CHAPTER 9

Liposomes in Biology and Medicine

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Abstract

Drug delivery systems (DDS) have become important tools for the specific delivery of a large number of drug molecules. Since their discovery in the 1960s liposomes were recognized as models to study biological membranes and as versatile DDS of both hydrophilic and lipophilic molecules. Liposomes—nanosized unilamellar phospholipid bilayer vesicles—undoubtedly represent the most extensively studied and advanced drug delivery vehicles. After a long period of research and development efforts, liposome-formulated drugs have now entered the clinics to treat cancer and systemic or local fungal infections, mainly because they are biologically inert and biocompatible and practically do not cause unwanted toxic or antigenic reactions. A novel, up-coming and promising therapy approach for the treatment of solid tumors is the depletion of macrophages, particularly tumor associated macrophages with bisphosphonate-containing liposomes. In the advent of the use of genetic material as therapeutic molecules the development of delivery systems to target such novel drug molecules to cells or to target organs becomes increasingly important. Liposomes, in particular lipid-DNA complexes termed lipoplexes, compete successfully with viral gene transfection systems in this field of application. Future DDS will mostly be based on protein, peptide and DNA therapeutics and their next generation analogs and derivatives. Due to their versatility and vast body of known properties liposome-based formulations will continue to occupy a leading role among the large selection of emerging DDS.

State of the Art of Nanosized Drug Delivery Systems

The first microencapsulated drugs were introduced in the 1950s and polymer based slow release systems appeared shortly thereafter. Soon after their discovery in the 1960s by A.D. Bangham and colleagues, liposomes—phospholipid bilayer nanocontainers with spherical shape properties—were recognized as potential drug delivery systems (DDS).[1,2] Since then a tremendous amount of work on applications of liposomes has been accomplished. Due to their versatility nanosized small unilamellar liposomes are used as models to study biological and biophysical membrane properties and as carriers of drugs for therapeutic applications. Liposomes undoubtedly represent today the most extensively and advanced drug delivery vehicles. Liposome-formulated drugs have entered the clinics to treat cancer and systemic or local fungal infections, mainly because they are biologically inert, biocompatible and practically do not cause unwanted toxic or antigenic reactions and, most importantly, industrial large-scale production of liposome formulated drugs has allowed their advance in the pharmaceutical industry.[3-5]

In the advent of the use of genetic material (DNA, ribozymes, DNAzymes, aptamers, (antisense-) oligonucleotides, small interfering RNAs) as therapeutic molecules the development of delivery systems to target these molecules to cells or to target organs becomes increasingly

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Bio-Applications of Nanoparticles, edited by Warren C.W. Chan. ©2007 Landes Bioscience and Springer Science+Business Media.

