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Review Article

What We Need to Know about Liposomes as Drug Nanocarriers: An Updated Review

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Abstract

Liposomes have been attracted considerable attention as phospholipid spherical vesicles, over the past 40 years. These lipid vesicles are valued in biomedical application due to their ability to carry both hydrophobic and hydrophilic agents, high biocompatibility and biodegradability. Until now, various methods have been used for the synthesis of liposomes and the numerous modifications have been performed to improve liposomes characteristics such as surface charge, size, number of their layers, and length of circulation in biological fluids. This article provides an overview of the significant advances in synthesis of liposomes *via* active or passive drug loading methods, as well as describes some strategies developed to fabricate their targeted formulations to overcome limitations of the "first-generation" liposomes.

Introduction

Nowadays, the use of nanomaterials as a system for drug-delivery has been widely considered, specially, in cancer therapy.[1] It has been proved that materials in nanoscale (<200 nm) can prolong the circulation time in body as well as entering the cells *via* endocytosis; consequently, cause intracellular absorption.[2,3] Different nanomaterials such as micelles,[4] dendrimers,[5,6] superparamagnetic iron oxide nanoparticles (SPIONs),[7] mesoporous silica nanoparticles,[8] gold nanoparticles (GNPs),[9] quantum dots,[10] carbon nanotubes,[11] and liposomes have been used in drug delivery systems.[12] Among them liposomes are the most common nanocarriers due to their inherent advantages such as high biocompatibility, low immunogenicity, cell-like membrane, low toxicity, and ability to protect drugs from hydrolysis and prolong their biological half-life. They are able to encapsulate either hydrophobic or hydrophilic molecules and control the drug release.[3,13,14] Besides, many efforts have been made in developing of smart drug carriers that deliver their cargo in response to an external or internal trigger. In this regard, liposomes are recognized as one of the most successful drug delivery systems.[15,16]

In general, liposomes are sphere-shaped microscopic vesicles with the hydrophilic portion completely enclosed by one or more phospholipid bilayers (Figure 1).[17] Due to the amphiphilic nature of phospholipids, they favor to assemble as closed bilayer structures in such a way that minimize the confrontation between aqueous and hydrophobic domains. So, the lowest free energy state and the maximum stability to self-assembled structures are achieved. Besides, the hydrodynamic and other destabilizing forces can cause the fragmentation of the bilayer to form the smaller liposomes.[18,19] *In vivo* and *in vitro* stability of liposomes are controlled by their physical and chemical characteristics such as lipid composition, size, charge, number of lamellae and surface modifications.[20] Up to now, numerous researches related to liposomes have been performed owing to their importance in the nanomedicine field. Loaded drugs on liposomes can include a wide range of anti-cancer drugs, antibiotics, small interfering RNAs (siRNA), antisense oligonucleotides, and bacterial plasmids carrying therapeutic genes.[21] Similarity of phospholipids to cell membrane facilitates passage of liposome through some membrane barriers for distribution in tissues and removal from the elimination organs. Besides, modification of liposomes with various ligands and polymers improves drug uptake and increases circulation time of drug in the blood.[12,22] After the clinical approval of PEGylated

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liposomal doxorubicin (Doxil®) as the first nanodrug by US FDA in 1995, 19 liposomal formulations have been clinically approved for the treatment of various diseases. Nevertheless, there are major concerns about their stability, controlled and predictable pharmacokinetics and pharmacodynamics as well as reproducible production in large scale that needs improvement.[14,20,23] One of the challenges in application of liposomes in clinical use is the interaction of liposome constituents with the immune system. Liposome components can induce antibody production which leads to reduction of their efficacy.[24] In addition, the lack of established techniques for large-scale production of liposomes, and suitable models that exactly imitate tumor heterogeneity, are limitations for clinical development of liposomes.[25]

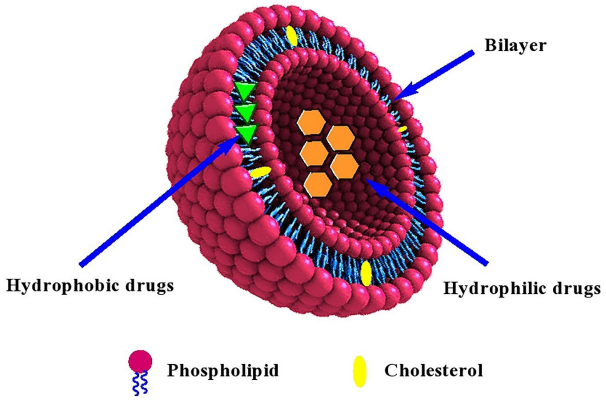

Figure 1. Schematic presentation of structure of a typical liposome loaded with hydrophilic and hydrophobic drugs

Several review articles have been published describing liposomal structures, preparation methods and their application.[12,15,17,19,24-31] The present review, besides the general aspects liposomes, focuses on the significant advances in their synthesis *via* active or passive drug loading methods, as well as describing some strategies developed to fabricate new-generation liposomes with target-specificity and stimuli-sensitivity.

Structural units of liposomes

In general, the structure of liposomes consists of two parts; phospholipid and cholesterol. Phospholipids are the major component of liposomal structure and cholesterol improves their stability. The hydrophilic head of these fats is a phosphate group that is joined to hydrophobic components by a water-soluble molecule like glycerol and can be natural or synthetic.[18,22] A list of different types of phospholipids is presented in Table 1. Choosing the proper phospholipid for achieving the desired therapeutic goals is essential.[32-34] Cholesterol incorporates within phospholipid bilayer because it cannot form liposomes alone. It is essential for the consolidation of bilayers, increasing the packaging of phospholipid molecules, controlling drug retention, and reducing the permeability of the bilayers.[17,32,35,36]

Characteristics of liposomes

The performance of liposomes depends on their size, number of layers, shape, and surface charge. Therefore, estimating and characterizing these properties is essential

