequent clearance of liposomes [70]. PEGylated liposomes have acquired a prolonged circulation time in the body, reduced clearance by the reticuloendothelial system (RES), and allow liposomes to cross the BBB (PMC2526358). For example, Caelyx[®] (pegylated liposomal formulation of doxorubicin) is exploited in clinical practice, demonstrating effectiveness in glioblastomas and metastatic tumours [71].

6. Receptor-mediated transcytosis

Receptor-mediated drug delivery systems are highly considered for administering therapeutic molecules such as genetic materials, hormones, peptides, and chemotherapeutics across the BBB and delivered into the brain [48, 72]. For this, drug-loaded liposomes are surface-functionalized or conjugated with suitable ligands (macromolecules such as peptides, antibodies, and aptamers), which enhance, at least in part, the pharmacokinetics and bio-distribution of liposomes [73]. The surface-functionalized ligand recognizes and binds to the specific receptors expressed on the brain endothelial cells' surface and penetrates across the BBB [74, 75]. Commonly used receptors for brain targeting with liposomes are insulin, glucose, transferrin, l-type amino acid

transporter-1, low-density lipoproteins, folate-receptors, etc. [76]. These receptors can specifically recognize and bind the corresponding ligands and triggers internalization into cells [77]. The macromolecule's internalization from plasma to cellular was mediated by a panel of the vesicular system [78]. Three types of endocytic vesicles have been discovered in the brain endothelial cells: clathrin-mediated endocytosis, micropinocytosis, and caveolae, which all play a significant role in the liposome's internalization [79]. The receptor-mediated endocytosis transverse BBB and endothelial cells are represented in Figure 2.

7. Why conventional drugs for the liposomal formulation for treating neurological disorders?

Several pharmaceutical drugs are used for the treatment of neurological diseases or disorders, especially brain tumor (Temozolomide), AD (donepezil, memantine, rivastigmine, galantamine, tacrine), PD (levodopa, entacapone, pramipexole, ropinirole, benserazide, carbidopa,

tolcapone, entacapone, selegiline, rasagiline, safinamide) are orally administered. Of note, the required concentration of the administered drugs did not reach the target site (brain) due to the liver's partial or complete metabolism [80]. This leads to an upsurge in the dosage of drugs that results in toxic effects on the liver, kidney, lungs, and heart [81]. Since conventional drugs are not designed to reach specific cells, their distribution affects both needed and healthy cells. Ultimately, the unsolicited distribution develops multi-drug resistance against neurological diseases [73]. Delivery of therapeutic molecules with faint hydrophilic properties across BBB is highly challenging, though their efficacy was proved *in vitro* [82]. These challenges can be overcome by re-formulating the molecules with liposomes [83]. Liposome's structure mimics cell membrane, which receives widespread attention as a drug delivery vehicle of most therapeutic agents due to their unique characteristics such as bio-compatible, lower toxicity, and delivering both lipophilic and hydrophilic drugs [84]. Liposomes are nano or micro-sized closed spherical vesicles comprised of mono or bilayer of phospholipids