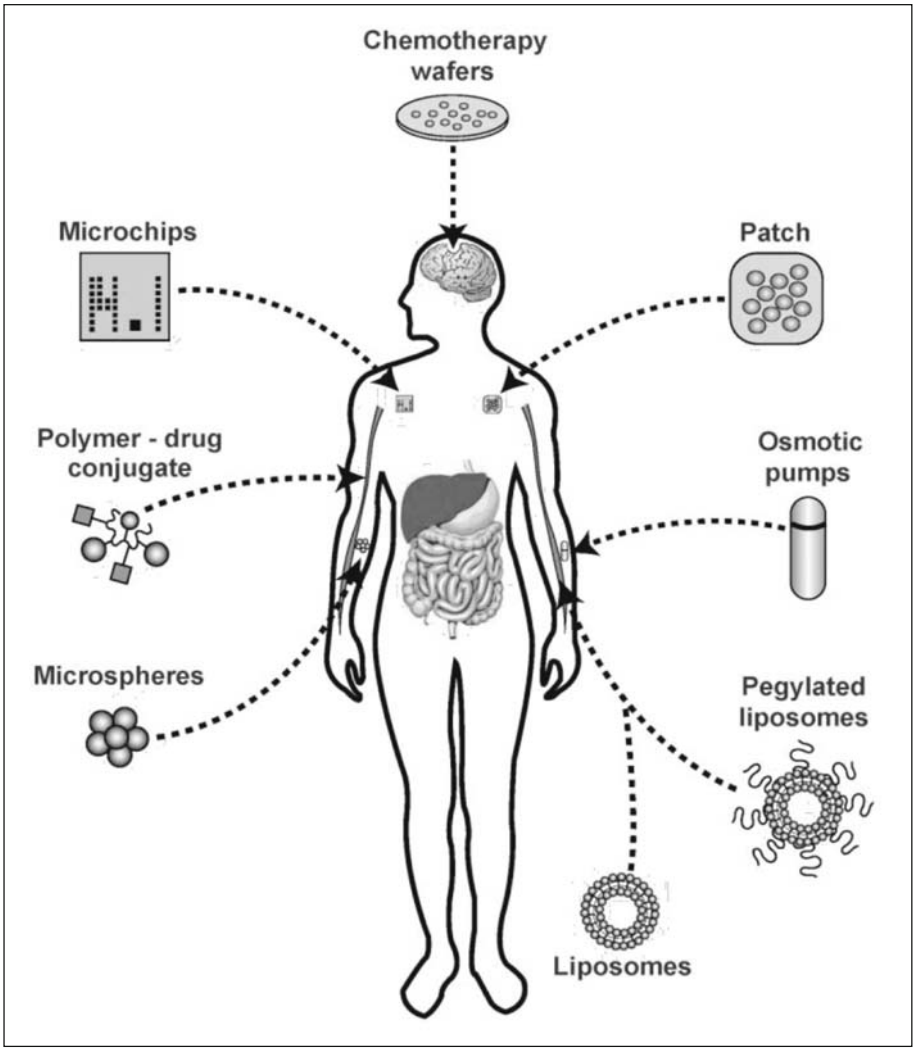

Figure 1. Modern drug delivery systems. There are a variety of different delivery strategies that are either currently being used or are in the testing stage to treat human cancers and other diseases. Examples of these include polymer microspheres, polymer wafers, osmotic pumps, liposomal systems, polymer/drug targeting moiety conjugates, and controlled release microchips. Reprinted from: Moses MA et al. Cancer Cell 4:337-341; ©2003, with permission from Elsevier.[12]

important. In this field the liposomes, in particular lipid-DNA complexes termed lipoplexes (see below), compete with viral gene transfection systems. Nanoparticles, nanospheres, polymersomes, nanogels, micelles, dendrimers, and virosomes are other main types of nanocarrier systems used for drug delivery.[6-11] As schematically shown in Figure 1, modern DDS including polymer-drug conjugates, liposomes, osmotic pumps, microchips, wafers, transdermal patches and other systems vary in their concepts, compositions, shapes, sizes, drug loading capacity as well as in their pharmacokinetic and organ distribution properties.[12] All DDS, however, pursue the aim of improving drug delivery for the benefit of the patient.

The major applications of DDS comprise drugs that possess nonideal physico-chemical and pharmacological properties such as (1) Poor solubility; (2) Tissue damage caused by unintentional extravasation of drugs; (3) Loss of drug activity following administration; (4) Unfavorable pharmacokinetic properties and poor biodistribution and (5) Lack of selectivity for target organs or tissues. Systemic drug distribution may cause toxic side effects and low drug concentrations at target tissues; this could lead to suboptimal therapeutic effects.

The formulation of pharmacologically active drug molecules in DDS can improve or abolish these unfavorable properties. However, there are also disadvantages in the development of particulate drug carriers, such as system complexity, unwanted biologic and immunologic effects, stability, costs of development and scale-up, as well as intellectual property issues. In the limited format of this chapter it is not possible to cover all methods and references from the vast field of liposome technology. Hence, we concentrate on summarizing use and properties of liposomes as DDS for the delivery of cytotoxic molecules for cancer therapy.