Table 1. Various types of phospholipids used in the preparation of liposomes

Chemical name	Abbrev.	Formula	Source	Ref.
Phosphatidylcholine	PC	$C_{42}H_{82}NO_8P$	Egg yolk, soybeans	37
Phosphatidylethanolamine	PE	$C_7H_{12}NO_8PR_2$	Chocolate, soybean milk	38
1,2-Distearoyl-sn-glycero-3-phosphoethanolamine	DSPE	$C_{41}H_{82}NO_8P$	Synthetic	39
Dimyristoyl phosphatidylcholine	DMPC	$C_{36}H_{72}NO_8P$	synthetic	29
Dimyristoyl phosphatidylglycerol	DMPG	$C_{34}H_{67}O_{10}P$	synthetic	29
Dipalmitoylphosphatidylcholine	DPPC	C40H80NO8P	cell membranes, pulmonary surfactant	29
Dioleoyphosphatidyl choline	DOPC	$C_{44}H_{84}NO_8P$	synthetic	29
dipalmitoyl phosphatidyl glycerol	DPPG	$C_{38}H_{75}O_{10}P$	mitochondrial membranes, pulmonary surfactant	29
2,3-dioleyloxy-N-[2(sperminecarbox amido)ethyl]-N,N-dimethyl-1-propanammonium trifluoroacetate	DOSPA	$C_{56}H_{111}F_3N_6O_5$	synthetic	29
1,2-bis(oleoyloxy)-3-(trim ethylammonio)propane	DOTAP	$C_{10}H_{25}N_3$	synthetic	29
1,2-dimystyloxypropyl-3-dimethyl hydroxyethyl ammonium bromide	DMRE	$C_{20}H_{29}BrN_2$	synthetic	29
3β[N-(N',N'-dimethylaminoethane)-carbomoyl] cholesterol	DC-CHOL	$C_{32}H_{56}N_2O_2$	synthetic	29
Dioctadecylamino-glycyl-spermine	DOGS	$C_{10}H_{26}N_4$	As polycation in eukaryotic cells	29
Phosphatidylinositol	PI	$C_{47}H_{83}O_{13}P$	Endoplasmic reticulum	40
Phosphatidylserine	PS	$C_{13}H_{24}NO_{10}P$	Soy, white beans, egg yolks, chicken liver, beef liver	41
Phosphatidic acid	PA	$C_{39}H_{77}O_8P$	Cabbage and radish leaves, *Mallotus japonicas*	42
Phosphatidylglycerol	PG	$C_{40}H_{77}O_{10}P$	Live amniotic fluid surfactant	43
Cardiolipin	CL	$C_{81}H_{158}O_{17}P_2$	Mammalian and plant cells, inner mitochondrial membrane	44

for clinical application as well as in determining their half-life.[19] These colloidal vesicles have different number of layers are classified based on size and number of bilayers (Figure S1). They can be unilamellar (UV) or multilayer. UVs are also classified into four subgroups of small (SUVs), medium (MUVs), large (LUVs), and giant (GUVs) vesicles according to their size. The multilayer liposomes are divided to oligolamellar (OLVs), multilamellar (MLVs), and multivesicular (MVVs) vesicles.[45,46] The zeta potential is the electrostatic charge of the particle surface that prevents the proximity and aggregation of particles.[47] Zeta potential can provide perception about circulation times, stability, circulation times, and biocompatibility of nanoparticles.[48] Moreover, Zeta potential is important factor in the initial adsorption of liposome onto the surface of cells.[49]

Synthesis methods of liposomes

Up to now, many methods have been reported for the production of liposomes which can be divided to conventional and novel techniques. Conventional strategies include thin-film hydration (Figure S2),[50] reverse phase evaporation (Figure S3),[26] ethanol injection (Figure S4),[51] ether injection (Figure S5, Supplementary file 1),[52] electro-formation,[53] and detergent depletion methods.[54] These methods are easy to implement and do not require complicated equipment; however, scale-up for industrial manufacture and scale-down for point-of-care applications are challenging issues of them.[19,55] In addition, limitations in process control, poor reproducibility, and inefficient use of materials and reagents are other significant problems.[56] For overcoming these problems, some *new methods* have been developed for preparation of liposome.

Microfluidic methods

Microfluidic techniques refer to the strategies in which the procedures are performed in a small volume, typically in sub-millimeter scales and low Reynolds Numbers. By exploiting these microfluidic techniques the laboratory procedures can be performed in planar chips or other small devices result in reducing cost of chemical and biological experimentation.[56,57] A number of microfluidic methods have been developed called modified Electro-formation,[58] lipid hydration,[59] micro hydrodynamic focusing,[60] Pulsed jetting,[61] double emulsion templates,[62] lipid coated ice droplet hydration,[63] transient membrane ejection[56,64] modified droplet emulsion transfer[65] either as modification of macro-scale techniques or as completely novel methods.

Supercritical fluid (SCF) based methods

Some new methods exploit SCF which is increasingly replacing organic solvents due to its ability for efficient separation and purification. There are several strategies for liposome preparation using SCF method. In a technique, a compressed mixture of the lipids, SCF and organic co-solvent is injected into the aqueous phase, and sprayed into water to form liposomes (injection method).

Whereas, in another approach, the compressed phase composed of lipid, SCF as well as aqueous phase is sprayed into air through a nozzle (decompression method). The size of the obtained vesicles is related to the rate of depressurization. It has been claimed that through these methods sterile, solvent free and pharmaceutical grade liposomes having a narrow particle size distribution can be produced. The incorporation of aqueous phase is the major difference between these approaches.[19,54] In another method, supercritical reverse phase evaporation (scRPE), a mixture of lipid, organic co-solvent and compressed gas are put in a stirred, variable volume cell above the lipid phase transition temperature, and then an aqueous solution is slowly introduced to the cell. The liposomes are formed upon the pressure is reduced by the release of the compressed gas. The principle of the scRPE method is similar to the decompression method. However, in this method the depressurization occurs by the release of the dense gas from a variable volume cell.[54,66] In another method called supercritical anti-solvent precipitation, the phospholipid dissolved in an organic co-solvent is sprayed into the SCF as an anti-solvent, resulting in formation of micronized particles. The size of the particles depends on the droplet size of the spray and the concentration of the lipid in the co-solvent. After hydration of the particles in an aqueous buffer the liposomes are formed. It was reported that increase in the pressure of the system or the SCF/co-solvent ratio causes the reduction in the fraction of small liposomes in the system.[67,68] It has been claimed that the scaling-up of the SCF methods can be implemented with less problems.[19]

