CHEMICAL REVIEWS

Review

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New Developments in Liposomal Drug Delivery

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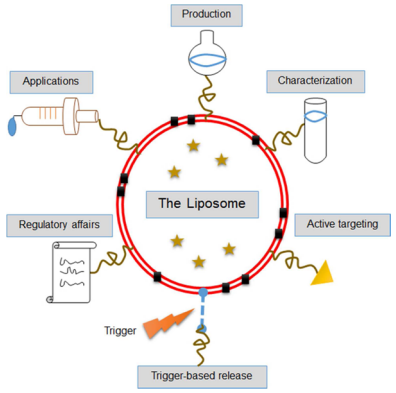

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1. INTRODUCTION

Described first in the 1960s by Bangham[1] and understood as a potential drug delivery system in the early 1970s,[2−4] the liposome has since become integral to research and clinical applications in the field of nanomedicine. Five decades of research in the field of liposome research have shown their prospective benefits in the medical and cosmetic[5−7] as well as the food industry.[8,9] Several promising small molecule drugs and genes previously deemed less than useful due to problems of stability, solubility, and nonspecific toxicity can now be delivered to the intended sites of action with the help of nanocarriers like micelles, nanoparticles, and liposomes.[10]

Liposomes are composed of phospholipids, which self-enclose to form spheres of lipid bilayers and an aqueous core within the bilayers. Ampiphilic in nature, the phospholipids result in polar shells in aqueous solutions (Figure 1) due to the hydrophobic effect of the hydrophobic acyl chains with the surrounding aqueous medium. This is a thermodynamically favorable formation further enhanced by hydrogen bonding, van der Waals forces, and other electrostatic interactions.[11,12] Because of the presence of an aqueous core and a lipid bilayer, liposomes can incorporate hydrophilic as well as lipophilic molecules. The solubility and the in vivo fate of the incorporated molecules become dependent on the liposomes employed. Advantages of the liposomes include the following:

- Improved solubility of the encapsulated drugs
- Prevention of chemical and biological degradation under storage conditions of agents and during patient administration
- Reduction of the nonspecific side-effects and toxicity of encapsulated drugs, thus improving their efficacy and therapeutic index
- Versatility when chemically modified with attached specific surface ligands for targeting
- Compatibility with biodegradable and nontoxic materials

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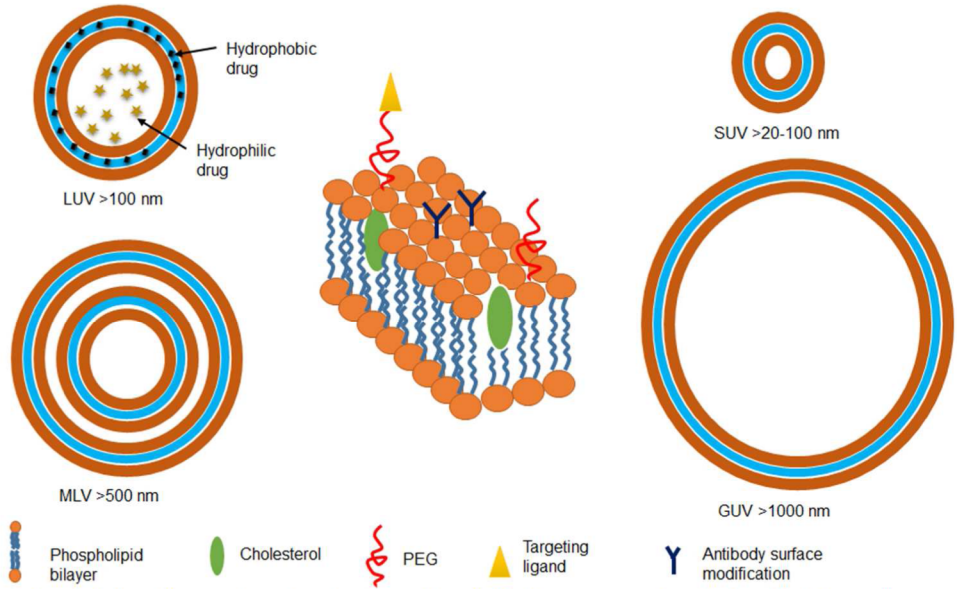

Figure 1. Different liposome types based on size and lamellarity. Also shown is a portion of a typical lipid bilayer with multifunctional surface modifications. Size not to scale.

Table 1. Currently Approved Applications of Liposomal Drug Formulations

product name	drug delivered	approved treatment	ref
Myocet	doxorubicin	metastatic breast cancer	15
Doxil	doxorubicin	Kaposi's sarcoma, ovarian, and breast cancer	16−18
Lipodox	doxorubicin	Kaposi's sarcoma, ovarian, and breast cancer	19
DaunoXome	daunorubicin	hematological malignancy	20
Marqibo	vincristine sulfate	acute lymphoblastic leukemia	21
Ambisome, Abelcet, Amphotec	amphotericin B	fungal infections	22−24
Depocyt	cytarabine	neoplastic meningitis and lymphomatous meningitis	25
Visudyne	verteporfin	age-related molecular degeneration	26−28
DepoDur	morphine sulfate	pain	29,30
Epaxal	inactivated hepatitis A viral strain RG-SB	hepatitis A	31
Inflexal V	inactivated hemaglutinin of influenza virus strains A and B	influenza	32

Table 2. Liposome-Based Formulations in Clinical Trials

product name	drug delivered	indication	trial phase	ref
LEP-ETU	paclitaxel	ovarian, breast, and lung cancer	Phase I/II	33,34
EndoTAG-I	paclitaxel	breast and pancreatic cancer with antiangiogenic properties	Phase II	35−37
ThermoDox	doxorubicin	nonresectable hepatocellular carcinoma, breast cancer	Phase II, III	38
Anti-EGFR immunoliposomes	doxorubicin	solid tumors	Phase I	39
MM-398	irinotecan	recurrent solid tumors, colorectal, breast, pancreatic, and ovarian cancer	Phase I/III	40−42
Liposomal Grb-2	*Grb2* antisense oligodeoxynucleotide	acute myeloid leukemia, chronic myelogenous leukemia	Phase I	43
SPI-077	cisplatin	lung, head, and neck cancer	Phase I/II	44,45
Lipoplatin	cisplatin	pancreatic, breast, non-small cell lung, head, and neck cancer	Phase III	46−50
LEM-ETU	mitoxantrone	breast, stomach, liver, and ovarian cancer, leukemia	Phase I	51
Stimuvax	BLP25 lipopeptide	non-small cell lung cancer	Phase III	52
Liposome-annamycin	annamycin	breast cancer, acute lymphocytic leukemia	Phase I/II	53,54
INX-0076	topotecan	advanced solid tumors	Phase I	55
INX-0125	vinorelbine	advanced solid tumors	Phase I	56
Arikace	amikacin	lung infection	Phase III	57
2B3-101	doxorubicin	solid tumors/recurrent malignant glioma	Phase I	58
Pulmaquin/Lipoquin	ciprofloxacin	non-cystic fibrosis bronchiectasis	Phase II/III	59

These properties of liposomes have led to many successful applications (Table 1) and clinical trials (Table 2). Liposomes can be classified in multiple ways depending on their size and number of bilayers and their composition (Table 3). It is important to understand the properties of the liposomes used to select a suitable route of administration as well as judge the

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Table 3. Classification of Liposomes

based on lamellarity and size	based on composition
small unilamellar vesicles (SUV); 20−100 nm	conventional liposomes
large unilamellar vesicles (LUV); >100 nm	long-circulating liposomes
giant unilamellar vesicles (GUV); >1000 nm	cationic liposomes
oligolamellar vesicles (OLV); 100−1000 nm	stimuli-sensitive liposomes (pH, temperature, magnetic field)
multilamellar vesicles (MLV); >500 nm	immunoliposomes

pharmacokinetic fate of the drug delivery system. To illustrate, the number of bilayers directly influences drug entrapment capacity, the addition of surface bound ligands, drug release from the system, storage characteristics,[13] as well as liposome−cell interaction and internalization.[14]

There has been much research in the field of liposomes, and multiple reviews of the research have been composed.[60−65] This Review is an attempt to provide a comprehensive insight into the liposome technology with recent advances in the liposome formulations and their applications in medicine.

2. LIPOSOMES: COMPOSITION, PREPARATION, AND CHARACTERIZATION

2.1. Composition of Liposomes

Liposomes are composed of naturally occurring and/or synthetic phospholipids such as phophatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine, and phosphatidylglycerol. The phase transition temperatures (T_M) of the phospholipids are especially important. The lipids exist in either a fluid state ($T > T_M$) or a gel state ($T < T_M$), depending on their temperature. The fluid state of the lipids is more permeable to water and can be exploited to encapsulate drugs during liposome production. At body temperatures ($T \approx 37$ °C), a fluid state will make the liposomes leaky, and the encapsulated drugs are likely to escape before reaching the site of action. Thus, choosing phospholipids with gel states at physiological conditions is often desirable to stabilize liposomes.

The phospholipids have an intrinsic natural flip-flop, or rotational freedom, which also promotes leakiness of liposomes. To stabilize the bilayer, cholesterol is generally added to the formulations of liposomes. The addition of cholesterol in different concentrations has various effects on the capacity of the liposomes to encapsulate and deliver a drug.[66−68] In an experiment conducted on the effect of cholesterol in egg phosphatidylcholine (ePC) liposomes, high cholesterol content preserved the stability of the liposomes in mice regardless of route of parenteral administration for a considerably longer time than cholesterol-poor liposomes.[69] In another study, the addition of the cholesterol led to increased vesicle size in a concentration-dependent manner.[70] Thus, consideration of the amount of cholesterol employed is necessary to formulate liposomes of desired characteristics.

The charge of the liposome can play an important role in the fate of the liposomes.[71,72] Liposomes can be either negatively, neutrally, or positively charged depending on the additives in their composition. For example, oleic acid addition generates negatively charged liposomes, while addition of *N*-[1(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTAP) makes the liposomes positively charged. Charged liposomes exhibit electrostatic repulsion, and thus do not aggregate quickly in storage conditions. Because the cell membranes are negatively charged, there is electrostatic attraction with cationic liposomes that increases cell−liposome interaction and internalization.[73,74] Cationic liposomes are generally more useful for loading nucleic acids because of their negative charge, and they can be loaded electrostatically. The composition of the liposomes also affects the cargo release profile of the system. A recent study explored the use of surfactants, commonly used to solubilize the liposomes, to modify the encapsulation and release properties of the liposomes.[75]

2.2. Methods of Liposome Preparation

There are several methods to prepare liposomes with each influencing liposome properties including size, lamellarity, and encapsulation efficiency (EE). While some approaches are easy, especially at laboratory scale, others are more useful for scale-up but require special equipment. As we shall see, the methods can be categorized as conventional or novel. Techniques for drug loading into the liposomes and for sterilizing the liposome preparations will be addressed.

2.2.1. Conventional Methods. The Bangham method or thin lipid film hydration method was the first described method for preparing liposomes.[76] This simple method involves creating a thin film of lipids in a round-bottom flask by evaporating the organic solvents and a freeze-drying procedure to ensure the complete removal of organic solvents. The liposomes form after rehydration with aqueous solvents. With vigorous shaking at rehydration, multilamellar vesicles (MLVs) with heterogeneous size distribution are formed, while a gentle hydration will generate giant unilamellar vesicles (GUVs).[77] With this method, additional size reduction techniques may be needed. Small unilamellar vesicles (SUVs) with homogeneous size can be generated with sonication. Probe sonication, using a titanium probe inserted into the lipid preparation, generates SUVs very effectively and can be adjusted to obtain liposomes of a required size. The drawbacks of this method involve sonicator contact with the liposomes, risk of high temperature exposure that may result in phospholipid/active ingredient damage, low encapsulation, as well as metal contamination from the probe. Water bath sonicators are a convenient alternative with the liposomes isolated from the surrounding water. With appropriate temperature and sonication parameters, homogeneous SUVs can be obtained.[78−80] Another method of reducing the size is by multiple extrusions through a polycarbonate membrane. The degree of size reduction is dependent on the number of extrusion cycles and the size of polycarbonate membrane pores.[81−83] A drawback of the thin film hydration technique is low aqueous core entrapment and subsequently low drug EE.

