REVIEW

Liposomes as nanomedical devices

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Abstract: Since their discovery in the 1960s, liposomes have been studied in depth, and they continue to constitute a field of intense research. Liposomes are valued for their biological and technological advantages, and are considered to be the most successful drug-carrier system known to date. Notable progress has been made, and several biomedical applications of liposomes are either in clinical trials, are about to be put on the market, or have already been approved for public use. In this review, we briefly analyze how the efficacy of liposomes depends on the nature of their components and their size, surface charge, and lipidic organization. Moreover, we discuss the influence of the physicochemical properties of liposomes on their interaction with cells, half-life, ability to enter tissues, and final fate in vivo. Finally, we describe some strategies developed to overcome limitations of the "first-generation" liposomes, and liposome-based drugs on the market and in clinical trials.

Keywords: liposomes, nanomedicine, drug delivery, ultrastructure

Introduction

Liposomes were discovered by Alec D Bangham in the 1960s at the Babraham Institute, University of Cambridge, and consist of single or multiple concentric lipid bilayers encapsulating an aqueous compartment (Figure 1).[1,2] The first formulations were composed solely of natural lipids; at present they can include natural and/or synthetic lipids and surfactants. They have the capability of entrapping both lipophilic and hydrophilic agents, in the lipid membrane and in the aqueous core, respectively. The size of these nearly spherical lipid vesicles can range from a few nanometers to several micrometers. However, liposomes applied to medical use range between 50 and 450 nm.[3]

Liposomes seem to be an almost ideal drug-carrier system, since their morphology is similar to that of cellular membranes and because of their ability to incorporate various substances. Therefore, for the last 50 years liposomes have been widely investigated and they continue to be the subject of intense research. They are valued for their biological and technological advantages as optimal delivery systems for biologically active substances, both in vitro and in vivo, and are considered to be the most successful drug-carrier system known to date.[4] During the two last decades, notable progress has been made, and several biomedical applications of liposomes are either in clinical trials or are about to be put on the market, while others have already been approved for public use.[5]

Therefore, the goal of this review is not to undertake an exhaustive report on the plethora of data published on liposomes since their first synthesis in the 1960s, but to focus on some points that play a key role in the development of liposomal formulation for therapy.

We briefly analyze how the efficacy of liposomes depends on the physicochemical properties of their membranes, on the nature of their components, and on their size,

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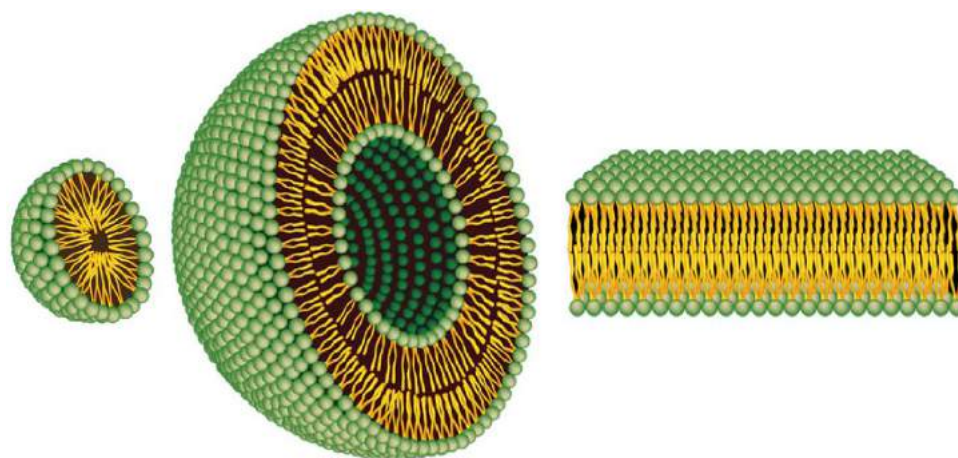Fax +39 06 4990 2137
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Figure 1 Representation of the steric organization of a micelle (left), a liposome (center), and a lipid bilayer (right). Whereas liposomes are composed of a lipid bilayer, micelles are constructed of one lipid layer in which the apolar section turns inward and the polar heads interact with the environment. These two different organizations mean that the space enclosed in the micelles is much more limited to that available in liposomes.
Note: Adapted from Bitounis D, Fanciullino R, Iliadis A, Ciccolini J. Optimizing druggability through liposomal formulations: new approaches to an old concept. *ISRN Pharm*. 2012;2012:738432.[14]

surface charge, and lipid organization. In addition, we discuss how the physicochemical properties of liposomes influence their stability in the bloodstream, their ability to enter various tissues, their interaction with cells, and their final fate in vivo. We also describe some strategies developed to overcome the limitations of the "first-generation" liposomes, and how this has been crucial in opening the way from the laboratory bench to clinical trials or to the market.

The physicochemistry of liposomes

The adequacy of liposomes as a carrier system for drugs strictly depends on the physicochemical properties of their membranes, on the nature of their components, on their size, surface charge, and lipid organization.[6]

Liposomes are mainly composed of phospholipids, amphiphilic molecules that have a hydrophilic head and two apolar hydrophobic chains. When phospholipids are dispersed in aqueous solutions, due to their amphipathic nature they have a strong tendency to form membranes.[7] On the one hand, their polar heads prefer to interact with the aqueous environment; on the other, their long apolar aliphatic chains promote interaction with one another. In aqueous solution, these dual properties favor the formation of two lipid layers. The hydrophobic chains of each layer face each other and constitute a lipophilic inner compartment that acts as a permeability barrier, both inward and outward. Hydrophobic interactions are behind the formation of these lipid bilayers, and van der Waals forces keep the long hydrocarbon tails together, thus strengthening this architecture. Lastly, hydrogen bonds and polar interactions between the water molecules of the aqueous environment and the polar heads

of lipids stabilize this organization. The final organization of lipids depends on their nature, concentration, temperature, and geometric form.[8] If ions or molecules are present during the formulation process, they can be encapsulated inside these membranes.

Liposomes can be classified on the basis of the preparation method (reverse-phase evaporation vesicles or vesicle extruded technique), size (small, intermediate, or large), and lamellarity (uni-, oligo-, and multilamellar vesicles). The formation of unilamellar vesicles (ULVs) or multilamellar vesicles (MLVs) depends on the synthesis methods and postformation processing used for their preparation (refer to the "Methods for the preparation of liposomes" section for more details). Since ULVs (one lipid bilayer, 50–250 nm) enclose a large aqueous core, they are ideally suited for the encapsulation of hydrophilic drugs. On the other hand, MLVs (two or more concentric lipid bilayers organized like an onion-skin, 1–5 μm) preferentially entrap lipid-soluble drugs.[9] In addition to the ability to entrap drugs with different solubility characteristics, it has been hypothesized that ULVs and MLVs have different release kinetics. In general, MLVs are formed more easily at larger hydrodynamic diameters, and thus have greater entrapped volume than ULVs. As a result, unilamellar liposomes with a hydrodynamic diameter of 130 nm exhibit a much faster release rate than MLVs with two to three lamellar bilayers and a hydrodynamic diameter of 250 nm.[10,11] The difference in the release rate is due overall to the number of phospholipid bilayer that it have to cross before being released.

The ongoing interest of researchers in liposome characteristics, such as stability, pharmacokinetic properties, and

therapeutic efficacy, has led to second-generation liposomes by the modulation of lipid composition, size, and the charge of the vesicle. The addition of cholesterol to the lipid bilayer of liposomes reduces their permeability and increases their in vivo and in vitro stability, because the presence of cholesterol induces a dense packing of phospholipids and inhibits their transfer to high-density lipoprotein (HDL) and low-density lipoprotein (LDL). In fact, cholesterol is a hydrophobic molecule and preferentially interacts with the core of the membrane, thus stabilizing it. Further, cholesterol can be used to anchor other molecules, such as polyethylene glycol (PEG) or deoxyribonucleic acid (DNA), to the liposomes for their application in biosensing or as "stealth" drug carriers[12] (reviewed in Hosta-Rigau et al).[13] Finally, the use of phosphatidylcholine with saturated fatty acyl chains and materials that stretch the transition temperature beyond 37°C offered even greater stabilization.[14] For prolongation of the in vivo liposome circulation time, a milestone is the inclusion of hydrophilic carbohydrates or polymers, such as monosialoganglioside (G_{M1}) and PEG in liposome composition. G_{M1} decreases the blood proteins absorbed on the liposomal surface and improves the half-life of liposomes in the blood.[15,16] Similarly, the PEGylation of the liposomal carrier proved to extend the blood-circulation time while diminishing the uptake by the reticuloendothelial system (RES). Further, by modifying the PEG-molecule terminus, liposomes can be actively addressed with specific ligands or monoclonal antibodies (more details are reported in the section "Pharmacokinetics of liposomes").[17]

Methods for the preparation of liposomes

There are many different methods for the preparation of liposomes. The choice of the appropriate method depends on several factors: 1) the physicochemical characteristics of the liposome components and those of the drug to be loaded; 2) the toxicity and the concentration of the loaded substance; 3) the type of the medium in which the liposomes are dispersed; 4) the additional processes during the application/delivery of the liposomes; 5) the size and the half-life desired for the successful application; and 6) the costs, reproducibility, and applicability regarding large-scale production for clinical purpose and good manufacturing practice-relevant issues.[18–20]

One of the most widely used techniques for liposome-manufacture preparation is the thin-film hydration or Bangham method.[21,22] Briefly, this method involves dissolution of the lipid in an organic solvent, evaporation of the solvent, and the dispersion of the obtained lipid film in

aqueous media. The drug to be entrapped can be included in the aqueous media (for hydrophilic drugs) or in the lipid film (for lipophilic drugs). However, the encapsulation efficiency of water-soluble drugs is low (5%–15%). Moreover, this method produces large and nonhomogeneous MLVs that require sonication or extrusion processes to be produced in homogeneous small ULVs.

