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Novelistic Frontiers in Fusogenic Liposomal Drug Delivery system: An Outlook

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ABSTRACT

The term liposomes (i.e. lipid body) is derived on the basis of names of sub cellular particles like lysosome and ribosomes. It is a sphere vesicle of lipid bilayers enclosing an aqueous compartment. The lipid most commonly used is phospholipids. Because of their availability in various sizes, ability to incorporate both water as well as oil soluble drugs, their inertness and their ability to protect labile drugs, liposomes are versatile carriers for parenteral drug delivery system. Liposomes have been realized as extremely useful carrier systems, additives and tools in various scientific domains. Liposomes over the years have been investigated as the major drug delivery systems due to their flexibility to be tailored for varied desirable purposes. They can be administrated parenterally, topically, by inhalation and possibly by other routes of administration, but the current products are administrated parenterally. Fusogenic liposomes are specially engineered liposomes that fuse and merge with cell membranes and directly introduce molecules (entrapped or anchored) into cytoplasm thus avoiding the route followed by conventional liposonmes, i.e., internalization via endocytic compartments into lysosomes. The fusogenic liposomes mimic the way by which several viruses (HIV, Sendai virus) bind and merge with cell membranes at neutral pH and subsequently release their genome into the cytoplasm.

Keywords: Novel Drug delivery, Targeted drug delivery, Nano-technology.

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INTRODUCTION

Lipids, along with proteins and nucleic acids, are essential biomolecules for the structure and function of living matter. Most lipids are fats and waxes, but here we focus on so-called amphiphilic lipids. This type of lipid is the predominant building block of biological membranes, as well as liposomes. Liposomes were discovered in the mid 1960s (Bangham *et al* 1965) and originally studies as cell membrane models. They have become very versatile tools in biology, biochemistry and medicine. Liposomes are spherical self-closed structures, composed of curved lipid bilayers, which enclose part of the surrounding solvent micrometers and they may be composed of one or several concentric membranes, each with a thickness of about 4 nm. Liposomes possess unique properties owing to the amphiphilic character of the lipids, which make them suitable for drug delivery. A schematic picture of a liposome is shown in figure 1.

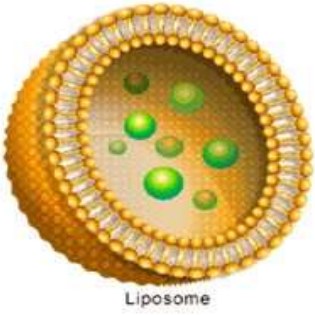


Figure 1: Liposome Structure [1]

Liposomes are the smallest artificial vesicles of spherical shape that the can be produced from natural untoxic phospholipids and cholesterol. As shown in the following schematic drawing of liposomes, the vesicles can be used as drug carriers and loaded with a great variety of molecules, such as small drug molecules, protein, nucleotides and even plasmids. Liposomes are extremely versatile and due to the variability of their composition as shown below in a figure, which we adapted from science-medicine they can be used for a large no of application.

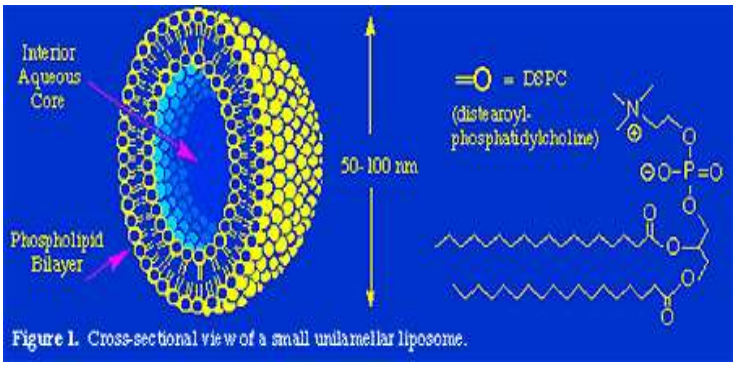


Figure 2:Cross-sectional view of a liposome. [2]