A generally employed preparative alternative is the reverse phase evaporation technique. This consists of initially forming inverted micelles or water-in-oil emulsions where the water phase carries the drug of interest and the organic phase is comprised of the lipids for the liposome bilayer formation. Briefly, the lipid mixture is added to a flask, and after evaporating the solvents, lipid films are formed, which are redissolved in an organic phase consisting mostly of diethyl ether and/or isopropyl ether. Addition of the aqueous phase results in a two-phase system, which can be sonicated briefly to form a homogeneous dispersion. Slow evaporation of the organic solvent under reduced pressure initially leads to

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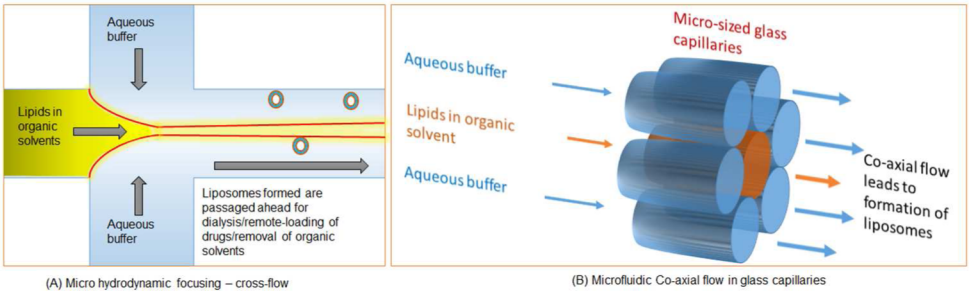

Figure 2. Microfluidics technology to produce liposomes with high precision and efficiency. (A) Cross-flow where the aqueous buffer is introduced in a different axis to the lipids in organic solvents. (B) Coaxial flow through glass capillaries.

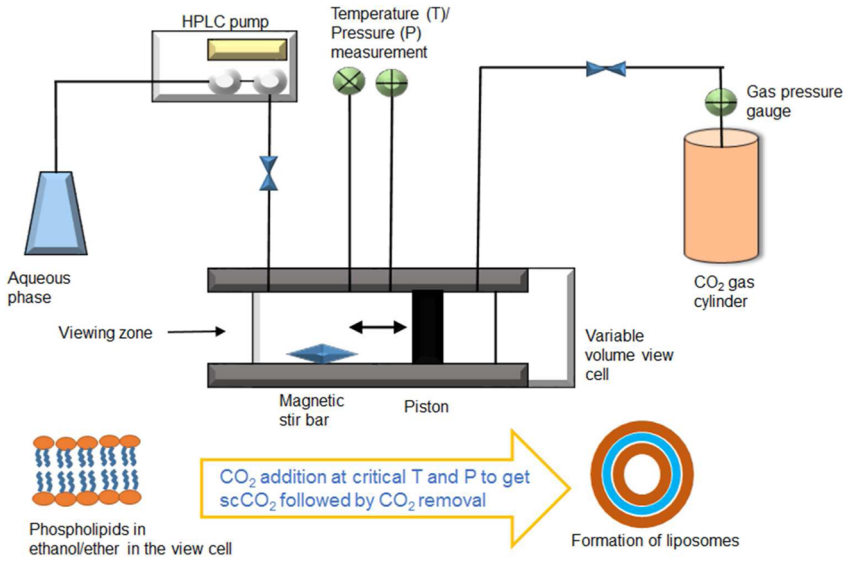

Figure 3. Schematics of supercritical reverse phase evaporation (SRPE). Reprinted with permission from ref 99. Copyright 2001 American Chemical Society.

conversion of the system into a viscous gel, which results in an aqueous suspension containing the liposomes. This method results in higher internal aqueous loading as compared to the thin film hydration method. The remaining solvent can be removed by dialysis, centrifugation, or passage through a Sepharose 4B column.[84] However, trace elements of the organic solvent may remain, which can interact with the lipids and the drugs/genes.

Injection of phospholipids dissolved in an organic phase (ethanol or ether) into a drug-containing aqueous phase also leads to formation of liposomes. This is termed the solvent injection technique. On injection of ethanol, its dilution results in instant liposome formation.[85] By comparison, because ether is immiscible with water, the ether is added to a prewarmed aqueous phase at ~60 °C, resulting in removal of ether and formation of the liposomes.[86,87] Solvent injection techniques result in the formation of a heterogeneous species of liposomes. The drawback with these methods involves incomplete removal of the organic phases especially ethanol. Contact with the organic phase as well as high temperatures during ether injection may be detrimental to cargos.[88]

Detergent dialysis uses phospholipids, dissolved in detergent micelles, which are added to the aqueous media. On removal of the detergent by either dialysis or size exclusion gel chromatography, the phospholipids coalesce together to form large unilamellar vesicles (LUVs).[89−91]

The conventional techniques for the liposome formation, although straightforward for small-scale preparations, are not convenient for industrial scale due to disadvantages including broad size distribution and inconsistent encapsulation efficiency, constant contact of the lipid/drug cargo with organic phases, along with difficulty in effective sterilization. With more recent advances in technology, novel methods have been studied for efficient generation of liposomes that can also be scaled up and applied to a broad range of phospholipids, additives, drugs, and genes. These are presented in the following section.

2.2.2. Novel Methods. First proposed by Jahn et al., a micro hydrodynamic focusing (MHF) method has been successfully used to form monodisperse liposomes using microfluidic technology.[92] Typically, in small microfluidic channels of varying diameters up to 500 μm, streams of aqueous phase result in laminar flow. A perpendicular flow of phospholipids in an organic phase (ethanol, isopropyl alcohol, etc.) results in diffusive mixing and local dilution of the organic phase. As a result, the phospholipids self-assemble into liposomes and are collected (Figure 2a). Parameters such as aqueous buffer-to-organic phase flow rate ratio (FRR), size of the microchannels, and concentration of phospholipids in the organic phase can be adjusted to achieve different liposome sizes and encapsulation efficiencies.[93] On increasing the FRR from 5:1 to 50:1, Jahn et al. changed the liposome size from 140 to 40 nm.[94] A recent analysis conducted on the effect of

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FRR on poly(ethylene) glycol (PEG) and folate incorporation onto the surface of liposomes found that an increase in FRR was inversely related to the incorporation efficiency of the PEG and folate.[95]

The ability to continuously produce liposomes for medical applications was studied using single hydrodynamic focusing (SHF) and double hydrodynamic focusing (DHF). While the SHF injected a single central stream of lipids in ethanol, which was hydrodynamically compressed by two aqueous streams, the DHF used two streams of lipids in ethanol. This increased the mass diffusion and the surface area between the aqueous and ethanol layers. The DHF device allowed for increased fluid flow velocity that resulted in higher production of unilamellar cationic liposomes with good size distribution for gene delivery.[96] An additional study modified the traditional ethanol injection method using two different approaches for microfluidic injection of the organic phase (a) via a microengineered nickel membrane maintained under shear-stress settings or (b) through a tapered-end glass capillary into coflowing aqueous stream with coaxial arrangements of glass capillaries (Figure 2b). These methods resulted in a larger surface area of contact between the ethanol and aqueous phases, avoiding the formation of organic phase droplets (emulsification) in the process and giving a precise control over the liposome size and polydispersity.[97]

The NanoAssemblr platform and the NanoAssemblr Scale-Up platform developed by Precision Nanosystems, Inc., Canada, utilize microfluidics to produce liposomes, among other nanomedicines, at a rapid and reproducible level, both at laboratory (milliliters) and at clinical scales (liters). This technology was recently analyzed to establish the mathematical parameters of total flow rate and the FRR to predict the impact on liposome size, polydispersity, and trapping efficiency.[98]

The supercritical reverse phase evaporation (SRPE) method, introduced in 2001, uses supercritical CO_2 ($scCO_2$) to dissolve the phospholipids.[99,100] At supercritical values, the CO_2 is dense and noncondensable and act as a convenient solvent with temperature- and pressure-dependent solvent properties. As illustrated (Figure 3), a variable volume view cell with a magnetic stirrer consists of phospholipids in an ethanol solution wherein a high pressure pump propels gaseous CO_2. The temperature and pressure inside the view cell are increased to a supercritical value for CO_2 with the temperature higher than the T_M of the phospholipids. After some seconds, aqueous drug-containing solution is introduced slowly into the cell using the HPLC pump until the desired drug concentration is reached. The next step involves reducing the pressure to release the CO_2 and formation of a liposomal dispersion. A 5-fold higher EE was reported as a result for a hydrophilic solute as compared to the traditional thin film hydration method. Transmission electron microscopy (TEM) revealed that the liposomes formed were LUVs with sizes of 0.1−1.2 μm.[99] The same group modified the method by concurrently putting the aqueous phase with the solid lipid materials in the view cell before introduction of the CO_2, resulting in an improved entrapment efficiency.[101] A benefit of this technology's use is that the $scCO_2$ is environment-friendly, cheap, and eliminates drug−organic solvent contact.

A recent study described modifications in the SRPE process that generated liposomes ranging between 130 ± 62 and 294 ± 144 nm, much below those obtained by the conventional SRPE technique. The supercritical assisted liposome formation, or SuperLip, utilized atomization of the water phase containing bovine serum albumin (BSA) into a high pressure vessel filled with $scCO_2$ and the phospholipid−ethanol mixture. The atomized water droplets were quickly surrounded by the lipid layer to form a water in CO_2 emulsion resulting in liposomes collected in a water pool at the bottom of the vessel. Different liposome sizes and size distribution could be obtained by modifying the process parameters including temperature, pressure, and the FRR between CO_2 and ethanol.[102]

In contrast, another group[103] utilized the $scCO_2$ as an antisolvent, which when in contact with phospholipids-containing organic solvent caused the phospholipids to precipitate. On hydration with aqueous buffer, liposomes were obtained. This method is known as the supercritical antisolvent (SAS) method.[104] Similarly, using the $scCO_2$ as an antisolvent, cyclosporine A was entrapped in liposomes composed of ePC with high efficiency and improved stability in comparison to the conventional Bangham technique.[105]

Freeze-drying uses lyophilization of a dispersion of liposomes in the presence of suitable water-soluble carriers such as sucrose, mannose, or lactose, which act as lyoprotectants. Briefly, the liposome preparation with the water-soluble carriers is sterilized by filtration and subjected to freeze-drying. This lyophilized liposome powder can be stored indefinitely and on rehydration with appropriate aqueous buffer can generate a spontaneous liposomal colloidal suspension.[106−108] In the absence of lyoprotectants, the lipid bilayers of the liposomes in an aqueous dispersion are in a loose configuration and result in a compact state after freeze-drying. The liposomes in such a dry state may generate packing issues with chances of liposome aggregation and leakage of encapsulated material, especially if the liposomes are neutrally charged. However, lyoprotectant addition before the freeze-drying step results in the insertion of the lyoprotectants on the surface of the bilayers in place of the water molecules. This interaction preserves the necessary headgroup spacing and reduces the van der Waals interactions between the acyl chains of the phospholipids after lyophilization. It thus prevents aggregation during rehydration and, because the packing of the lipid bilayer is stable, the content leakage is reduced.[109,110] Giving slight negative charge to the liposomes also helps to prevent aggregation during rehydration.[111] A comprehensive review on the lyophilization of liposomes, including the effects of several parameters such as lipid composition, lipid/carrier ratio, particle size, freeze-drying technology, and storage conditions on the final product, is presented.[112]

Spray drying has been widely used in the pharmaceutical industry for several purposes such as granulation for tablet manufacturing, drying of active pharmaceutical ingredients or large molecules such as proteins, inhalable powders, etc.[113] For liposome preparations, spray drying is also a convenient, single-step procedure that meets both laboratory scale and clinical scale requirements.[114] Preparation of spray-dried liposomes usually requires core carriers such as mannitol and lactose. Proliposomes are basically dry powders for inhalation, which on reconstitution with either aqueous buffers or biological fluids form liposomes. Rifapentine (an antitubercular drug) was loaded into such proliposomes for inhalation using a one-step spray drying technique. Basically, lipids (HSPC/Chol/stearyl amine) in ethanol and respitose SV010 (inhalable lactose) and L-leucine in double distilled water were heated to 60 °C to get a homogeneous solution on mixing. Subsequently, the drug in ethanol was added to form a clear solution and was spray dried at specific conditions of inlet air temperature (120 ± 5 °C),

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outlet temperature (60−65 °C), feed flow rate (1.2 mL/min), aspirator capacity (35%), and nozzle air pressure (1.8 bar). The spray-dried proliposomes were obtained immediately.[115] In another investigation, isoniazid proliposome powders for inhalation were prepared using mannitol as the core carrier. The conditions described in this method were inlet air temperature (90 °C), outlet temperature (70 ± 1 °C), feed flow rate (3 mL/min), and atomizer pressure (8 bar).[116] Thus, depending on the drug and the lipids used, the spray-drying conditions can be optimized case-by-case.