The reverse-phase evaporation and solvent-injection methods provide hydration of the lipids directly from an organic solvent, and achieve an aqueous suspension of MLVs and ULVs, respectively.[23–25] The use of these methods is affected by the solubility of lipids in the organic solvent and the elimination of the latter from the products. Nevertheless, these procedures guarantee higher encapsulation efficiency than thin-film hydration.

The detergent-depletion method involves the hydration of a lipid film with a detergent solution and leads to the formation of large MLVs.[22,26,27] This method is rarely used, because it needs long preparation time and poor trapping efficiency. On the contrary, the dehydration–rehydration technique described by Kirby and Gregoriadis provides high drug-encapsulation efficiency and is widely used in nanomedicine. This procedure induces the fusion of preformed vesicles by means of dehydration and controlled rehydration.[28]

To ensure the desired size, lamellarity, and homogeneity properties of liposomes manufactured with the aforementioned techniques, postformation processing is required. The most common methods for postformation processing are sonication, extrusion, and high-pressure homogenization. Sonication is used to reduce the size of the vesicles and give energy to lipid suspension. This can be obtained by applying an ultrasonic irradiation to the suspensions of MLVs.[29] The resulting small ULVs are purified by ultracentrifugation. The membrane-extrusion method reduces the size of the liposomes (large ULVs or MLVs) by passing them through a membrane filter with a defined pore size.[30–32] The high-pressure homogenization is a fluid mechanical process that involves the subdivision of vesicles into smaller sizes and occurs in a special homogenizing valve.[33]

Since industrial-scale production of liposomes has become a reality, the range of liposome-preparation methods has been extended by a number of techniques. Among these are the heating method, spray-drying, freeze-drying, supercritical reverse-phase evaporation, and several modified ethanol-injection techniques that are increasingly attractive, as extensively reviewed in Laouini et al.[34]

Finally, the microfluidic-based method is an emerging technology for liposome synthesis that allows strict control

of the lipid-hydration process. Microfluidics is a method to manipulate liquid flows in channels with dimensions of tens to hundreds of micrometers.[35,36] The characteristic of laminar flow and rapid and tunable mixing has some advantages in liposome formation over traditional methods, such as thin-film hydration and reverse-phase evaporation. In continuous micro-fluidic flow systems, precise control of liposome size and size distribution can be implemented by controlling flow and mixing conditions. However, the problem of scaling up liposome production needs to be addressed during the implementation of microfluidic technology for practical application. Additionally, the ultrafine structure of liposomes produced with the microfluidic method needs to be further investigated.[37]

Microfluidic remote loading for rapid single-step liposomal drug preparation has been recently reported. In this method, microfluidic-directed formation of liposomes is combined with in-line sample purification and remote drug loading for single-step, continuous-flow synthesis of nanoscale vesicles containing high concentrations of stably loaded drug compounds. Using an on-chip microdialysis element, the system enables rapid formation of large transmembrane pH and ion gradients, followed by immediate introduction of amphipathic drugs for real-time remote loading into the liposomes. The microfluidic process enables in-line formation of drug-laden liposomes with drug:lipid molar ratios of up to 1.3, and a total on-chip residence time of approximately 3 minutes, representing a significant improvement over conventional bulk-scale methods, which require hours to days for combined liposome synthesis and remote drug loading. The microfluidic platform may be further optimized to support real-time generation of purified liposomal drug formulations with high concentrations of drugs and minimal reagent waste for effective liposomal drug preparation at or near the point of care.[38]

Real-time, highly sensitive, and low-cost monitoring of drug-loading and delivery dynamics in different pharmaceutical and biomedical environments could be very useful both for pharmaceutical manufacturing and for quality-assurance assays applied to liposomal formulations. Currently, the techniques used to investigate liposomal structures and their stability in different environments, as well as drug-loading and -delivery mechanisms, operate basically off-line and/or with prepared sampling. Organic electrochemical transistors (OECTs), promising devices for applications in bioelectronics and nanomedicine, have been recently proposed as ideally suitable for sensing and real-time monitoring of liposome-based structures. These systems seem to be particularly suited for real-time monitoring of liposomes in solution. OECTs

are sensitive devices for detecting liposomes on a wide dynamic range down to 10^{-5} mg/mL (with a lowest detection limit, assessed in real-time monitoring, of 10^{-7} mg/mL), thus matching the needs of typical drug-loading/drug-delivery conditions. Furthermore, OECTs proved to be able to sense and discriminate successive injections of different liposomes, and so could be good candidates in quality-control assays or in the pharmaceutical industry.[39]

Parameters including shape, size, surface features, and lamellarity strongly influence the biological behavior of liposomes. Therefore, they have to be extensively characterized prior to their use in order to ensure in vitro and in vivo performance. Liposome shape can be assessed using electron microscopy techniques. Lamellarity of liposomes can be determined by using negative staining and/or freeze-fracturing for transmission electron microscopy (TEM) and P-31 nuclear magnetic resonance analysis.[40]

Several techniques are available for the determination of size and size distribution, among which the most widely applied include dynamic light scattering (DLS), size-exclusion chromatography, and field-flow fractionation (FFF).[41–44] Various microscopy techniques, including cryo-TEM, have also been found useful for the characterization of liposome size.[45,46]

DLS measures the time-dependent fluctuations in the intensity of scattered light, which occur because particles (liposomes) in a suspension undergo random Brownian motion due to collisions between suspended particles and solvent molecules. An analysis of the intensity fluctuations allows the determination of the distribution of the diffusion coefficients of the liposomes, which are converted into a size distribution using established theories. DLS is a simple and rapid method, but it provides an average property of liposome bulk.

On the contrary, electron microscopy techniques, such as cryo-TEM and TEM using freeze-fracturing, provide a precise determination of liposome size, since they allow for the visualization of single liposomes and can resolve particles of varying size. The result is exact information about the profile of the liposome population over the whole range of sizes. Unfortunately, these techniques are very expensive and require specific equipment.[45] Another microscopic technique utilized to analyze liposome morphology and size is atomic force microscopy, which provides information with high resolution on the three-dimensional profile of liposomes without removing them from their native environment.[47,48]

Size-exclusion chromatography is a technique that can separate and quantify liposome populations by exploiting

the time-based resolution of hydrodynamic size. The process of separation is based on large-particle elution before that of smaller particles. The large particles are left out from the internal pore volume of the porous substrate used in this technique, and are eluted more quickly from the column.[43,49]

FFF is a separation technique based on the laminar flow of particles in a solution, and uses a semipermeable membrane that allows only the carrier fluid to pass through the membrane. FFF separates liposomes based on size, and can separate materials over a wide colloidal size range while maintaining high resolution.[50]

Liposomes as nanocarriers for drug delivery

Despite considerable progress in recent years, the diagnosis and treatment of various diseases, especially cancer, continue to present constraints, such as low sensitivity or specificity, drug toxicity, and severe side effects. In particular, many therapeutic agents have a very narrow window, ie, the therapeutic dose is not much lower than a toxic one. In many cases, the employment of an appropriate drug carrier can reduce the toxicity by changing the temporal and spatial distribution of a drug.

Since they were first described in the 1960s, liposomes have long been recognized as drug-delivery vehicles. They are very appropriate for this aim, due to their biocompatibility and biodegradability.[9] Due to their nature, liposomes are in fact considered safe nanocarriers. However, the addition of nonphysiological additives can induce chemical modifications that are useful to improve efficacy in drug delivery but potentially toxigenic.

In addition to being biocompatible, all liposomes have in common a structure that gives them the ability to contain both hydrophilic and hydrophobic drugs. The encapsulation of the active form of a drug into the lipid bilayer protects it against naturally occurring phenomena, such as enzymatic degradation and immunologic and chemical inactivation. Therefore, liposomes prevent a drug from being metabolized prior to reaching target tissues, and simultaneously they minimize exposure of healthy tissue to the encapsulated drug during its circulation in the blood. Both of these effects contribute to increase the therapeutic index. In fact, high levels of the active form of a drug are delivered to the tumor site so that the expected cytotoxic effect can be achieved. Meanwhile, any undesirable side effects of the encapsulated drug are substantially reduced when compared to the free form.

The delivery of the encapsulated drug depends on the nature of the lipid bilayer, the size of the drug molecules, their partition coefficient in oil/water, and their interactions with the lipid membrane. The mechanism and extent of liposome-delivery is also strongly influenced by the nature and density of the charge (ζ-potential) of the liposome surface (refer to the "Charged liposomes for drug delivery" section for more details).

The encapsulation efficiency of a molecule in a liposome depends on its polarity and partition coefficient, which also determines its localization in the liposomal membrane. If a drug is hydrophobic in nature, it resides in the acyl hydrocarbon chain of the liposome, and hence encapsulation is dependent on the properties of the acyl chains of the liposome, such as length and packing density. It is also expected that the encapsulation efficiency of hydrophobic molecules is influenced by changes in the drug-to-lipid ratio. On the other hand, if a drug is polar/hydrophilic, it tends to localize in the aqueous core or adjacent to the water–lipid interface, near the polar head groups of the liposome. Therefore, its encapsulation efficiency does not exhibit a strong dependence on the drug-to-lipid ratio. On the contrary, the introduction of hydrophilic chains in the liposome surface favors greater entrapment of hydrophilic molecules when compared with hydrophobic molecules.[51]

Drugs loaded in liposomes are not bioavailable; they only become bioavailable when they are released. Therefore, optimizing the release rate of a liposome-vehicle drug is strategic to reach a level within its therapeutic window and at a sufficient rate for a sufficient period to have optimal therapeutic activity. On the other hand, premature drug release should be avoided. To overcome this inconvenience, several experimental approaches have been pursued, either by modifying the lipid bilayer or entrapping drugs suitable for the purpose.