Other new methods

In the method called "freeze-drying double emulsions", preparation of liposomes is accomplished by the lyophilization of double emulsions (W1/O/W2) containing disaccharides as lyoprotectants in both the inner and outer aqueous phase, by a two-step emulsification procedure at room temperature.[69] "Membrane contactor" is a modified ethanol injection method in which phospholipid solution in alcohol was extruded through a membrane contactor into an aqueous solution and the liposomes are formed.[70] In the method "hydration of deposited phospholipids on nanostructures" phospholipids are deposited on amphiphilic electrospun nanofibres composed of polyvinylpyrrolidone and soybean lecithin. The liposomes self-assembled upon addition of the nanofibers into water.[71] In the other method namely "Curvature-tuning", the phenomenon of spontaneous vesiculation and theory of curvature have been taken into consideration in solvent-free liposome preparation procedure. In this method, rapid pH change followed by a defined period of equilibration is exploited for the preparation of stable,

monodisperse, and unilamellar liposomes. Further, by direct addition of the lipids into an aqueous buffer, there is no need to first preparation of MLVs suspension. The size, shape, and dispersity of the liposomes are affected by some critical factors such as time interval of pH increase, time of equilibration, temperature, and type of lipid.[19,72,73]

Large-scale techniques for liposome production

Application of liposomal formulation in industrial scale has two challenging issues including; poor capability of transferring from academic bench to highly regulated technology and stability of liposomes.[74,75] Ethanol injection method is one of the interesting methods for scaling-up production of liposomes owing to reproducibility, fast implementation, and simplicity. Moreover, this technique did not cause oxidation and degradation of lipids. It has been reported that by use of this method, 0.5 to 12 kg of liposomes can be obtained from batches.[76] Microfluidic is another effective reproducible method for scale up of liposomes. This method has a high potential to achieve more control over the physical properties of the end product, especially in terms of size distribution, lamellarity, and high encapsulation efficiency.[75,77,78]

Modifications to conventional liposomes

Vesicles with simple structure including; cholesterol and phospholipid are named conventional liposomes or "first-generation liposomes". These liposomes have some drawbacks like fast release of drug, rapid elimination from the blood, capture by the mononuclear phagocyte system, and low entrapment efficiency of water–soluble drugs.[45,79] To overcome the mentioned deficiencies some new strategies have been developed in liposome preparations and novel generations of these vesicles have been emerged.[30,80]

Fusogenic liposomes (FLs)

Conventional liposomes are usually taken up into cells by phagocytosis or endocytosis and the main part of their content such as macromolecules might be degraded before reaching the cytoplasm.[79,81] The induction of membrane fusion between liposomes and the cell membrane can overcome to this problem. FLs are nanocarriers which may fuse with biological membranes, thereby increasing drug contact and delivery into cells. FLs are composed of lipids, such as dioleoyl-phosphatidylethanolamine (DOPE) and cholesterylhemisuccinate (CHEMS), which cause increased fluidity in the lipid bilayer and can destabilize biological membranes.[82] Due to their composition, the bilayer structure of FLs is efficiently fused with the cellular plasma membrane of cell to deliver the content of liposomes into the cytoplasm without degradation.[79,83]

One of the most interesting types of FLs is virosomes. These FLs are prepared by incorporation of conventional liposomes-based phospholipid with UV-disabled Sendai virus. The presence of the Sendai virus allows liposomes to rapidly and directly transfer their contents into the cells by membrane fusion. Therefore, these liposomes can be used as drug carriers for specific purposes.[45,84,85]

pH-sensitive liposomes

To date, various triggered release models are widely researched and reported in order to increase the therapeutic index of pharmaceutical or other materials encapsulated within liposomes. Liposome composition can be modified to obtain triggered release in response to environmental conditions. The pH-sensitive liposomes are designed to control the release of their contents in response to acidic pH of the endosomal system. These liposomes have obviously improved the intracellular delivery of a variety of materials such as anti-cancer drugs, toxins, proteins, and DNA.[86-88] The typical lipids used to prepare pH-sensitive liposomes are phosphatidylethanolamine (PE) and its derivatives including; diacetylenic phosphatidylethanolamine (DAPE), phosphatidylethanolamine (POPE) and DOPE. They are mixed with the compounds containing an acidic group that acts as a stabilizer at neutral pH. DOPE is usually combined with gently acidic amphiphiles such as oleic acid, CHEMS, and palmitoyl homocysteine.[89,90] The most commonly used lipid combination is DOPE with CHEMS. Recently, a pH-responsive liposome has been prepared from 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-liposome) for endosomal escape mediated drug delivery. Doxorubicin-loaded DC-liposome has exhibited higher cytotoxicity effect than free drug which supporting the endosomal escape of pH-responsive DC-liposome.[91]

The pH-sensitive liposomes are stable at neutral pH. In this condition, amphiphilic acid molecules cause the electrostatic repulsion between carboxylate and phosphate groups resulting in the formation of lamellar phases. However, an acidic medium (in pH less than the normal physiological value), either in endosomal vesicles or in the extracellular tumor environment, causes the protonation of the carboxylate groups triggering a transition from lamellar to hexagonal phase leading the release of loaded drugs.[89,92] The surface of pH-sensitive liposomes can be coated by PEG to prolong the circulation time. Therefore, the liposomes are prevented from rapid clearance via the reticuloendothelial system (RES).[93]

Cationic liposomes

Cationic liposomes are vesicles that are constructed from positively charged lipids and have increasingly been used in gene therapy because of their interactions with negatively charged DNA.[94-97] It is notable that the negatively charged genetic material is not encapsulated in liposomes but form complex with cationic empty liposomes by electrostatic interactions whereas total surface charge of DNA/liposome remains positive.[81] DNA-cationic liposome complexes (lipoplexes) enter the cell by fusion with the

plasma or endosome membrane. Conventional liposomes are negatively charged and may release their contents in the circulation and/or extracellularly after interaction with blood components and tissues due to their weak affinity for cell membrane. However, unlike these vesicles, cationic liposomes with positive charge are highly interactive with cells (with negatively charged biological membrane) and can deliver contents into cells by fusion with cell membranes. They are usually constructed from a neutral phospholipid (DOPE) and a positive derivative such as stearylamine, dimethyldioctadecylammonium bromide, dimethyl-aminoethane carbamoyl cholesterol (DC-chol), and Dioleoyl-3-trimethylammonium propane (DOTAP).[98,99]