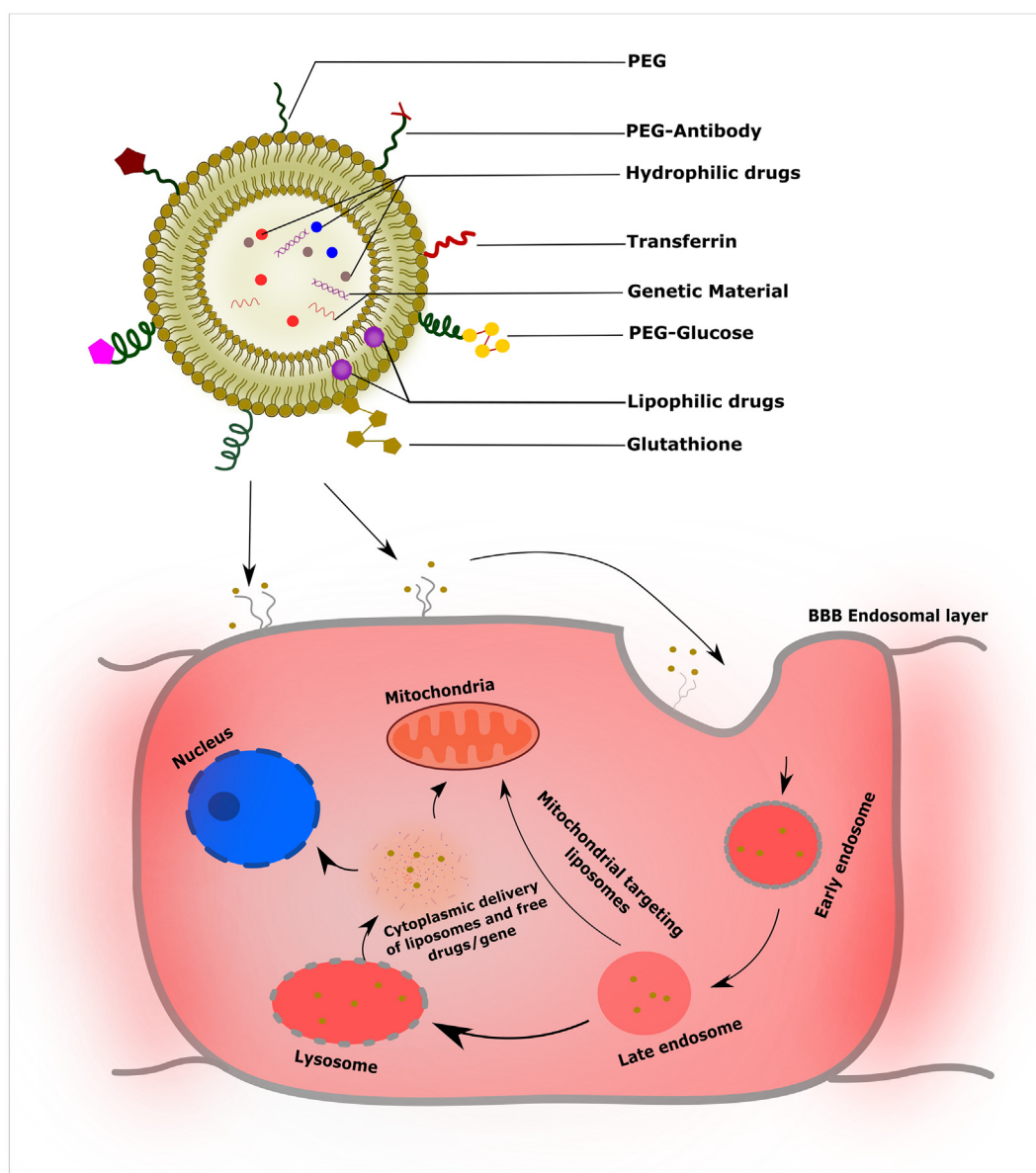


Figure 2. Represents the entry of liposomes across BBB. The liposomes can be loaded with hydrophobic- and hydrophilic drugs, molecules, and genetic materials, etc. The surface of liposomes is conjugated with receptor-targeting molecules to facilitate the BBB penetration and target brain cells. Through the formation of early- and late-endosomes, the cargo of liposomes is processed, and released in the cytoplasm. Further, the drugs or molecules progress towards mitochondria, or nucleus, which ultimately leads to action on specific metabolism.

or cholesterol with a hydrophobic tail, and hydrophilic head, which can shield their cargo from degradation by plasma enzymes can deliver their loaded drugs into the brain [85].

8. Liposome delivery for other malignancies

Liposomes are explored for various tumours and malignancies, including breast-, colorectal, and lung cancers. Amid breast cancer is the most common and major reason for casualty among women. Though chemotherapy is prescribed primarily, nano-based medicines are also available commercially, such as Doxil[®], Myocet[®], Abraxane[®], Onivyde[®], DaunoXome[®], and ThermoDox[®] and many other formulations are under clinical trial [86]. In an approach toward targeting breast cancer, LinTT1 peptide was conjugated on liposomes; Sorafenib (SRF) and doxorubicin hydrochloride (DOX) were co-loaded to enhance the cytotoxic effect of liposomal formulation. The efficacy of SRF and DOX were increased substantially while administered with liposomal formulation, and it can be used for the therapy of triple-negative breast (TNB) cancer [87]. A multistage vector composed of mesoporous silica nanoparticles (MSPs) with PEGylated liposomes loaded with oxaliplatin (oxa) was analyzed for their activity against colorectal cancer (CRC). A novel lyophilization strategy was employed to fabricate the MSV to compensate for the limitations that resulted in better drug targeting and release to the intracellular of desired cells [88]. Hesperetin liposome formulations were analyzed for their activity against MDA-MB-231 cells (breast cancer) and H441 (lung cancer) cells. Hesperetin is a natural molecule with multiple anticancer properties; however, its hydrophobic nature impedes its therapeutic activity. Hesperetin delivered with liposomes displayed enhanced efficiency in breast and lung cancer cells [89]. The benefit of non-damaging mild hyperthermia treatment (MHT) was analyzed on tumor transport alteration and therapeutic efficacy of liposomal gemcitabine in pancreatic ductal adenocarcinoma. Under the tumor microenvironment, mild hyperthermia enhances the tumor transport properties and improves chemotherapeutics' delivery, circulation, and efficacy [90]. A novel stable carrier system called super stealth liposomes is generated with the combinations of mPEG-dendron-phospholipids to prevent macrophage clearance and enhance the drug accumulation at the target site. Doxorubicin-loaded mPEG-dendron-phospholipid formulation proved anti-colorectal cancer activity *in vitro*, where the drug usage was 1000-fold lower than conventional stealth liposomes. This improved the therapeutic efficacy with minimal drug requirement and extended circulation [91].

