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Review

Liposomal drug delivery, a novel approach: PLARosomes★✪

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Almost from the time of their rediscovery in the 60's and the demonstration of their entrapment potential, liposomal vesicles have drawn attention of researchers as potential carriers of various bioactive molecules that could be used for therapeutic applications in humans and animals. Several commercial liposome-based drugs have already been discovered, registered and introduced with great success on the pharmaceutical market. However, further studies, focusing on the elaboration of more efficient and stable amphiphile-based vesicular (or non-viral) drug carriers are still under investigation. In this review we present the achievements of our group in this field. We have discovered that natural amphiphilic dihydroxyphenols and their semisynthetic derivatives are promising additives to liposomal lipid compositions. The presence of these compounds in lipid composition enhances liposomal drug encapsulation, reduces the amount of the lipid carrier necessary for efficient entrapment of anthracycline drugs by a factor of two, stabilizes liposomal formulation of the drug (both in suspension and in a lyophilized powder), does not influence liposomal fate in the blood circulation system and benefits from other biological activities of their resorcinolic lipid modifiers.

Although the formation of vesicular structures from lecithin upon its hydration was demonstrated by Lehmann at the beginning of the passing century (for a review see [1]), their potential applications were fully recognized only in the mid 60's by Bangham and his coworkers [2]. This group of researchers demonstrated that those particles, produced upon

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★75th Anniversary of Membrane Lipid Bilayer Concept.

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Abbreviations: AR, alkylresorcinol; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; MSAR, *O*-myristoyl-*O′*-sulphoalkylresorcinol; PLARosomes, phospholipid-alkylresorcinol liposomes; SUV, small unilamellar vesicles.

swelling of a lipid film in water during agitation, sequestered part of the solution (and the solutes in it) into their interior and, what is more important, that they are surrounded by the lipid layer that functions as a permeability barrier [2]. This barrier made their properties similar to osmometers and isolated cells. These structures were finally named liposomes, in Greek "fat bodies". Since this discovery, two main streams of studies have been created, basic studies on liposomes as model membranes and research on the practical application of these structures in various aspects of human life. This second stream of studies resulted in the development of numerous small high-tech liposome-oriented pharmaceutical companies. As a result, there are already several commercially available pharmaceutical products based on drug-in-liposome formulations. Most of them concern anticancer drugs that, administered in their free form, are toxic or exhibit serious side-effects and their encapsulation into liposomal vesicles significantly diminishes these unwanted properties. In such cases, liposomes serve as a reservoir for the drug. The milestones in liposome technology were the development and introduction on the market in 1995–1997 of the liposome-based drugs: DAUNOXOME®, DOXIL® and AmBisome®. The accumulation of many novel experiences in the practical aspects of liposomes, together with new developments in basic research, will bring the field of liposome biotechnology to the place it deserves in the future.

LIPOSOMES AS DRUG DELIVERY SYSTEMS

Liposomal vesicles were prepared in the early years of their history from various lipid classes identical to those present in most biological membranes. Basic studies on liposomal vesicles resulted in numerous methods of their preparation and characterization. Liposomes are broadly defined as lipid bilayers surrounding an aqueous space. Multilamellar vesicles (MLV) consist of several (up to 14) lipid layers (in an onion-like arrangement) separated from one another by a layer of aqueous solution. These vesicles are over several hundred nanometers in diameter. Small unilamellar vesicles (SUV) are surrounded by a single lipid layer and are 25–50 nm (according to some authors up to 100 nm) in diameter. Large unilamellar vesicles (LUV) are, in fact, a very heterogenous group of vesicles that, like the SUVs, are surrounded by a single lipid layer. The diameter of these liposomes is very broad, from 100 nm up to cell size (giant vesicles) [3]. Besides the technique used for their formation the lipid composition of liposomes is also, in most cases, very important. For some bioactive compounds the presence of net charged lipids not only prevents spontaneous aggregation of liposomes but also determines the effectiveness of the entrapment of the solute into the liposomal vesicles. Natural lipids, particularly those, with aliphatic chains attached to the backbone by means of ester or amide bonds (phospholipids, sphingolipids and glycolipids) are often subject to the action of various hydrolytic (lipolytic) enzymes when injected into the animal or human body. These enzymes cleave off acyl chains and the resulting lysolipids have destabilising properties for the lipid layer and cause the release of the entrapped bioactive component(s). As a result new types of vesicles, that should merely bear the name of liposomes as their components are lipids only by similarity of their properties to natural (phospho)lipids, have been elaborated. These vesicles, still named liposomes, are made of various amphiphile molecules (the list of components is long). The crucial feature of these molecules is that upon hydration they are able to form aggregation structures resembling an array and have properties of natural phospholipid bilayers. Among such molecules, various amphiphiles have been employed, such as ether lipids [4–7], fluorinated lipid [8–10], synthetic dou-