Switching from a fluid-phase phospholipid bilayer to a solid-phase bilayer, eg, by incorporating cholesterol (bilayer-tightening effect) or sphingomyelin into liposomes, the retaining of cargo into liposomes is increased.[52–54] Another approach to control the release rate of entrapped substances is to choose drugs with physical characteristics favoring retention in the lipid nanovector. Similarly to biological membranes, liposomes have high permeability to hydrophobic drugs and low permeability to hydrophilic drugs. Anticancer drugs of high hydrophilicity retained in the aqueous internal compartment of liposomes (albeit with low trapping efficiency) are slowly released from the liposomes over several hours to several days.[55,56] Drugs of high hydrophobicity

efficiently inserted into the fatty acyl chain region of the lipid bilayer can be easily released. Retention of highly hydrophobic drugs, such as paclitaxel, in liposomes is problematic, and many formulations and their pharmacokinetics have been studied to increase drug–liposome associations.[57]

Many anticancer drugs are of intermediate solubility and readily partition between the liposome bilayer and the exterior or interior aqueous phase, resulting in their rapid release from liposomes. However, manipulation of the interior pH of the liposomes or the formation of molecular complexes within the liposomes can result in excellent retention of weak bases, such as doxorubicin (Dox) or daunorubicin, in liposomes.[58,59] Drug retention can be improved by loading drugs to achieve high intraliposomal drug concentrations above their solubility limits, thus enhancing precipitation, or by encapsulating polyanions, such as dextran sulfate.[60,61] Drugs that are not weak bases, such as docetaxel, can be converted to weak-base prodrugs, thus allowing encapsulation and liposomal retention.[62]

Liposomes should store, protect, and transfer substantial quantities of drugs while being well tolerated in patients receiving the cure. These unique characteristics could provide for an improved biopharmaceutical profile through reduced toxicity, favorable pharmacokinetic behavior, and an enhanced therapeutic index in comparison to the free-form drug. Liposomes as drug-delivery systems could show several advantages over conventional dosage forms, particularly for parenteral (ie, local or systemic injection or infusion), topical, and pulmonary routes of administration.

Several clinical studies have demonstrated that liposomal encapsulation of drugs typically leads to a change in toxicity profiles. When used in clinical settings, liposomal treatments proved to improve patient outcome dramatically, reducing some of the side effects associated with chemotherapy, such as cardiotoxicity, nausea, and vomiting, when compared to unencapsulated drugs.[63,64] Therapeutic activity of vincristine, widely used in the treatment of a number of human carcinomas, significantly increased after its encapsulation in appropriately designed liposomal systems. In fact, vincristine sulfate-liposome injection improved the therapeutic index by facilitating increased dose intensification, while maintaining a predictable and manageable safety profile. This effect is a consequence of a lower clearance and a higher area under the curve compared with conventional free vincristine sulfate.[65] In the field of antimicrobial agents, liposomal amphotericin B showed better tolerance and higher efficacy than the antibiotic amphotericin B deoxycholate. At present, liposomal amphotericin B is the drug of choice for the treatment of patients with disseminated histoplasmosis and acquired immunodeficiency syndrome (AIDS).[66] Nystatin entrapped in pH-sensitive liposomes enhanced anticryptococcal efficacy in a murine model.[67]

Antitumoral anthracyclines, such as Dox, daunorubicin, and epirubicin, achieve highly efficient encapsulation. Liposomal anthracyclines proved to be effective and showed reduced cardiotoxicity when compared to free agents, either as a single agent or in combination with other drugs.[68,69] A meta-analysis study compared the safety and toxicity of liposomal Dox versus conventional anthracyclines. Both liposomal Dox and PEGylated liposomal Dox (PLD) showed favorable toxicity profiles, with better cardiac safety and less myelosuppression, alopecia, nausea, and vomiting compared with conventional anthracyclines, making them a favorable choice over conventional anthracyclines in elderly patients, patients with risk factors for cardiac disease, and patients with prior use of anthracyclines.[70]

Among the pharmaceutical options available for treatment of ovarian cancer, increasing attention has been progressively focused on PLD, whose unique formulation prolongs the persistence of the drug in the circulation and potentiates intratumor accumulation. PLD has become a major component in the routine management of epithelial ovarian cancer (extensively reviewed in Pisano et al).[71] Nonrandomized Phase II trials of PLD in platinum-resistant ovarian cancer patients documented the biological activity of this agent in this clinical setting, with objective response rates of approximately 10%–20% being reported in several trials.[72–74] Data indicated that palmar–plantar erythrodysesthesia (hand–foot syndrome), toxic acral erythema, and mucositis were the most common toxicities of PLD, reported in up to 50% of treated patients. Although not life-threatening, palmar–plantar erythrodysesthesia can negatively impact quality of life, and it is a major cause of both dose reduction and treatment discontinuation.[75,76] As regards cardiac toxicity, in several trials PLD formulation has been related to a better safety profile compared to conventional Dox.[77] Compared to the 7.5% incidence of irreversible cardiotoxicity at cumulative doses of 400–550 mg/m^2 reported with Dox, most of the studies of PLD showed a lower incidence of cardiac failure even at doses higher than 500 mg/m^2.[78–80] In a prospective trial performed on patients with advanced gynecological malignancies treated with PLD, cardiac safety was further assessed at histology (endomyocardial biopsies), showing no myocardial damage in patients treated with PLD (median PLD dose of 708 mg/m^2).[81] Therefore, the optimal cardiac safety profile of PLD may allow prolonged treatment. Encouraging results

from a Phase II trial in AIDS-related Kaposi's sarcoma patients treated with PLD up to a 2,360 mg/m^{2} cumulative dose have been reported.[82] In metastatic breast cancer patients also, doses greater than 450 mg/m^{2} were not associated with a significant decrease in left ventricular ejection fraction from baseline compared to conventional Dox.[80] In a relapsed ovarian cancer patient responding to second-line chemotherapy, maintenance therapy with PLD for more than 1 year was reported to be safe by Andreopoulou et al with no cardiac events reported.[83]

Effect of size on liposome fate

Pathological tissues, such as inflammatory or solid tumor tissues, are characterized by increased vascular permeability.[84] The enhanced permeation and retention (EPR) effect is the phenomenon characterizing malignant tissues by which nanocarriers of an appropriate size can pass through tumor-vessel walls and enter the neoplastic lesion (Figure 2). More particularly, solid tumors that undergo angiogenesis develop a discontinuous endothelium, with large fenestrations allowing molecules of up to approximately 4,000 kDa, or 500 nm, to enter the interstitial space.[85,86] Liposomes can satisfy the size conditions needed to pass through tumor vessels and concentrate in the target site. This mechanism represents the major targeting principle for intravenously administered long-circulating liposomes. However, because no specific targeting ligands are used to interact with the tumor target site, this tumor-localization process is referred to as "passive targeting."

This passive targeting depends on the mechanical and physical properties of liposomes. More importantly, once liposomes have entered the tumoral tissue, they are retained from the malfunctioning lymphatic system. Therefore, after its release, the drug encapsulated into liposomal carriers can exert its therapeutic effect.[9]

The overall size of the liposome-based drugs is an essential physical aspect that determines the clinical successes of the nanocarriers. Experimenting with variations in liposomal size, researchers observed that liposomes smaller than 100 nm in diameter interacted less with plasma proteins, evaded capture by the RES, had a longer half-life in the blood, and accumulated passively at the tumoral site.[5] Conversely, it was found that larger liposomes were eliminated more rapidly from blood circulation and did not escape RES uptake. On the other hand, it was observed that small liposomes had reduced drug-storage capacities.[17]

Even if the size of these drug-delivery systems can be easily modulated, theoretically an ideal liposome designed for the delivery of chemotherapeutics should be of 50–100 nm in diameter. The lower size limit (50 nm) should prevent intravenous-based nanocarriers from randomly penetrating normal vessel walls while in circulation.[87] However, an upper size limit to these systems could also exist. In order to gain access to tumor tissue, nanocarriers should retain the ability to extravasate from vessels through the large vascular fenestrations (250 nm or larger) that are present in and around tumor sites and are attributed to ongoing angiogenesis.[84,87,88] At the tissue level, many nanomedicine products attempt to target sites passively through the EPR effect, with feature sizes typically in the 100–200 nm range, but particles up to 400 nm have demonstrated extravasation and accumulation in tumors (although this is an extreme case). However, when size is increased, capture by the RES also increases.[68,89] For example, a previous study reported that PEGylated liposomes 250 nm in diameter were removed from circulation more than twice as fast as liposomes 100 nm in diameter with similar lipid composition.[90] This is particularly problematic, since it is imperative that liposomes loaded, eg, with an antitumoral agent, will remain in circulation until they accumulate within tumor tissue and release the active molecule at a concentration sufficient to exert a therapeutic effect.

Pharmacokinetics of liposomes

The pharmacokinetics of liposomes focuses on their distribution throughout the body fluids and tissues and their metabolism. The latter mainly includes liposome chemical degradation and excretion, which is achieved through their uptake and clearance by the RES.