Evolution of Liposomes in Cancer Therapy

Liposomes have become known as one of the most versatile tools for the delivery of pharmacologically active molecules. Since their discovery in the 1970s their potential for the delivery of cytotoxic drugs in cancer therapy has been recognized.[4,5,11,13,14]

Liposomes are spherical vesicles that consist of an aqueous compartment enclosed by a phospholipid bilayer. If multiple bilayers of lipids are formed around a primary core, the structures that are generated are termed multilamellar vesicles (MLVs). MLVs are formed spontaneously upon reconstitution of dry lipid films in aqueous media. Small (nanosized) unilamellar vesicles (SUVs) of scaleable mean diameters of 20 to 500 nanometers are produced by high pressure extrusion of MLVs through polycarbonate membranes. SUVs are also obtained by ultrasonication, by detergent dialysis and by many other, less important methods. Hydrophilic and hydrophobic drugs can both be entrapped in liposomes. Since the composition of the liposome bilayers can be varied with a huge selection of different phospholipids and additional intercalating molecules, liposomal delivery systems are of high versatility and customized formulations can easily be engineered to obtain desired sizes, surface charge, membrane composition and morphology providing them with high versatility such as long circulation half-life, sustained and targeted drug delivery or diagnostic imaging properties.[11,15-19]

As schematically shown in Figure 2, the liposomes evolved from rather simple compositions (Fig. 2A,B) to highly sophisticated multi-component systems. The state-of-the-art liposomes used for parenteral drug delivery are the long circulating ("stealth") liposomes (Fig. 2C-E). Stealth liposomes are sterically stabilized formulations that include polyethylene glycol (PEG)-conjugated lipids or other hydrophilic coating molecules. The surface grafted polymers create an impermeable, highly hydrophilic layer on the outer liposome surface. The prominent properties of long-circulating liposomes are dose-independent, nonsaturable, log-linear pharmacokinetics and increased bioavailability. Pegylation prevents or retards opsonization and recognition of the liposomal vesicles by the monocytic phagocyte system (MPS).[11] Due to their long circulation time in blood and the enhanced drug permeability and retention effect in tumor tissues, PEG-liposomes accumulate at high concentrations (up to 10% of the injected dose per organ) in tumors.[20-24]

Immunoliposomes (Fig. 2B,D) are complex drug or gene delivery systems that are developed for specific cell targeting by attachment of functionalized antibodies or antibody fragments to the outer surface of the liposomes. The modern immunoliposomes are PEG-liposomes to which receptor specific molecules are attached, preferably at the distal tips of the PEG chains (Fig. 2E,J). Immunoliposomes target cell specific receptors and facilitate receptor-mediated endocytosis for cell uptake.[25]

A variety of tumor-specific antibodies have been used for targeting of liposomes to tumor cells or molecules located in the tumor stroma. In earlier studies whole IgG antibodies were linked to the liposome surface by various coupling methods.[26] Today the most advanced