Liposomes as a drug delivery system

The applicability of drugs is always a compromise between their therapeutic effect and side effects. Liposomal drug delivery system not only enables the delivery of higher drug concentration (Woodle *et al* 1997), but also a possible targeting of specific cells or organs. Harmful side effects can therefore be reduced owing to minimized distribution of the drug to non-targeted tissues. Like all other carrier systems, the use of liposomes in drug delivery has advantages and disadvantages. The amphiphilic character of the liposomes, with the hydro-phobic bilayer and the hydrophilic inner core, enables solubilization or encapsulation of both hydrophobic drugs. Along with their good solubilization power, a relatively easy preparation and a rich selection of physicochemical properties have made liposomes attractive drug carrier systems. However a complete saturation of the immune system and interaction with lipoproteins are some examples of potentially toxic and adverse effects. Efficient drug delivery systems based on liposomes need to process a large number of special qualities. First, good colloidal, chemical and biological stability is required. A colloidally stable non-equilibrium structure is less sensitive to external change than equilibrium structures, such as micelles. Hence, colloidally stable liposomes after work well in pharmaceutical application. Biological stability includes control over the rate of clearance of liposomes from the circulatory system or compartments of the body, if the drug has been administered locally. The rate of clearance is dose dependent and varies according to the size and surface change of the liposomes. Biological stability also comprises retention of the drug by the carrier en route to its destination (a phenomenon known as sustained release).

Objective of the study:

The term liposomes (meaning lipid body) is derived on the basis of names of sub cellular particles like lysosome and ribosomes (Brahmankar *et al*. 2006). The main objective of the study is to know the recent scientific development on liposomal drug delivery. It covers the various research articles and review articles regarding this study. From this study, will able to know that liposomes are most widely studies modern drug delivery system that can be administered parentrally topically, by inhalation and possibly by other routes of administration. But the current products are administered parentrally only, which are used for the formulation of sustained release, triggered release and targeted release drug products. Here we will cover the preparation, classification, drug loading, drug targeting, marketed products, and evaluation and review articles of liposomes that can help the readers to know the recent scientific development in the field of liposomes.

Classification of Liposomes

The classification of liposomes is based on their pharmaceutical and therapeutical aspects. One way of their classification relies upon the number of bilayers formed and diameters of the resultant vesicles (Vyas and Khar).

Classification of Liposomes

A. **Based on structural parameters**

MLV-Multilamellar large vesicles - > 0.5 µm

OLV- Oligolamellar vesicles – 0.1-0.1 µm

UV- Unilamellar vesicles – All size range

SUV -Small unilamellar vesicles – 20-40 nm

MUV- Medium unilamellar vesicles – 40-80 nm

LUV - Large unilamellar vesicles – 100 – 1000 nm

GUV- Giant unilamellar vesicles - > 1000 nm

B. **Based on Method of liposome Preparation**

REV -Single or oligolamellar vesicles made by reverse phase evaporation method – 0.5 µm

MLV-REV- Multilamellar Vesicles made by reverse phase evaporation method

SPLV - Stable plurilamellar vesicles – 100 nm - 2µm

FATMLV - Frozen and thawed MLV

VET- Vesicles prepared by extraction technique – 100nm – 1 µm

DRV- Dehydration – rehydration method - > 1 µm

C. **Based upon composition and application**

Conventional liposomes (CL) -Neutral or negatively charged phospholipids and cholesterol

Fusogenic liposomes (RSVE) - Reconstituted sendai virus envelops

Cationic liposomes - Cationic lipids with DOPE

Long circulatory (stealth) - Neutral high Tc, Chol and 5-10% of

Liposomes (LCL)- PEG – DSPE or GMI

Immuno -liposomes -CL or LCL with attached monoclonal antibody or recognition sequence.

Figure 3: Unilamellar Liposome

Methods of liposome preparation and drug loading:

Liposomes are manufactured in majority using various procedures in which the water soluble (hydrophilic) materials are entrapped by using aqueous solution of these materials as hydrating fluid or by the addition of drug/drug solution at some stage during the manufacturing of liposomes. The lipids soluble (lipophilic) materials are solubilized in the organic solution of the constitutive lipids(s) and then evaporated to a dry drug containing lipid film followed by its hydration. These methods involve the loading of the entrapped agents before or during the manufacturing procedure (Vyas and Khar).

Figure 4: Multilamellar Liposome[32]

All the methods of preparing liposomes involve three or four basic stages :

a. Drying down lipids from organic solvent
b. Dispersion of lipids in aqueous medium
c. Purification of resultant liposomes and
d. Analysis of the final product.

