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Development of a New Generation of Drug Dosage Forms by Construction of Liposomal Nanocontainers

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Abstract—Various methods of construction of liposomal nanocontainers are studied. Parameters of liposomes are determined. Advantages of phase transformation method are demonstrated. The routes of liposome delivery to the organism are determined.

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INTRODUCTION

In mid-1960s, the British researcher Alec Bangham studied the structure of colloid systems formed on swelling of phospholipids in water to find out their role in blood coagulation [1]. The electron microphotographs showed lamellar particles remarkably similar to cellular membranes [2]. Further research showed that during swelling of phospholipids the inorganic ions pass from the solution into these particles and are retained therein for a long time exchange with the ions in the solution with a very low rate [3]. It was thus discovered that phospholipids, the main components of cellular membranes, are capable of spontaneously forming in water closed membrane shells which can entrap part of their surrounding aqueous solution. The resulting phospholipid membrane functions as a semipermeable barrier: It allows water to pass through readily but prevents diffusion of solutes.

This discovery had a great impact on further development of membrane biology as a whole. The lipid particles described by Bangham and co-workers proved to be a maximally simplified model of cellular membranes. These particles were given the name "liposomes" and they became a favorable object of researchers focusing on the properties of biological membranes.

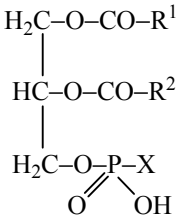
The increasing present interest in liposomes is associated with their unique physicochemical and biological properties *in vivo* and *in vitro*. These include chemical intertness, biocompatibility, biodegradability, almost lacking toxicity, antigenicity, and allergic reactions of the organism in response to introduction of liposomes, and, which is especially important, ability to effectively and not infrequently specifically interacting with certain cells in the organism, thus ensuring delivery into cells of liposome-entrapped drugs and prolonging their biological action [4]. These advantages make liposomes candidates for wide applications in the production of radically new therapeutic and preventive preparations [5].

Structure and Properties of Liposomes

Liposomes (lipid vesicles) are specially prepared, artificial particles formed by lipid bilayers. They are usually obtained from phospholipids. Detailed study of physicochemical and biological properties of liposomes showed that they can be used in biotechnological processes as models of cellular systems and biological transporters of various substances into cells, organs, and tissues.

Cellular membranes contain phospholipids of two types: glycerophospholipids and sphingophospholipids [6].

Glycerophospholipids are esters of glycerol, higher fatty acids, and phosphoric acid:



where R^1 and R^2 are the hydrocarbon radicals of higher fatty acids, and X is a base.

The principal glycerophospholipids include:

phosphatidylcholines, X = $CH_2CH_2N^+(CH_3)_3$;

phosphatidylethanolamines, X = $CH_2CH_2NH_3$;

phosphatidylserines, X = $CH_2CH(NH_2)COOH$;

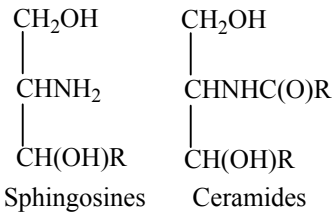
phosphatidylthreonines, X = $CH(CH_3)CH(NH_2)\cdot COOH$;

phosphatidylglycerols, X = $CH_2CH(OH)CH_2(OH)$;

diphosphatidylglycerols (cardiolipins), X = $CH_2CH\cdot(OH)CH_2OP(O)(OH)OCH_2CH(OCOR')CH_2OCOR''$;

and phosphatidylinositols, X = inosityl.

The group of sphingophospholipids (sphingomyelins) includes lipids containing the amino alcohol sphingosine; apart from that, sphingophospholipids are structurally similar to glycerophospholipids. Sphingophospholipids are derivatives of ceramides, viz. N-acyl derivatives of sphingosine:



where R is the hydrocarbon radical of a fatty acid.

Phospholipids used to produce liposomes are isolated from different mammalian organs and tissues, as well as from plant raw materials [4, 7, 8].

In terms of chemical structure, phospholipids are classed with amphiphilic compounds. The polar heads of phospholipid molecules are hydrophilic, while their nonpolar tails are hydrophobic**.** The tendency of lipids to prevent, as far as possible, the nonpolar chains from contact with water results in that the bilayer, provided it is extended enough, closes itself to form hollow shell-type structures which were given the name vesicles.