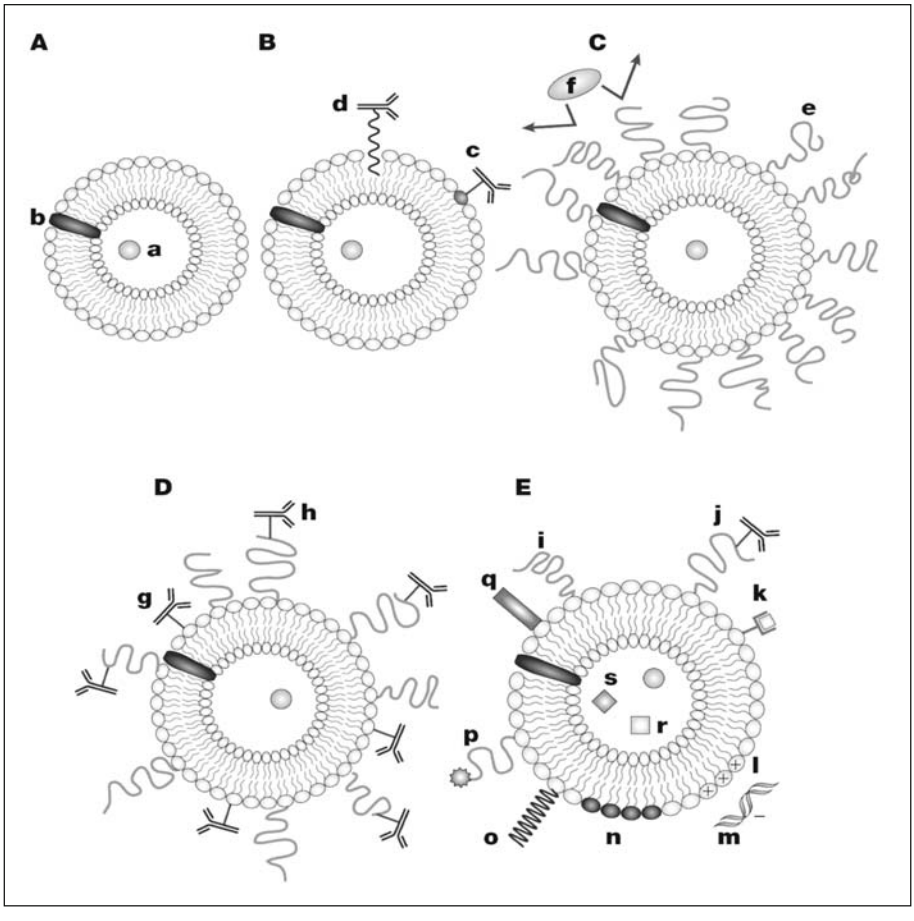

Figure 2. Evolution of liposomes. A) Early traditional 'plain' liposomes with water soluble drug (a) entrapped into the aqueous liposome interior, and lipophilic drug (b) incorporated into the liposomal membrane. B) Antibody-targeted immunoliposome with antibody covalently coupled (c) to the reactive phospholipids in the membrane, or hydrophobically anchored (d) into the liposomal membrane after preliminary modification with a hydrophobic moiety. C) Long-circulating liposome grafted with a protective polymer (e) such as PEG, which shields the liposome surface from the interaction with opsonizing proteins (f). D) Long-circulating immunoliposome simultaneously bearing both protective polymer and antibody, which can be attached to the liposome surface (g) or, preferably, to the distal end of the grafted polymeric chain (h). E) New-generation liposome, the surface of which can be modified (separately or simultaneously) by different ways. Among these modifications are: the attachment of protective polymer (i) or protective polymer and targeting ligand, such as antibody (j); the attachment/incorporation of a diagnostic label (k); the incorporation of positively charged lipids (l) allowing for the complexation with DNA yielding lipoplex structures (m); the incorporation of stimuli-sensitive lipids (n); the attachment of a stimuli-sensitive polymer (o); the attachment of a cell-penetrating peptide (p); the incorporation of viral components (q). In addition to a drug, liposomes can be loaded with magnetic particles (r) for magnetic targeting and/or with colloidal gold, silver particles or fluorescent molecules (s) for microscopic analysis. Adapted from: Torchilin VP. Nat Rev Drug Discov 4:145-160; ©2005, with permission from Nature Publishing Group.[11]

immunoliposomes are the anti-p185/HER2 liposomes that target the herceptin receptor which is over-expressed in various cancers, especially breast cancer. Long-circulating immunoliposomes targeted to HER2 (ErbB2, Neu) have been prepared by conjugation of anti-HER2 MAb fragments (Fab' or single chain Fv, scFv) to liposome-grafted polyethylene glycol chains. MAb fragment conjugation did not affect the biodistribution or long-circulating properties of i.v.-administered liposomes.[27-29] The epidermal growth factor receptor (EGFR) is another target for immunoliposomes that bind to and internalize in tumor cells that over-express EGFR. Anti-EGFR immunoliposomes have been constructed modularly with Fab' fragments of the antibody cetuximab.[30-32] A large number of antibodies directed against other target molecules expressed on colon,[33] B-cell lymphoma,[34] and neuroblastoma[35] tumors have been used for the preparation of immunoliposomes. In order to target the ED-B isoform of fibronectin, which is exclusively expressed in the extracellular matrix of solid tumors, we constructed immunoliposomes decorated with scFv antibody fragments directed against ED-B fibronectin and successfully used these DDS for targeted delivery of cytotoxic drugs into tumors in vivo.[36] We also developed specific antibodies and immunoliposomes for specific targeting of tumor endothelial marker (TEM1) and the vascular endothelial growth factor receptor-2 (VEGFR-2).[37,38] Tissue-specific gene delivery using immunoliposomes has also been achieved with folate[39,40] and transferrin[41] receptor specific immunoliposomes. Additionally, tumor vasculature targeted immunoliposome therapy was shown to be effective with liposomal doxorubicin.[42-44]