9. Liposomal approaches to brain tumor

Various nanomaterials have been reported and are under development to target brain disorders. The popular strategy for brain selectivity is targeting receptors or transporters expressed at BBB extensively [32]. Nanomaterials aimed to cross BBB should have the capability to target specific cells, including neurons, astrocytes, oligodendrocytes, and microglia; also, they need to encounter the extracellular molecules [33]. BBB comprises brain capillary endothelial cells and possesses low permeability, making a selective entry of molecules [92]. Except for water, glucose, and amino acids, most molecules are impermeable to BBB, which affects the treatment strategy for CNS disorders [93].

The surface of the nanomaterials needs to be orchestrated with adsorptive peptides to achieve receptor or transporter-mediated transcytosis. Electrostatic attachment to BBB and penetration were demonstrated with cell-penetrating peptide TAT and albumin (with a positive charge) [94, 95, 96]. SynB peptides RGRLSYSRRRFSTSTGR, Penetration, and Mastoparan enhanced the drug permeability in the co-cultured BBB model and two-fold drug transport *in vivo* [97]. Conversely, the stability of the cell-penetrating peptides was reduced *in vivo*, which ultimately affected the activity of the drug and practical applications.

Geniposide-liposome (GE-LP) conjugate has been experimented with for cerebral ischemia-reperfusion injury (CIRI) therapy, followed by

evaluation such as pharmacokinetics, brain targeting, and neuro-protective effects *in vivo*. Although GE possesses a splendid neuro-protective effect, it has a short half-life in circulation and lacks brain targeting. The formulation was characterized by 223.8 nm. Subsequently, the *in vivo* experiments revealed three-fold longer availability and bio-distribution of GE-LP than the GE solution. Also, the middle cerebral artery occlusion (MCAO) rat model contemplated the neuro-protective effect of GE by preventing the injury of CIRI [98]. The liposomal formulations experimented with for AD and PD are discussed in the following sections and represented in Figure 3.

10. Liposomal approaches to Alzheimer's disease

Alzheimer's disease (AD) is a debilitating central nervous system (CNS) disease that is expected to impact 115 million individuals globally by the year 2050. The influence of AD will be pronounced widely in the future. AD is connected with the accretion of misfolded and aggregated proteins such as β -amyloid and tau in the senile plaques [32]. It is categorized as an irreversible chronic neurodegenerative disorder that leads to a tremendous economic burden [99]. Though AD and its symptoms were discovered 100 years ago, FDA approved drugs can treat only the disease symptoms. There are no preventive or long term solutions available [100]. The commercial drugs prescribed for AD alleviate the symptoms temporarily and do not suppress the progression of the disease. Enhanced levels of $A\beta$ protein influence sporadic AD, and several studies indicated that the declining concentration of $A\beta$ protein is perhaps advantageous in AD [101]. Experimental evidence supports the role of apolipoprotein E (ApoE) as a crucial factor associated with $A\beta$ level and amyloid burden in the AD progression [102, 103, 104]. Liposomes delivered plasmid encoded ApoE2 (pApoE2) for the effective treatment of AD. Before encapsulation, chitosan was applied to facilitate DNA condensation and prevent endolysosomal degradation [22].

Further, the surface of liposomes was functionalized with Glut-1 substrate mannose (MAN) to target glucose transporter-1 (glut-1) of BBB [30, 105]. In addition, penetratin (Pen) of rabies virus glycoprotein peptide (RVG) was used along with MAN for the enhanced penetration of liposomes [106]. The liposomes modified with RVGMAN and PenMAN significantly enhanced the transport and transfection of ApoE2 genes across the BBB. Of note, the dual functionalized liposomes were to be effective in brain targeting and also prevent endonuclease digestion [106].