As mentioned previously, one important goal of the design of a new liposomal carrier is the modulation of the pharmacokinetic profiles of a drug. The advantages of liposomal-based drugs should be greater solubility of the cargo, increased half-life, selective delivery to the site of action, improved therapeutic index, and the ability to overcome resistance against chemotherapeutics. When a therapeutic agent is loaded into liposomes, it adopts the carrier's pharmacokinetics until it is delivered. As a result, liposomes modify both the tissue distribution and the rate of clearance of the loaded drug.[91] The pharmacokinetics of liposomal-based drugs depends on the physicochemical characteristics of the lipid vehicle, such as lipid composition, size, membrane lipid packing, steric stabilization, surface charge, dose, and route of administration. It has been reported that the primary sites of accumulation of carrier-mediated agents are the tumor, liver, and spleen, compared with noncarrier formulations. Factors affecting the pharmacokinetic and pharmacodynamic
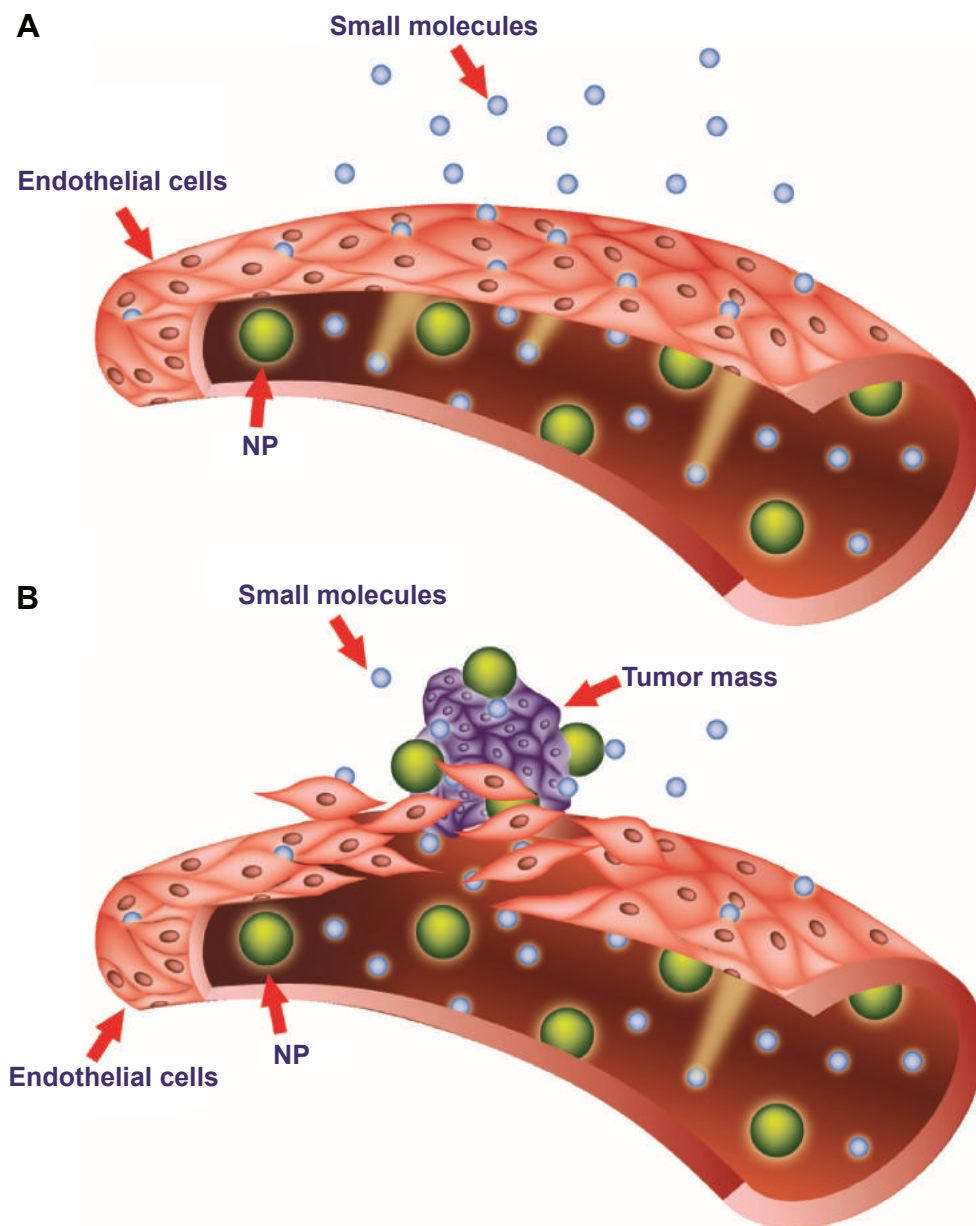
Figure 2 Targeting of nanomedicines by the enhanced permeability and retention (EPR) effect.
Notes: Differences between normal (**A**) and tumor (**B**) vessels are depicted. Tumor vessels contain large fenestrations between the endothelial cells: this structural characteristic allows the nanoparticles (NPs) to reach the matrix and the tumor cells by the EPR effect. Conversely, normal tissue contains tightly joined endothelial cells: this prevents the diffusion of NPs outside the blood vessels.

variability of these agents remain unclear, but most likely include the RES.[63]

Once inside the organism, liposomes' stability in the bloodstream, as well as their capacity to enter target tissues, determines the fate of liposomes. During their circulation in the blood, liposomes meet plasma proteins, such as opsonins and HDLs and LDLs. Opsonins include various protein types, like immunoglobulins and fibronectin, which help RES recognize and eliminate liposomes. Blood carrying HDL and LDL interacts with liposomes and reduces their stability.

The interaction with lipoproteins causes lipid transfers and rearrangements on the surface of liposomes. This frequently induces lipid depletion, liposome breakdown, and rapid release of the cargo to the plasma.[5] By modulation of lipid composition, this effect can be avoided (see later in this paper for more details).

After interaction with target cells, the delivery of the encapsulated drug depends on the nature of the lipid bilayer, the size of the drug molecules, their partition coefficient in oil/water, and their interactions with the lipid membrane.

The final fate of the drug, ie, the extracellular fluid or the cytoplasm of the target cell, depends on the molecular architecture, mechanism of release, and the composition of the carrier.[63]

Elimination of liposomes takes place in different ways. The first involves absorption of plasma proteins on the surface of liposomes and then their recognition by the RES. This event results in the excretion of the cargo at the hepatic level and its subsequent metabolism by Kupffer cells. In the second way, liposomes are metabolized by splenic macrophages. Finally, after their accumulation, they are metabolized and eliminated by the target tissues.[5,92,93]

Despite all the hopes invested in conventional liposomes, they have presented various problems and pharmacological implications over the years. A major drawback of conventional liposomes is their quick capture by the RES.[94] Liposomes are mainly accumulated in the liver and the spleen, due to their generous blood irroration and the abundance of tissue-resident phagocytic cells.[63,92,93] This is extremely advantageous in the case of local infections: the high concentration of antimicrobial agents in the RES can help treat infective pathogens. However, during chemotherapy, it may lead to partial depletion of the macrophages and interfere with the important host-defense functions of this cell type.[95] On the other hand, the marked increase in retention and accumulation of liposomal drugs in such organs as the spleen and the liver may lead to the delayed removal of lipophilic anticancer drugs from the circulation.[96]

A number of different strategies were then tested in the following years in order to overcome the aforementioned limitations, giving rise to a "second generation" of liposomes. The best strategy was described in the early 1990s, when experiments carried out by several groups of scientists demonstrated that PEGylation of the liposome surface was able to improve the stability and circulation time of liposomes dramatically after intravenous administration, by rendering the liposomes invisible to macrophages.[50] These "long-circulating liposomes" were then named Stealth liposomes because of their ability to evade the immune system; this results in a significant increase in blood-circulation time in vivo.[97–99]

PEG is a linear or branched polyether diol with many useful properties, such as biocompatibility, solubility in aqueous and organic media, lack of toxicity, very low immunogenicity and antigenicity, and good excretion kinetics. These properties permit the employment of PEG in a variety of applications, including the biomedical field, after US Food and Drug Administration (FDA) approval for internal administration (http://www.accessdata.fda.gov/scripts/cder/drugsatfda).

PEG chains protect liposomes from mononuclear phagocytic system cells by building a protective, hydrophilic film on the liposomal surface. Their presence prevents the interaction of liposomes with other molecules, such as various serum components. One possible explanation for the impaired interaction is the PEG-induced "steric hindrance." The mechanism of steric hindrance by the PEG-modified surface has been thoroughly examined.[100] The water molecules form a structured shell through hydrogen bonding to the ether oxygen molecules of PEG. The tightly bound water forms a hydrated film around the particle and repels the protein interactions.[101] In addition, the presence of PEG on the surface may also increase the hydrodynamic size of the particle, decreasing its clearance, a process that is dependent on molecular size as well as particle volume.[102]

PEG-bearing liposomes are not opsonized or affected by complement components, and consequently evade capture by mononuclear phagocytic system cells.[103,104] Finally, the presence of PEG in liposome formulations prevents aggregation, favors the formation of small, monodisperse particles, and increases the EPR effect, due to the extended circulation time and escape from the RES.[105]

The practical consequences of these phenomena are evident when the biopharmaceutical profiles of Stealth liposomes and conventional liposomes are contemplated. Stealth liposomes have a longer half-life (which leads to longer blood-circulation times), low systemic plasma clearance, and low volume of distribution (minimal interaction with nondiseased tissue). This results in multiple-fold greater area-under-the-curve values (drug concentration–time profile) and improved tissue distribution (targeting of target sites).[90]

The advantage of PEGylation is credible when the relative half-lives of non-PEGylated and PEGylated liposomes are compared. In fact, the half-life of liposomes after PEGylation increases from a few hours to 45 hours.[106] Therefore, it is not surprising that the clinically approved antitumoral drug Doxil® is PEGylated in order to improve tumor-site accumulation of the drug.[107] However, while PEG coating increases liposome-circulation times, it could also negatively influence the uptake by target cells due to PEG-induced steric hindrance.[97]