ble chain amphiphiles [11] as well as single chain amphiphiles, such as N-alkylindoles [12], polyhydroxyl lipids [13], polyhedral non-ionic surfactants [11, 14–16], polymerized liposomes [17, 18], cationic amphiphiles [16], plasmalogens [19] and others [20–27]. This resulted in various new formulations of vesicle compositions and new names given to them, such as niosomes, lederosomes, archeosomes, etc.

The common feature of classical liposomes, i.e., made preferentially of phospholipids, and of vesicles made of amphiphilic molecules, was their ability to form dynamic lamellar structures with barrier properties separating the interior of the vesicles from the outside medium. One may conclude that, at present, the term “liposomes” covers not only phospholipid-based vesicles but also other vesicular structures with properties identical or similar to those of classical, natural phospholipid based liposomes.

In the early 70’s the use of liposomes as a drug carrier system was proposed by Gregoriadis & Ryman [28]. Since this first report, liposomes were developed as an advanced drug delivery vehicle. They are generally considered non-toxic, biodegradable and non-immunogenic. Associating a drug with liposomes markedly changes its pharmacokinetics and lowers systemic toxicity; furthermore, the drug is prevented from early degradation and/or inactivation after introduction to the target organism [29–34].

The use of liposomes or, in general, vesicular structures for the delivery of various active compounds is recognized in relation to water solubility of the compound. When the compound is water soluble, the size and volume of the aqueous compartment of the vesicle is crucial. In contrast, hydrophobic compounds will prefer incorporation into the lipid (amphiphile) layer that constructs the vesicle. In such a case, the size of the aqueous compartment is not important. Depending on the need, one can use SUV type or MLV type vesicles for effective entrapment and delivery of the drug to the target tissues or cells [35, 36]. Nevertheless, charge properties and interactions of the active compound with vesicle forming molecules will determine the effectivity of entrapment, i.e., the amount of the compound that can be “loaded” into a single vesicle [37–40]. On the other hand, the composition of the molecules used for the formation of the vesicular structure will, at least, affect the fate of vesicles from the site of their introduction as well as the interaction with components of the body (e.g., surface charge [37–39], serum proteins, lipoproteins, opsonin system [40, 41], phagocytic system [42] and finally target cells [41, 43, 44]). In the earlier studies, when therapeutically active substance were not easily available, most of the experiments were done using a marker compound. The results, however, were not the same as those obtained in experiments in which an active substance was used and the conditions were more related to the real situation (*ex vivo, in vivo*). These findings implicate the necessity for studies in which an active substance is used and the conditions of the experiments resemble, as closely as possible, those of therapeutic liposomal (vesicular) drug application.

The benefits of liposomal formulations were already demonstrated clinically and stimulate many laboratories (research and pharmaceutical) in their efforts to introduce new liposomal/vesicular drugs. These can be illustrated with the data presented in Table 1.