Bi-functionalized liposomes (mApoE-PA-LIP) targeting AD have been designed. Its therapeutic effectiveness experimented with a transgenic mouse model (early-onset APP/PS1) with the outcome of a reduction in amyloid burden and memory improvement. The liposomes were fabricated to cross BBB, impeding the formation of brain $A\beta$ aggregates and enhancing their clearance from the brain. To achieve this, the liposomes were bi-functionalized with sphingomyelin (Sm) and cholesterol (Chol) with phosphatidic acid (PA) with the mission of binding to $A\beta$ [107] and crossing the BBB [108, 109]. Treatment with mApoE-PA-LIP, enhanced the reduction in the brain plaques and slow-down the neurodegeneration process [110]. Glutathione targeted two different liposomal formulations were designed to off-load amyloid-targeting antibody fragments across the BBB. 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and egg-yolk phosphatidylcholine (EYPC) based glutathione targeted two different liposomal formulations were fabricated to off-load amyloid-targeting antibody fragments (VHH-pa2H) across the BBB. The antibody fragments were pre-labelled with radioisotope indium-111. The retention of VHH encapsulated with GSH-PEG liposomes was significantly higher in the brains of transgenic mice than wild-type controls. The results indicated that targeting glutathione is suitable for the specific delivery of targeted drugs and proteins [111]. Imatinib mesylate (IM)-liposomes formulations have been reported for a nose to brain delivery. IM is an anticancer agent that was revealed to treat AD. The drug was loaded into liposomes with particle size less than 150 nm, and the drug release was observed up to 96

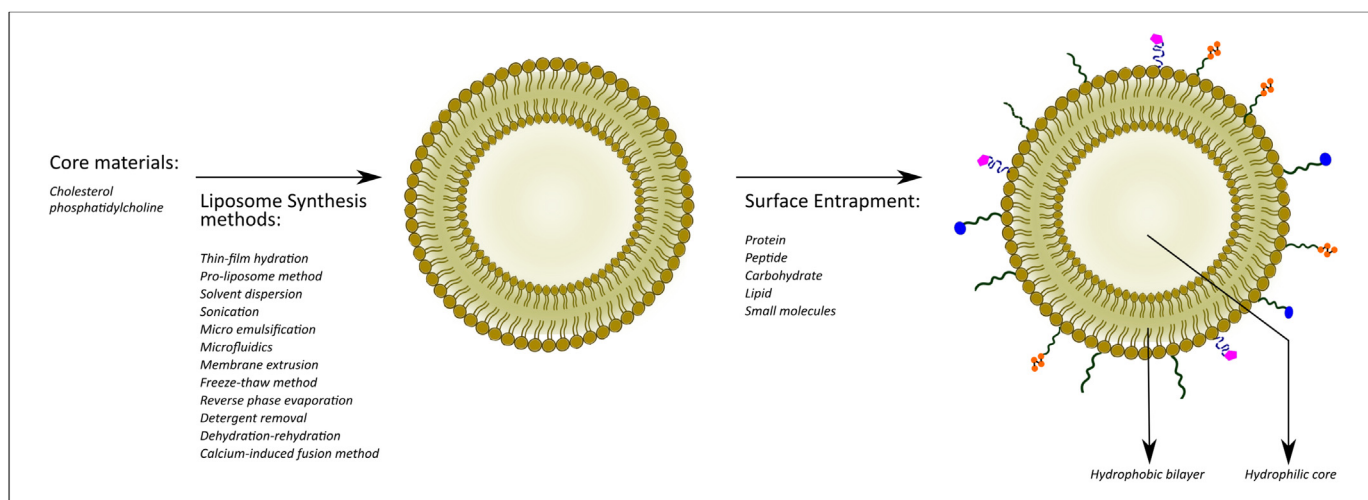


Figure 3. Denotes the synthesis of liposomes by various methods. Surface orchestration by peptide, protein, carbohydrates, lipids, and small molecules empower liposomes to target specific receptors and to cross BBB.

h. The liposome formulation enhanced the brain deposition of IM *in vivo* while compared to the bare IM solution. The nose to brain delivery method of IM helps significant delivery and retention time of drugs than oral and intranasal routes [112]. Osthole (Ost), a coumarin compound with limited solubility, bio-availability and low permeability, was delivered across BBB by liposomes (CXCR4-Ost-Lips). The liposome was surface-functionalized with Ost and CXC chemokine receptor 4 (CXCR4). The intracellular uptake and cytoprotective effect were demonstrated *in vitro* by APP-SH-SY5Y cells. Besides, the brain distribution, prolonged circulation, intensified accumulation of CXCR4-Ost-Lips was witnessed, along with alleviating AD-related pathologies [113].