Although clinically useful activity has been demonstrated, currently available liposome-based therapies do not exhibit active targeting at the cellular level. Existing FDA-approved liposome technologies against cancer rely on passive accumulation through the EPR effect.[108] In other words, these nanomedicines possess no functionality to actuate release other than passive efflux from the liposome

at the tumor site. However, this uncontrolled, passive release in some cases results in suboptimal pharmacokinetics or reduced efficacy, as observed with cisplatin-loaded liposomes.[109–111] An additional level of sophistication and specificity for the target cell can be achieved through ligand-mediated targeting, which is defined as active targeting. The goal is to develop platforms with improved biodistribution, pharmacokinetic properties, and active targeting. The properties of such targeted liposomes can be modulated and adapted to different needs. Peptides, carbohydrates, glycoproteins, receptor ligands, monoclonal antibodies, and growth factors have been applied as ligands. Ligand-targeted liposomes can selectively recognize the antigens or the receptors located on the surface of target cells. Due to this high selectivity toward cancer cells, almost all of the administered liposomal drug would accumulate at the tumor site, leaving uninjured healthy bystander cells. That way, the required dose for the expected cytotoxic effect will be significantly smaller when compared to nontargeted therapies: this contributes to a better therapeutic index, with higher drug efficacy and fewer side effects.[112–115]

As extensively reviewed in Noble et al[116] ligand-targeted liposomes have demonstrated improved efficacy over passively targeted equivalents through enhanced targeting and intracellular uptake, but they have raised new challenges, such as hindered diffusion and penetration through the target tissue, immune recognition, and deactivation of targeting through the nonspecific binding of serum proteins. As a result, ligand-targeted liposome systems have not demonstrated consistently successful outcomes in preclinical settings, and other studies are necessary to address issues related to their efficiency.[116]

Charged liposomes

When a liposome interacts with a cell, the delivery of the drug and its distribution in the target cell can occur in several ways. Liposomes can adsorb into the membrane of cells, where the lipid bilayer of the carrier is degraded by enzymes, such as lipases, or by mechanical strain. This leads to the release of the active ingredients into the extracellular fluid, where they can diffuse through the cell membrane and cytoplasm. However, the latter process cannot easily occur when the loaded molecules are hydrophilic. A second way requires the fusion of the liposomal membrane with the plasma membrane of the target cell: this phenomenon causes the release of liposomal content directly into the cytoplasm. The third and most frequent way is receptor-mediated endocytosis. This process only regards vesicles of a maximum

diameter of 150 nm and active ingredients that can endure the acidic environment of lysosomes, where liposomes are enzymatically processed. Phagocytosis can also occur, but involves liposomes of a diameter larger than 150 nm. These large liposomes are phagocytosed by specialized cells of the immune system, such as macrophages, monocytes, and Kupffer cells (Figure 3).[5,84,117]

The mechanism and extent of liposome–cell interaction is strongly influenced by the nature and density of the charge of the liposomes surface. By changing the lipid composition, both of these parameters can be modified. The liposomes can include charged components that confer them an overall neutral, positive, or negative charge. Lack of surface charge (neutral liposomes) increases the aggregation of liposomes, reducing their physical stability. Moreover, neutral liposomes do not interact significantly with cells, and this causes drug release from the liposomes in the extracellular space.[118,119] On the other hand, charged liposomes present numerous advantages compared with neutral liposomes. For example, the presence of a charge on the surface induces electrostatic repulsion among liposomes by creating a ζ-potential, positive or negative, that
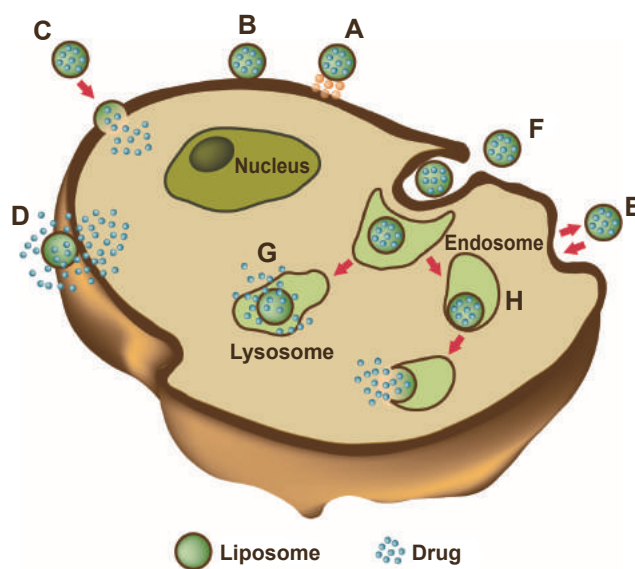

Figure 3 Liposome–cell interaction.
Notes: Liposomes loaded with a drug interact with the cell, binding to the surface through receptors (**A**). Absorption onto the plasma membrane can also occur by electrostatic interactions (**B**). The delivery of the cargo into the cell cytoplasm can take place through different modes. Lipid nanocarriers fuse with the plasma membrane and discharge drugs into the cell (**C**). After the interaction with the cell, the structure of the liposome bilayer can be affected and the cargo is released (**D**). Exchange of carrier-lipid components with the cell membrane can also occur (**E**). Liposomes internalized by endocytosis (**F**) can have different fates depending on physicochemical characteristics. Endosomes fuse with lysosomes (**G**): in this case, the low pH induces the degradation of the liposome membrane and the drug is released. Endosomes follow another route (**H**): liposomes release their cargo after fusion or the destabilization of the endocytic vesicle.

prevents their aggregation and flocculation. Moreover, a high electrostatic surface charge could promote the interaction of liposomes with cells.

The literature includes several studies carried out on the potential use of charged liposomes for biomedical applications. However, the great majority of these studies focused on positively charged liposomes, due to the encouraging results obtained in in vitro and in vivo experimentations.

Negatively charged liposomes are generally constituted by anionic lipids, such as dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol. Literature data show that the negative liposomes, due to their electrostatic properties, are less stable than neutral and positive liposomes when injected into the blood circulation. In fact, anionic liposomes rapidly interact with the biological system subsequently to their opsonization with complement and other circulating proteins.[120–122] Such an interaction has at least two acute consequences: a rapid uptake by the RES, and toxic effects, such as pseudoallergy that is manifested as vasoconstriction, pulmonary hypertension, dyspnea, and drop in circulating platelets and leukocytes.[123] For this reason, anionic liposomes have not been widely used as drug-delivery systems for intravenous administration. Moreover, several studies examining the effect of negative and positive charge on the adjuvant activities of liposomes have been carried out, but the results obtained were rather conflicting and inconclusive.[124–127] However, in recent years, there has been increased interest in developing charged liposomes as carriers for transdermal drug delivery, due to their enhanced penetration properties through the skin.[128] To clarify the effect of the surface charge on percutaneous absorption, histological studies revealed that the negatively charged liposomes diffused to the dermis and the lower portion of hair follicles through the stratum corneum and the follicles much more quickly than the positive vesicles. Therefore, the rapid penetration of negatively charged liposomes would contribute to increased permeation of drugs through the skin.[129]

Cationic liposomes (CLPs), first described in 1987 by Felgner et al are typically used for gene delivery, based on the electrostatics between positively charged lipids and negatively charged nucleic acids.[130] They consist of natural neutral phospholipids and positively charged lipids. Commonly used neutral phospholipids include dioleoyl phosphatidylethanolamine (DOPE) or dioleoyl phosphatidylcholine, and are in most cases required for the stabilization of the liposome/DNA complex. A variety of lipid formulations with a positive charge are already on the market, such as DC-cholesterol HCl, (3β-[N-(N′,N′-dimethylaminoethane)-carbamyl]

cholesterol hydrochloride), DOTAP (1,2-dioleoyl-3-trimethylammonium-propane [chloride salt]), DOBAQ (N-[4-carboxybenzyl]-N,N-dimethyl-2,3-bis(oleoyloxy) propan-1-aminium), DDAB (dimethyldioctadecylammonium [bromide salt]), and MLV5 (N1-[2-((1S)-1-[(3-aminopropyl) amino]-4-[di(3-amino-propyl)amino]butylcarboxamido) ethyl]-3,4-di[oleyloxy]-benzamide), and many others are under development.

In the case of CLP–cell interaction, the endocytic pathway seems to be the preferential route of internalization. The pathway of internalization is of the utmost importance in the final fate of the drug loaded in the lipid carriers, as, eg, in the case of drugs sensitive to an acidic environment. Both the interaction with the cell membrane and the pathway of internalization are strictly dependent on the physicochemistry of liposomes. Morphological and ultrastructural studies are invaluable tools to study the mechanisms of liposome–cell interaction and analyze at nanoresolution the temporal and spatial parameters involved. As an example, morphological and ultrastructural studies by the freeze-fracturing technique on the interaction of CLPs with tumor cells are reported in Figures 4 and 5. In these experiments, CLPs formulated as described in Bombelli et al were used.[131] Freeze-fracturing is an election technique used to study plasma-membrane architecture and its modifications occurring upon the interaction with xenobiotics, microbial agents, nanomaterials, and so on. As shown by the replica