A NOVEL APPROACH TO LIPOSOMAL ANTHRACYCLINE DRUGS: PLARosomes

In contrast to the thoroughly studied application of various derivatives of lipid and synthetic amphiphiles for the encapsulation of various drugs [9, 12, 13, 15, 23, 45–50], the idea of modifying the vesicle’s lipid barrier/encapsulation properties by means of lipid layer modifying molecules is relatively

Table 1. Examples of drugs in liposomal formulations

Drug	Application	Commercial name	Composition of liposomes
Amikacin	Bacterial infections	MiKasome	HSPC/CH/DSPG
Adriamycin (doxorubicin)	Stomach cancer	–	DPPC/CH
Ampicilin	Listeria monocytogenes	–	CH/PC/PS 5:4:1 CH:DSPC:DPPG 10:10:1
Annamycin	Kaposi's sarcoma, Breast cancer, Leukemia	Annamycin	Liposomes
Amphotericin B	Systemic fungal infections	AmBisome	HSPC/CH/DSPG
All–*trans*-retinoic acid	Acute promyelocytic leukemia, Lymphoma, Prostate cancer	ATRAGEN	Liposomes
Muramyl dipeptide	Immunostimulator	–	DSPC/PS 1:1
1-β-D-Arabino-furanozidecytozine	Leukemia	–	SM/PC/CH 1:1:1
Ciprofloxacine	*Pseudomonas aeruginosa*	–	DPPC
Clodronate	Macrophage suppression	–	PC/CH
Cis-diaminodichloro-platinum(II)	Cancers	–	Liposomes
Cyclosporin	Immunosuppressor	–	PC/CH
Chloroquine	Malaria	–	PC/PG/CH 10:1:5
Cu/Zn superoxide dismutase	Antiinflammatory	–	Liposomes
Doxorubicin	Cancers	Doxil	HSPC/CH/PEG–DSPE
Doxorubicin	Breast cancer	EVACET	
Daunorubicin	Cancers	DaunoXome	DSPC/CH
Ganciclovir	Cytomegalovirus retinitis	–	Liposomes
Interleukin 2	Immunostimulant	–	DMPC
Leukotriene A4	Not estimated	–	PC/DCP/CH 7:2:1
Lipid A	Immunoadjuwant	–	Liposomes
Mitoxantron	Colon cancer	–	PC/Ch 7:1
Methotrexate	Cancers	–	DPPC/PI 18:2w/w
Nystatin	Systemic fungal infections	NYOTRAN	Liposomes
$Na_3(B_{20}H_{17}NH_3)$	Cancers	–	DSPC/CH
Pentostam	Leishmanioses	–	Niosomes
Platinum drugs e.g., cisplatin	Mezotelioma	PLATAR	Liposomes
Lurtotecan	Cancers	NX 211	Liposomes
Oligonucleotides against *c–myc*	Cancers	INXC–6295	Liposomes TCS
Prostaglandin E1	Anti-inflammatory	–	PC
Ribavirin	Herpes simplex	–	Liposomes
Streptosotocin	Lymphocyte activator	–	DMPC/CH 2:1
Suramin	Trypanosomes	–	DPPC

Muramyl tripeptide	Immunostimulant		Liposomes
Ether lipids	Non-small-cell lung cancer, Melanoma, Leukemia, Human prostate cancer	TLC ELL12	Liposomes
Vincristin	Cancers	VincaXome	DSPC/CH
Vincristin	Cancers, Lymphoma	Onco TCS	Liposomes TCS
Various drugs and contrasts	Diagnostics of various diseases	lipoMASC	PEG–liposomes

Abbreviations to Table 1: HSPC, hydrogenated soya phosphatidylcholine (hydrogenated soya lecithin); CH, cholesterol; DSPG, distearoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin; PI, phosphatidylinositol; DCP, dicetylphosphate; PEG-DSPE, polyethylene glycol-phosphatidylethanolamine derivative; liposomes TCS, commercial composition of liposome-forming lipids; PEG-liposomes, liposomes modified with components containing a PEG (polyethylene glycol); Liposomes, liposomes with composition not defined.

less exploited. Our research focusses on the biological properties of natural single chain amphiphilic compounds belonging to the group of non-isoprenoic phenolic lipids [51]. They are amphiphilic in nature due to the non-isoprenoid side chains attached to the hydroxybenzene ring and are believed to be derived from the polyketide (acetate) pathway, as for example 6-pentadecylsalicylic acid. Resorcinolic lipids, alternatively called alkylresorcinols or 5-alkylresorcinols that are derivatives of resorcinol or higher homologs of orcinol (1,3-dihydroxy-5-methylbenzene), are of interest from the biopharmacological, biomedical and biotechnological points of view. The occurrence in their molecules of a benzene ring with two hydrophilic OH attachments and long hydrocarbon chains (in most cases C15–C21) determines their ability to interact strongly with biological membranes and phospholipid bilayers.