A. Passive loading techniques

It include three different groups of methods working on different principles namely – Mechanical dispersion, Solvent dispersion, Detergent removal (Vyas &Khar).

a. Mechanical dispersion method of passive loading

It begins with a lipid solution in organic solvent and end up with lipid dispersion in water. The various components are typically combined by co-dissolving the lipids in an organic solvent and the solvent is removed, the solid lipid mixture is hydrated using aqueous buffer. The lipids spontaneously swell and hydrate to form liposomes. Post hydration treatments include sonication, freeze thawing and high – pressure extrusion (Vyas and Khar).

b. Solvent dispersion method for passive loading

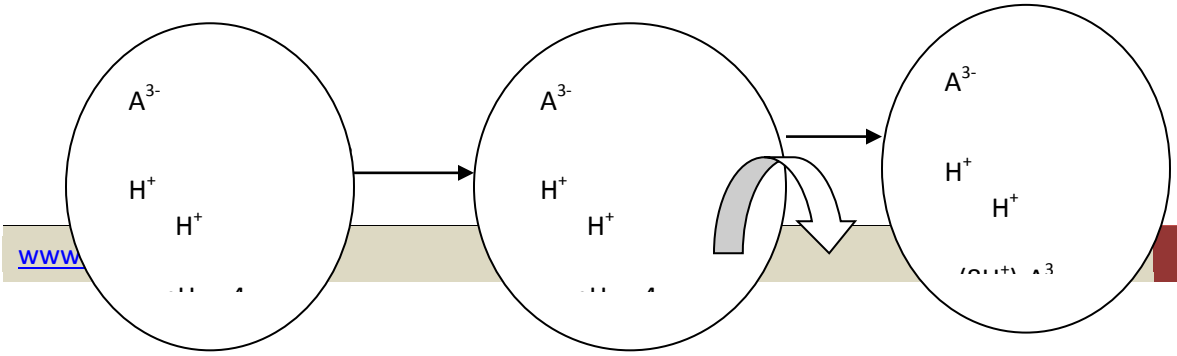
In this method, lipids are first dissolved in an organic solution, which is then brought into contact with an aqueous phase containing materials to be entrapped within the liposomes. The lipids align phase themselves at the interface of organic and aqueous phase forming monolayer of phospholipids, which forms the half of the miscibility of the organic solvent and aqueous solution.

c. Detergent Removal Methods of Passive Loading

In these methods, the phospholipids are brought into intimate contact with the aqueous phase via detergents, which associate with phospholipids molecules and serve to screen the hydrophobic portions of the molecule from water. The structures formed as a result of this association are known as micelles. Their shape and size depend on chemical nature of he detergent, the concentration and other lipids involved. The concentration of detergent in water at which micelles just start to form is known as the "critical micelle concentration". (CMC). Below the CMC, the detergent molecules exist entirely in free solution. If the concentration of detergent in water is higher than CMC, micelles form in more numbers. In these methods, the basic feature is to remove the detergent from perform spontaneously. Three methods are applied for the removal of detergent and transition of mixed micelles to concentric bilayered form-Dialysis, Column Chromatography and the use of Biobeads (Vyas and Khar).

B. Active (remote) loading

Remote loading can be used for loading of weakly acidic of alkaline drug molecule. The basic idea of the method is that neutral molecules are shuttled, due to the different pH between the inside and the outside of the membrane, into liposomes where they become charged. Charged drug molecules will thereby be trapped in the liposomal interior, owing to a low diffusion rate of charged molecules over the membrane **(Vyas and Khar).**



Drug=B

Figure 4: The pH gradient method, for loading of weak hydrophilic alkaline drugs

Figure 4 Shows remote loading of a weak base into preformed liposome is lower than the pH outside and the weak base B, which is in equilibrium with its protonated form, passes through the membrane in its unchanged form. Inside, it is, however protonated and thereby inhibited to leave the liposome (Vyas and Khar).

LIPOSOME: SUSTAINED, TRIGGERED RELEASE AND SITE SPECIFIC TARGETING.

Sustained release:

A sustained release of encapsulated drugs, i.e. retention of the drug enroute to its destination, in combination with a long circulation time, makes the liposomes useful as a targeted drug delivery system. Controlling the permeability of the liposome membrane, and thus avoiding the drug release will minimize the negative side effects caused by freely circulating drug molecules and the permeability of a bilayer is strongly influenced by its constituents (Kante *et al* 1998).

Membrane Permeability

Liposome membranes are semi permeable in that the rate of diffusion of molecules and ions across the membrane varies considerably. For molecules with high solubility in both organic and aqueous media, a phospholipids membrane clearly constitutes a very tenuous barrier, while polar solutes and high molecular weight compounds pass across the membrane very slowly. The generally accepted leakage mechanisms, for polar solutes is via defects or temporary openings (pores in the membrane).The frequency of pore formation in a membrane is mainly determined by the state of the membrane. Comparing the permeability of liposomal membranes in a liquid crystalline state with a more ordered state, the former display a higher leakage rate of encapsulated hydrophilic substances. In pharmaceutical application, liposomes usually contain about 40 mol % cholesterol, since cholesterol is known to increase the bilayer packing order. The result is a lipid membrane with reduced permeability.