Figure 4 Replica of a cryofixed and cross-fractured human glioblastoma cell, interacting with dimyristoyl-sn-glycero-phosphatidylcholine liposomes. Liposomes appear mainly clustered on a pole of the cell (arrows). The fracture passes through the cytoplasm, and reveals the nucleus (asterisk) and the cytoplasmic organelles (arrowhead).
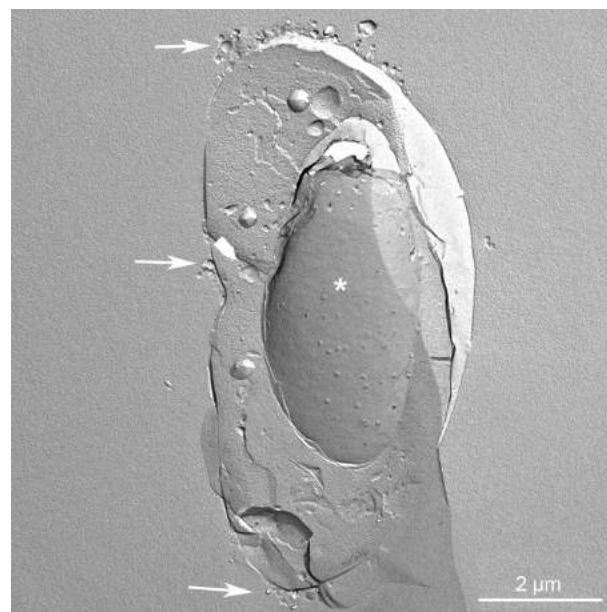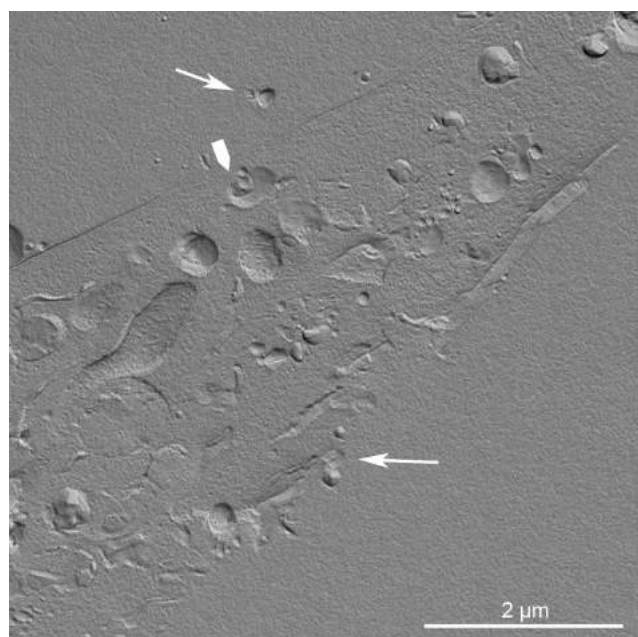
Figure 5 Replica of a cryofixed and cross-fractured human glioblastoma cell, interacting with dimyristoyl-*sn*-glycero-phosphatidylcholine liposomes. Liposomes (arrows) appear in the extracellular space and the cytoplasm of the cell. Some of them are interacting with cytoplasmic organelles (arrowhead).

performed on cryofixed and cross-fractured samples, before being internalized, CLPs appear clustered on the plasma membrane of the glioblastoma cell. The capping phenomenon strongly suggests the involvement of an endocytic pathway (Figure 4). The replica of cross-fractured tumor cells allows the visualization of liposomes interacting with cytoplasmic organelles, very likely endosomes (Figure 5).

Since the overall positive charge of CLPs enhances the transfection of anionic animal target cells, liposome/DNA complexes are commonly used for delivering genes. However, preclinical and clinical results encourage the use of CLPs for the delivery of antitumoral agents to the tumor vasculature. In fact, it has been demonstrated that the presence of anionic sites in angiogenic endothelial cells (such as anionic phospholipids, proteoglycans, hyperglycosylated and hypersialylated membrane proteins, and so on) makes newly formed tumor vessels selective targeting sites for CLPs. It has been reported that such drugs as paclitaxel, Dox, and oxaliplatin carried by CLPs showed enhanced antitumor efficacy associated with a reduced functionality of the tumor microvasculature.[132,133] Campbell et al demonstrated that the presence of a positive charge on liposomes is needed to improve their interactions with the glycoproteins of the endothelial cell membranes.[134] These data support the use of CLPs to target cytotoxic drugs preferentially to the tumor vascular endothelium, and to achieve long circulation half-lives.[135]

An important goal of CLPs is to cross the blood–brain barrier (BBB) and achieve brain drug concentration. The BBB is composed of specific structures formed by brain-capillary endothelial cells and basement membrane, sheathed with astrocytic end-feet. BBB impedes drug penetration into the central nervous system, and for this reason many brain drug-delivery strategies have focused on crossing it. The mechanism by which CLPs have been shown to cross the BBB easily is either absorptive-mediated transcytosis or receptor-mediated transcytosis. Both mechanisms are triggered by the electrostatic interaction between liposome cationic components and membrane anionic microdomains of the brain's capillary endothelial cells.[136]

Even if an increased amount of cationic lipids in liposomes may enhance affinity for angiogenic endothelial cells and the subsequent anticancer effect, some points need to be considered. The increased positive charge on the unshielded CLP surface could result in their aggregation in the bloodstream through electrostatic interactions with anionic species in the blood, in an enhanced uptake by the RES, and in reduced accumulation in the tumor.[122,133,137–141] These effects negatively influence the fate and therapeutic efficacy of CLPs in vivo.

Even in the case of CLPs, PEG protects from circulating proteins, improving their plasma clearance and enhancing their anticancer effects. PEG-modified CLPs have also been shown to improve oligonucleotide loading and delivery and the solubility of various therapeutic agents.[142,143]

The use of CLPs may improve the efficacy and intrinsic safety of photodynamic therapy, a protocol that involves the administration of a photosensitizer and its irradiation in light of the appropriate wavelength (near infrared radiation) to excite and activate it and to induce the formation of cytotoxic species. In fact, liposomes can efficiently solubilize hydrophobic photosensitizers, thus increasing the photoactive population. Further, they can improve the accumulation in tumors and the pharmacokinetics of the photosensitizer, thus reducing the side effect of skin photosensitization due to poor target specificity.[144] In our studies, CLPs formulated with dimyristoyl-*sn*-glycero-phosphatidylcholine (DMPC) and the cationic gemini surfactant (*S,S*)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N,N*-dimethylammonio)butane bromide 1a (DMPC/1a) was developed to deliver the photosensitizer *m*-tetrahydroxyphenylchlorin (*m*-THPC) to human colon adenocarcinoma and glioblastoma cells.[131,145] The presence of gemini surfactant strongly influenced the interaction of liposomes with the cell membrane and the delivery efficacy of CLPs. It significantly increased the cell uptake of *m*-THPC

and the cytotoxic effect of the photosensitizer after the irradiation, when compared with the related pharmaceutical formulation Foscan.[145] A subsequent study demonstrated that the stereochemistry of the gemini spacer strongly influenced the physicochemistry of liposomal formulations and the final intracellular fate of the loaded drug.[146]

Stimuli-responsive liposomes

Conventional and long-circulating liposomes may present a slow release of the loaded drug or may be unable to fuse with the endosome after internalization. To overcome these problems, stimuli-responsive liposomes have been developed. Lipids in these liposomes generally include a triggerable component that is responsible for gating the stability and/or permeability of the lipid bilayer. Stimuli-responsive liposomes are capable of reacting when triggered by stimuli from target tissues (pH, redox potential) or applied from outside the organism (hyperthermia, ultrasound, and [electro]magnetic field).[147,148] pH-sensitive, redox potential-sensitive, temperature-sensitive, magnetic field-sensitive, and ultrasound-sensitive liposomes have been produced.

The development of pH-sensitive liposomes was planned after the consideration that some pathological tissues, including tumors or areas of inflammation and infection, display an acidic environment compared with normal tissues.[149] A pH-sensitive liposome is generally stable at physiological pH, but can be subjected to destabilization and acquire fusogenic properties under acid conditions, thus leading to the release of its aqueous contents.[150] To achieve the pH-sensitive release of liposome content, liposomes are formulated with pH-sensitive components. After being endocytosed in an intact form, these fuse with the endovacuolar membrane as a result of the lower pH inside the endosome, and release their contents into the cytoplasm. Long-circulating PEGylated pH-sensitive liposomes, despite having decreased pH sensitivity, still effectively deliver their contents into the cytoplasm.[151]

Redox potential-sensitive liposomes take advantage of the high redox-potential difference that exists between the reducing intracellular space and the oxidizing extracellular space. Redox potential-sensitive liposomes release their content inside cells when disulfide bonds present in lipids or other components are reduced by glutathione to thiol groups. Consequently, the integrity of the liposome structure, which is maintained under normal conditions by disulfide bonds, is compromised, and the entrapped cargo can be released. Endogenous triggering of liposomal payload release by overexpressed enzyme activity in affected tissues offers the possibility of active and site-specific release. Redox-triggered

content release from liposomes was reported when applying liposomes made from quinone-DOPE (Q-DOPE) lipids. Complete payload release occurs upon their redox activation when the quinone head group possesses a "trimethyl-locked" quinone redox switch, attached to the N-terminus of DOPE lipids, that undergoes a cleavage event upon two-electron reduction.[152] The authors expect that Q-DOPE liposomes and their variants will be important in treating diseases with associated tissues that overexpress quinone reductases, such as cancers and inflammatory diseases, because the quinone redox switch is a known substrate for this group of reductase.[152]

Thermosensitive liposomes have been widely investigated since 1978.[153] In the last decade, there has been increased interest in delivery mediated by temperature-sensitive liposomes, in part due to advances in image-guided hyperthermia applicators. Temperature-sensitive liposomes in combination with heating the target region can selectively enhance the bioavailability of the drug locally while minimizing systemic exposure. Temperature-sensitive liposomes promptly discharge their cargo upon heating (within seconds to minutes), while at body temperature the payload is somewhat stably encapsulated.[154–156] Temperature-sensitive liposomes release their encapsulated drugs at the melting-phase transition temperature (T_m) of the lipid bilayer. At this T_m, the lipid membrane changes its permeability because of the transition from the gel to the liquid crystalline phase.[157,158] Temperature-sensitive liposomes frequently include dipalmitoylphosphatidylcholine as the key component, because the gel-to-liquid crystalline phase transition occurs for these lipids at 41°C, as extensively reviewed in Kono.[159]

Temperature-sensitive liposomes have been successfully applied in both preclinical and clinical studies in combination with heat-based thermal therapies, including radiofrequency ablation, ultrasound hyperthermia, and microwave hyperthermia.[160–163]

A formulation based on these thermosensitive liposomes took the brand name ThermoDox® and was further developed by Celsion Corporation. ThermoDox liposomes can be triggered to release their payload by any heat-based treatment, such as radiofrequency thermal ablation, microwave hyperthermia, and high-intensity-focused ultrasound.[157]