Membrane contactor technology uses a simple design unit where pressurized ethanol and the dissolved phospholipids are passed through a membrane with defined pore size into a column that contains a tangentially flowing aqueous phase. As the aqueous phase flows perpendicular to the membrane, it dilutes the ethanol locally and instantly results in self-assembled liposomes carried forward into the collector. The liposome dispersion in the collector is subjected to rotary evaporation to remove the ethanol. Studies carried out with this technology resulted in 100 nm liposomes and high entrapment for several lipophilic drugs, beclomethasone dipropionate, and spironolactone.[117,118] Ease of modification of the product by adjusting either the aqueous phase flow rate or the pump pressure for the organic phase makes it applicable for industrial scale-up.[119]

The crossflow injection technique is a method that can be used to refine the conventional detergent depletion method along with easy scale-up options.[120] The crossflow injection unit uses a starting material of micelles dissolved in the detergent similar to the conventional model. However, instead of dialysis, it employs tangential filtration under pressure to remove the detergent more effectively via a membrane to obtain the liposomal preparations. The advantages of this technique include homogeneously sized liposomes and a short duration of operation with options for a continuous operation. Moreover, it is a cost-effective method, where the waste filtrate consisting of the detergents can be processed and reused.[121−123]

2.2.3. Drug Loading in Liposomes. Liposomes are useful drug delivery systems for carrying hydrophilic (in the aqueous core), lipophilic (in the lipid bilayer), and amphiphilic (partitioned at the surface of the bilayers) drugs.[62] In the classical thin film hydration technique, the lipophilic drugs become highly embedded in the lipid bilayers when liposomes self-assemble. Yet, the hydrophilic drugs may not be incorporated with high efficiency because the volume of hydration is larger on the outside of the liposomes rather than the core with its limited aqueous volume. As a result of such variations, it is imperative to understand the physicochemical properties of the drug and the lipids to improve the drug loading in the liposomes. The method of liposome preparation also plays a role as noted in the previous sections.

Passive loading relates to drug loading during the method of preparation without any additional steps. As mentioned before, the degree of drug loading depends on the drugs and the composition of the liposomes (lipids, lipid/cholesterol ratio, drug/lipid ratio, and charge of the liposomes) as well as the method used for preparation. A recent study examined the effect of different phospholipid/cholesterol concentrations on the encapsulation efficiency of a RIP II protein, mistletoe lectin. Varying the concentrations as well as the use of different lipids (ePC, DOPC, DPPC) had differing effects on the protein encapsulation.[124] Efforts were also undertaken to increase the aqueous core volume, and thus the EE, by using different methods of liposome formation.[84,101,125]

The use of active loading procedures results in improved efficiency of entrapment as compared to passive methods. The basis of active loading usually takes advantage of diffusion properties when a gradient is established across the lipid bilayers. The liposomes are initially hydrated, first with a buffer of known pH, followed by dialysis in an excess of another buffer with a different pH to replace the buffer outside the liposomes to establish a transmembrane pH gradient. The second buffer is chosen with the intention of having an uncharged drug then enter the liposome and become charged in the first buffer of the aqueous core. The drug remains entrapped in the liposomes because the charged molecules cannot diffuse through the lipid layer.[126,127] This method can also be modified using different buffers to generate gradients of salts.[128,129] The active drug loading of doxorubicin (DOX) in marketed preparations (Doxil and Myocet) is a working example. With Doxil, a transmembrane ammonium sulfate gradient was established with higher ammonium sulfate inside the liposomes and DOX was encapsulated at higher loads. Once inside, the DOX precipitated as a sulfate salt.[16] With Myocet, a proton gradient was formed with an acidic buffer inside and basic buffer outside the liposomes as the driving force for the DOX loading.

For a gene therapy study, liposomes were explored as delivery vehicles in comparison with viral vectors. The cationic lipids/polymers are usually used with negatively charged nucleic acids (DNA, RNA, antisense oligonucleotides) to form cationic liposome/gene complexes.[130−132] DOTAP was used with ursodeoxycholic acid to make cationic liposomes for oligonucleotide delivery.[133]

The amount of drug loading is different for each case and must be studied carefully to optimize formulation designs.

2.2.4. Sterilization of Liposomes. Basically, while liposomes are made of phospholipids and cholesterol, they may also contain additional ligands and molecules for imparting special properties to liposomes. All of these constituents are potential sources of contaminants such as bacteria, viruses, endotoxins, and pyrogens. They are of concern because parenteral injections are the most common route of liposomal administration. Unlike traditional pharmaceutical products, the liposomes present a challenge in terms of sterilization because of their unique composition as well as their special requirements for manufacture.[134] For instance, the phospholipids can be affected by the surrounding temperatures, and a phase transition from gel to liquid can occur at temperatures above T_M. This may result in a nonrecoverable product loss. Additionally, the lipid constituents have the potential of chemical degradation via oxidation, hydrolysis, and aggregation.[135−139]

While filtration of the nanosized liposomes (<200 nm in size) through a membrane filter of 200 nm under pressure removes most bacterial contaminations, it is a labor- and time-intensive procedure. Sterile filtration of liposomes does not subject the liposomes and its contents to excess temperature, irradiation, or light exposure.

Steam sterilization typically utilizes steam at 121 °C for 15 min to eradicate microbial contamination. With liposomes, however, exposure to the high temperatures may result in a gel−fluid phase transition for the phospholipid content and in hydrolysis, resulting in damage to the lipid bilayers.[140] Still, with an appropriate use of a dispersion buffer and pH, the hydrolysis and aggregation can be minimized.[140,141] Even if dry heat

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sterilization is employed, the high temperatures required results in damage to most liposome dispersions by inducing gel−fluid shifts or even oxidation of the phospholipid content. When considering heat trigger-sensitive liposome preparations, heat-based sterilization techniques are inappropriate.

Chemical sterilization, for example, ethylene oxide purge, results in an effective removal of all contaminants. As this technique does not involve heat, it is useful for liposome applications. Additionally, ethylene oxide does not affect the liposome bilayers. However, complete removal of ethylene oxide after sterilization is necessary because it is a known carcinogen.[142] This technique is more appropriate for lyophilized liposomes as compared to liposome dispersions because it has the potential to form toxic byproducts with the aqueous buffers.[143]

Instead, γ-irradiation can be employed for sterilization of liposomes, especially those lyophilized. Initial work reported damage by oxidation and hydrolysis of the phospholipids and cholesterol after γ-irradiation.[136] Later, it was found that the liposomes could be protected from damage with antioxidants during the irradiation or by freeze-drying the liposomes prior to irradiation.[143] Still, a careful choice of additives was necessary before γ-irradiation. Zuidam et al. compared the damage by γ-irradiation on nonfrozen, frozen, and freeze-dried liposomes in the absence or presence of trehalose (a cryoprotectant). The liposomes composed of either dipalmitoylphosphatidylcholine/dipalmitoylphosphatidylglycerol (DPPC/DPPG) or egg phosphatidylcholine/egg phosphatidylglycerol (ePC/ePG) in 10 mM phosphate buffer (pH 7.4) without trehalose degraded considerably as compared to the trehalose-treated ones. Yet, the trehalose reacted strongly with the species that resulted from γ-irradiation-induced damage to the liposomes, and thus limited the use of γ-irradiation for frozen or freeze-dried liposomes.[144]

An alternative to the terminal sterilization techniques shown above is aseptic manufacturing.[134] Basically, the raw materials are sterile filtered, and the equipment to manufacture and store the liposomes is decontaminated thoroughly prior to use. The whole operation of liposome preparation is carried out in aseptic conditions. Appropriate HVAC and personnel clothing care is taken during the manufacturing, filing, and storage procedures with the intention of avoiding external contamination. The biggest disadvantage in aseptic manufacturing is the associated high cost. Moreover, this system works on the assumption that the contamination has been avoided because there is no final step of sterilization. The chance that viruses as well as toxins present in the raw materials prior to filtration may carry through the whole procedure must be considered. It has been typical to use aseptic manufacturing only as the last option.

In liposome preparations where the SRPE or SAS method is employed, the $scCO_2$ acts not just as a solvent but also as a germicidal agent. Csaba et al. showed that the $scCO_2$ can pass through the microbial cell walls and dissolve in intracellular water to form carbonic acid (H_2CO_3), which lowers the pH in the bacterial cytoplasm and leads to metabolically adverse conditions. Ionization in cytoplasmic water as HCO_3^- and CO_3^{2-} disrupts the cytoplasm of the bacteria adding to the killing action.[145] In a separate study, Fages et al. showed the mechanism of inactivation of viruses in human bone tissue by $scCO_2$, a method that can be applicable to the SRPE/SAS technique.[146] It was also reported that $scCO_2$ rapidly inactivated bacterial endospores.[147] It has been shown with $scCO_2$-based microencapsulation of indomethacin in poly-(lactic acid-*co*-glycolic acid) (PLGA) block copolymers that sterile products can be obtained,[148,149] which suggests that this technology may possibly be used for liposome sterilization.

2.3. Characterization of Liposomes

The fate of the liposomes under storage conditions and the various clinical applications can be estimated by knowing the properties of the liposomal formulations. Described in a review by Crommelin and Storm[150] are several ways to characterize the liposomes, some of which are presented in the following section.

2.3.1. Size and Polydispersity. Size and polydispersity determination provide an important tool to characterize liposomes because the liposome size is critical for parenteral administrations. They also provide a quick estimate of the batch quality and variations in manufacture. Size can be measured by several techniques including dynamic light scattering (DLS), size exclusion chromatography (SEC), nuclear magnetic resonance (NMR) and microscope technologies including transmission electron microscopy (TEM), cryogenic-TEM (Cryo-TEM), and atomic force microscopy (AFM).

The easiest and most widely used technique to quickly measure the liposome size and size distribution is DLS. DLS analyzes the Brownian motion of the colliding particles resulting in scattering of the incident light. The scattering of light is dependent on the refractive index difference between the suspended particles and the solvent. The amount of light scattered is calculated and evaluated to give a mean particle size of the suspended liposomes. The advantages of this system are ease of use, wide range of measurement capability (20−1000 nm), and the ability to analyze the liposomes in their native environments. However, there are a few disadvantages including the system's inability to resolve the difference between an individual liposome and an aggregate. Given that it considers an aggregate as a single particle, false readings may be obtained. This technique is also sensitive even to low amounts of impurities.[151,152]

High resolution with low sample requirements is associated with HPLC-SEC size analysis. The samples are passed, under pressure with HPLC pumps, through columns with appropriate porous packing. The ability to separate particles ranges from low to moderate sizes. It is a reliable and reproducible method, which can be combined with DLS. Although the separation is based on size, the chemistry of the liposomes as well as the column packing can play a role in the assay. If the liposomes are composed of deformable lipids, they can squeeze through the pores and give false readings. On the other hand, it is possible that the liposomes become attached to the lipid packing in the columns, leading to recovery issues.[153,154]

Microscopy is a unique way to visualize the liposomes as well as measure their size. TEM, cryo-TEM procedures have been extensively implemented for generating liposomal images. For negative staining TEM, the liposomes are placed on a small dry copper grid where the aqueous buffer is allowed to dry and a negative stain (uranyl acetate, phosphotungstic acid) is used to mark the background such that the liposomes appear as bright vesicles.[105,155] Because this technique depends on removal of liposomes from their native environment, the lipid chemistry may be disturbed, leading to artifacts in the image generated. In a recent work, negative staining of liposomes prepared for aerosols was avoided during the TEM imaging to avoid induced artifacts.[156] Alternatively, cryo-TEM can be used to keep the liposomes close to their native state and prevent damage by

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shrinkage or shape distortion.[157] The preparative steps involve flash-freezing of the liposomes using liquid nitrogen and then transferring them to the controlled environment in the cryo-TEM unit. However, the analysis works best with samples in the lower nanometer range. Larger liposomes may be removed from the sample film during the blotting step. A review on the cryo-TEM method with several sample and sample preparation parameters is described.[158] AFM is a quick and reliable, high-resolution method of measuring the size of liposomes without sample modifications.[159]

A recently developed technique, nanoparticle tracking analysis (NTA), detects the nanoparticles by the light scattering when illuminated by laser lights. The liposomes in their native buffer environment are injected into a view cell illuminated by a laser beam. As individual liposomes move in the medium, their motions are captured by a digital camera and traced from frame to frame. The rate of particle movement is related to the sphere equivalent hydrodynamic radius calculated using the Stokes–Einstein equation.[160,161] The instrument software computes liposome size on an individual particle basis and gives it an advantage over other systems. Nanosight LM10, NS300, and NS500 (Nanosight, Amesbury, UK) developed on principles of NTA can rapidly analyze liposomes and other nanoparticles from 10 to 2000 nm in size.[162,163]

2.3.2. Zeta Potential. Zeta potential measurement involves calculating the charge of the liposomes in a dispersion. Each liposome carries a charge, negative, positive, or neutral, depending on its composition and associated ligands. The stability of the liposomes in the medium can be estimated by the zeta potential. Liposomes uncharged or with low charge tend to aggregate over time, whereas the liposomes with a higher negative or positive charge will have repulsive forces in the medium that discourage agglomeration.