Historically, the name liposomes was first given to particles formed upon mechanical dispersion of a suspension of phospholipids swollen in water. These are multilayer particles and, therefore, they are sometimes called multilamellar vesicles. They consist of several tens of lipid bilayers with aqueous interspaces and are fairly coarse particles (up to 50 μm). The opposite pole of the large liposome family is occupied by small unilamellar vesicles (diameter ~20 nm) formed by a single lipid bilayer. Between these two extremes there is a great variety of liposomal structures differing from each other in size, shape, number of lipid bilayers, and core structure. Liposomes not always look like globular structures. Sometimes they take a flattened, disc-like shape (discomes) or look like very long and thin tubes (tubular liposomes) [9].

The hydrocarbon chains of phospholipids packed to form bilayer membrane can, depending on temperature, undergo a gel-to-liquid crystal transition, and, therewith, this phase transition occurs stepwise. The temperature of the main phase transition which resembles the melting process depends on the nature of the polar heads of phospholipid molecules, as well as on the length and saturation degree of fatty acid residues [10].

Liposomes are formed in the aqueous phase which is a constituent part of the polar part of the lipid bilayer. Therewith, each molecule of phosphatidylcholine which has a higher affinity to water than phosphatidylethanolamine and phosphatidylserine strongly binds 2.5−10 water molecules and weakly binds 10−20 water molecules in the space between the lipid bilayers. The quantity of water bound with the lipid bilayers can be varied by introduction into them of certain lipids, for example, cholesterol or glycolipids which can enhance hydration of polar phospholipid heads.

Summarizing the principal paroperties of liposomes, which make possible their use as nanocontainers for delivery into body cells and tissues of drugs and biologically active substances, we would like to dwell on the following issues.

Model membrane systems can be formed from egg lecithin with cholesterol or without it, with long-chain anion or cation additives, as well as from combine lipid extracts of the plant and/or animal origin [11].

Positively charged phospholipid membranes are sufficiently permeable for Li, Na, K, Pb, and Ca cations (50% diffusion ion exchange is reached within 100 h at 22°C).

Liposomal membranes are ~10^5 times more permeable for water than for cations (50% diffusion exchange is reached within 1 min at 22°C). Anions rapidly diffuse through charged membranes and pure lecithin membranes (no net charge). Membranes are selective with respect to anions.

Liposomes are capable of entrapping and retaining substances of various natures. The efficiency of drugs and biologically active substances entrapped in liposomes, at the level of cells or the whole body depends on their stability, ability to interact with and penetrate cellular membranes, as well as on the lack of side effects [10]. Liposomal drugs exhibit enhanced stability. The large specific volume of vesicles ensure a high degree (78−88%) of entrapment of biologically active and therapeutic substances [12].

It was found that liposomal drugs lose only little active substances after storage for 12 months and preserve their high pharmacotherapeutic efficincy [13].

The high promise liposomes hold in drug therapy is associated with the possibility of targeted delivery of drugs to diseased organs and inside cells, decrease toxicity of drugs, prolong their action 4−7 times, and decrease drug consumption (5−7 times per treatment course).

Thus, liposomal drugs favor enhanced efficiency of treatment. At present such drugs are the only remedy against intracellular infections and oncologic diseases.

Technology of Liposome Construction

There is a great variety of technologies of liposome production, which provide vesicles of different sizes, compositions, structures, and core volumes, and a various technologies of immobilization of substances in them are known.

Liposomes are always generated in water as the dispersion medium which strongly affects the formation of the bilayer lipid membrane.

Production of Liposomes by Hydration of Phospholipids

Fairly simple phospholipid hydration technologies are known, which provide giant unilamellar vesicles. This, one of the approaches involves addition of distilled water or a salt solution into a flask with walls coated with a film of phospholipids, obtained by evaporation of an organic solution of the latter, with the subsequent heating for a few hours at 70°C. During heating the film with liposomes that form separates from the flask walls. After short shaking the phospholipid aggregates are destroyed to form giant unilamellar liposomes [14].

To drive and optimize the hydration process, the phospholipid layer is suggested to apply onto a material capable of swelling in water, as well as onto glass balls. This favors breakage of the lipid layer and liposome formation [15].