A complete regression of local cancer using temperature-sensitive liposomes combined with ultrasound-mediated hyperthermia was recently reported by Kheirolomoom et al.[164] These authors employed temperature-sensitive liposomes containing lysolipid, loaded with a pH-sensitive complex formed by Dox and copper, ie, CuDox. The complex remains associated at neutral pH, but dissociates to give free Dox

in lower-pH environments. The resulting liposomes were injected intravenously into a syngeneic murine breast cancer model. Successively, the intravascular release of the drug was triggered by ultrasound. The entire tumor was insonified for 5 minutes prior to drug administration and 20 minutes after drug injection. A single-dose administration of CuDox lysolipid-containing temperature-sensitive liposomes (LTSLs) combined with insonation suppressed tumor growth. Moreover, after twice-weekly treatment over a period of 28 days, a complete response was achieved in which the NDL tumor cells and the tumor interstitium could no longer be detected. All mice treated with ultrasound combined with CuDox LTSLs survived, and the tumor was undetectable at 8 months posttreatment. Iron- and copper-laden macrophages were observed at early time points following treatment with this temperature-sensitive formulation. Systemic toxicity indicators, such as cardiac hypertrophy, leukopenia, and weight and hair loss, were not detected with CuDox LTSLs after the 28-day therapy.[164]

Liposomes in theranostics

Nanotechnology gives the opportunity to assemble therapeutic and diagnostic agents as a single theranostic platform, ie, a molecular platform that simultaneously integrates diagnosis and therapy.[165–168] The main goal is to diagnose and treat the diseases at their earliest stage. A theranostic platform is multifunctional in nature, able to detect and specifically deliver therapeutic agents to the diseased cells with the help of targeting ligands and biomarkers.[168–171]

Liposomes are a valid platform for theranostic nanomedicine, owing to their size, hydrophobic and hydrophilic character, biocompatibility, biodegradability, low toxicity, and immunogenicity. In 2007, the concept of liposome–nanoparticle hybrids was presented by Al-Jamal and Kostarelos as a general methodology to be used as a platform for the delivery of novel nanoparticles. Such hybrid constructs present great opportunities to engineer theranostic nanoscale delivery systems.[172] Liposome–nanoparticle hybrids can be designed by embedding, encapsulation, or conjugation of nanoparticles onto various types of liposomes. The theranostic potential of such hybrids is illustrated in Al-Jamal and Kostarelos.[173] The authors described, in particular, Dox-loaded, lipid bilayer-embedded quantum-dot vesicle hybrids capable of chemotherapy (cytotoxic activity of Dox) and optical imaging (embedded quantum dots).

For imaging purposes, nanosize diagnostic agents can be entrapped within the theranostic liposomes, and the therapeutic agent can be either encapsulated in the core or embedded in the lipophilic bilayer shell.[173–178] For example,

for magnetic resonance imaging, superparamagnetic iron oxides can either be coated with a lipid layer (small magnetoliposomes [MLs]) or several superparamagnetic iron oxides or gadolinium(III) chelates can be entrapped into the aqueous core of liposomes (large MLs). These large MLs provide additional cargo space for drug encapsulation, into either the core or the lipid membrane, rendering MLs theranostic agents.[179,180] Multimodal imaging properties have been obtained by loading quantum dots or fluorescent dyes, such as calcein, into the lipid membrane of liposomes. For example, recently, Li et al constructed a multifunctional liposome containing gadolinium-DOTA lipids for magnetic resonance imaging, a lipidized near-infrared dye for near-infrared fluorescence imaging, Dox loading for therapeutic activity, and radiolabeling with ^{99m}Tc and ^{64}Cu for single-photon emission computed tomography and positron-emission tomography imaging. These liposomes were applied in vivo to a squamous cell carcinoma of head and neck tumor xenograft in nude rats after intratumoral injection.[181]

Muthu et al prepared tocopheryl polyethylene glycol 1000 succinate-coated theranostic liposomes containing Dox and quantum dots with and without targeting moieties.[182] Folic acid was used as the targeting probe to target folate receptors overexpressing MCF-7 breast cancer cell lines. Along similar lines, Wen et al developed quantum dots and apomorphine-incorporated theranostic liposomes to eliminate uptake by the liver and to enhance brain targeting.[183] In their most recent work, Wen et al prepared theranostic liposomes loaded with quantum dots, camptothecin, and irinotecan for simultaneous bioimaging and drug delivery.[184]

Smith et al used heat-sensitive liposomes with modified HER2 affisomes (HER2$^+$ affisomes).[185] Affibody® affinity ligands are innovative protein-engineering technologies. The liposomes were either loaded with rhodamine phosphatidylethanolamine and calcein or with Dox. HER2$^+$ cells and HER2$^-$ cells were both incubated with the liposomes.[186]

As stated by Svenson, even if liposomes are an ideal platform for theranostics, several issues remain to be addressed. Nanocarrier polydispersity is an issue for clinical translation and regulatory approval of nanocarriers. Random entrapment and surface conjugation of diagnostic and therapeutic agents into liposomes or other nanocarriers is a tempting and easily achievable approach. However, the polydispersity of the resulting nanocarriers and questionable reproducibility of the approach will create high hurdles for clinical translation and regulatory approval. Adding active targeting ligands to a nanocarrier not only adds at least another step to its production but also adds to polydispersity, complicates regulatory

evaluation and approval, increases the costs of goods, and can have negative biological outcomes because of multivalency binding and enhanced recognition by the RES with reduced circulation time.[187]

Liposomes on the market or in clinical trials

Doxil (100 nm) was the first pharmaceutical product in a PEGylated liposomal formulation that received FDA approval (1995) for the treatment of chemotherapy-refractory Kaposi's sarcoma in AIDS patients, and more recently for recurrent epithelial ovarian cancer.[188] Currently, several liposome-based drugs are approved for clinical practice; many others are still in the various stages of clinical trials (Tables 1 and 2).[189] Most liposomal drug formulations, such as Doxil and Myocet® (190 nm), have been approved for intravenous application.[190] Generally, liposome products have a longer circulating half-life when compared with respective unencapsulated drugs. The time of circulation in the blood depends on size, charge density, fluidity of the lipid bilayer, or coating by PEG. For the delivery of surface antigens derived from the influenza virus (Inflexal® V) or hepatitis A (Epaxal®), intramuscular delivery has been approved. Inflexal V and Epaxal are both vaccine products: it has been reported that cell-mediated and humoral immune response is potentiated when viral membrane proteins or peptide antigens are incorporated into liposomes.[191,192] For the administration of liposomal vaccines, oral delivery has also been considered; however, this is more problematic, due to the potential for liposome breakdown following exposure to bile salts. Therefore, injections remain the best route of administration for therapeutic peptides.[193] Since the first liposomal pharmaceutical product, Doxil, liposomes have been widely utilized as carriers for various therapeutic agents in clinical trials (extensively reviewed in Chang and Yeh).[189] Until now, virosomes (Epaxal and Inflexal V), CLPs (EndoTAG1-1®), PEGylated liposomes (Doxil and Lipodox), and temperature-sensitive liposomes (ThermoDox) have been considered for clinical use. In contrast with the liposome-based drugs on the market, liposome-based drugs in clinical trials display a large variety of loaded drugs (eg, cisplatin, BLP25 lipopeptide, Grb2 antisense oligodeoxynucleotide, bacteriophage T4 endonuclease 5) for several therapeutic applications (from topical delivery systems to portable aerosol delivery systems). As previously described, PEGylation may extend the blood-circulation time of liposomes, modify drug distribution in the body, and hence reduce related adverse effects (eg, cardiotoxicity). However, a significant incidence of stomatitis in clinical trials contemplating the use of PEGylated liposomes (Doxil and Lipodox) has been reported.[189]

In addition, it has been observed that some of the new-generation liposomes demonstrated only comparable or even poor therapeutic efficiency when they were compared with relative free drugs or conventional vesicles in clinical trials. In comparison with Doxil, ThermoDox showed a significant decrease of Dox accumulation in mouse tumors at 24 hours after administration.[162,189] EndoTAG-1 plus gemcitabine showed beneficial survival and efficacy in a randomized controlled Phase II clinical trial in advanced pancreatic cancer and triple receptor-negative breast cancer.[194] In addition, a positive efficacy trend of the EndoTAG-1 combination therapy for triple receptor-negative breast cancer was reported by MediGene (http://www.medigene.com). SPI-77, the first liposomes loaded with cisplatin, showed limited clinical efficacy in a Phase II clinical trial of advanced non-small-cell lung cancer. On the other hand, the same formulation induced increased cisplatin tumor accumulation in preclinical models.[195] Similarly to SPI-77, a Phase II study demonstrated that liposomal annamycin had no detectable antitumor activity in the treatment of Dox-resistant breast cancer.[196]

Conclusion

Due to their biocompatibility and biodegradability, liposomes were the first drug-delivery system approved for clinical purposes. Despite their long history in the field of research and development, there are still unresolved problems that limit their ultimate therapeutic outcome.

The advantages of liposomal-based drugs should be greater solubility of the cargo, increased half-life, selective delivery to the site of action, and the ability to overcome resistance against chemotherapeutics. The consequential pharmacokinetic changes could result in a reduction of adverse effects and an improvement in the therapeutic index of the encapsulated drugs. To reach these therapeutic outcomes, liposomes were firstly modified (PEGylated) in order to solve pharmacological challenges, such as destabilization by blood lipoproteins, uptake by RES, and rapid clearance from blood circulation.

PEGylated liposomes have been approved and are on the market, but their clinical success is hampered by some limitations, such as a lack of specificity. To increase their target-specificity and the amount of released therapeutic agent at the site of disease, stimuli-sensitive liposomes and multifunctional carriers for theranostics have been designed.