Temperature-sensitive liposomes

The temperature-sensitive liposomes (TSLs) are vesicles that their content release behavior is controlled with temperature changes. TSLs rapidly release the loaded drug at few degrees above physiological temperature or hyperthermic conditions.[100-103] The release of encapsulated hydrophilic drugs is related to the melting phase transition temperature (Tm) of the lipid bilayer; the temperature that the structure of the lipid bilayer changes from solid gel phase to liquid-crystalline phase. In the liquid-crystalline phase, the membrane of TSLs is more permeable to water and hydrophilic drugs than in the gel phase. In the most TSLs, the major component for liposome formulation is 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) with Tm of 41.4°C. To prevent the drug leakage at body temperature, DPPC can be mixed with small amounts of other phospholipids, such as 1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC; Tm = 54.9°C). The composition of the mixed phospholipids specifies the Tm of the formulation. Further, TSLs can be constructed by modification of conventional liposomes with thermosensitive polymers.[104-106] TSLs in combination with local hyperthermia or high intensity focused ultrasound are concerned as effective route for external targeting of anti-cancer drugs to solid tumors.[107,108]

Stealth liposomes

Stealth liposomes (long circulating liposomes) namely "second-generation liposomes" are obtained by modifying the surface of the vesicles with an inert molecule.[29] At first, liposomes with modified surfaces were developed using several molecules, such as glycolipids or sialic acids. However, with inclusion of the synthetic polymer polyethylene glycol (PEG) in the liposome composition, the long-circulating pegylated liposomes as a new generation were emerged. It has been proved that such surface modification extends blood-circulation time of liposomes and stabilizes these nanocarriers by minimizing their interaction with the RES.[45,109] So far, this technology has been used to formulate a large number of liposomes containing various drugs or other biomolecules with high efficiency and activity. Moreover, by combining of the terminal PEG with appropriate compound, long-circulating liposomes can be synthesized to target on specified cells.[109]

Magnetoliposomes

Combination of liposomes and SPIONs or other magnetic nanoparticles,[110,111] that called magnetoliposomes (MLs) is an interesting strategy creating vesicles with the potential for the application in controlled drug delivery systems and diagnostic imaging. They are promising nanocarriers for the development of the selective and site-specific drug delivery systems in the cancer therapy which can effectively deliver the drug towards tumor cells by applying a magnetic field.[112,113] MLs are widely exploited as contrast agents in magnetic resonance imaging and as chemotherapeutic agents.[114] There are three different approaches in associating the SPIONs to liposomes: (i) encapsulation of magnetic nanoparticles directly within the liposome lumen,[115-117] (ii) embedding them in between the lipid bilayer,[118-120] and (iii) directly conjugating magnetic nanoparticles to the liposome surface.[121] The different types of MLs are depicted in Figure 2.

Photosensitive liposomes

In the contrary to internal triggerable liposomes like pH-sensitive liposomes, external triggering system exploit the outside factors such as light, heat or magnetic field to release the liposomal cargo. On the other hand, the thermo-sensitive liposomes undergo phase transition of the phospholipids, while photo-triggerable liposomes are composed of a light-sensitive group engineered into the vesicle. Function of light-triggering liposome is based on two approaches: (i) photo-destabilization of liposome membrane to promote cargo release and (ii) light absorption of metal nanoparticles such as gold nanoparticles.[122] Different photosensitive molecules can induce membrane destabilization and permeabilization. They can locate in the liposomal structure according to their intrinsic polarity. Phospholipid molecule modification can be performed in potential sites, namely, head group, glycerol backbone and fatty acyl chains.[123-126] The various mechanisms for cargo release from photosensitive liposomes including; light-induced

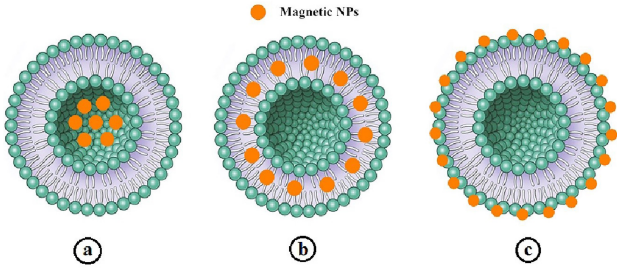

Figure 2. Schematic illustration of three approaches in associating of SPIONs to the liposomes: a) Encapsulation directly within liposome lumen, b) embedding in between the lipid bilayer, and (c) directly conjugating to the liposome surface[115]

oxidation, photocrosslinking, photoisomerization, photocleavage, and photothermal release have been extensively reviewed by Miranda and Lovell.[124]

In case of incorporation of metal nanoparticles like GNPs in liposomal structure, they can localize within lipid bilayer, into the lumen, and on the surface of liposomes, aggregate with liposomes or be free in liposome solution (Figure 3). With irradiation of liposomes, GNPs convert the photo energy to thermal energy, inducing the instability of liposome membrane; therefore, the entrapped drug is released. Photo-responsive liposomes are powerful carriers for topical and transdermal drug delivery to superficial tissues like skin, eyes, and mucous membranes.[16,127]

Liposomes as targeting nanocarriers

Cancer therapy using liposomes can be accomplished through two main approaches including; passive and active targeting.

Passive targeting of liposomes

In passive targeting, the nanocarriers are transported into the tumor interstitium and cells through leaky tumor capillary fenestrations by convection or passive diffusion.[128] In general, all nanoparticle-based drug delivery systems use the tumor characteristics for targeting. The angiogenesis phenomenon in tumor tissues causes the irregularity of endothelial cells with pore sizes of 100 nm to 2 μm. The different pore sizes between the endothelial cells of the tumor microvasculature and the tighter structures of normal cells causes the nanoparticles such as liposomes have better and more access to the cancerous sites. The "enhanced permeability and retention effect" (EPR) causes the increased drug delivery to the affected tissues with a much less return of the fluids to the lymphatic circulation.[129,130] All nanocarriers benefit from the EPR effect in passive targeting so that of drug-loaded nanocarriers in tumor site are 10-50 folds higher than in normal tissue within 1-2 days.[131] The nanocarriers must have at least three characteristics to exploit in passive drug delivery system: (i) The size of nanocarrier should be much less than 400 nm, and being in the range of 10-100 nm which is ideal for efficient extravasation to tumor site, (ii) having neutral or anionic charge for the nanocarriers is necessary to avoid the renal elimination, and (iii) the nanocarriers should be protected from the RES.[128,131,132] The mechanism of passive targeting is illustrated in Figure 4. A number of successful results have been obtained from passive targeting property of liposomes in cancer therapy.[133-138]