The thickness of the liposomal membrane formed on phospholipid hydration is usually larger than 0.2 μm [16].

Production of Unilamellar Liposomes by the Sonication Technology

Sonication destroys multilamellar liposomes obtained by the hydration technology or the phospholipid film swollen in the aqueous phase to form unilamellar vesicles. Sometimes sonication is performed in the presence of a hydrophobic organic solvent which is further removed [17]. Therewith, liposomes get smaller and more uniform. As shown in [18], liposome dimensions stabilize after short unltrasonic treatment and are only slightly affected by subsequent sonications.

Sonication is also used for immobilization of various substances in liposomes. This method is reproducible and readily accomplished.

Injection Technology

Phospholipids dissolved in certain organic solvents can form various-size unilamellar liposomes when injected under pressure into water [19]. The principal disadvantage of this technology is that the resulting material retains some organic solvent which is hard to remove even if low-boiling solvents are used.

Extrusion Technology

Stable and pure unilamellar liposomes with a high concentration of lipids can be produced by passing an aqueous dispersion of multilamellar liposomes under a high pressure through small holes (French press). Already the first extrusion cycle is fairly successful: 70% of lipids fall on unilamellar liposomes, and after the second extrusion cycle their yield is higher than 95% [20].

The disadvantages of this technology include its high labor consumption and high losses of lipids (96.5%) remaining on the filter [21].

Reverse-Phase Evaporation Technology

The essence of this technology consists in the following. Phospholipids are dissolved in ether,

Sizes of liposomes and degrees of immobilization of substances into liposomal vesicles obtained by different methods

Method	Liposomal vesicle size, μm	Immobilization degree, %
Phospholipid hydration and sonication	1–50	up to 1
Injection	0.07	up to 2.5
Extrusion through ultra filter membranes	0.015–0.05	up to3.5
Reverse-phase evaporation	up to 1.2	67–78
Freeze−thaw	1–50	up to 98.8
Solubilisation and detergent removal	0.5–30	up to 60

chloroform or a 2:1 ether−chloroform mixture, after which a phosphate buffer solution (pH 7.5) of a substance to be included in liposomes is added. The mixture is subjected to sonicated (frequency 20 kHz, power 200 W) for a few minutes until a water-in-oil emulsion form. Then the organic solvent is removed completely by gradually reducing in the flask in such a way as to prevent the solvent from boiling. Completion of evaporation is judged about by gel formation (and disappearance of the smell of the organic solvent). During evaporation the mixture temperature is maintained slightly above the phase-transition temperature of phospholipids. The resulting gel is diluted with phosphate buffer, and the flask is shaken until homogeneous liposomes.

In our opinion this technology holds the greatest promise for production of liposomal concentrates. It gives oligolamellar liposomes with high degrees of immobilization of substances in the bilayer membrane and liposome core, which depend on the hydrophilicity/hydrophobicity and other properties of the substances [22–24]. This technology is readily automated. The use of chloroform at the stage of liposome construction ensures sterility of the product [13].

To obtain more size-uniform liposomes, reverse-phase evaporation technology is combined with extrusion through polycarbonate membranes (the second procedure provides liposomes whose core has a lower capacity to entrap substances).

Freeze−Thaw Technology

Multiply repeated cycles of freezing phospholipid dispersion systems and subsequent thawing form liposomes with high contents of immobilized substances. Therewith, liposomes merge together and get larger. The freeze−thaw cycle is repeated six times. The mixture thawed after the last cycle is diluted with a 4-fold volume of a 0.14 M solution of NaCl to suspend large unilamellar liposomes [25].

Preparation of Unilamellar Liposomes Using Detergents

The method is based on the ability of a mixture of phospholipids, protein, and detergent to form bilayer vesicles with an immobilized material as the detergent is removed by dialysis [26]. Vesicles are formed gradually. Therewith, hydrophilic materials are entrapped in the vesicle core, whereas materials having hydrophobic moieties are incorporated into the liposomal membrane.

This method provides liposomes with a high degree of inclusion of necessary materials, but they retain some detergent which can adversely affect the entrapped substances.

The possibilities of the above-described technologies are shown in the table.