Liposomes in a sample cell are illuminated by incident light, and the zeta potential is measured by fluctuations in the scattered light as the liposomes move due to the application of an electric field. The mobility of the liposomes is proportional to the associated charge.[164] A Doppler shift in frequency of the detected laser light is generated by the mobilization of the liposomes.[165,166]

2.3.3. Encapsulation Efficiency (EE). To investigate the EE, entrapped drug must first be removed from the liposomes. This can be done by replacing the aqueous media with an organic phase (acetonitrile, ethanol, methanol, Triton X-100). Depending on the drug under study, several techniques can be employed to estimate the concentration including UV and/or fluorescence spectroscopy, enzyme- or protein-based assays, and gel electrophoresis. Prior to the EE estimation, the unencapsulated (free) drug must be removed from the formulation. This may be done by ultracentrifugation, dialysis, or column separation. The measurements of EE can also be aided by the use of instruments such as HPLC, UPLC, and LC–MS.[167]

2.3.4. Lamellarity Assays. As indicated previously, the number of bilayers highly influences liposomal in vivo fate and applications. While several chemical-based techniques[168–170] use labeled reagents or radiolabeled ions to estimate the number of layers or the amount of lipids in the surface, often the predictions assume that the reagents were evenly distributed on the surface of the outer layer. This may lead to false readings.[171] A commonly used method for study of the liposomal lamellarity and morphology is by cryo-TEM analysis. One-dimensional ^{31}P NMR has also been used to determine the lamallerity,[172] specifically, the ratio of the amount of phospholipids in the outer to inner layers.[14] It has been noted that the MLV and SUV give a broad and narrow line spectra on the NMR, respectively. When paramagnetic ions such as Mn^{2+}, Co^{2+}, and Pr^{3+} are added to the NMR sample preparation, there is a shift (broadening, downfield and upfield, respectively) in the detected resonance due to interactions of the ions with the bilayers. Comparison of the spectra before and after the addition of Mn^{2+} can be used to estimate the lamellarity.[14] Techniques such as small-angle X-ray scattering (SAXS)[173–175] and trapped volume measurement[176] have also been used.

2.3.5. In Vitro Drug Release. This analysis is simple to perform and involves dialysis conditions. Liposome samples are put into dialysis bags with appropriate molecular weight cut-offs and stirred continuously in a dissolution medium. The medium is usually a buffered saline at pH 7.4, and the entire setup is kept in enclosed conditions at 37 °C to mimic an in vivo environment. At defined time intervals, samples are taken and analyzed by methods including UV/fluorescence spectroscopy and HPLC specific to the drug under study. The volume of the samples is kept constant by replacing with fresh dissolution media. A plot of release profile gives an estimate of the drug release by the liposomal carrier.

In addition to the above characteristics, formulations of proliposomes, liposomal dry powder for inhalation (DPI), etc., require supplementary analysis to fully determine the characteristics of the formulation. In case of proliposomes for oral and skin delivery via tablets or gels, angle of repose, flow-ability by bulk/tapped density, moisture content, proliposomal granule size by sieve analysis, rheological behavior, and conversion rate to form liposomes from the proliposomes on rehydration are also measured.[115,177–179] Formulations for skin delivery are also subjected to rheological and viscosity analysis, deformability, and even ex vivo permeability/diffusion analysis.[180–182]

3. LIPOSOMAL DRUG DELIVERY: PASSIVE, LONG-CIRCULATING LIPOSOMES

Liposomes mimic the cell membrane. However, liposomes are rapidly acted upon by the plasma proteins and macrophages, predominantly in the liver and the spleen, leading to a very short half-life. Another issue with the use of liposomes is that their drug retention after in vivo administration is also short. The need for improved circulation of the liposomes and drug retention led to the development of long-circulating liposomes. In some circumstances, liposome uptake by the macrophages is preferred when they are the therapeutic target, as in infections and diseases affecting the macrophages.[183]

The first strategy used to generate long-circulating liposomes was to adjust the properties of the liposomes including their composition and size. It was noted that small liposomes avoided the reticulo-endothelial system (RES) better than their larger counterparts. Allen and Everest observed that circulation times of small unilamellar vesicles were higher than those of multilamellar vesicles larger in size.[184] Another study determined the liposomes of a 100 nm size were optimum for tumor delivery.[185] Yet, when the composition of the liposomes was adjusted, especially by using saturated phospholipids with higher T_M as compared to the unsaturated phospholipids, the circulation time as well as drug retention also improved for larger vesicles too.[186,187]

Surface modification of the liposomes was another strategy developed to avoid the RES uptake. Initially, ganglioside and sialic derivatives like GM_1 (monosialoganglioside) were used to

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mimic the erythrocyte membrane surface,[188] and later hydrophilic polymers like PEG[189,190] were found to be useful to provide long circulation properties to the liposomes. The mechanism of improving their circulation time was attributed to modifications that provided a steric boundary to the liposomes and prevented the plasma proteins binding and RES uptake.[191−193] Such surface modifications enabled the development of so-called "stealth liposomes", which function more effectively by mimicry of the biomembranes. Doxil is a typical stealth liposome with a PEG surface coating that leads to an improved circulation time and safety profile in DOX chemotherapy.

Passive delivery of the liposomes is often useful for diseased areas with altered structures and function. Tumor tissues have a well-known characteristic of upregulated angiogenesis. However, the blood vessels are not formed in an orderly fashion. This haphazard formation comes with a leaky vasculature with enlarged endothelial cell gaps convenient for passage of liposomes and other nanocarriers of up to moderate nanometer sizes by extravasation into the tumor interstitium.[194,195] Tumor tissues also tend to have poor lymphatic drainage. The combination of these histological features makes tumors susceptible to increased cargo delivery by long-circulating liposomes. This effect is termed the enhanced permeation and retention (EPR) effect.[196−198] Similar leaky tissue morphology has also been observed in the inflamed tissues of inflammatory bowel disease and inflammatory rheumatoid arthritis.[199] In short, liposomes will more readily extrude and accumulate in the tumor interstitium from the blood circulation to create a local depot of drug. The increased local tissue concentration of the drug will promote therapeutic effect. Commercially available liposome-based formulations take advantage of this enhanced passive delivery to preferred sites of action.

Oftentimes it occurs that the PEGylated liposomes do concentrate in a targeted area by the EPR effect, but are unable to efficiently release the drug. It has also been observed that the PEG coating may inhibit endosomal escape of the drugs after endocytosis by the cells.[200] It is also known that each tumor type carries its individual identity and has idiosyncrasies in the development of its blood vessel supply. A study conducted to compare the distribution of PEGylated liposomal DOX in different murine tumors (4T1, breast and 3LL, lung) in vivo revealed that the liposomes accumulated significantly more in the 4T1 as compared to the 3LL cells, demonstrating that the vascular permeability probably was higher in 4T1.[201] Thus, a homogeneous distribution of the liposomes throughout the tumor also may not be possible. Even after the drugs are released from the liposomes, most agents must enter the cells to exhibit a cytotoxic effect. With complex tumor microenvironments, it is possible that the drugs are unable to produce a sufficient treatment. The compounding effect of these situations can also lead to a rise in multidrug resistance by the tumor.[202−204]

An improved strategic requirement for drug delivery to a targeted site has resulted in the development of active drug targeting as well as triggered/stimuli-sensitivity-based targeting techniques. Often these methods are used together to promote efficient chemotherapy.

4. LIPOSOMAL DRUG DELIVERY: ACTIVE AND TRIGGER-BASED TARGETING

4.1. Active Targeting Strategies

Active targeting can be achieved by surface modifications of the liposomes with target-specific ligands and antibodies.[205] Factors that play a key role in mediating active targeting include:

- Manner of ligand/antibody attachment to the liposomes
- The targeted receptor/antigen presentation by the diseased tissue as compared to normal tissues
- Internalizing properties of the target ligand
- Long-circulation capabilities of a liposome bearing the targeting moiety

The ligands can be attached either directly to the surface of a non-PEGylated liposome or PEGylated liposome or to the free end of the PEG in a PEGylated liposome. Care should be taken with surface-modification of liposomes with ligands, especially antibodies or peptides, so that the conjugation does not alter ligand structure or otherwise negatively impact the activity.[206] Also, an investigation on a case-by-case basis should be conducted on the optimum ratio of targeting ligand to liposome surface to achieve optimum targeting. Several studies have been conducted on how the ligand attachment affects the efficiency of the targeted drug delivery system (TDDS).[207−209] Generally, ligand/antibody presentation at the distal ends of the PEG is more efficient for TDDSs. Usually, the targeted liposomes should be prepared with the targeting ligand shielded during circulation but presentable once it reaches a targeted site. Strategies to chemically enhance the PEG for targeting have been presented in this Review.[210] An understanding of the targeted receptors/antigen representation is essential for developing a TDDS. The targeting ligand should distinguish the target cells from the normal cells to avoid nonspecific binding and toxic side-effects. Either the receptor/antigen should be exclusively expressed on the target tissue or the target should have relatively high expression as compared to the normal cells.

Likewise, it is preferable if the ligand induces receptor-mediated endocytosis or fusion of the liposome with the membrane. A ligand that binds to the receptor without any internalization is of little use and will remain in the interstitial space.[211,212] It is also preferable for a TDDS to possess a long circulation time, unless the targeted cells in question are the macrophages. The pharmacokinetics of targeted PEGylated liposomes are the same for nontargeted PEGylated liposomes. This is because the liposomes depend on the blood circulation and the EPR effect to arrive at the target site. Only when they reach the targeted tissue does the targeting ligand play a role. TDDS interact with cell receptors and may then be internalized into the cells, while the nontargeted liposomes accumulate in the tumor interstitium.[213]

To date, antibody surface-modified liposomes have formed the major part of active targeting studies.[214,215] Specific antibody fragments (Fc, F(ab′)) and monoclonal antibodies (mAb) attached to the liposome surface have served as TDDS. Highly upregulated HER2/neu growth factor receptors on breast cancer cells have been targeted with anti-HER2 immunoliposomes. Patients with advanced HER2+ breast cancer were treated with HER2-targeted DOX-loaded immunoliposomes (MM-302) in a Phase I study. MM-302 bound specifically, entered the tumor cells, while sparing normal cells that expressed HER2 at low levels.[216−218] Many

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tumor types also overexpress surface-bound nucleosomes, which can be targeted effectively using the 2C5 mAb. Our work involved binding of the mAb 2C5 to PEGylated DOX liposomes (Doxil). Binding of the 2C5 resulted in no loss of DOX from the liposomes. In vitro and in vivo studies demonstrated enhanced binding to a wide variety of cancer cells when compared to the nontargeted liposomes.[219,220] Similarly, DOX-loaded PEGylated immunoliposomes targeted with the F(ab′) fragment of a tumor-specific human mAb-GAH were well tolerated in patients with metastatic cancer in Phase I trials.[221]