Various cell uptake mechanisms for liposomes have been described.[8,45] Due to their particulate properties, phagocytic uptake mechanisms (phagocytose, endocytose, pinocytose) are predominant, however cell membrane adhesion and fusion can also occur. In the phagocytic uptake pathway liposomes are captured at the cell surface followed by endosomal and lysosomal uptake. Drug liberation into the cytoplasm depends on the lipid composition of the liposomes. To release encapsulated material into the cytoplasm of a cell, pH-sensitive liposomes can be generated by addition of dioleylphosphatidyl-ethanolamine (DOPE) to liposomes composed of acidic lipids such as cholesterylhemisuccinate (CHEMS) or oleic acid and other lipids. At a pH of 7, these lipids possess the typical bilayer structure; however, upon endosomal compartmentalization (pH becomes more acidic) they undergo protonation and collapse into nonbilayer structures. This leads to the disruption and destabilization of the endosomal membrane, which in turn promotes rapid release of encapsulated molecules into the cytoplasm.[8]

Cell penetrating peptides (CPPs) have proven to be efficient intracellular delivery systems overcoming the lipophilic barrier of cell membranes. CPPs can deliver a wide range of large cargo molecules such as proteins, peptides, oligonucleotides and even small nanoparticles as liposomes to a variety of cell types and to different cellular compartments. The CPPs are basic, lysine- or arginine- rich amphipathic peptides originating from different sources. CPPs can either form complexes with many different types of molecules (peptides, proteins, plasmids, oligonucleotides, siRNA, dyes etc.) or they can be covalently linked to these cargo molecules.[46,47] Liposomes have also been decorated with the TAT[48] or pAntp CPPs,[49] demonstrating higher cell uptake rates in vitro. Regrettably, their usefulness as drug delivery systems is hampered by their ability to penetrate virtually any cell type both in vitro and in vivo in a nonspecific mode. This feature complicates CPP applications as target specific drug delivery systems; therefore therapeutic applications seem unlikely, unless their target cell specificity can be significantly improved.

Liposomes as Carriers of Lipophilic and Amphiphilic Nucleoside Analogs

The majority of applications of liposomes as therapeutic DDS are based on the encapsulation of water soluble cytotoxic molecules within the trapped aqueous volume of the liposomes. Liposomes loaded with cytotoxic anti-tumor drugs doxorubicine, mitoxantrone, topotecan, irinotecan and cytarabine are examples of clinically applied chemotherapeutic liposome

formulations.[5,11,15,16,22,50-55] For a current summary of clinically used liposomal anti-cancer formulations (see ref. 22). In contrast to the extensive exploitation of the trapped aqueous volume of the liposomes that serves as nanocontainer for water soluble molecules, the phospholipid bilayer has not been given the same attention for its use as carrier matrix for lipophilic drugs. Hence, the development of liposomal drug formulations with lipophilic drugs is less popular. This difference may have several reasons with the main reason being that the chemistry required to transform water soluble molecules into lipophilic compounds allowing incorporation into the lipid bilayer core is difficult. The most favorable chemical modifications consist in the attachment of long chain fatty acyl or alkyl residues, for example saturated or unsaturated fatty acids, preferably palmitic or stearic acid and alkylamines, preferably hexadecyl- or octadecylamine to a suitable functional group of the hydrophilic part of the drug molecule. Some recent examples of lipophilic modifications of antitumor drugs and their formulation in liposomes are gemcitabine, 5-iodo-2'-deoxyuridine, methotrexate, paclitaxel and a lipophilic topoisomerase inhibitor.[56-62]