The search for optimal ways of delivery of liposome-encapsulated substances to microorganism cells is based on known mechanisms of interaction of bilayer lipid vesicles and cells. The experimental system “cell−liposome” is an important tool for research on various aspects of functioning of biological membranes. The most complete information on the mechanisms of functioning of biological membranes is contained in the monograph of Margolis and Bergel’son [27] who summarized the numerous research results in this field, reported by mid-1980s. These mechanisms are quite important to know for developing methods for delivering liposome-encapsulated substances to target cell areas.

There are several ways of interaction of liposomes with cells in the culture.

(1) Merging of liposomes with the plasmatic membrane, as a result of which the substances encapsulated in liposomes enter the cell cytoplasm, while the liposomal membrane is incorporated into the cellular plasmatic membrane. The components of the liposomal membrane, incorporated by merging into the

plasmatic membrane can either remain in the cellular membrane or penetrate inside cell due to endocytosis and then interact with lysosomes. (2) Liposomes enter inside cells by their merging with the endocytic membranes of vacuoles. Digestive and contractile or pulsating vacuoles (which control osmotic pressure and act to excrete metabolism products from the body) are differentiated. If merging increases the permeability of membranes, that the liposome content can enter the cell cytoplasm. (3) Endocytosis of uni- and multilamellar liposomes followed by merging of endocytal vacuoles with lysosomes. (4) Adsoprtion of liposomes on the cell surface, involving no real inclusion of their content in the cell. (5) Adsoprtion of liposomes on the cell surface, which affects permeability of liposomal and cellular membranes. (6) Mutual exchange of components between adsorbed liposomes and plasmatic membrane due to exchange diffusion [28].

Transport of Drugs Immobilized in Liposomes

Research on the pharmacokinetics of liposomal drugs demonstrated the possibility of their targeted transport to separate organs, tissues, and cells of a macroorganism at different routes of their administration.

On subcutaneous application of liposomal drugs as ointments or lotions the active substances enter through skin. Certain authors suggest that drugs penetrate the corneal layer and epidermis, whose total thickness is 0.1 mm, but do not reach the lower dermal layer.

The molecules of many drug components are much larger than the distances between corneal layer cells, and, therefore, such molecules hardly reach “live” dermal layers. Liposomal drugs are free of this disadvantage. Так, the degree of penetration through human skin of the triamcinolone ointment incurporated in bilayer lipid vesicles is 3.5 times higher compared with intact preparation [10]. Histological studies showed that liposomal triamcinolone penetrates inside cells [13].

Liposomal gels containing a lipophilic extract of magnolia-vine, laurel, melilot, and chamomile, immobilized in the liposomal membrane or a hydrophilic extract of ginseng, plantain, and licorice, included into the vesicle core, exhibit anti-inflammatory, reparative, venotonic, and anticellulitis effects [13].

Inhalational liposomal drugs have an expressed broncholytic effect and therapeutic action on bronchial asthma [29].

Liposomal drugs found application in ophthalmology. Thus, repeated instillations in rabbits of cyclosporine encapsulated in bilayer phosphatidylcholine vesicles cause no irritation of the ocular tissue and facilitate corneal epithelization, hinder formation of new blood vessels in cornea and transplant upon burns and keratoplastics, favor transplant acceptance in the case of keratoplastics with enhanced threat of development of transplant diseases, and efficient against choroidal inflammation and herpetic keratitis (corneal inflammation). The use of the liposomal form allows the concentration of cyclosporine to be decreased 10 times compared with that in oil eye drops [30]. The same preparation in the form of eye drops showed a high efficiency in treatment of choroidal and corneal inflammations which are underlain by autoimmune processes, as well as in treatment of patients after keratoplastics. The patients all tolerated treatment quite well, and no local or general allergic reactions were observed [31].

The resulting data gave evidence showing that liposomal drugs can be widely used in dermatology, ophthalmology, and cosmetology.

There is increasing evidence for successful use of oral liposomal forms of drugs, including those tending to degrade in stomach in the intact form. The available information shows that liposomes can protect their entrapped substances from chemically unfavorable factors on oral administration. The substances introduced in bilayer lipid membranes include enzymes, polypeptide hormones (insulin, parathormone, calcitonin), certain proteins, etc. [32]. At the same time, the fine mechanisms underlying the ability of bilayer lipid vesicles to persist in the digestive tract are still unknown.