The transferrin receptor highly expressed in tumors is associated with their higher metabolic requirements for iron. This receptor can be targeted with antitransferrin receptor antibodies or transferrin itself. A phase I study with 39 patients with different tumor types (colorectal, pancreatic, and neuroendocrine) was performed with transferrin-conjugated oxaliplatin-loaded liposomes (MBP-426). The formulation depends on the EPR effect to first reach the tumor and then it binds to the transferrin receptors. After initial dose-limiting toxicity was observed, an adjusted dose was applied and investigated in patients with gastro-esophageal adenocarcinoma in combination with 5-fluorouracil/leucovorin in Phase II.[222,223] Folate receptor has also been a target of interest in several in vitro/in vivo applications because it is overexpressed by many tumor types.[224−226] A recently published U.S. patent (US 20130071321 A1) notes that folate-targeted liposomes effectively delivered anti-inflammatory agents to inflamed tissues, because their folate receptor is upregulated.[227]

Active targeting in combination with cell penetrating peptides (CPP) has also generated much interest recently. The CPP are protein translocation domains, which translocate through the cell membranes and facilitate the transport of associated cargo. Usually the CPPs are short peptides with less than 40 amino acids. They are transported across the cell membrane by several mechanisms including endocytosis and even by a direct entry through the cell membrane.[228] Transactivator of transcription protein (TATp) of the HIV virus, octa-arginine (R8), and amphipathic model peptide (MAP) are well-studied CPPs that have been shown effective for drug and gene delivery.[229−231] Our studies have focused on liposomes surface-modified with TATp or R8 peptide. TATp was used to transfect the pEGFP plasmid (expressing green fluorescent protein) in liposome/DNA complexes in mouse spleen-derived antigen presenting cells, mouse NIH/3T3 fibroblasts, and rat H9C2 cardiomyocytes without cytotoxicity. The TATp-modified liposomes efficiently transfected the pEGFP in LLC tumors and U-87 MG intracranial tumors in separate studies in mice.[232−234] The R8 peptide was conjugated to poly(ethylene) glycol-phosphatidylethanolamine (PEG-PE) and attached to the DOX-loaded PEGylated liposome surface by the post-insertion technique.[235] These liposomes induced tumor growth inhibition in non-small cell lung tumor xenografts in mice when compared to nonsurface modified PEGylated liposomes.[236]

4.2. Trigger-Based Targeting

As mentioned previously, tumor physiology is highly variable. The presence of an abnormal vascular system among tumors can result in drugs not reaching their target in an effective concentration. Even when liposomes enter the tumor interstitium, they may not release the drug in sufficient amounts. An ideal drug delivery system restricts drug release in the circulation and delivers maximum payload only at the targeted site. Triggered release of stimuli-sensitive targeting devices enables regulation of the release of the drugs from the liposomes. Different strategies for triggered release include those physiology-dependent (pH or enzyme-based) and those that are external stimuli-dependent (ultrasound, thermosensitive, photosensitive, magnetic field-based).

4.2.1. Physiology-Dependent Release. Abnormal tissues often exhibit differences in their local environment as compared to normal tissues, which can be used to develop target tissue-specific triggered release systems.

pH-sensitive liposomes (PSL) exploit the lower pH conditions present in tumors[202] or sites of inflammation.[237] PSL systems are designed to be stable at pH of the blood (7.4) and degrade at lower pH (≤6.0). One approach in the design of these systems is the use of fusogenic peptides, also known as pH (low) insert peptides (pHLIP), for example, GALA (glutamic acid-alanine-leucine-alanine).[238] At neutral and high pH conditions, these peptides are monomeric and water-soluble, whereas at acidic pH, the peptides become hydrophobic and assume a monomeric transmembrane helix structure that inserts into the membrane, leading to fusion of the liposomes with the cell membrane. At pH of 7.4, the peptide ensures that liposomes will not fuse with cells. However, in areas of low pH (a tumor, inflammation, or ischemic myocardium), the peptide's effect will enhance the cellular uptake by fusion of the liposomes and the release of cargo.[239−241]

Another approach to designing PSL is the use of functional groups/moieties between the PEG coating and the lipid bilayers. Attachment of PEG to bilayer groups such as ester (C═O) or hydrazone (Hz; C═N−NH_2) provides advantages derived from a stable liposome formulation at neutral pH and a long-circulation via the PEG coating. Hydrolysis of the functional groups at lower pH (≤6) releases the PEG and makes the liposomes available for local drug delivery.[242] Improved transfection of GFP plasmid DNA encapsulated in pH-sensitive TATp-modified liposomes was observed in tumor-bearing mice.[243] For another recent analysis, a multifunctional pH-sensitive liposome was made by conjugating DOX-loaded liposomes with TATp linked by a PEG_{1000}-PE and a pH-sensitive polymer with longer PEG chain PEG_{2000}-Hz-PE. A mAb (2C5) was attached to the longer PEG chain for added targeting. The principle behind this system is that (a) the conjugated antibodies provide targeting, (b) the longer PEG chain shields not only the liposome, but also the TATp on the shorter PEG-PE from the plasma proteins and the RES, and (c) at the tumor, the longer PEG chain hydrolyzes, exposing the TATp to generate additional liposome uptake by the cells. Significant tumor reduction was obtained in mouse studies when compared to unmodified liposomes treatments.[244] Watarai et al. developed PSLs, modified with the fusogenic polymers, succinylated poly(glycidol) (SucPG) and 3-methylglutarylated poly(glycidol) (MGluPG)), as mucosal vaccines. In both PSLs, the induction of immune responses was better as compared to the unmodified liposomes.[245]

In some cases, the pH in the tumor microenvironment is not radically lower (not less than 6.5) than the normal tissue pH.[246] Designing liposomes that remain stable at pH 7.4 but destabilize at 6.5 is difficult and may lead to premature dumping of the cargo in the blood. The use of fusogenic lipids in the liposomes to target the endosomes/lysosome with an internal pH ≈ 5.0 is more reasonable. The fusogenic lipids are stable at neutral pH, while at low pH (acidic enzymes in

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endosomes) they undergo conformational changes, destabilizing the endosome membrane with release of the cargo into the cytoplasm. This mechanism is especially useful for transport of genes like siRNA or plasmid DNA whose locale of action is cytosolic.[247,248] Dioleylphosphoethanolamine (DOPE) and cholesterylhemisuccinate (CHEMS) have been used to prepare fusogenic PSL for endosomal/lysosomal escape. DOPE with its small polar headgroup and longer lipophilic tails has a conical shape, while CHEMS has the inverted conical shape with a larger headgroup. In mildly acidic conditions, the CHEMS loses its shape due to the weakly ionized headgroup, causing membrane destabilization and release of the drug/gene cargo.[249] Recent studies with different lipids have been carried out on design of PSLs. To improve plasmid DNA gene delivery to tumors, a complex mixture of cationic and pH-sensitive liposomes was prepared.[250] Cationic liposomes composed of 3β-{*N*-(*N*′,*N*′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) and DOPE were used to complex the plasmid DNA and combined in different ratios with separately prepared pH-sensitive liposomes composed of DOPE/CHEMS. The resulting complexes (300−400 nm) had high transfection efficiencies in tumor studies in vivo. Another group synthesized a phospholipid with a cationic headgroup combined with a cholesterol-based tail with a pH-sensitive ortho ester linker incorporated into DOPE-based liposomes complexed with pDNA encoding for a luciferase reporter gene. In vitro and in vivo studies reported stable and 5−10-fold higher transfection as compared to pH-insensitive DC-CHOL/DOPE/DNA lipoplexes.[251] Zwitterionic oligopeptide lipids, 1,5-dioctadecyl-L-glutamyl 2-histidyl-hexahydrobenzoic acid ($HHG2C_{18}$) and 1,5-distearyl *N*-(*N*-α-(4-mPEG_{2000}) butanedione)-histidyl-L-glutamate ($PEGHG2C_{18}$), were employed to prepare liposomes with multistage (tumor microenvironment and endosome/lysosome) pH-sensitivity.[252]

Overexpressed enzymes in certain disease states can be exploited for enzyme-sensitive triggered release. Diseases like cancer, inflammatory arthritis, and others overexpress various enzymes including phospholipase A_2, matrix metalloproteinases (MMP), glutathione transferase, sirtuins (SIRT), lysozymes, and cathepsins.[253−256] Such altered physiological conditions provide an opportunity for the use of enzyme-based liposomal targeting. MMPs, which are not known to cleave the lipids, can be conjugated with the liposomes. For instance, MMP-substrate peptide/cholesterol was used as an enzyme cleavable linker for PEG to lipids and inserted into anionic liposomal adenoviral (Ad) vectors. Gene expression with this formulation was higher as compared to noncleavable formulations in the in vitro analysis.[257] In a similar fashion, additional studies synthesized MMP-sensitive peptides to get PEG-MMP cleavable peptide-lipid-based liposomes for tumor targeting.[258,259]

4.2.2. External Stimuli-Dependent Release. External stimuli-mediated drug delivery depends on advancements in the composition of liposomes as well as the instrumental modalities needed to precisely generate the necessary trigger required at a desired site.

Thermosensitive liposomes (TSL) are heat-trigger-based liposomes. It should be noted that all liposomes are inherently thermosensitive. The phospholipids have a T_M below which they are in a gel state and above which they are in a fluid state. To be applicable for drug delivery, liposomes are formulated with the aim of phospholipids in a gel state at about 37 °C. Locally applied mild hyperthermia is already used for combination chemotherapy.[260,261] Hyperthermia is chemotherapeutic by not only being directly cytotoxic on the cells (heat ablation), but also by increasing the vascular permeability (improved liposomal extravasation) and increasing cellular membrane fluidity (improved diffusion of drugs).

Use of TSL composed with either lysolipids (LTSL) or polymers (PTSL) can thus be advantageous for enhanced cytotoxic effects. Lysolipids are structurally different from the generally used phospholipids, in that they possess a large headgroup and a single hydrophobic tail. On their own, they tend to form micelles. When heat is applied to LTSL, their lipid bilayers enter a gel−fluid transition state with the lysolipids accumulating at transition spots to form stable pores by inverting into micelle-like structures. These pore assemblies strongly destabilize the liposomes, leading to drug release.[262,263] A commonly used lysolipid is monopalmitoylphosphatidylcholine (MSPC) whose incorporation in DOX-loaded LTSL - ThermoDox [DPPC:MPPC:DSPE-PEG_{2000} (86:10:4)] is now being investigated at different stages in clinical trials for several tumors indications.[264,265] Besides causing lipid bilayer destabilization, the lysolipids also lower the T_M of the thermosensitive phospholipids to a clinically applicable 39−40 °C. Mild hyperthermia for even 20−30 s caused the liposomes to deliver almost 50% of their dose.[266] However, the lysolipids have also been observed to dissociate from the liposomes rapidly after intravenous administration due to the destabilizing action of plasma proteins with premature release of the cargo.[267,268] This phenomenon was cited as the reason for the unsatisfactory results with ThermoDox in clinical trials. Considerable efforts have been made to improve the formulation. One approach has been to replace the lysolipid with a surfactant Brij78 in a new formulation as DPPC:Brij78 (96:4 mol %). Stability at 37 °C in comparison to the earlier formulation was improved. The DOX was delivered to tumors in mice efficiently and safely, and it also led to tumor growth regression.[269−271] In another study, hydrogenated soy phosphatidylcholine (HSPC) was added to formulations to achieve highly temperature-sensitive liposomes [DPPC:HSPC:MSPC:DSPE-PEG_{2000} (73.6:18.4:4:4)]. Increasing the T_M of the liposomes led to significantly higher uptake of brucine at tumor site heated to 43 °C as compared to a brucine solution.[272] Use of 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP) to add a cationic charge to thermosensitive liposomes (DPPC/DSPC/DPTAP/DSPE-PEG_{2000}) successfully led to targeting the tumor vasculature in B16 tumor-bearing mice as a function of the degree of cationic charge and the release of encapsulated DOX via a heat-trigger.[273] Interestingly, Chen et al. prepared thermosensitive liposomes with DPPC/Chol/DSPE-PEG_{2000} (60:40:5) and no lysolipids. Instead, for DOX encapsulation, an ammonium bicarbonate (NH_4HCO_3) core was used to create a transmembrane ammonium bicarbonate gradient. Heat application to around 42 °C released CO_2 bubbles from the NH_4HCO_3, which induced defects in the lipid bilayer and a rapid release of the encapsulated DOX.[274]