We have demonstrated that these compounds act differentially upon lipid bilayer permeability depending on their localization [52]. When they were introduced into a suspension of liposomes made of phospholipids, they caused an increase of membrane permeability and the release of liposomal contents. On the other hand, when introduced into the lipid mixture prior to the formation of the liposomal structures, they induced stabilization of the liposomal membrane, similarly to the effect observed for cholesterol [52, 53]. Furthermore, an enhancement of liposomal entrapment and simultaneously a decrease of vesicle diameter were observed [52, 54, 55]. The entrapment of various markers (Patent Blue Violet, 5,6-carboxyfluorescein, calcein, potassium chromate) into liposomes made of phosphatidylcholine, phosphatidylethanolamine, their mixtures and mixtures of phosphatidylcholine/cholesterol varied significantly. It was dependent both on the amount of the modifier in the liposomal lipid mixture (at 10% of C15 resorcinolic lipid a twofold increase of captured volume was already noted) and on the length of the aliphatic chain. The most effective were 5-*n*-nonadecylresorcinol and resorcinolic lipids isolated from cereal bran with mean chain length of about C18. It should be pointed out that the similarity of the resorcinolic lipid composition between natural preparations was very high, as it has been determined. Liposomal vesicles obtained from phospholipid-resorcinolic lipids mixtures only by application of the freezing and thawing were stable and of mean diameter 250–280 nm. This stability is probably due to the inverted cone-like shape of a resorcinolic lipid molecule and the related increase of the bilayer curvature. Liposomes modified with resorcinolic lipids, are easily calibrated by extrusion through polycarbonate membranes and their size remains stable (within 25%) over weeks, both at 4°C and at room temperature. Modified liposomal vesicles composed of

lecithin, phosphatidylethanolamine or mixtures of these lipids were also stable with respect to the leakage of their contents and they did not differ significantly from phosphatidylcholine/cholesterol (50:50, molar ratio) liposomes. Generally, in terms of stability of size and contents, the resorcinolic lipids-modified liposomes behaved similarly to conventional phospholipid or phospholipid/cholesterol vesicles *in vitro* and *in vivo* [54]. A similar tendency of resorcinolic lipids was observed when sphingolipids (ceramides, sphingomyelin), cholesterol and free fatty acids (palmitic and linoleic) containing liposomes were studied [56].

In another approach, we have demonstrated that resorcinolic lipids at high pH form, alone or in mixtures with free fatty acids or cholesterol, vesicular structures (110–190 nm mean diameter) of captured volume varying from 4–8 L/mol. The size of most of the vesicles was stable at 4°C for at least 3–6 months [56].

According to this, and the results mentioned earlier, the introduction of negative charges into the liposomal membrane seems to be one of the crucial factors governing the effective encapsulation and binding of anthracycline drugs (one of the most exploited and used anticancer drugs). A derivative of resorcinolic lipid with a strong negative charge was synthesised. To change the inverted conical shape of the molecule, one of the hydroxyl groups was esterified with myristoyl chloride. The second hydroxyl group was modified by sulfonation, thus a strong negative charge was introduced. This compound we named MSAR (*O*-Myristoyl-*O'*-Sulfo-AlkylResorcinol) [54, 55]. This compound, as expected, formed, upon hydration, vesicular structures capable of efficient entrapment of the solutes present in the aqueous phase. The introduction of this compound into the liposome-forming lipid mixture containing egg phosphatidylcholine and hydrogenated phosphatidylcholine, at a level not exceeding 50% (w/w), resulted in a several-fold enhancement of the liposomal drug's encapsulation. The encapsulation of both markers as well as the anthracycline drugs (doxorubicin, mitoxantrone) was higher than in the case of conventional liposomes — the drug to lipid ratio was lowered from 15 to 8 with the encapsulation efficiency between 90 and 99% [54]. For such a high encapsulation efficiency no additional procedures such as gradient loading, is needed. These new liposomes showed enchanced shelf-life and stability of size both at room temperature and at about 4°C. Additionally, formulation of anthracycline drugs in modified liposomes allowed the production of the dry (lyophilized) form of the active substance with size and stability after rehydration identical to those before this process (Gubernator & Kozubek, unpublished). Therefore, we have named vesicles modified with resorcinolic lipids or their derivatives PLARosomes abbreviated from PhosphoLipid-AlkylResorcinol liposomes.