Dutch and Japanese researchers could reach positive results on treatment of patients with hemophilia A by oral administration of antihemolytic globulin (factor VIII) immobilized in liposomes [33].

Of particular interest are studies on the use of liposomal insulin for treatment of insular diabetes. It was found that liposomal insulin is effective on oral administration. In this respect liposomal insulin is preferred over intact insulin, since the latter is only introduced parentherally because of destruction in stomach. Evidence for perspectiveness of liposomal insulin comes from the fact that, even though the results of its application for treatment of insular diabetes are not unambiguous and the possibility of

adjuvant action is not excluded, certain companies plan to commercialize this preparation [32].

Parentheral (subcutaneous, intraperitoneal, intramuscular) introduction of liposomal drugs hold some promise.

The distribution of liposomes in a macro-organism on their parentheral injection depends not only on their dose and physicochemical and biological properties, but also on the administration route. The drugs from liposomes enter the organism directly as the liposome shell destroys in blood plasma, lymph, and tissues, and after entering macrophages [34].

When injected subcutaneously, small liposomes much faster enter the blood stream through lymphatic nodes than large vesicles which are localized preferentially in regional lymphatic nodes. Most subcutaneously injected liposomes are deposited in the injection site and are eliminated from there primarily via the lymphogenic route. Therewith, their content in liver remains unchanged, unlike what occurs at different introduction routes, at a level of 0.2% of the total injected. Local injection is an optimal method ensuring delivery of liposomes to regional lymphatic nodes [35]. The inclusion of liposomes containing drugs into a collagen gel matrix much prolongs their biologic effect [36].

Experimental animal study showed that the distribution of large unilamellar liposomes in organs and tissues upon subcutaneous and intraperitoneal injections depends on the introduction route. The drug was injected in 100-μl portions subcutaneously and intraperitoneally in white mice; the animals were killed within a certain lapse of time and measured blood and tissue contents of the radioactive marker fixed in liposomes [37]. Upon subcutaneous injection, a fairly high level of liposomes in blood was reached within 24 h, whereas their maximum blood concentration upon intraperitoneal injection was observed only during the first four hours. According to [38], liposomes are transported from the peritoneal cavity to blood by lymph. Upon cutting the lymphatic thoracic duct, liposomes no longer enter the blood stream.

Intramuscular injection makes it possible to localize the action of liposomes in their injection site for several days, even though they are spread in part into other organs with the blood stream. Small bilayer lipid vesicles, unlike large ones, much faster enter the blood stream upon intraperitoneal and intramuscular injection. This finding points to a limited ability of large vesicles to penetrate capillaries and vascular membranes. Upon intravenous injection, small liposomes are slower removed from the blood stream than large ones [31].

Bilayer vesicles obtained from soya lipids and containing 87% of phosphatidylcholine, as well as lysophosphatidylcholine, and glycolipids, 30 min after intravenous injection in guinea pigs, are much more (150 times) accumulated in myocardium than liposomes obtained from castor oil. On the other hand, human, pig, or bovine protein glycophorin which is not a structural component of lipid vesicle membranes strongly affects the distribution of liposomes in an organism by decreasing their concentration in liver and lungs and increasing their concentration in blood [39].

At the same time, liposomal drugs not always are more active than intact, even though their total toxicity is lower. As an example we would like to consider the results of research on liposomal actinomycin as a drug for treatment of osteosarcoma in mice. Liposomes, when injected parentherally and, especially, intravenously, into a macro-organism, are fairly rapidly absorbed by reticuloendothelial cells, which makes these cells and organs they contain potential targets for directed therapy by means of liposomal drugs [40].

The inclusion in liposomal membranes of lipid A which is a fragment of the lipopolyssacharide of gram-negative bacteria endows lipid vesicles with enhanced affinity to liver on intravenous injection [41]. Enhanced affinity to liver is observed in liposomes containing in their membrane synthetic palmitoyl derivatives of certain amino acids or acylated peptides. Study of the distribution of intravenously injected liposomes containing phosphatidylcholine, cholesterol, and glycolipids showed that the best glycolipid for liposomes in terms of their delivery to brain and liver tissues is sulfatide, to spleen tissues, gangliosides, and to lung tissues, sphingomyelin [42].