Thermosensitive polymers in liposomes have also been explored to make TSLs. The polymers used are water-soluble at about 37 °C and become water-insoluble on application of heat, leading to a hydrophobic state. This characteristic is due to different states of the H-bonding between the polymers and the adjacent water molecules that depend on the lower critical solution temperatures of the polymer.[275] The temperature-dependent phase transition of such polymers disrupts the liposomal membrane to release the drugs. Naturally occurring polymers like cellulose, chitosan, and gelatin or synthetic

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polymers including poly(*N*-isopropylacrylamide) (pNiPAAm), poly(*N*-vinylethers), and poly(*N*-vinylalkylamides) are commonly employed for production of thermosensitive nanoparticles.[276−278]

Various methods have been employed for local heating including water bath (typically applicable for animal studies and superficial tumors), externally applied electromagnetic pulses, minimally invasive heat applicators, focused ultrasound, and MRI guided focused ultrasound.[278] It is also useful to consider the appropriate timing for application of heat considering that the effects on nontarget areas must be considered. Hyperthermia's application has been studied with two-step hyperthermia and compared to a one-step application. The two steps consisted of preheating the local area to 41 °C and administering the DOX-loaded TSL to allow maximal extravasation and accumulation followed by the second step of increasing the temperature to 42 °C.[279]

Ultrasound (US)-triggered release with echogenic liposomes (ELIP) encapsulating air (oxygen, CO_2, nitrogen), originally used for imaging, has also been studied.[280,281] Focused ultrasound can also be used to cause reversible disturbances in the endothelium of blood vessels to encourage drug entry to the target tissue interstitium. Enhanced permeation of the blood−brain barrier has also been observed.[282,283]

An US stimulus transfers energy to the encapsulated gas pockets in the ELIPs, leading to bubble expansion and collapse causing disruptions to the liposome bilayer membrane[284,285] and release of the encapsulated materials. NF-κB oligonucleotides were encapsulated in ELIPs (800 nm), which released the oligonucleotides efficiently on application of a 1 MHz continuous wave of US.[286] However, Kopechek et al. observed no drug release from ELIPs even after generation of cavitation in the liposome membranes. The study, conducted with very short bursts of low frequency US, demonstrated that assessing cavitation activity alone was not a reliable assay for drug release from ELIPs.[287]

Echogenic liposomes have been considered for treatment of several diseases including thrombosis, atherosclerosis, and cancer. Plasmin (fibrinolytic) was loaded into ELIPs, and an in vitro comparison of US-mediated thrombolytic efficacy against non-US Plasmin-ELIP and recombinant tissue plasminogen activator (rtPA; FDA-approved) was carried out. The mean clot lysis due to Plasmin-ELIP with US exposure was 31% higher than Plasmin-ELIP without US exposure and 15% higher than rtPA, demonstrating a benefit of US-mediated release of plasmin from the liposomes.[288] Recently, US-aided Xenon-loaded ELIPs were responsible for a reduced early brain injury caused by experimentally induced subarachnoid hemorrhage in rats, leading to long-term neuroprotection.[289] ELIPs were also used for encapsulating bevacizumab, a mAb specific for VEGF, to study US-facilitated inhibition of atheroma. In vitro analysis demonstrated 90% and 70% inhibition of VEGF and 64% and 45% cytotoxicity relative to untreated controls and non-US bevacizumab-ELIPs, respectively.[290] An interesting study on the features of the vascular architecture in B16-F10 tumor-bearing mice was carried out by injecting 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)-based liposomes and subsequent US analysis.[291] Lee et al. demonstrated the potential cytotoxicity advantage with high concentrations of nitric oxide (NO) presented by US-mediated release from NO-ELIPs in the in vitro breast cancer models.[292] DOX-liposome-microbubble complexes stimulated by US, sensitized MDR MCF-7 breast cancer cells via an increase of reactive oxygen species, promoted DNA damage and downregulated P-glycoprotein expression.[293]

Because liposomes encapsulating bubbles typically have a large size, the EPR effect is very limited. A novel formulation was prepared using biocompatible perfluoropentane (PFC5) emulsions coencapsulated with either calcein or GFP plasmid-loaded folate-targeted liposomes. The size of the final liposome formulations prepared by extrusion ranged from 150 to 200 nm, and cryo-TEM images indicated up to 3 emulsions encapsulated in the liposomes.[294] US stimulus transformation of the emulsion droplets from a liquid to gas phase ruptured the lipid bilayer of the liposomes and released drug and plasmid in significantly higher amounts as compared to the controls. In vitro studies in HeLa cells also demonstrated that the expansion of the PCF5 emulsion droplets possibly disrupted both the liposomes and the endosomes, generating elevated drug concentrations and plasmid transfections in the cells.[295] Subsequent studies with DOX loaded PCF5-liposomes proved significantly cytotoxic to HeLa tumor cells with low intensity US stimulations as compared to free drug, PCF5-liposomes alone, and conventional ELIPs.[296] It would be of considerable interest to see in vivo applications of this formulation.

Magnetically sensitive liposomes (MLs) containing stabilized iron oxide particles allow for targeting and triggered release of encapsulated drugs on stimulation by a magnetic field.[297] MLs have found applications in molecular imaging and chemotherapies.[298,299] DC dipole electromagnets or alternating magnetic fields (AMF) of low or high frequency have been used in MLs treatments. For instance, DOX-loaded magnetite (Fe_3O_4) nanoparticles containing MLs were used to treat osteosarcoma-bearing hamsters. A DC dipole magnet applying a magnetic field strength of 0.4 T for 60 min maximized DOX concentrations in the tumor.[300] Nappini et al. applied low-frequency AMF (0.2−6 kHz) to successfully trigger the release of carboxyfluorescein from cobalt ferrite nanoparticle-embedded phosphatidylcholine MLs.[301] High-frequency AMF is usually associated with hyperthermia at the targeted area. This property can be beneficial if the targeted area alone is affected while the surrounding normal tissue is spared from heating.[302] Dual function MLs can be prepared, which when triggered by magnetic fields can release cargo and also cause thermal ablation-related damage in the target tissue. AMF-controlled calcein release from sodium bis(2-ethylhexyl) sulfosuccinate-coated superparamagnetic ferriferous oxide (Fe_3O_4) hydrophobic nanoparticles embedded in the liposomal bilayers of lecithin liposomes was investigated. An insight into the AMF-stimulated magnetic nanoparticles' magneto-thermal effects leading to a gel−liquid transition phase in the bilayers was provided by time-resolved anisotropy measurements.[303,304] Superparamagnetic iron oxide nanoparticles incorporated into PEGylated MLs generated stealth liposomes with increased stability.[305] High-frequency AMF produced transient disturbance in the lipid bilayers, increasing their permeability for the cargo, and could be applied in a periodic release schedule as the liposomes retained their structure. As an alternative, short magnetic pulses have been applied to rapidly release cargo from DPPC:DSPC:Chol (88:1:10) MLs, incorporating iron oxide nanoparticles.[306] This work also revealed that the magnetic nanoparticles generated US played a role in the content release from the MLs. Lately, MLs capable of encapsulating hydrophilic and lipophilic drugs have been prepared with a lipid bilayer embedded with a hydrophobic magnetite coated with oleic acid.[307]

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Finally, light-sensitive liposomes (LSLs) combining the use of the liposomes with photosensitizing agents are now being considered. Photosensitizers can be either light-sensitive lipids or other light-sensitive molecules that can be incorporated within the LSLs. Serum-stable DOX-loaded PEGylated LSLs were prepared using DPPC and diacetylene phospholipid ($DC_{8,9}PC$). On application of 514 nm laser treatment, DOX was released with up to a 3-fold improved cytotoxicity in cancer cells as compared to nonirradiated samples.[308] Embedding photosensitive molecules such as porphyrin derivatives, chlorins, or phthalocyanines into liposomes can be used for photodynamic therapy. On laser irradiation, these molecules cause disruption of the lipid bilayers as well as generate reactive oxygen species to kill target cells.[309] Visudyne, a commercially available product composed of dimyristoylphosphatidylcholine and egg yolk phosphatidylglycerol liposomes with liposome-embedded benzoporphyrin derivative monoacid ring A (BPD-MA; Verteporfin), is indicated for photodynamic therapy of age-related macular degeneration and other serious eye ailments.[310] An in vitro synergistic effect of Doxil combined with photodynamic therapy was recently noted with two-step treatment using MCF-7 cells.[311]

Multitargeted or multisensitive liposomes have been used to combine the specificities provided by individual modifications. Such techniques can lead to enhanced specificity and sensitivity of the formulations. A recent work with a novel liposome formulation modified with a membrane-disruptive copolymer [*N*-isopropylacrylamide-*co*-propylacrylic acid] imparted both pH and temperature sensitivity to the formulations; MR-guided focused US stimulation enabled more than 50% release of the encapsulated DOX. Tumor growth inhibition in rats with the dual sensitive liposomes was significantly more effective accompanied by improved tumor penetration, ECM remodeling, and cell destruction as compared to free drug and traditional thermosensitive DOX liposomes.[312] Similarly, pH- and temperature-sensitive liposomes modified with copolymer [poly(*N*-isopropylacrylamide-*co*-methacrylic acid-*co*-octadecyl acrylate)] were prepared for triggered delivery of berberine hydrochloride, an antibacterial/antifungal isoquinoline alkaloid.[313] A recent study described encapsulating an ammonium bicarbonate (NH_4HCO_3) precursor to confer pH-sensitivity to folate receptor-targeted liposomes. The principle involved endocytosis of the folate targeted liposomes and release of the DOX cargo by generation of CO_2 bubbles inside the liposomes in the presence of the endosomal acidic pH and simultaneously applied US.[314]

5. LIPOSOMAL DELIVERY: HYBRID LIPOSOMES

This section discusses the development of hybrid liposomes (HL) for drug delivery. In HL, the benefits of the liposomes are enhanced by those of another delivery system/polymer/nanoparticle by complex formation or encapsulation. In many cases, the individual drawbacks of the two systems can also be avoided. The simplest example of a hybrid liposome can be that of magnetic nanoparticles encapsulated in liposomes.

The work of the Matsumoto and Ueoka group over the past two decades in the development of hybrid liposomes is very noteworthy. The majority of their work involves L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylene dodecyl ether ($C_{12}(EO)_n$) where n = 4, 8, 10, or 21−25. The simple method of making HL adopted was with 90−95 mol % of DMPC combined with 5−10 mol % of $C_{12}(EO)_n$ in 5% glucose solution, ultrasonication at 45 °C and 300 W, followed by filtration with 200 nm filters. With increase of n, the membrane fluidity of the HL increased, which played a role in the cytotoxic effects shown in various in vitro and in vivo studies. HLs were cytotoxic by themselves, without the need of any drug. Initial studies with HL composed of L-α-dimyristoylphosphatidylglycerol [DMPG:$C_{12}(EO)_{10}$ (90:10)] indicated tumor growth inhibition of lung adenocarcinoma and stomach tumor cells without any toxic effects in normal cells in vitro.[315] Subsequent studies aimed to describe the mechanism of the HL cytotoxicity on cancer cells. Fusion and accumulation of the HLs occurred in the tumor cell membranes followed by an apoptotic signal induced through the activation of the caspase pathway. Again, the normal cells and normal animals were spared by the HLs representing their specificity for cancer cells.[316,317] The mechanism behind the specificity of the HLs for the cancer cells involves the greater fluidity of the cancer cell membranes as compared to the normal cells, which was the probable trigger for the HLs to fuse and accumulate in the tumors.[318] Several in vivo studies were carried out in tumor-bearing mouse models, including lung metastasis of LM8 (murine osteosarcoma),[319] human B lymphoma (RAJI) in SCID mice,[320,321] hepatic metastasis, acute lymphatic leukemia, human breast tumor (MDA-MB-231),[318] and human colorectal cancer (WiDR) xenografts.[322] The work shows the versatility of the HLs in treatment of several cancer types. Clinically, HL [DMPC:$C_{12}(EO)_{23}$ (95:5)] administration in 10 patients with various tumor types resulted in prolonged survival of one patient with advanced stage B-lymphoma who did not respond to traditional chemotherapeutics. The patient's solid lymph node tumor reduced, and there were no apparent side effects.[320]