11. Liposomal approaches to Parkinson's disease

The occurrence of PD relies on the assembly of α -Synuclein protein in the brain and irregular protein clusters [114]. PD is diagnosed with characteristics such as selective loss of dopaminergic neurons and neuroinflammation in the substantia nigra (SN) [115]. Existing treatment strategies are not definite to reduce disease progression. Administration of levodopa (L-dopa) is the primary treatment for PD by restoring striatal dopamine levels [116]. Previous studies support that the interfacial reactions of α -Synuclein and nanomaterials make the nanoformulation more functional against fibrils or α -Synuclein fibrillation [117].

The development of a novel microbubble (MB) liposomal complex has been demonstrated as a gene carrier and triggered the BBB opening during the ultrasound-targeted microbubble destruction (UTMD) process for CNS gene delivery. The clinically approved MB formulation is coupled with neurotrophic factors (NF)-loaded PEGylated liposome to carry versatile genes across BBB [118]. NF has been reported to reduce a progressive neuronal loss [119] and plays a significant role in the central and peripheral nervous systems [120]. Glia cell-derived neurotrophic factors (GDNF) and brain-derived neurotrophic factors (BDNF) levels are crucial therapeutic targets for neuronal cells [121]. The study revealed that either GDNF or BDNF gene carriers are related to behavioral correction and dopaminergic neuronal protection. Monumental evidence supports the role of GDNF or BDNF in neuroprotection, neurotransmission, and enhancement of dopaminergic neuronal loss in MPTP animals [119, 121, 122]. A brain targeting liposome formulated with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), Chol, and L- α -phosphatidic acid (PA) in a molar ratio of 5.5:4.0:0.5 has experimented in murine models with PD. The liposomes were devised with a moiety of diacylglycerol and coupled to a peptide of 5 amino acids by a linker. The amino acids were chosen from the amyloid precursor protein (APP) to target BBB-specific receptors. APP-targeted liposomes injected with

dopamine (800 μ g/kg) resulted in a substantial increase of striatal DA in amphetamine-treated mice within 5 min. The enhanced striatal DA content was observed in circulation 3 h post-administration, representing the sustained release of DA from the liposomes [123].

12. Dual targeting liposomal drug delivery

The decoration of the liposomal surface with two ligands is a current trend in liposome surface functionalization. Dual-targeted liposomes have significant advantages, such as targeting two or more receptors and transporting more drugs to cells. Another advantage of dual targeting is the synergistic therapeutic effect of the loaded drugs and reduced toxicity in normal tissues [124]. The use of liposomes sparked early attention, leading to the approval of several liposomal medicines to treat a variety of autoimmune diseases and malignancies. Inserting ligands into the particle surface improves the delivery of nanocarriers to tumor cells or other structures, allows active targeting. For example, antibodies or antibody fragments coupled to PEG chains can be inserted into the lipid bilayer to create sterically stabilized immunoliposomes. Another essential prerequisite is the efficient internalization of the drug-loaded carrier and intracellular release from the endosome, which can be achieved by encapsulating ligands or antibodies binding to rapidly internalizing receptors with stimuli-responsive liposomes such as pH-sensitive liposomes [125, 126, 127]. These liposomes are called immunoliposomes. Mumbengegwi and Allen designed an immunoliposome with anti-CD19 and anti-CD20 IgG coupled to maleimide-PEG-DSPE for CD19 and CD20 directed cellular uptake. The results indicated a considerable reduction in the cellular cytotoxicity when compared to doxorubicin-loaded liposomes [128].

Similarly, natural ligands and synthetic peptides can also be used to generate double-targeting nanoliposomes. For example, liposomes functionalized with folate and anti-EGFR antibody, or incorporating liposomes with various molecules such as α v β 3 recognizing arginine-glycine-aspartate (RGD) peptide with neurophilin, transferrin, galectin-1, and so on [129, 130]. Several research groups have created liposome formulations with dual ligands. Ke et al. have demonstrated around 90% entrapment efficiency (EE) and strong bond targeting effect of Asp8 (aspartate) and folate modified doxorubicin (DOX)-loaded liposomes [131]. Peng Y et al. demonstrated the efficiency of dual-targeting moiety glucose-vitamin C (Glu-Vc-Lip) as liposomes to deliver Paclitaxel (PTX) into the brain effectively. Glu-Vc-Lip could be identified on the surface of cells by glucose transporters GLUT1 and SVCT2 through residues on the surface and then energy-dependently absorbed by the synthetic endocytic route that included clathrin-mediated, caveolae-mediated, and micropinocytosis-mediated endocytosis [132].