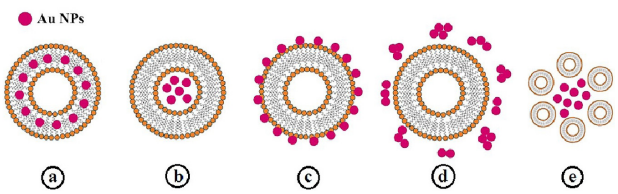

Figure 3. Different types of GNPs incorporation in liposome structure: (a) in the lipid bilayer, (b) in the lumen, (c) on the surface of liposomes, (d) free in the liposome solution, and (e) aggregate with liposomes[16]

Active targeting of liposomes

The reduction of drug toxicity and increase the therapeutic index can be implemented by the site-specific delivery. Nanocarriers can reach tumor microenvironment passively through the EPR effect, whereas the surface engineered nanomedicine acts through binding to the receptors over-expressed by cancer or angiogenic endothelial cells such as epidermal growth factor, fibroblast growth factor, folate, transferrin, and nuleolin receptors (Figure 5). Targeting these overexpressed receptors to increase the anti-cancer agents up taken by cancer cell as well as accumulation in cancer microenvironment is a vital approach.[31] Surface modification of a variety of nanocarriers such as liposomes with antibodies specified for cancer cells, is a more common method. In addition to antibodies, the other molecules or biomaterials with the various strategies for the conjugation have been attached to PEGylated liposomes which enabling them to be actively taken up by the target cells *via* receptor-mediated endocytosis.[128,139,140] Active targeting and efficient ligand receptor interaction are related to some factors such as the extent of expression of the receptor on tumor cells relative to non-target cells, availability of the receptor

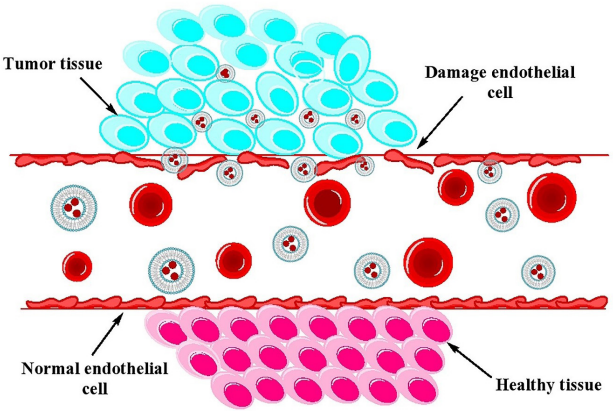

Figure 4. Schematic illustration of the mechanism of passive targeting

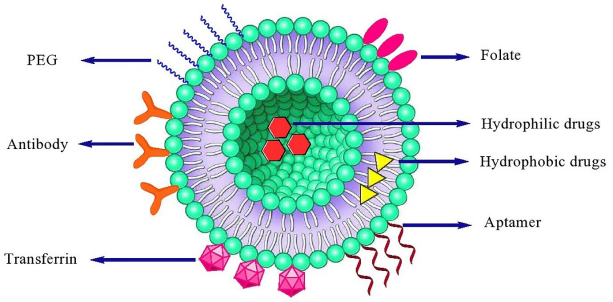

Figure 5. A schematic presentation of various ligands used in liposome targeting

on the surface of target cells, the internalization rate, and heterogeneous expression of tumor receptor.[141] Monoclonal antibodies, transferrin,[1] folic acid,[34] and aptamers[142,143] have been frequently used for surface modification of nanocarriers such as liposomes.[128,130,144,145] A schematic presentation of entrance of these ligands into the cell is shown in Figure 6. A number of researches have been performed in this area. Recently, functionalization of liposomal surface and targeting strategies in treatment of solid tumors are extensively investigated. Recently, mannosylated liposomes have been developed to encapsulate Chlorogenic acid as a targeted delivery system

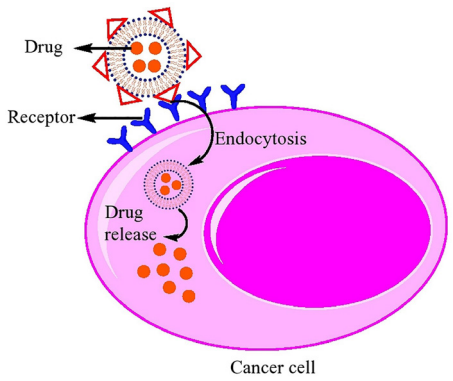

Figure 6. Schematic representation of active targeting using surface engineered liposomes

to tumor-associated macrophages (TAMs) for cancer immunotherapy. It has been reported that chlorogenic acid-loaded liposomes conjugated with mannose exhibited superior accumulation in tumors through the mannose receptor-mediated TAMs-targeting effects.[146] Cancer cells overexpress α5β1, αvβ3 and αvβ5 integrins and it has been observed that the cyclic RGD (cRGD) can strongly attach to αvβ3 and αvβ5 integrins on cancer cells. cRGD-PEG liposomes loaded miR-34a have been developed for suppressing microRNA in breast cancer cells.[147] In one study, it has been reported that tyrosine-modified irinotecan-loaded liposomes exhibited more cellular uptake in MCF-7 and BxPC-3 cells due to highly expressed $ATB^{0,+}$ and LAT1 in cancer cells.[148] In another study, the liposomes modified by glutamic hexapeptide and folic acid were designed for bone metastatic breast cancer. The results showed that paclitaxel loaded in co-modified liposomes presented high stability, more hydroxyapatite binding efficiency and also improved cytotoxic activity of the drug.[149] Some of the more recent published works in this field are summarized in Table 2.