Drugs that are highly lipophilic by their own nature, e.g., taxanes and epothilones can only be used therapeutically by addition of possibly toxic solubilizing agents (e.g., Cremophor EL) in complex pharmaceutical formulations.[63,64] One of several feasible means of obtaining nontoxic parenterally applicable formulations of such drugs is their incorporation into the bilayer matrix of phospholipid liposomes.[65]

Nucleoside analogs are a major class of chemotherapeutic agents for the treatment of cancer and viral diseases. Natural endogenous nucleosides must be phosphorylated to corresponding 5'-triphosphates in order to be incorporated into the DNA or RNA synthesised within the cell. Nucleoside analogs are in essence prodrugs since they must undergo the same transformations in the cytoplasm similarly to the natural nucleosides before becoming active. We chose the approach of chemical transformation of water-soluble nucleosides of known cytotoxic and antiviral properties into lipophilic drugs or prodrugs, thus reversing the paradigm of transforming lipophilic molecules into hydrophilic derivatives. The first cytotoxic nucleoside we chose is 1-β-D-arabinofuranosyl cytosine (ara-C) because its major clinical disadvantages are a very short plasma half-life and rapid inactivation. To reduce these limitations, a large number of 5'- and N^4-substituted ara-C derivatives have been synthesized and characterized in the past (reviewed in ref. 66). Of a series of N^4-alkyl-ara-C derivatives with alkyl chain lengths ranging between 6 and 22 C-atoms, N^4-octadecyl-ara-C (NOAC) exerted the strongest anti-tumor activity after oral and parenteral therapy in several mouse tumor models and showed to have distinct pharmacological properties compared to ara-C.[67,68] Of note, (ref. 69) provides an excellent review of the topic.

Consequently, we further modified NOAC by the synthesis of a new generation of lipophilic/amphiphilic heterodinucleoside phosphate derivatives, termed "duplex drugs" that combine the clinically used antitumor drugs ara-C and 5-fluorodeoxyuridine (5-FdU) with NOAC to the heterodinucleoside phosphates arabinocytidylyl-N^4-octadecyl-1-β-D-arabinofuranosyl cytosine (ara-C-NOAC) and 2'-deoxy-5-fluorouridylyl-N^4-octadecyl-1-β-D-arabinofuranosyl cytosine (5-FdU-NOAC).[70,71] Ethynylcytidine (1-(3-C-ethynyl-β-D-ribo-pentafuranosyl)-cytosine, ETC) is a novel nucleoside that was found to be highly cytotoxic.[72] Its combination with NOAC yields the lipophilic duplex drug ETC-NOAC (3'-C-ethynylcytidylyl-(5'→5')-N^4-octadecyl-1-β-D-arabinofuranosyl cytosine). Due to the combination of the effects of both active molecules that can be released into the cytoplasm as monomers or as the corresponding monophosphates (MP), the cytotoxic activity of the duplex drugs is expected to be more pronounced as compared to the monomeric drugs. Further, it can be anticipated that the monophosphorylated nucleosides ara-CMP, 5-FdU-MP and ETC-MP, respectively, are directly released in the cell after enzymatic cleavage of the parent drugs. Thus, monophosphorylated molecules do not need to pass the first phosphorylation step, which is known to be rate limiting.[73,74] The lipophilic side chains warrant a stable incorporation of

these duplex drugs into liposomes, allowing the exploitation of the liposome formulations advantages.

We conclude that the chemical modification of water-soluble molecules by attachment of long lipophilic chains and their stable incorporation into bilayer membranes of small unilamellar liposomes represent very promising examples of taking advantage of the high loading capacity lipid bilayers offer for lipophilic drugs. The combination of chemical modifications of water soluble drugs with their pharmaceutical formulation in liposomes is a valuable method for the development of novel pharmaceutical preparations not only for the treatment of tumors or infectious diseases, but also for many other disorders.