Xue Ying et al. synthesized and evaluated the drug delivery efficiency across the BBB of dual-targeting daunorubicin liposomes modified with MAN (4-aminophenyl- α -D-manno-pyranoside) and transferrin (TF) in mice. Compared to free daunorubicin, dual-targeting daunorubicin liposomes enhances daunorubicin circulation time in the blood and increases the daunorubicin transport over the BBB [124]. In another study conducted by Lakkadwala et al., the liposome functionalized with TF and a cell-penetrating peptide (CPP) showed a 10-fold and 3-fold increase in DOX and erlotinib accumulation, respectively in mice brains [133]. Similarly, Pu et al. developed and evaluated the efficiency of dual functionalized liposomes targeting TNBC (triple-negative breast cancer). The liposome contained fructose and RGD peptides to recognize GLUT5 and α v β 3 actively. The dual functionalized liposome loaded with PTX showed elevated growth inhibition of MDA-MB-231 and 4T1 cells [134].

13. Drawbacks and barriers

Liposomes have the potential to become a fantastic drug delivery vehicle, but they are not without flaws. Like every foreign particle that enters the body, liposomes are met by a variety of defense systems geared at detecting, neutralizing, and eliminating the invading molecules. Disadvantages include variability instability, drug-encapsulation, short life span, batch-to-batch reproducibility, and sterilization. However, as seen by an increasing number of clinical investigations, most of the faults have been rectified, albeit certain concerns remain.

14. Activation of immune system and clearance of liposomes

The RES includes phagocytic cells that help eliminate particles and soluble chemicals from the bloodstream and tissues. Immune complexes, germs, poisons, and foreign antigens are just a few of the eliminated substances. Internalization happens via non-specific endocytosis, non-immune receptor-mediated phagocytosis, or immunocytotoxic actions mediated by binding to Fc or complement receptors. Following systemic injection of liposomes, RES is the primary location of liposome accumulation. Kupffer cells in the liver, microglia in the brain, alveolar macrophages, bone marrow lymph nodes, macrophages in the colon, and other organs make up the RES. The liver, lungs, bone marrow, spleen, kidney, and lymph nodes are the primary organs linked with RES [135].

The liver has the most liposomal absorption capability, followed by the spleen, which may store liposomes up to 10-fold more than other RES organs. Liposomes are removed in the RES by resident macrophages' direct interaction with phagocytic cells. Liposomes uptake by RES is usually triggered by vesicle opsonization, i.e., adsorption of plasma proteins such as immunoglobulin, fibronectin, lipoproteins, or complement proteins onto the phospholipid membrane. Several studies have also demonstrated liposomal clearance in the absence of plasma protein [136].

Because RES cells are also part of the innate immune system, it's unclear if liposome saturation of macrophages causes immunosuppression and raises the risk of infection. Excessive liposome deposition in macrophages may decrease phagocytic capability or affect other cellular activities; nevertheless, clinically meaningful immune suppression at therapeutic doses of non-cytotoxic liposomes has yet to be reported [137].

Anticancer liposomes containing cytotoxic medicines capable of causing macrophage destruction are in a distinct scenario. Although clinically significant macrophage function inhibition in humans has yet to be established, few indirect indicators imply immune suppression. For example, due to partial blockage of RES in the liver, administration of PEGylated liposomal doxorubicin (PLD) in mice resulted in a dose-dependent clearance saturation effect. This effect was not observed in drug-free liposomes after a comparable amount of free doxorubicin or phospholipid was administered. Furthermore, PLD delivery to mice was found to interfere with bacterial clearance from the blood, attributed to macrophage suppression [138].

PEG polymer conjugation to the liposomal membrane is important for increasing circulation times and inhibiting RES elimination via steric stability. PEGylation results in a localized concentration of highly hydrated groups on the surface of plasma proteins or cells, which sterically limits both electrostatic and hydrophobic interactions with plasma proteins and/or cells, reducing liposomal absorption by RES. The addition of PEG reduces but does not eliminate liposomal absorption by RES—pathways other than opsonization are also feasible [139, 140].

The methoxy form of PEG has a single hydroxyl group that can be coupled with various entities such as small drugs, proteins, polymers, and lipids [141]. The stealth shielding and increased circulation time are the outcomes of the PEGylation [142]. The development of a dense hydrophilic barrier on the surface of the PEGylated carrier reduced their interactions with the reticular-endothelial system. Also, PEGylation enhances the hydrodynamic size of the drug delivery system, thus resulting in reduced clearance from the circulation [91, 143]. However, modifications in the PEG chain may occur while the plasma proteins of the blood start to interact with the surface of the nanomaterials [144]. Also, incomplete PEG shielding over the surface of the carrier or nanomaterial may pave the way to opsonization [145]. Though several studies supported as PEG is poorly immunogenic, the development of immunogenic responses [146], and complement activation [147], antibody production [148] against the systemic administration of PEG were reported. Traces of naïve-anti-PEG antibodies have been found in healthy patients who have never been medicated with PEGylated drugs [149]. Increased hydrophobicity of the PEG end-group is considered as the reason for the activation of immunoglobulins (IgG, and IgM) [20], and ethylene oxide units of PEG can be recognized by the antibodies [150, 151].