Studies have also been conducted with HLs composed of DMPC and surfactants.[323,324] HLs composed of different types of polymers and phospholipids have also recently been utilized for several drug and gene delivery purposes.[325−328] The robustness and chemical versatility of these polymerosomes were combined with the biocompatibility and flexibility of liposomes to prepare hybrid phospholipid/block copolymer vesicles. Different ratios of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC; phospholipid component) and polybutadiene-*b*-poly(ethylene oxide) (PB-PEO; block copolymer) resulted in self-assembled nanoscale HLs using the thin-film hydration and extrusion techniques.[329] Quinteros et al. used either mucoadhesive polymers (sodium hyaluronate or carboxymethylcellulose) or poloxamers to prepare HLs encapsulating the hypotensive melatonin analogue 5-methoxycarbonylamino-*N*-acetyltryptamine. The HLs composed of a bioadhesive 0.2% sodium hyaluronate proved to be most beneficial at reduction of the intraocular pressure in rabbit eyes. The inclusion of such polymers enhanced the total rheological and biophysical properties of the preparation with long-lasting hydration and lubrication of the ocular surface.[330]

Hybrid liposomes have also been prepared with metallic nanoparticles including silver and gold. In separate studies, PEGylated HLs were effectively prepared with gold nanoparticles by exploiting the plasmonic properties of metallic nanoparticles and the long-circulating properties of the PEGylated liposomes for imaging and chemotherapeutic applications. While one study encapsulated the gold nanoparticles in the core, another one involved the gold nanoparticles decorating the outer surface of the cationic liposomes.[331,332] Three techniques to prepare hybrid liposome/nanoparticles have been described: (a) classical thin-film

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Table 4. Recently Conducted In Vivo Studies with Liposome-Based Drug Delivery

drug/gene	indication	liposome configuration and route of administration	ref
Paclitaxel	cancer	Paclitaxel-conjugated gold nanoparticles in HLs;[a] iv[b]	348
		high-density lipoproteins; iv	349
	glioblastoma	mannose-Vit E or dequalinium-lipid conjugated lip[c] coloaded with artemether; iv	350
	pulmonary arterial hypertension	R8-modified stealth lip; iv	351
Doxorubicin	cancer	prostate cancer specific RNA aptamer-conjugated lip; iv	352
	metastatic cancer	Lyp-1-targeted PEGylated lip; sc[d]	353
	brain glioma	transferrin-TATp-conjugated PEGylated lip; iv	354
Vincristine	MDR cancer	folate targeted PEGylated lip; iv	355
	PK studies in beagle dogs	soy PC/Chol lip; iv	356
Docetaxel	CNS delivery study	glucoside-modified lip for BBB targeting; iv	357
Docetaxel + anti-BCL-2 siRNA	MDR cancer	PEGylated cationic lip; iv	358
Docetaxel + anti VEGF siRNA	glioblastoma	dual targeted peptide-modified lip; iv and intratumoral	359
Anti-BCL-2 oligonucleotide	cancer	hybrid anionic copolymer/cationic lip complex; intratumoral	360
Anti EphA2 siRNA	cancer	multistage vector lip; iv	361
O^6-methylguanine-DNA methyltransferase-siRNA	glioblastoma	siRNA cationic lip complex in combination with/without Temozolomide; convenction-enhanced delivery directly to the brain	362
Antigenic ovalbumin	cancer immunotherapy	polymer-modified PSL for cancer immunotherapy; sc	363
Cyclic di-GMP	cancer immunotherapy	fusogenic PSLs for cancer immunotherapy; sc	364
Indole-3-carbinol	lung cancer	lip dispersion; intranasal	365
Ciprofloxacin	tularemia (rabbit fever)	lip ciprofloxacin; iv/aerosolized inhaler	366
Primaquine	malaria	heparin-targeted cationic lip; iv	367
B-methasone prodrug − β-methasone hemisuccinate	cerebral malaria	nonsterically stabilized lip; iv	368,369
Rifampicin, Isoniazid, Pyrazinamide	tuberculosis	alveolar macrophage-specific targeted lip; dry (liposomal) powder for inhalation	370
Pyrazinamide, Levofloxacin	tuberculosis	proliposomal dry (liposomal) powder for inhalation	371,372
Amikacin	pulmonary infection	liquid dispersion of amikacin-loaded lip for inhalation	373
Prostaglandin E2	idiopathic pulmonary fibrosis	aerosolized lip for inhalation	374
Amphotericin B	disseminated aspergillosis	erythropoietin combined with Ambisome; iv	375
Lovastatin	hypolipidemic agent	proliposomes in capsules or compressed in tablets; oral	376
Nimodipine	cerebrovascular spasm, stroke, migraine	proliposomes in gelatin capsules hydrated with distilled water before administration; oral	178
Recombinant human insulin	diabetes	lip containing bile salts; oral	377
Insulin		biotinylated lip; oral	378
Salmon calcitonin	hypercalcemia, osteoporosis	mucoadhesive polymer - chitosan-thioglycolic acid coated lip; oral	379
Ketoprofen	nonsteroidal anti-inflammatory	proliposome powders; oral	380
Curcumin	anti-inflammatory	propylene glycol lip and ethosomes; transdermal	381
Diclofenac	anti-inflammatory	penetration enhancer-containing vesicles (lip); dermal	382
Lidocaine	local anesthetic	TATp-conjugated octadecyl-quaternized, lysine-modified chitosan lip; transdermal	383
Psoralen	psoriasis therapy	lip and ethosomes; dermal/transdermal	384

[a]HLs = hybrid liposomes. [b]iv = intravenous administration. [c]lip = liposome. [d]sc = subcutaneous administration.

hydration followed by sonication/extrusion, (b) one-step nanoprecipitation using microfluidic technology, and (c) a modified emulsification method (reverse phase evaporation).[333]

Quite a few studies have described additional methods to prepare HLs for drug delivery applications. Cyclodextrin-in-liposomes were used to improve the loading of both hydrophilic and lipophilic drugs as well as improve the drug/drug delivery system stability in biological fluids. One method involved heating the prepared liposomes at 65 °C in a PBS solution containing drugs encapsulated in cyclodextrin. The heat increased the fluidity and thus permeability of the liposomes toward the cyclodextrin (remote loading technique).[334] The other method employed a dehydration−rehydration technique.[335] Liposome−quantum dots hybrids,[336] hybrid capsules of alginate plus guar gum encapsulated with liposomes,[337] and hydrogel-in liposomes[338] to encapsulate several drugs have been described in separate studies.

6. LIPOSOMAL APPLICATIONS: DISEASES AND ROUTE OF ADMINISTRATION

Liposomes have been used for several medical indications including cancer, infections, and skin disorders. Most of the research with liposomes has been carried out in the field of cancer. Liposomes, being chemically versatile, have also been developed for different routes of administration such as parenteral, dermal/transdermal,[339,340] pulmonary,[341−343] as well as oral.[344−346] Each route possesses its advantages and disadvantages, and the liposomes need to be designed accordingly. For instance, liposomes for systemic administration are useful for solubilizing and stabilizing several drugs by protecting them from the biological fluid environment, reducing nonspecific toxicity of free drug, drug/gene targeting, as well as by promoting intracellular delivery of cargos.

However, parenteral administration often presents distress and noncompliance in patients, especially in diseases like cancer

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where they undergo multiple infusion treatments. In those instances, pulmonary or oral liposomal delivery can be of great use. Pulmonary delivery can be particularly beneficial for lung cancer and infections such as tuberculosis or pneumonia. Topical administration of drugs/genes has also been included in liposomes for skin cancer, acne vulgaris, hydration, and psoriasis.

Investigations on liposomal drug/gene targeting to the central nervous system have aimed to circumvent the blood−brain barrier and deliver the drugs in significant concentrations for brain cancer therapy and disorders such as Parkinson's and Alzheimer's disease therapy.[347]

Table 4 compiles some of the extensive recent work involving liposomes used for different diseases with their configuration and route of administration.

7. LIPOSOMAL APPLICATIONS: OTHERS

While the technology and the applications of the liposomes in drug delivery have been described at length, liposomes also play a major role in the fields of molecular imaging, vaccines, and analytical sciences. On their own, each of these applications deserves an extensive assessment, which is beyond the scope of this Review. However, the general features and some investigations thereof are presented.

7.1. Molecular Imaging

Molecular imaging is applied widely for diagnosis and for monitoring of treatment progression of diseases. In addition to being a versatile system for labeling with the radionuclides or contrast enhancement agents, the liposomes can also be passively or actively targeted to the target tissue. Because the radionuclides used have a short half-life, it is imperative that the liposomes are PEGylated to confer long-circulation and time for the liposomes to reach the desired area of interest. This property is also useful to enhance the signal intensity, because more liposomes will accumulate at a targeted site and improve the signal-to-background noise ratio. The radiolabels can be incorporated with the liposomes by (a) encapsulation into the aqueous core, (b) dissolution in the lipid bilayer of liposomes, (c) surface chelation of the liposomes, or (d) remote loading into the core of the liposomes by generation of a transmembrane gradient.[385] To improve the signal intensity by high incorporation efficiency and quick loading, surface chelation or remote loading techniques on preformed liposomes are usually preferred. With the remote loading technique, chelators are often used inside the core of the liposomes, which trap the radiolabels, improve stability, and provide an opportunity for high signal intensity.[386] The roles of the difference in liposome composition and the transmembrane gradients employed for the remote loading of ^{99m}Tc-*N*,*N*-bis(2-mercaptoethyl)-*N*′,*N*′-diethyl-ethylenediamine (^{99m}Tc-BMEDA) complex were recently compared in terms of radiolabeling efficiency and stability of the formulation.[387] Erdogan et al. incorporated a polychelating ampiphilic polymer to attach gadolinium (Gd) in 2C5 mAb-targeted PEGylated liposomes and successfully imaged tumors in mice as early as 4 h postinjection.[388] To improve the incorporation efficiency along with the additional benefit of high relaxivity for signaling, Gd with attached dextran was embedded in the porous lipid bilayer of [1,2′-bis[10-(2′,4′-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphocholine] (bis-SorbPC) liposomes in another work.[389] Indocyanine green label in lung-specific surface-modified liposomes enabled Murata et al. to perform noninvasive, real-time, near-infrared optical imaging of these pulmonary administered liposomes in rat lungs.[390]

Techniques employed for the detection of radionuclides (^{99m}Tc, ^{111}In, ^{123}I, ^{18}F, ^{68}Ga, etc.) include single-photon emission computed tomography (SPECT) and positron emission tomography (PET). Proper optimization of each radionuclide and corresponding detection technology is necessary for reliable imaging.[391,392] Likewise, contrast agents for CT imaging, paramagnetic metals for MRI, or microbubbles for US sonography are also used widely. The choice of the imaging system including the imaging agent and the instrumentation depends highly on the imaging properties, availability, ease of use, and cost.