However, the transfer to large-scale production and to the clinic of these liposomal formulations suffers drawbacks,

Table 1 Liposomes on market or in clinical trials

Product name	Drug	Indication	Route of injection	Nanoscale dimensions (nm)	Status	References
Abelcet	Amphotericin B	Fungal infections	Intravenous	1,600–11,000	Approved	197–200
AmBisome	Amphotericin B	Fungal infections	Intravenous	45–80	Approved	197–199,201
Amphocil	Amphotericin B	Fungal infections	Intravenous	110–114	Approved	202,203
DaunoXome	Daunorubicin citrate	Kaposi sarcoma	Intravenous	45	Approved	197,198,204,205
DepoCyt	Cytarabine	Lymphomatous meningitis	Intravenous	20	Approved	197–200
Doxil	Doxorubicin	Kaposi's sarcoma	Intravenous	87	Approved	197–199,206,207
Visudyne	Verteporfin	PDT sensitizer	Intravenous	100	Approved	197,199,208
Evacet	Doxorubicin	Ovarian cancer	Intravenous	150	Approved	199,209
Lipo-Dox	Doxorubicin	Solid tumors	Intravenous	20	Approved	210
Epaxal	Inactivated hepatitis A virus (strain RG-SB)	Hepatitis A	Intramuscular		Approved	211
Inflexal	Inactivated hemagglutinin of influenza virus strains A and B	Influenza	Intramuscular		Approved	211
DepoDur	Morphine sulfate	Pain management	Epidural		Approved	212,213
Nyotran	Nystatin	Solid tumors	Intravenous	110–135	Terminated	214,215
Alocrest	Vinorelbine	Solid tumors	Intravenous	100	Investigational	216,217
AmBiL	Amphotericin B	Fungal infections	Intravenous	130	Investigational	213,218
Aroplatin	Cisplatin and its analog	Colorectal neoplasms	Intravenous/ Intrapleural		Investigational	219–221
ATI-1123	Docetaxel	Solid tumors	Intravenous	60–80	Investigational	222,223
Atragen	Tretinoin	Solid tumors	Intravenous		Investigational	224,225
Atu027	siRNA	Solid tumors	Intravenous	120	Investigational	226–228
BAY 79-4980	Kogenate FS	Hemophilia a	Intravenous	80–110	Investigational	229,230
BP-100-1.01	Grb-2	Leukemia	Intravenous		Investigational	231
BP-100-1.02	Bcl-2	Lymphoma	Intravenous		Investigational	232
BP-100-2.01	siRNA	Ovarian cancer	Intravenous		Investigational	232
Brakiva	Topotecan	Solid tumors	Intravenous	100	Investigational	233,217
CPX-1	Irinotecan	Solid tumors	Intravenous	100	Investigational	234,235
CPX-351	Cytarabine and daunorubicin	Acute myeloid leukemia	Intravenous	100	Investigational	235,236
C-VI SA bikDD	BikDD	Pancreatic cancer	Intravenous	405	Investigational	237,238
Doxisome	Doxorubicin	Solid tumors	Intravenous		Investigational	213
EndoTAG-1	Paclitaxel	Solid tumors	Intravenous	180–200	Investigational	239,240
I HL-305	Irinotecan	Solid tumors	Intravenous	100	Investigational	241
I NGN-401	DOTAP: Chol-fus1	Lung cancer	Intravenous	375	Investigational	198,242
JVRS-100	Immunostimulatory DNA	Leukemia	Intravenous	120	Investigational	243,244
L-annamycin	Annamycin	Acute lymphocytic leukemia	Intravenous	150	Investigational	245,246
LE-DT	Docetaxel	Solid tumors	Intravenous	<200	Investigational	247
LE-M	Mitoxantrone	Solid tumors	Intravenous	<200	Investigational	247,248
LEP-ETU	Paclitaxel	Solid tumors	Intravenous	150	Investigational	249,250
LE-rafAON	LErafAON-ETU	Neoplasms	Intravenous	400	Investigational	251–253
LE-SN38	SN-38	Solid tumors	Intravenous	150–200	Investigational	254,255
L-Grb-2	Grb2 antisense oligodeoxynucleotide	Leukemia	Intravenous	90	Investigational	256,257
Lipoplatin	Cisplatin and its analog	Solid tumors	Intravenous	110	Investigational	258–260
Liposomal alendronate	Alendronate	Coronary artery stenosis	Intravenous	148–180	Investigational	261
Lipotecan	Camptothecin	Solid tumors	Intravenous	180–200	Investigational	213,262,263

Abbreviations: PDT, photodynamic therapy; siRNA, small interfering ribonucleic acid; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt); Chol, cholesterol; DNA, deoxyribonucleic acid; LErafAON, liposome-entrapped, end-modified raf antisense oligonucleotide; ETU, easy to use.

Table 2 Liposomes on market or in clinical trials

Product name	Drug	Indication	Route of injection	Nanoscale dimensions (nm)	Status	References
Lipovaxin-MM	Vaccine	Melanoma	Intravenous	240	Investigational	264,265
Liprostin	Prostaglandin	Peripheral vascular disease	Intravenous	100–200	Investigational	266
L-MTP-PE	L-MTP-PE	Osteosarcoma	Intravenous	710	Investigational	267,268
Marqibo	Vincristine	Solid tumors	Intravenous	100	Investigational	217,269
MBP-426	Oxaliplatin	Solid tumors	Intravenous	180	Investigational	239,270,271
MBP-Y003	Methotrexate	Lymphoma	Intravenous		Investigational	271
MBP-Y004	Docetaxel	Solid tumors	Intravenous		Investigational	271
MBP-Y005	Gemcitabine	Solid tumors	Intravenous		Investigational	271
MCC-465	Doxorubicin	Stomach cancer	Intravenous	143	Investigational	239,272
Myocet	Doxorubicin citrate	Breast cancer	Intravenous	190	Investigational	197,198,273
Nanocort	Prednisolone	Rheumatoid arthritis	Intravenous	<150	Investigational	274
NanoVNB	Vinorelbine	Colon cancer	Intravenous	95, 2	Investigational	213,275
ONCO-TCS™	Vincristine	Solid tumors	Intravenous	120	Investigational	276,277
OSI-211	Lurtotecan	Solid tumors	Intravenous	100–200	Investigational	278
PEP02	Irinotecan	Solid tumors	Intravenous	100	Investigational	279
PNT2258	Oligonucleotide	Cancer	Intravenous	100	Investigational	280
RVCLUV	Ropivacaine	Anesthetic	Intravenous	130	Investigational	281
SapC-DOPS	Saposin C	Solid tumors	Intravenous	190	Investigational	282–284
S-CKD602	Camptothecin analog	Advanced malignancies	Intravenous	100	Investigational	285
Stimuvax	BLP25 vaccine	Solid tumors	Intravenous	150–580	Investigational	286
ThermoDox	Doxorubicin, lyso-thermosensitive	Solid tumors	Intravenous	175	Investigational	287,288
TKM-ApoB	siRNA	Hypercholesterolemia	Intravenous	120	Investigational	289,290
TKM-Ebola	siRNA	Ebola	Intravenous		Investigational	291
TKM-PLK1	siRNA	Solid tumors	Intravenous		Investigational	291
Dimericine	T4N5	Precancerous condition	Topical	200	Investigational	292,293
Lip glucantime	Meglumine antimoniate	Cutaneous leishmaniasis	Topical	400	Investigational	294
NanoDOX™	Doxycycline monohydrate	Foot ulcer, diabetic	Topical	200–350	Investigational	295,296
T4N5 liposomal lotion	Bacteriophage T4 endonuclease 5	Skin cancers	Topical		Investigational	297,298
AeroLEF	Fentanyl	Pain relief	Aerosol		Investigational	299
Arikace	Amikacin	Cystic fibrosis	Aerosol	375	Investigational	300
L9NC	L9NC	Solid tumors	Aerosol	100–300	Investigational	301
VaxiSome	Vaccine	Influenza	Intramuscular		Investigational	302
CAF01	Vaccine	Tuberculosis	Intramuscular	450	Investigational	303
RTS S/AS02	FMP2.1/AS02A	Malaria	Intramuscular	20	Investigational	197,304
CFTR gene liposome	CFTR gene	Cystic fibrosis	Nasal		Investigational	305
OX-NLA	Cetirizine HCl	Allergic rhinitis	Nasal		Investigational	306,307
pGT-1 gene liposome	pGT-1 gene	Cystic fibrosis	Nasal		Investigational	308
DPX-0907	Cancer vaccine	Neoplasms	Subcutaneous		Investigational	309
IL-2 LIPO	Interleukin 2	Melanoma	Subcutaneous		Investigational	310
PLCLUV	Prilocaine	Dental anesthesia	Interstitial	400	Investigational	311,312
L-CsA	Cyclosporine	Bronchiolitis obliterans	Interstitial	40–50	Investigational	313,314
Telintra	TLK199 HCl	Myelodysplastic syndromes	Oral		Investigational	315

Abbreviations: L-MTP-PE, liposome-encapsulated muramyl tripeptide phosphatidylethanolamine; siRNA, small interfering ribonucleic acid.

such as instability, polydispersity, toxicity at repeated administration, and capability of inducing immunostimulation and complement activation. The precise control of liposome size and distribution can be optimized by new preparation methods, such us microfluidic-based methods and microfluidic remote loading (rapid single-step liposomal drug preparation). For pharmaceutical manufacturing and for quality-assurance assays, we can foresee progress in automation and control of processes of conventional liposomes as well as multifunctional liposomes. OECTs have been recently proposed for real-time monitoring of liposome-based structures.

The increase of complexity of liposomal formulation even more needs accurate in vitro and in vivo preclinical studies before transfer to the clinic. The analysis of physicochemical characteristics, toxicity, hematocompatibility, delivery, and therapeutic efficiency is mandatory. Dialogue between scientists, clinicians, and industry is indispensable in the design phase of new liposomal formulations to increase the success rate for liposomes as nanomedical formulations.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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