Triggered release :

Considering the multitude of factors that might cause biological destabilization of liposomes, it may seem to be a simple task to obtain drug release from liposomes.

pH-triggered Release

In many applications the liposome-encapsulated drug needs to be delivered to a specific site but as long as the drug remains trapped inside the liposome, it stays inactive. A slow drug release in

most cases not sufficient for an efficient treatment. Different types of liposomes such as temperature and pH sensitive liposomes have been triggering the liposomal membrane to structural rearrangement that includes a leakage of the encapsulated substance. The use of PEG – lipids as stabilizers of DOPE liposomes serves dual purpose – liposome formation is facilitated and at the same time the PEG-lipids provide steric stabilization. Mildly acidic amphiphiles, such as oleic acid (OA) and cholesteryl hemisuccinate (CHEMS) are other stabilizers that are commonly used in triggered release systems of DOPE liposomes. Thin layer chromatography (TLC) indicated that the hydrolysis of the acid labile linkage was a slow process at pH 4.5. This was further confirmed by the slow leakage and lipid mixing of DOPE liposomes containing 1 mol% of DHCho MPEG 5000.

Release Mechanism

The use of pH sensitive liposomal systems requires targeting of liposomes to specific cells that are capable of internalizing substance filled liposomes by means of endocytosis. (Liposomes internalized via endocytosis will experience a gradual pH decrease (96-100) and this environmental change constitutes the basic idea for triggered release from pH-sensitive liposomes. A cytosolic delivery depends first of all on the chemical properties of the drug. A drug that is suitable for hydrophilic/hydrophobic properties will be able to cross the endosome membrane by simple diffusion. There are some other mechanisms also.

A) The fusion or the“microinjection” mechanism: In which liposomes, upon acidification fused with the endosomal membrane. This would lead to a ‘microinjection’ of the drug into the cytosol.

B) The destabilization mechanism: In which the liposomes, as result of a lowered pH, first collapse and release their contents into the endosomal compartment. In a second step the DOPE molecules; initially situated in the liposomes, any interact with the endosome membrane, which could lead to higher permeability and also major structural rearrangement of the endosome membrane.

Site Specific Targeting of Liposomes:

The development of a targeted nuclide therapy originates from the fact that conventional methods like surgery, chemo and external radiotheray, all have limitations. In an ideal therapy for cancer, the treatment should be able to completely eradicate the tumor cells without injuring normal tissues. In tumor tissue and at sites of inflammation the blood vessels are rather leaky. This helps to bring a large fraction of small liposomes loaded with the drug to these sites. However, this passive accumulation does not mean that the liposomes are internalized into the

tumor cells but rather that they slowly leak their content in the tumor area. In BNC a specific targeting towards the tumor cells and cell cytoplasm is necessary. To increase the accumulation of the liposomes in the tumor area, receptor targeted sterically stabilized liposomes have been developed.

Receptor-mediated targeting

A characteristic difference between normal and tumor cells is essential for targeting. Antibodies are the most commonly used biomolecules in targeting against tumor – associated antigens. Other types of biomolecules are hormones or ligands, which are directed towards cells. An example of such a biomolecule is the epidermal growth factor, ECF.

EGF – labeled liposomes

EGF has been suggested as a good candidate for tumor targeting in BNCT for several reasons. The ECG receptor is over expresses in many cancer forms, including breast, colon and prostate cancer. ECG was conjugated to the distal end of PEG -DSPE lipids in a miceller solution. The ECF was conjugated to the distal end of PEG-DSPE lipids in a miceller solution. The ECF – PEG-DSPE micelles were then mixed with preformed liposomes, either empty or loaded with WSA. By this "micelle-transfer" method the EGF – lipids became incorporated into the liposome membrane. For the optimized EGF – labeled liposomes 10-15 EGF molecules liposome was obtained and this amount is assumed to be enough to achieve a satisfactory cellular uptake of antibody – targeted liposomes. The drug to lipid ratio was set to 0.2. This amount of WSA corresponds to about 10^5 to 10^6 boron atoms in each liposome.

BNCT with EGF – labeled liposomes

Two important requirements must be met for BNCT to be efficient. First the $^{10}\beta$ – compounds must be distributed specifically to the tumor cells and, second, a high concentration of $^{10}\beta$ must be delivered to each cell. These two obstacles can be overcome by a concept involving two-step targeting. Preliminary results indicate that neutron activation of cytoplasmic WSA in cultured glioma cells, internalized by receptor mediated endocytosis of EGF-labeled liposomes, gives a therapeutic effect when WSA was exchanged for the conventional cytotoxic drug doxorubicin the two step targeting concept was proven.