Methods for encapsulating materials into liposomes

Various methods of liposome loading largely depend on the physicochemical characteristics of the loaded agents. In general, the encapsulation strategies are divided in two categories: passive and active loading.[166,167]

Table 2. Some recently published researches in active targeting of liposomes for cancer therapy

Targeting ligand	Drug	Liposome type	Preparation method	Loading method	Cancer treated	Ref.
Monoclonal antibodies	Glycosylated paclitaxel	Immunoliposomes	Thin Film	Passive	Ovarian	150
Monoclonal antibodies	Curcumin	Cationic liposome	Thin Film	Passive	Breast	151
Monoclonal antibodies	Doxorubicin	PEG-liposome	Ethanol injection	Passive	Breast	152
Monoclonal antibodies	Doxorubicin	PEG-liposome	Thin Film	Passive	Breast	153
Folate	Oleuropein	PEG-liposome	Thin Film	Passive	Prostate	154
Folate	Gold nanorods and doxorubicin	PEG-liposome	Thin Film	Passive	Breast	155
Folate	Rapamycin	PEG-liposome	Thin Film	Passive	Bladder	156
Folate	Arsenic trioxide	PEG-liposome	Thin Film	Active	Cervical	157
Transferrin	Doxorubicin and Sorafenib tosylate	PEG-liposome	Thin Film	Passive	Breast	158
Transferrin	Doxorubicin	Cationic liposome	Ethanol injection	Passive	Glioma	159
Transferrin	Plumbagin	PEG-liposome	Thin Film	Passive	Carcinoma, glioblastoma	160
Transferrin	Resveratrol	PEG-liposome	Thin Film	Passive	Glioblastoma	161
Aptamer	A-particle generator 225Ac	PEG-liposome	Thin Film	Passive	Prostate	162
Aptamer	All-trans retinoic acid	PEG-liposome	Thin Film	Passive	Bone	163
Aptamer	MiR-139-5p	Cationic Liposome	Thin Film	Passive	Colorectal	164
Aptamer	Paclitaxel and siRNA	Cationic Liposome	Thin Film	Passive	Breast	165
Mannose	chlorogenic acid	PEG-liposome	Thin Film	-	glioblastoma	146
RGD	microRNA	PEG liposomes	Thin Film	-	Breast	147
Amino acid	irinotecan	Liposome	Thin Film	-	Breast	148
Glutamic hexapeptide and folic acid	paclitaxel	liposome	Thin Film	-	Bone metastatic breast cancer	149

Passive loading

In the all techniques where the lipids and encapsulating agents are introduced in an aqueous buffer solution and the entrapment is achieved while the liposomes are being formed, a passive trapping is occurred. Passive loading of pharmaceutical agents into the liposomes are implemented by two ways including (i) entrapment in liposomal membrane (bilayer) by hydrophobic interaction, electrostatic interaction or combination of these two mechanisms, or (ii) entrapment of hydrophilic substances such as salts (ionic compounds), amino acids, antibiotics, and proteins in intra-liposome aqueous phase (shown in Figure 1). However, the lipophilic substances are added in the organic solvent containing lipids, before formation of liposomes.[168] In both routes, encapsulation is occurred simultaneously with the lipid self-assembly and liposome formation.[169] In this method the loading efficiency, and therefore, drug/lipid ratio (D/L) is usually low (10-50%). However, some additional procedures such as freeze-thaw and dehydration-rehydration can provide higher encapsulation efficiencies. Besides, encapsulation efficiency depends on some factors such as the lipid amount and concentration, and the solubility of the entrapping agent in aqueous phase.[166,170-172]

Active loading

Passive encapsulation method presents some disadvantages including; low entrapment efficiency (20-30%), non-loaded drug loss, organic solvent impurities, and fast release of drug. Hydrophilic small molecules are usually passively loaded during the phospholipid self-assembly. However, amphiphilic substances can be actively loaded into liposomes after liposome formation without diffusing back out. In active encapsulation, the molecules cross through the lipid bilayer into the internal aqueous compartment of liposomes, and cannot diffuse back out or return into the external aqueous solution. The active loading methods are typically based on two phenomena: (i) a given lipophilic molecule diffuse the lipid bilayer and gain a charge upon entering the liposomal core and (ii) the molecule as an ion cannot be able to cross the bilayer and accumulation of the encapsulated agent is achieved.[167,173] Some frequently used active drug loading methods are summarized in the following sections.

The pH gradient method

In this method, penetration of the drug into the preformed liposomes is driven by a transmembrane pH gradient. In order to achieve the efficient active loading, the aqueous solubility of the encapsulating drug and the presence of ionizable functional groups in its structure (e.g. amine group in weak bases) are necessary.[174] In the pH of the extra-liposome aqueous phase the drug exists in the unionized form and is able to migrate across the liposome bilayer. Upon translocation into the liposomal core, the drug changes to ionized form due to the differing pH and retained there. Thus, for amphiphilic drugs which are weak bases or acids, a pH gradient can be the driving force to translocate and retain in liposomes. A pH gradient of 3 units can cause a 1000-fold higher concentration of a substance within the liposome core in comparison to the external aqueous phase.[167,173-175]

Citrate method

In this approach pH of intra-liposomal core is 4 owing to the presence of citrate buffer and extra-liposome aqueous phase has a pH equal to 7.4 which is adjusted with HEPES buffer.[176] Therefore, a proton gradient is observed when substances such as biogenic amines and anti-cancer doxorubicin are present in extra-liposomal aqueous solution. In the presence of HEPES buffer (pH 7.4), the compounds containing the amine groups are in the neutral form and therefore, are able to cross the lipid bilayer. By entering the amines inside the liposome, they produce low-soluble citrate salts due to the presence of citrate ions. This method has been used for remote loading of anthracycline into the liposomes with coffee bean liposome appearance.[177,178] Citrate method was successfully exploited in commercially manufacturing of doxorubicin and daunorubicin products namely Myocet and DaunoXome, respectively.[173]

Ammonium sulfate gradient method

The remote loading strategy that called transmembrane ammonium sulfate gradient method was introduced by Haran et al. for the encapsulation of amines.[179] In this method, there is no need to prepare the liposomes in the acidic pH and alkalinize of extra-liposomal aqueous phase.[180] Ammonium ion gradient is generated *via* its counter-ion sulfate which stabilizes anthracyclines by aggregation and gelation as anthracycline sulfate salt. Firstly, the empty liposomes are formed in ammonium sulfate solution using thin-layer method. Then, the liposomes are dialyzed in PBS solution to form ammonium sulfate gradient inside and outside of liposomes. After the formation of ammonium sulfate gradient, remote encapsulation is carried out by incubation of liposomes with drug solution. The neutral ammonia molecules (permeability coefficient 0.13 cm/s) are diffused towards extra-liposomal solution and left behind a proton due to the higher concentration of ammonium ions in aqueous core of liposome; therefore, the pH gradient is formed. The drug including; amine group in its neutral form (in pH 7.4 buffer) penetrates the bilayer and precipitation of the drug as sulfate salt is occurred. Doxil as the first commercially available long circulating liposomal doxorubicin was produced using ammonium sulfate gradient method.[173,179-181]