The pharmacokinetics and bio-distribution of PEGylated liposomes (2nd dose) were affected in rats and rhesus monkeys followed by the initial administration of the same formulation. Ultimately, the half-life and circulation of PEGylated liposomes were affected when they were administered a second time, and anticipatedly accumulation in the liver and spleen increased proportionally [152, 153]. Similarly, the degree of anti-PEG IgM production and the ABC phenomenon are drastically reduced in splenectomized rats, suggesting that this immune response is mediated by the spleen. Higher dosages of the first PEGylated liposomes, on the other hand, have been demonstrated to limit the degree of ABC phenomenon. It has been proposed that increasing the phospholipid dosage causes PEG-reactive B cells to be apoptotic and, as a result lowers anti-PEG IgM production and therefore abating the ABC phenomenon [148, 154, 155]. ABC is an unexpected immunogenic response to the repeated administration of PEG-conjugated substances and PEGylated nanocarriers such as PEGylated liposomes, PEGylated nanomaterials, PEGylated micelles, and other PEGylated nanocarriers leads to increased clearance and decreased efficacy of PEG-conjugated substances or PEGylated nanocarriers. The ABC phenomenon is a major issue for PEGylated formulations that require multiple dosage regimens in clinical practice.

Apart, the complement system's activation against the PEGylated liposome administration was accounted for by the *in vitro* and *in vivo* studies [156, 157, 158]. Doxil, composed of PEGylated liposomes loaded with crystallized doxorubicin hydrochloride in the aqueous compartment, was taken to study the effect of complement activation. Among 29 patients enrolled for the clinical study, 13 patients showed clinical reaction upon the exposition of Doxil, high levels of SC5b-9 complex were quantified in 21 patients, and 9 patients showed complement activation without any symptom [159]. Though various studies support the hypothesis of PEG derivatives can activate the complement pathway, further studies are needed to prove the mechanism of complement activation [160].

15. Complement activation-related pseudoallergy (CARPA)

One of the main goals of current pharmacotechnology is to improve the drug therapeutic index (TI) by adopting nanoparticulate vehicle

systems to assure delayed or tailored drug delivery. Some liposomal systems and medications can activate the innate immune system, resulting in complement system activation and an acute hypersensitivity condition called complement activation–related pseudoallergy (CARPA). CARPA is a pseudo-allergic, non-IgE-mediated hypersensitivity response (HSR) induced by C activation. CARPA is a kind of biological stress that occurs due to immune system stress. It can have adverse side effects such as fast medication clearance, reduced efficacy, producing an acute disease such as anaphylactic shock and antibody formation against pharmaceuticals, therefore eliminating their therapeutic utility [161, 162, 163].

Haematological abnormalities, such as thrombocytopenia, leukocytosis, and leukopenia with or without compensatory leukocytosis are signs of CARPA. Infusion-related hypersensitivity events to liposomal drug treatment have been documented in many patients (2–45%). CARPA has also been found in liposomal formulations that are both experimentally and clinically acceptable. This pseudoallergy is caused in part by complement activation, which results in the production of C3 split and anaphylatoxins C3a and C5a. Mast cells, basophil leukocytes, secretory macrophages, WBC, and platelets are stimulated to release a wide range of vasoactive and inflammatory mediators by the anaphylatoxins C3a and C5a through their anaphylatoxin receptors. Capillary leakage, bronchoconstriction, pulmonary vasoconstriction, coronary vasoconstriction, and systemic vasodilation can all be caused by this inflammatory cascade, which can also affect autonomic effector cells such as smooth muscle cells and endothelial cells. A variety of vasoactive mediators are released when anaphylatoxins bind to their specialized receptors on immune cells, including histamine, tryptase, platelet-activating factor (PAF), leukotrienes (LT), thromboxane-A2 (TXA2), and prostaglandins (PGs). It should be emphasized, however, that the sensitivity of various species to liposomal CARPA varies significantly, with certain species (dogs and pigs) demonstrating tachyphylaxis (tolerance induction) in response to further doses [164, 165, 166].