Liposome-Mediated Depletion of Tumor Associated Macrophages

The physical depletion of macrophages located in organs of the monocytic phagocyte system (MPS; spleen, liver, lymph nodes, bone marrow) by liposome encapsulated clodronate (clodrolip) has become an important and reliable method to study the roles of macrophages in the immune system and in inflammatory processes.[75-78] Even though the infiltration of macrophages into solid tumors and their pro-tumorigenic function has been described three decades ago, their use as potential therapeutical targets is only now being discussed.[79-82] Tumor cells shed chemokines that attract macrophages from the peripheral circulation. These macrophages infiltrate the stroma of solid tumors and accumulate in hypoxic tumor tissue. Tumor associated macrophages (TAMs) play a pivotal role in tumor growth and metastasis by promoting tumor angiogenesis. Recently, we have investigated whether the depletion of TAMs would inhibit tumor angiogenesis and consequently tumor growth. We show that TAM depletion mediated by clodrolip inhibits tumor growth, presumably through blocking tumor angiogenesis and promoting tumor cell starving. Clodrolip are liposomes containing the drug dichloromethylenebisphosphonic acid (also known as clodronate). In our experiments, tumor bearing mice were treated with clodrolip as single therapy in comparison to free clodronate and in combination with anti-VEGF single chain fragment antibodies,

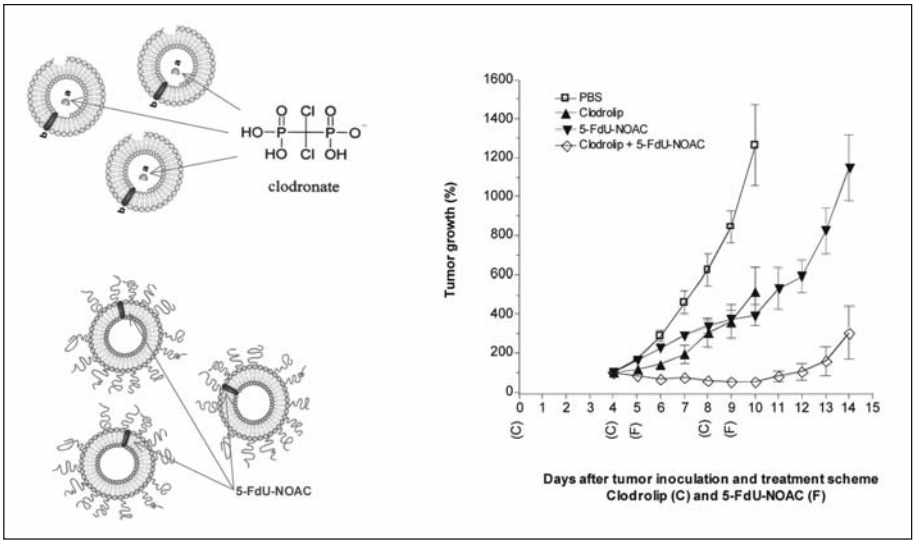

Figure 3. Depletion of tumor associated macrophages in combination with liposomal chemotherapy. Treatment of F9 teratocarcinoma tumors in syngeneic Sv129 mice, either with clodronate in plain liposomes (clodrolip) and 5-FdU-NOAC in pegylated long circulating liposomes alone (black triangles) or in combination (open diamonds). Phosphate buffer (PBS) treated controls are shown with open squares. The experiment was performed as described in reference 36.

resulting in drastic tumor growth inhibition and exhaustion of TAM cell populations.[83] In a representative experiment shown in Figure 3 we treated mice bearing syngeneic F9 teratocarcinoma tumors with clodronate and the lipophilic heterodinucleotide duplex drug 5-FdU-NOAC, both applied in liposome formulations. Macrophage depletion combined with a cytotoxic therapy was highly effective in this tumor model. Based on our results we conclude that clodrolip mediated depletion of TAMs in concert with cytotoxic or anti-angiogenic treatment regimens represents a new and highly effective therapeutic modality for the treatment of solid tumors and prevention of metastasis. Further, this is an interesting tool for the study of macrophage function in solid tumors.