Calcium acetate method

The transmembrane calcium acetate method is based

on different permeability coefficients of acetic acid and calcium ions. In this technique, the blank liposomes are prepared in calcium acetate solution. Whereas the calcium ions remain in the liposomal aqueous core, the acetic acid molecules act as proton shuttles. This generates a pH gradient, with higher pH value inside the liposomes, results in entrapping the weak amphiphilic acid molecules inside the liposomes in a way similar to that of weak amphiphilic bases.[173,181,182] In the another method, similar to the aforementioned strategy, arsenic trioxide as an anti-cancer agent has been actively loaded into the liposomes that contain acetate salts of bivalent cations such as Co(II), Ni(II), Cu(II), and Zn(II). The external neutral As $(OH)_3$ penetrates across the bilayer and forms the low-soluble heavy metal- arsenite complex in the liposomal core. On the other hand, the released protons associate with the acetate anions produce the weak acid (HOAC) which diffuses out of the liposome. Both phenomena: the formation of insoluble nickel (II) arsenite compound and the diffusion of the acetic acid out of the liposome drive drug uptake.[183]

Ionophore-mediated method

This protocol involved the use of ionophore agents such as antibiotic nigericin or A23187 which mediate the exchange of $K^+$and H^+across the liposomal bilayer generating pH gradient of about 2 units. When, loaded liposomes with K_2SO_4 are placed in the K^+-free aqueous phase containing an ionophore, the pH of the intra-liposomal aqueous solution decreases due to the release of potassium and the entry of protons.[184] This pH decrease results in the active encapsulation of weak bases. It is notable that nigericin cause a one-for-one exchange of $K^+$for H^+, whereas A23187 makes it possible to move two protons per every divalent metal cation such as Ca^{2+}, Mn^{2+}, or Mg^{2+}. In the use of divalent cations, the presence of EDTA as external chelator is required to bind with the released cations and complete the uptake process as well as prevent aggregation of liposomes.[173,185]

EDTA gradient method

It has been reported that EDTA can form low soluble precipitates with anthracyclines or other weak bases and the formation of low soluble EDTA-drug complexes inside of liposomes can lead to increased drug encapsulation and retention. The protocol is especially suggested for the encapsulation of idarubicin because of its very low solubility in EDTA solutions at acidic pHs. In this case, the liposomes are formed *via* hydration of the lipid film with EDTA disodium salt or EDTA di-ammonium salt solution and then, idarubicin hydrochloride solution is added to the liposomal suspension. The accumulation of idarubicin in liposomes is pH-dependent, so that in higher external pH (8.5) and lower internal pH (4) the drug is better accumulated and higher encapsulation efficiency is achieved.[173,186]

Phosphate gradient method

The main concept in transmembrane phosphate gradient strategy is the same as in the case of other pH gradient methods. In this case, the liposomes are prepared in $(NH_4)_2HPO_4$ solution. It is reported that a near 100% doxorubicin accumulation inside the liposomes *via* both protonation and precipitation of drug have been observed as in the case of other gradients. Besides, in pHs close to physiological level no drug leakage is observed from the liposomes. However, in acidic extra-liposomal medium accelerated drug leakage is achieved. It was suggested that doxorubicin can be retained in the hydrophilic liposomal core by protonation and precipitation, or incorporated in the bilayer. This remote loading process depends on various factors such as intra-liposomal salt concentration and pH value.[173,187]

Solvent-assisted active loading method

In all the aforementioned remote loading methods the compounds with high solubility and membrane permeability are solubilized in the exterior aqueous phase and penetrate through the liposome bilayer into the internal aqueous phase. However, a large number of drugs have low-water solubility and are passively encapsulated in the lipid bilayer of liposomes. The solvent-assisted active loading technology (SALT) has been used for remote encapsulating of poorly soluble drugs in the liposomal core in order to achieve the better loading efficiency and formulation stability. In this technique, a small volume (~5 v/v %) of a water miscible solvent is added to the loading solution for complete dissolution of the compound. Then, the dissolved compound diffuses into the internal compartment of liposome and interacts with a precipitating agent to form low-soluble precipitate. The solvent can be eliminated using dialysis or gel filtration techniques. It is reported that a rapid and complete encapsulation with high D/L ratio and improved circulation half-life are achieved by exploiting this method.[188]

Medical applications of liposomes

Liposomes can be used in the various clinical fields including; therapeutic systems, medical imaging, and cosmetic products which have been summarized below[13]:

Therapeutic systems

Liposomes are used in many fields of medical treatment such as cancer therapy, anti-infective therapy, protein or peptide drug delivery, gene delivery, macrophage activation, and vaccination.[13,15,27,45] The first application of liposomes as drug delivery system was the delivery of chemotherapeutic small molecules.[15] A large number of drugs are formulated into the liposomes taking advantage of high therapeutic efficiency and low systemic toxicity compared with the free drug. Anti-infective drugs and anti-tumor drugs are two major classes of small

molecules that can be loaded in liposomal formulations. After approval of liposomal systems for delivery of small molecules, delivery of macromolecules such as nucleic acid-based therapeutics (gene therapy agents) was noticed. Nucleic acid-based materials with high-molecular weight and highly charged molecules cannot cross cell membranes by passive diffusion. Moreover, applications of these materials as therapeutic agents are limited by their rapid enzymatic degradation, low selectivity, and poor cellular uptake. Lipoplex is a complex between negatively charged nucleic acid-based material and cationic liposome which can enter the cell *via* fusion with the plasma membrane. Thereby, gene therapy is performed through these liposomal formulations. Protein or peptide based therapeutic agents including; enzymes, peptide hormones, and cytokines are the other class of drugs that can be encapsulated in liposomes.[189,190] Incorporation of these agents into the liposomes resulted in some advantages such as improving therapeutic activity of protein and peptide drugs, reducing their side effects and modulating the immune response towards these proteins and peptides.[45]