Any form of liposomes such as liposome size, charge, surface properties, lipid composition, shape, bilayer packing, and even lipid dosage can trigger complement activation. The existence of aggregates, medicines that can bind to the aggregate, the presence of cholesterol in the bilayer membrane at 70%, and PEGylation with PEG-PE are all liposomal features that increase the tendency for complement activation. As a result, this should be considered while developing formulations and rigorously evaluated during clinical trials [137, 167].

16. Alternative strategies to overcome PEGylated Liposome's immunogenicity

At present, PEG is used to stabilize, enhance the half-life of nanocarriers, and augment the pharmacokinetics of nanomedicines. Indeed, the side effects and immunogenicity were reported in the patients administered with PEGylated drugs, which ultimately reduced nanocarriers' pharmacokinetics. To overcome these obstructions, research reports support the use of other biodegradable (sialic acid, ganglioside, poly-amino acids, and hyaluronic acid) and non-biodegradable polymers (vinyl polymers, poly (glycerol) (PG), and HDSA-SHP) for drug delivery. Liposomes coated with sialic acid made them long-circulating nanocarriers initially reported by Allen and co-workers [168]. Doxorubicin-loaded liposomes coated with sialic acid were explored for targeting Peripheral blood neutrophils (PBNs). The liposomes modified with sialic acid exhibit greater tumor targeting ability and strong therapeutic efficacy [169]. Various poly-amino acids were reported as an alternative to PEG and to synthesize stealth nanocarriers. Poly (hydroxyethyl-l-asparagine) (PHEA) and poly (hydroxyethyl-l-glutamine) (PHEG) were examined as drug delivery systems and to substitute PEG. The coating reduced the ABC phenomenon for the surface-modified liposomes [170]. Manipulating the physiochemical properties of nanocarriers may help to overcome their immunogenic response. PEGylated polymeric micelles below 30nm may not cause the

ABC phenomenon [171]. Neglected anti-PEG IgM production was observed upon systemic administration of PEGylated interferon drug, where the molecular weight of PEG is 40 kDa [172]. Based on the experimental results, surface modification with low molecular weight molecules may enhance the circulation time of liposomes and drug delivery.

17. Expert opinion on liposomal drug delivery for neurodegenerative disorders

The prevalence of brain disorders is increasing worldwide, and there is a need for new therapeutic strategies to reach brain cells. The neurodegenerative conditions, including AD and PD, result in atrophy of the brain and rob the cognitive function of elders. Though the liposomes are proved to be good carriers, and therapeutically experimented with for brain disorders, most of the studies were demonstrated on animal models. Most of the drugs are medicated orally for the management of neurodegenerative disorders. At the same time, they are aided in symptoms management, not reaching the target properly due to their metabolism by the liver [173]. The preset barriers of CNS are composed of the blood-cerebrospinal fluid barrier and the BBB [26]. These barriers not only impede the medications for AD or PD but also meningitis viral and bacterial infections, psychiatric disorders, and acquired immune deficiency syndrome [174]. The boundaries set around the BBB offer new limits for nanotherapeutics to surpass in the delivery system's domains such as affinity, absorption, and function. Site-specific delivery facilitates bypass of oral and intestinal absorption of drugs and enhances the bioavailability at the target site. The liposomal formulations need to be improved to enhance the efficacy and specificity of chemotherapeutics, subsequently reducing the adverse effects of drugs.

The surface modifications with peptides, proteins, aptamers, and antibodies perhaps enhance the BBB penetrating ability of liposomes; meanwhile, the formation of antibodies and clearance of liposomes from circulation need to be addressed. Albeit the liposome formulations are proven to treat brain disorders, and some of them are under clinical trials, there are some downsides from the view immune system. Liposomes and their formulations are readily cleared by the mechanism of opsonization, RES, ABC, and CARPA. Novel methodologies are needed to overcome or reduce the immunogenicity against the liposomes or PEGylated liposomes. Surface orchestration allows the nanomaterials to cross BBB and avoids the phagocytic opsonization process [175]. Rivastigmine-loaded liposomes stabilized with dodecyl dimethyl ammonium bromide for nasal delivery methods. The modifications enhanced the presence of rivastigmine at the plasma and brain levels [176]. Liposomal formulations enhance the bioavailability of agents, lower reticuloendothelial uptake, and ultimately reduces systemic toxicity. Though liposomal formulations need to cross sundry struggles, many are in USA clinical trials [177], and the successive outcomes will be a hope for the treatment of neurodegenerative disorders. In this purview, novel materials need to be discovered to obscure liposomes and formulations and to offer long circulation ability. Hence, additional investment and detailed research are needed to develop and take the formulations for the clinical trials stage of drug development.

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