Dopamine immobilized in liposomes, unlike intact dopamine, can reach brain and thus correct extrapyramidal disorders and prevent development of the parkinsonian syndrome (animal experiments) [43].

If lipids used for liposome production are isolated from some specific organs or tissues, the resulting bilayer vesicle have a much higher affinity to their cells, since each type of membranes is characterized by

its specific ratio of polar lipids. According to [44], lipids are able, to a certain extent, to recognize cells. An important role in this process also belongs to glycolipids (gangliosides) which are involved in intercellular interactions and act as specific receptors for certain biologically active substances [10].

Liposomes containing, along with drugs, immobilized magnetic materials can be magnetically controlled [45]. Such liposomes circulating in the blood stream can be fixed in a certain organ or tissue of a macro-organism by applying magnetic field. As liposomes degrade, drugs release from their lipid shells and thus concentrate in a certain site of the body.

Temperature-sensitive liposomes whose lipid membrane phase transition temperature is a few degrees higher than the body temperature hold great promise for drug targeting applications [46]. If a certain organ or body segment is heated by microwave irradiation or any other physical means, the membranes of liposomes circulating in blood start to "melt," and the drugs immobilized in bilayer vesicles release specifically in this site. Such technique creates an increased drug concentration in the site of local hyperthermia [27].

To endow liposomes with affinity to specific organs, tissues, and cells, antibodies against antigens of such specific formations and various compounds preventing absorption of lipid vesicles with macrophages and cells of liver and other parenchymatous organs (i.e. internal organs formed by a continuious tissue, such as liver, spleen, etc.) are immobilized on the liposome surface. For more specific interactions, monoclonal antibodies, as well as immunoglobulin fragments with a specific activity are used [47].

The identity of liposomal and cellular membranes makes bilayer lipid vesicles highly biocompatible and virtually nontoxic. At the same time, the organism is not completely insensitive to bilayer lipid vesicles, since their constituting lipid components take an active part in metabolic processes and can cause certain functional and biochemical alterations [11]. For example, already after double intravenous injection of 2 mg of liposomes comprising sphingomyelin and phosphatidylcholine (molar ratio 4:1) or distearoyl phosphatidylcholine and cholesterol (molar ratio 1:1), a granulomatous reaction of the liver tissue is observed, which disappears soon liposomes are no longer injected [41].

The available evidence shows that liposomal membrane components are actively involved in various metabolic processes. However, they are unlikely to entail some expressed pathologies, in view of the fact that the principal components of liposomal and cellular membranes are identical to each other. Phospholipids can enter into a macro-organism with food or are synthesized in it, and many drugs contain physiologically active lipids. In this connection some effort is undertaken to create cosmetic, X-ray contrasting, medicinal, diagnostic, and preventive preparations liposomal preparations for practical use in biology and medicine [4, 12]. Thus, the German firms Vitorgan and ReAm produce liposomal drugs for treatment of paradontosis and venous dilation, and the Italian firm Fidia produces the drug Liposom-forte injected intravenously to stimulate hypophysis. Many foreign firms specialize on the production of other liposomal drugs (vaccines, antibiotic, antiallergenes, and bronchodilatators) [48].

CONCLUSIONS

The available information on liposomes cannot be considered exhaustive. The problems concerning the assortment of and production technologies for liposomal drugs, including their sterilization and lyophilization, have just been raised. Even less is known about on standardization of liposomal drugs: The relevant information is scanty and insufficient for developing regulatory documents. Stabilization of liposomal drugs is scarcely reported, which complicates their commercialization in Russia, and, therefore, further experimental and theoretical research work on the development of original medical and preventive liposomal drugs remains an urgent issue.

At the same time, we would like to mention that at present there is experimental evidence showing that liposomes are efficient in treatment of various infectious diseases, including in animals, tumors, and burns. However, experiments are generally limited to pharmacological testing. Industrial production of liposomal medical and preventive preparations in Russia has still not been initiated. The development of such industry will make possible production of highly efficient domestic drugs for treatment of such diseases as cancer, intracellular infections, etc.

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