PLARosomes, when incubated with human plasma, were more stable than conventional lecithin/cholesterol or sphingomyelin/cholesterol liposomes. Conventional liposomes, in a two-week experiment, released up to 50% of their contents whereas PLARosomes, despite their composition, released only 3–20% of their contents. Liposomes modified with alkylresorcinol (AR) or MSAR are cleared from the circulation in a similar way as conventional liposomes. However, the clearance of PLARosomes with high load of MSAR (30%, w/w) from the circulation was drastically enhanced by their strong negative charge [56]. This results in their preferential localization in the liver (up to 60%, 24 h after injection). Similar liposomes, but modified with ARs, were found in almost equal amounts in the liver and spleen. Vesicles containing less modifier did not differ in their distribution from sphingomyelin/cholesterol liposomes, belonging to the longest circulating conventional liposomes. Approximately 12% of both types of liposomes were still present in the blood 24 h after injection [56].

What other benefits could be related to the new liposomes? Conventional liposomes, be-

sides their uptake by the reticulo-endothelial macrophage system, are also subject to the action of various lipolytic plasma enzymes, including phospholipase A_2. This enzyme alone will alter the barrier properties of the lipid bilayer by hydrolysis of the phospholipid components into lysophospholipids, responsible for the destruction of bilayer integrity. Resorcinolic lipids present in the bilayer alter phospholipase kinetics so that they may be considered functional inhibitors of the enzyme [57]. These properties might be responsible for the protection of liposomes from the action of these enzymes. Additionally, resorcinolic lipids, due to their phenolic nature, exhibit antioxidant properties [54, 58–60]. The use of natural antioxidants for the protection and stabilization of liposomal components against oxidative damage has been reported recently [49, 61]. Resorcinolic lipids, in the context of the above findings, would also play a role in such protection. On the other hand, resorcinolic lipids, besides their direct or indirect effect upon liposomal drug-entraping properties, display other properties crucial to the human organism. These compounds have been demonstrated as having antimutagenic properties [62], especially when metabolically activated mutagenic compounds are considered (e.g., benzopyrene) [63, 64].

Additionally, resorcinolic lipids are effective functional inhibitors of triglyceride synthesis and the enzymatic oxidation of unsaturated fatty acids [65, 66], which makes possible a synergistic effect of those compounds on the organisms into which they were introduced. Recent reports indicating the participation of resorcinolic lipids in DNA damage [67–71] and inhibition of its repair [72, 73] with parallel enhancement of anticancer drugs activity, strongly support the necessity for further studies of this interesting group of natural compounds. The concominant biological activities may be a key issue in creating a kind of universal vesicular systems based on natural and biodegradable modifiers. It may also be speculated that resorcinolic lipids, the compounds present in all whole grain-based products, and used for centuries in human nutrition, will participate in establishing a modern way of liposome medical applications.

CONCLUDING REMARKS

The ability of liposomes consisting of components other than phospholipids and cholesterol or their semisynthetic derivatives to enhance the encapsulation of bioactive substances provides new promising perspectives for establishing new, efficient and stable carriers for drug delivery. We hope that in the near future PLARosomes, the components of which are not directly toxic (unpublished data), will be used for the efficient entrapment and delivery of drugs to human or animal organisms. Resorcinolic lipids and modern studies on their biological activities are relatively new but show a tremendous potential not only as components of PLARosomes [72, 73]. PLARosomes may come into commercial use relatively soon because of the benefits anticipated from the knowledge generated through the use of conventional liposomes.

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