Diagnostic imaging

Apart from the method used, in diagnostic imaging the appropriate intensity of signal from an area of interest is needed to differentiate specified structures from surrounding tissues. On the other hand, molecular imaging has an important role in diagnosis and treatment tracing of diseases. Liposomes can be targeted to specified disease tissues by combining with specific targeting ligands and imaging molecular probes. These probes are loaded with liposomes in four ways: (i) incorporating into the liposome during its formation, (ii) penetration into the lipid bilayer of preformed liposome, (iii) encapsulation into the preformed liposome *via* various active methods, and (iv) attaching on the surface of preformed liposome.[45,191,192]

Cosmetics

Liposomes have been considered in the delivery of ingredients in cosmetics due to their unique physicochemical properties. Incorporation of liposomes in cosmetic formulations has shown some advantages such as increasing in skin moisture, improving the cell membrane fluidity, and causing deep penetration of oil or water-soluble cosmetic ingredients through the skin. Liposomal cosmetics have been manufactured by famous company namely Christian Dior, for first time in 1987. After that, some other liposomal cosmetic formulations are reported and manufactured.[13,45]

Future prospective of liposomal formulations

Today, many approved liposomal and nano-liposomal products entered the commercial market. Some of these products are presented in Table 3.[193-196] However, there are huge challenges in their clinical translation. It seems that the most important field of research ahead is related to solving problems related to the targeted liposomal formulations. Many of these nanocarriers have shown more efficiency in animal and *in vitro* studies; however; a few of them have been entered to clinical trials, and there is not any investigation and evaluation guideline for active targeting liposomal formulations in cancer treatment.[20] To achieve the successful clinical use of these formulations, many problems have to be solved. It has been shown that a number of factors such as liposome size and charge, type and amount of ligand, the ligand binding with the serum proteins, and the elimination by the body immune system can influence on the function of targeted liposomes.[197-202] Owing to the excellent clinical potential of targeted liposomes to improve the therapeutic index of drugs, further research is required for their clinical applications.

Conclusion

Liposomes have been recognized as therapeutic carriers in very diverse clinical fields because of their unique physicochemical properties. They are the first nano-delivery systems that some of them are already successfully translated into the clinical use, and some liposomal formulations are approved or under clinical trials. Employing of liposomes as drug delivery systems provide a platform for delivering of drugs with reducing side effects and increasing their efficacy, solubility, and bioavailability. Despite the improvements made to these carriers to reduce their adverse effects and increase the therapeutic index of the cargo, investigations to fabricate the liposomes with fewer deficiencies are ongoing. A number of synthesis methods have been developed to obtain liposomes with various structure, size, and polydispersity. Ethanol injection technique is one of the interesting methods for scaling-up production of liposomes due to simplicity, fast implementation, and reproducibility. To overcome some practical challenges such as precise process control, poor reproducibility, and inefficient use of materials and reagents novel strategies such as microfluidic and SCF based methods have been designed for preparation of liposomes. Microfluidic is effective reproducible method for scale-up of liposomes owing to achieve more control over the physical properties including; size distribution, lamellarity, and high encapsulation efficiency. Moreover, solvent free and pharmaceutical grade liposomes having a narrow particle size distribution can be produced by SCF method. Fabrication of the PEGylated liposomes is the important modification to solve the problem of uptake by RES and rapid clearance from bloodstream. However, the selective delivery of these liposomes to the action site is limited. Today, research in fabrication of stimuli-sensitive and functionalized liposomes are two forefront fields to increase of their target specificity. Besides, to overcome to the passive loading drawbacks

Table 3. List of some approved liposomal drugs

Trade name	Chemical name	Clinical uses	Ref.
Ambisome	Amphotericin B	Sever fungal infections	26
DaunoXome	Daunorubicin	Kaposi's sarcoma	26
Myocet	Doxorubicin	Breast cancer	13
DepoDur	Morphine	Pain following surgery	13
DepoCyt	Cytarabine	Neoplastic meningitis, lymphomatous meningitis	13
Visudyne	Vertporfin	Macular degeneration, pathologic myopia, ocular histoplasmosis	170
Abelcet	Amphotericin B	Sever fungal infections	26
Amphotec	Amphotericin B	Sever fungal infections	26
Doxil	Doxorubicin	Kaposi's sarcoma, ovarian and breast cancer	24
Onivyde	Irinotecan	Pancreatic cancer	13
ELA-Max	Lidocaine	Skin diseases	144
Newcastle	Disease vaccine	Chicken	144
Epaxal	A vaccine	Hepatitis	144
Lipodox	Doxorubicin	Anti-cancer	170
Marqibo	Vincristine	Acute lymphoblastic leukemia	13
EvacetTM	Doxorubicin	Metastatic breast cancer	203
Avian retrovirus vaccine	Killed avian retrovirus	Chicken pox	203
Novasome®	Smallpox vaccine	Smallpox	203
Topex-Br	Terbutaline sulphate	Asthma	203
Alectm	Dry protein free powder of DPPC-PG	Expanding lung diseases in babies	203
Ventustm	Prostaglandin-E1	Systemic inflammatory diseases	203
Fungizone®	Amphotericin-B	Fungal infections, Leishmaniasis	203
VincaXome	Vincristine	Solid Tumors	204
Autragen	Tretinoin	Kaposi's sarcoma	204
Shigella Flexneri 2A vaccine	Shigella flexneri 2A	Shigella flexneri 2A infections	204
Nyotran	Nystatin	Systemic fungal infections	204

such as low entrapment efficiency, non-loaded drug loss, and fast release of drug, several active loading methods have been developed. Furthermore, in order to enhance internalization of liposomes by the specified tissues, their surfaces can be modified with targeting ligands such as transferrin, integrins, polysaccharides, folic acid, aptamers and antibodies. However surely, liposomes have found their place in the modern pharmaceutics and their use is increasing day by day. Nowadays, many liposomal anti-cancer drugs have been used in the treatment of breast, ovarian cancers, and sarcoma. Due to the potential clinical applications of liposomes, challenges such as therapeutic and loading efficiency, stability, and scale up of industrial production with more clinical success, needs further investigation.

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Ethical Issues
Not applicable.

Conflict of Interest
The authors declare that they have no competing interests.

Supplementary Files
Supplementary file 1 contains Figures S1-S5.

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