The field of theranostics employs liposomes coloaded with drugs and imaging agents for simultaneous drug delivery and imaging measurements of the efficiency of the drug release and therapeutic effects.[393,394] It was recently determined in clinical settings that, in patients with advanced pleural mesothelioma, the individuals exhibiting higher ^{99m}Technetium (^{99m}Tc)-liposomal DOX uptake showed improved progression-free survival than those who did not. Correlation studies proved that the higher uptake, as measured by imaging ^{99m}Tc coadministered in the liposomes, was related to the improved efficiency of the chemotherapy.[395]

7.2. Vaccine Delivery

The capacity of the liposomes as potent vaccine delivery systems has been recognized.[396,397] Liposomes with surface-presented or encapsulated antigens can induce an immune response by being phagocytosed by the macrophages, processed, and presented on the macrophage surface with either the MHCI (major histocompatibility class I) complex (when the liposomes and antigens end up in cytoplasm) or the MHCII complex (when the liposomes and antigens are broken down in lysosomes). Subsequently, the cytotoxic T lymphocytes (CTL) recognize the antigen peptides on the surface of the macrophages and bind to the T-cells. The T-helper cells produce cytokines, interact with B-cells, and stimulate the secretion of antibodies. Additionally, the liposome also represents a good depot for gradual and continuous presentation of antigens. The liposomes provide a benefit from their larger antigen presenting (surface-bound or encapsulated) properties in comparison to commonly applied adjuvants including alum. Appropriate modifications in the size, charge, composition of the liposomes, and the antigen used can induce specific immune responses.[398,399]

Liposomes of trehalose 6,6′-dibehenate (TDB) with either dimethyldioctadecylammonium bromide (DDA) (cationic charged) or DSPC (neutral charged), in different composition ratios, were compared for their delivery of Ag85B-ESAT-6-Rv2660c (H56 antigen; tuberculosis vaccine) and the rate of cell-mediated and antibody immune response. The decrease in charge with replacement of DDA by DSPC adversely affected the surface binding of H56, and only the complete replacement of DDA with DSPC reduced the cell-mediated immune response. This study demonstrates the importance of composition and charge in the preparation of liposomes with adjuvant properties.[400] The liposomal adjuvant of DDA and TDB in a weight ratio of 5:1 is known as CAF01. The CAF01 with the Ag85B-ESAT-6 (H1; tuberculosis vaccine) was recently reported in a first-in-man trial at varying doses. A long-lasting immune response without toxicity was reported in two groups of the trial.[401]

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Antigens used with liposomes range from proteins[402] to peptides,[403] RNA,[404] DNA,[405] and synthetic human MUC1 peptides,[406] further establishing the versatility of liposomes as vaccine drug delivery vehicles. The antigen can be encapsulated in the liposome core or presented on the liposome surface via adsorption to the lipid bilayer or with a covalent/noncovalent conjugation to the bilayer surface. The size and lamellarity, charge, membrane fluidity, as well as fusogenic properties of the liposomes have different effects on their capacity to serve as vaccines.[398] Liposomes can act as immunostimulant adjuvants when incorporated with specific lipids or molecules such as phosphatidylserine,[407] DOTAP,[408] fatty acids,[409] and monophosphoryl lipid A,[410] among others.[411] Cationic liposomes with poly(inosinic-polycytidylic acid) (poly(I:C)), a Toll-like receptor ligand, encapsulating a model CTL epitope (SIINFEKL) presented by a synthetic long peptide (SLP), induced a 25-fold superior T-cell immune response in mice as compared to the poly(I:C)-adjuvanted soluble SLP.[412]

Epaxal and Inflexal V are clinically approved liposome-based vaccine products, classified as virosomes. Virosomes are viral liposomes reconstituted from virus membranes without the genetic information of the virus. Epaxal, a hepatitis A virus vaccine, is based on an inactivated hepatitis A antigen incorporated into the virosomes, while Inflexal V influenza virus vaccine is based on hemagglutinin and neuraminidase from inactivated influenza incorporated into virosomes with lecithin.[413] Both of the vaccines are well-tolerated, safe, and generate effective immune responses. In contrast, Stimuvax (L-BLP25), a liposome-based anticancer vaccine, which targeted the MUC1 tumor-associated antigen, was reported to fail in Phase III trials of non-small cell lung carcinoma (NSCLC) in patients.[414] Reasons cited for the failure encompassed the presence of advanced tumors and resistance of the NSCLC to immunotherapy, the inclusion of just one antigen to induce an immune response, and the design of the clinical trial itself. Thus, there is a long way to go before the true potential of the liposomes for successful vaccine formulations is realized.

7.3. Analytical Applications

Liposomes can be used in different analytical techniques, particularly liquid chromatography, immunoassays, and as biosensors.[415−417] Because of the large surface area and a relatively larger encapsulation volume of liposomes as compared to the commonly engaged analyte/antibody-conjugated signal probe, they can amplify the signal intensity by incorporating large amounts of signaling molecules in the core or bilayer. Often this enables achievement of considerably lower limits for detection in a sample providing an advantage with smaller volumes. Also, because the liposomes mimic the cell membrane, analyses of the drug/biomolecule/microbe interactions with the membrane can be simulated.

Immobilized liposome chromatography involves liposomes conjugated to gel beads (stationary phase) and can be considered for several applications such as drug partitioning, protein separation, or even the effect of the drugs on the membrane. Immunoassays, on the other hand, rely on the signals generated by the liposomes via fluorescence, chemiluminescence, colorimetry, or electrochemical detection. Generally, the liposomes-in immunoassay (LIA) employs the principles behind the enzyme-linked immunosorbent assay (ELISA) in a competitive or noncompetitive assay type. For instance, after an antibody is immobilized on the biosensor capillary strip, an antigen and a secondary-antibody tagged dye-containing liposome undergo capillary action either together or in sequence. In a fashion similar to a sandwich ELISA, the antigens in the sample are captured by immobilized antibody to which the secondary antibody-tagged liposomes get bound, and a signal is induced corresponding to the amount of antigen present in the sample. Further LIA techniques and their modifications are described expansively.[415−417]

Edwards and Baeumner recently described a new technique for detection of proteins, using maltose periplasmic binding protein (MBP) conjugated to fluorescent dye-encapsulating liposomes (MBP-FL). In microtiter plates, maltose and the MBP-FL were mixed with amylose magnetic beads. After competitive binding of the MBP-FL to the magnetic beads or to the maltose, the magnetic beads were held in the plates with a magnet while the unbound material was washed away. The liposomes remaining bound were lysed, and the fluorescent dye released from the liposomes was measured. The signal was inversely proportional to the amount of maltose in the sample. This method can be extended to various proteins. The limit of detection was 78 nM, thus displaying a capacity to measure nanoamounts of analyte in samples.[418] In another study, colorimetric and electrochemical strips were prepared for measuring the levels of progesterone. Antiprogesterone antibody was immobilized on both of the strips. Competitive binding was carried out between sample progesterone and methylene blue-encapsulated liposomal progesterone. The virtue of the liposomes encapsulating the dye in large amounts provides signal amplification for both colorimetric and electrochemical measurements that can be observed corresponding to the different amounts of the progesterone in the sample.[419]

The interactions between the protein hormone, recombinant human erythropoietin (rh-EPO) immobilized on disposable optical fiber streptavidin biosensor tips, and three different liposome formulations have been investigated by biolayer interferometry. The binding kinetics were measured on the basis of the attachment of liposomes that increase the thickness of the layer of protein-coated surface that is correlated directly to the spectral shift as measured by biolayer interferometry.[420] This method has considerable high-throughput capability for identification of lead biomolecular drugs. Similarly, viral interactions with receptors on host cell membranes can be mimicked using receptor-bound liposomes.[421] As well, liposomes have been used as models to understand the partitioning of drugs within phospholipid bilayers and the effects of free ions, membrane-bound detergents, and fatty acids on the partitioning.[422] Liposomes composed of negatively charged components as well as cholesterol model cell membranes appropriately[423] and thus can be used for drug/membrane interactions as well as potential lead drug screening.

Thus, liposomes can be used in several analytical applications including detection of analytes in samples, high-throughput screening of lead candidates, and clinical diagnosis.

8. REGULATORY AFFAIRS

One-half a decade of liposome research on drug/gene delivery has provided several advantages and opportunities for liposome applications. However, it has also brought about multiple regulatory issues. In general, not just liposomes, but the broader field of nanomedicine and micromedicine has been in a turmoil for some decades while setting up definitive parameters and programs for the regulation of nanoproducts. The market for nanomedicine is only going to go stronger. The projections for

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the year 2016 predict a $96.9 billion total global nanomedicine market.[424] This justifies the need for a strong globally accepted, stable regulatory system.

Some of the current issues[425,426] include the following.

The definition of nanomedicine: There is a lack of a consistent definition of nanotechnology and nanomaterials globally. According to the U.S. National Nanotechnology Initiative, nanotechnology is currently defined as the "understanding and control of matter at the nanoscale, at dimensions between approximately 1 and 100 nm, where unique phenomena enable novel applications."[427] Clearly, as we have seen in several studies above, liposome size ranges from nanometers to micrometers, which creates an issue of classification when filing for drug approval.

Regulatory guidelines regarding the characterization of liposomes and nanotechnology are ambiguous and depend on the first-in-class product filed. Because most of the applications involve a new technology, the regulatory offices may know less about the system than the sponsors, creating a gap in the requirements necessary for a predictable, safe, and stable drug delivery system.

The liposomes are often composed of exotic materials ranging from phospholipids, small molecule drugs, nucleic acids, to large molecule proteins and peptides. It becomes necessary for the applicant to conduct stability and toxicology studies of each component including the liposome carrier individually and in combination with the other components. Moreover, the same liposome formulation may possibly be manufactured by different techniques, which can have a significant impact on how it is best stored as well as its biological fate. This can be a special issue because a product can be a drug, a biological product, and/or a device, all of which are handled by different centers within the FDA.

Various government agencies for drug regulation have identified the issues and are making conscious efforts to create guidelines for nanomedicine approvals, especially because many of the nanomedicines target hard-to-treat diseases like cancer and tuberculosis.[425,426]

9. CONCLUSION

Applications of the liposomes as drug delivery systems are numerous, stemming from the fact that the liposomes are very versatile in use. Ease of chemical modifications, the ability to carry drugs/genes of different kinds, and the potential to be administered by different routes have made them especially attractive. The ability to safely transport drugs to the target site and even cause their trigger-based release is icing on the therapeutic cake. Newer applications in diagnosis and even theranostics have been realized. Certainly a great deal of advancement in liposome technology has been observed since their discovery in 1965. However, it is still a point of major concern that the full potential of the liposomes has not been realized. Successful bench to bedside applications have been few.

Limitations to successfully market developments include potential cytotoxic effects of the liposomes. It has been observed that the liposomes are leaky and can release quite a lot of their payload almost immediately on administration. This negates some of the advantages of their use. Some studies have found charged liposomes to be toxic.[428−430] Moreover, with some methods of liposome production, there are chances for the organic solvents (ethanol, ether) to be present in trace amounts in the final preparation. With trigger-release systems, particularly TSLs requiring an externally applied stimulus, the use of some invasive modalities is inconvenient for multiple dosage regimens. Additionally, low penetration depth of heat, insufficient heating, and local normal tissue damage have also limited their applications. Still, MR guided FUS has been applied with considerable benefits for the TSLs.

The liposomes also have some manufacturing-related issues like batch to batch reproducibility, low drug entrapment, effective sterilization methods, stability problems, and, most importantly, scale-up problems. Classical methods like transmembrane gradients and novel methods such as microfluidization, freeze−thaw cycles, SRPE, and spray-drying have improved the encapsulation efficiency and reproducibility with a better control over size. Also, lyophilization to make lyophilized liposomal powders for reconstitution can now be used to improve the stability profile of a formulation. Still, a universal method for sterilization of liposomes has not been achieved. The most commonly used sterilization technique is filtration with 0.22 μm filters, but it comes with issues related to limited batch size applications. Perhaps, the biggest issue relates to the scalability of liposome technology. Complications also arise when production of multifunctional liposomes is involved. These issues in combination with the costly raw materials result in the liposomes localized on the expensive side of the medicinal market.

Still, with the advancements in technology of the past decade, high hopes are justified for the continued development of liposomes, and nanomedicine in general, as drug delivery systems.

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The authors declare no competing financial interest.

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Vladimir V. Chupin graduated from the Moscow Institute of Fine Chemical Technology, Russia. He then worked at the same institute, at Utrecht State University, Netherlands, and at the Institute of Bioorganic Chemistry, Russia. Currently, he is head of the biophysics department of the Moscow Institute of Physics and Technology, Russia. The sphere of his scientific interests is lipid chemistry, membrane proteins, and nanobiotechnology.

Vladimir P. Torchilin is a University Distinguished Professor and Director, Center for Pharmaceutical Biotechnology and Nanomedicine, Northeastern University, Boston, MA. He has published more than 350 original papers, more than 150 reviews and book chapters, wrote and edited 10 books, and holds more than 40 patents. He is Editor-in-Chief of *Current Drug Discovery Technologies* and of *Drug Delivery* and on the Editorial Boards of many journals including *Journal of Controlled Release* (Review Editor). Professor Torchilin received the 1982 Lenin Prize in Science (the highest award in the former USSR). He is a Member of European Academy of Sciences (EAS) and Fellow of AIMBE, AAPS, and CRS, and received the 2005 Research Achievements in Pharmaceutics and Drug Delivery Award from the AAPS, 2007 Research Achievements Award from the Pharmaceutical Sciences World Congress, 2009 International Journal of Nanomedicine Distinguished Scientist Award, 2010 Controlled Release Society Founders Award, 2012 Alec Bangham Life Time Achievements Award, 2012 Journal of Drug Targeting Life Time Achievements Award, and 2013 Blaise Pascal Medal in Biomedicine from the EAS. In 2005 he was a President of the CRS. In 2011, Times Higher Education ranked him number 2 among the top world scientists in pharmacology for 2001−2010.

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