Cationic Liposomes and Lipoplexes as DNA Delivery Systems

Liposomes can be used as DNA drug delivery systems either by entrapping DNA-based therapeutics inside the aqueous liposome core or by complexing them to positively charged lipids (lipoplexes, see below). Liposomes offer significant advantages over viral delivery systems. They are generally nonimmunogenic because of the absence of protein components. Liposome encapsulated DNA molecules are protected from nuclease activity for enhanced biological stability. Cationic polymers have an enormous potential for DNA complexation and have shown to be useful as nonviral vectors for gene therapy applications. In past years, liposomes composed of cationic lipids, termed lipoplexes, have routinely been utilized for the delivery of nucleic acids such as plasmids, oligodeoxynucleotides and siRNA to cells in culture and in vivo. A large number of these reagents are commercially available or can be formulated in the laboratory.[7-9,84-91] The majority of cationic lipid-DNA complexes form a multilayered structure with DNA molecules intercalated between the cationic lipids. An inverted hexagonal structure with single DNA strands encapsulated in lipid tubules is observed rarely.[92] Together with other advantages the lipoplexes have the ability to transfer very large genes into cells. However, the understanding of their mechanisms of action is still incomplete and their cell transfection efficiencies remain low compared to those of viruses. Despite the appreciable success of cationic lipids in gene transfer, toxicity is a main issue for both in vitro and in vivo applications. Inflammatory toxicity represents a typical effect associated with systemic administration of lipoplexes. Recent results indicate that lipoplex gene delivery systems mediate uptake of plasmid DNA by the liver, mainly by the phagocytic Kupffer cells, in which a large amount of cytokines is produced.[93] In addition, these complexes are immunostimulatory, a property that may either be harmful or beneficial. Another disadvantageous property of lipoplex mediated gene transfer is the low transfection efficiency; this has been attributed to the heterogeneity and instability of the lipoplex formulations. Lipoplex size heterogeneity also adversely affects their quality control, scale-up, and long-term shelf stability, which are important issues for pharmaceutical development. Another unwanted property of cationic lipids is the rapid inactivation of their cargo in the presence of serum proteins.[94] Development of optimized cationic lipids that are safe to use for in vivo applications is an ongoing process. A cautionary note to the potential dangers of all viral gene products, transgenes, viral proteins and peptides and CpG DNA sequences in siRNA or plasmids formulated in liposomes or other DDS has to be given. Immune responses induced by these molecules may lead to problems such as transient gene expression, nonefficient readministration of the same vectors and to severe side-effects in clinical trials.[95] Due to their particulate nature, the DDS are recognized as foreign and thus elicit immune reactions of the host organism. However, the immunomodulating activities of the DDS depend largely on their composition, size and homogeneity. Synthetic polymers can exhibit significant immunomodulatory activity, whereas liposomes prepared with natural phospholipids and cholesterol are known to be less immunogenic.

Outlook and Future Directions

The development of DDS is an ongoing challenging venture that combines multidisciplinary research efforts in various areas including bioengineering, nanotechnology,

biomaterials, pharmaceutics, biochemistry, and cell and molecular biology. Specific characteristics of pathological processes and cell or tissue types that are subject of therapeutic interventions govern the path from target selection to the development of specific DDS formulations. The identification of novel cellular targets, for example easily accessible vascular endothelial cells, in contrast to tumor cells or other less targetable tissues, will lead to optimized pharmaceutical drug delivery formulations and preparation technologies. Refinement of DDS in order to overcome unwanted properties such as toxicity, nonspecific tissue distribution and uncontrolled release of entrapped active molecules will be the major challenges in the field. Future DDS will mostly be based on protein, peptide and DNA therapeutics and their next generation analogs and derivatives. Liposome-based formulations will continue to occupy a leading role among the large selection of emerging DDS due to their versatility and vast body of known properties.

Acknowledgements

The author wishes to acknowledge the contributions of Herbert Schott, Cornelia Marty and Steffen Zeisberger.

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