Biodistribution of EGF – labeled liposomes

One problem related to the use of EGF - mediated targeting is the toxic effects on normal cells expressing EGFR, e.g. liver. However recent distribution tests of EGF – labeled liposomes in mice indicate that when EGF is conjugated to stabilize liposomes with a diameter of about 100nm, the distribution to the liver is significantly reduced.

Antibody – labeled liposomes

Antibody-labeled liposomes have also been investigated with regard to the two step targeting concept. The antibody used was herceptin, which is specific towards HER – 2 receptors. HER – 2 is over expressed in several types of cancers, including breast, cancer lung cancer, gastric cancer and bladder carcinoma. Herceptin - labeled liposomes loaded with WSA showed a receptor specific binding and internalization. (Maruyama K. 1997).

FUSOGENIC LIPOSOMES FOR SPECIFIC DRUG TARGETING:

A liposome whose outer wall contains molecules (i.e.: the **F protein**) that cause cell fusion, especially fusion between somatic cells. (Angmuir, 2003)

Definition of F protein:

A protein derived from the Sendai virus which can be used in the laboratory to cause cell fusion between somatic cells (any cell that is not a gamete). It is also used to make fusogenic vesicles.

Techniques involved in Pre-Preparation:

The present invention provides a fusogenic liposome comprising a lipid capable of adopting a non-lamellar phase, yet capable of assuming a bilayer structure in the presence of a bilayer stabilizing component; and a bilayer stabilizing component reversibly associated with the lipid to stabilize the lipid in a bilayer structure. When a polyethyleneglycol-phosphatidyl ethanolamine conjugate or a polyethyleneglycol-ceramide conjugate is used as the bilayer stabilizing component, the rate at which the liposome becomes fusogenic can be varied, for example, by varying the concentration of the bilayer stabilizing component, by varying the molecular weight of the polyethyleneglycol, or by varying the chain length and degree of saturation of the acyl chain groups on the phosphatidylethanolamine or the ceramide. In addition, other variables including, for example, pH, temperature, ionic strength, etc. can be used to vary and/or control the rate at which the liposome becomes fusogenic. Such fusogenic liposomes are extremely advantageous because the rate at which they become fusogenic can be not only predetermined, but varied as required over a time scale ranging from minutes to days. Control of liposome fusion can be achieved by modulating the chemical stability and/or exchangeability of the bilayer stabilizing component(s). Lipids which can be used to form the fusogenic liposomes of the present invention are those which adopt a non-lamellar phase under physiological conditions or under specific physiological conditions, e.g., in the presence of calcium ions, but which are capable of assuming a bilayer structure in the presence of a bilayer stabilizing component. Lipids, which adopt a non-lamellar phase, include, but are not limited to, phosphatidylenthanolamines, ceramides, glycolipids, or mixtures thereof. Such lipids can be

stabilized in a bilayer structure by bilayer stabilizing components which are either bilayer forming themselves, or which are of a complementary dynamic molecular shape. More particularly, the bilayer stabilizing components of the present invention must be capable of stabilizing the lipid in a bilayer structure, yet they must be capable of exchanging out of the liposome, or of being chemically modified by endogenous systems so that, with time, they lose their ability to stabilize the lipid in a bilayer structure, thereby allowing liposome to become fusogenic. By controlling the composition and concentration of the bilayer stabilizing component, one can control the chemical stability of the bilayer stabilizing component and/or rate at which the bilayer stabilizing component exchanges out of the liposome and, in turn, the rate at which the liposome becomes fusogenic. In addition, other variables including, for example, Ph, temperature, ionic strength, etc. can be used to vary and/or control the rate at which the liposome becomes fusogenic. Administration may be by a variety of routes, but the therapeutic compounds are preferably given intravenously or parentrally. The fusogenic liposomes administered to the host may be unilamellar, having a mean diameter of 0.05 to 0.45 microns.

Methods of preparation of Fusogenic liposomes:

Suitable methods are sonication, extrusion, high pressure/homogenization, microfluidization, detergent dialysis, calcium-induced fusion of small liposome vesicles and ether-fusion method.

Preparation of multilamellar vesicles:

Hand shaking technique or Rotary flash evaporator produces multilamellar vesicles of heterogeneous sizes. In this method, the vesicle-forming lipids are dissolved in a suitable organic solvent or solvent system and dried under vacuum or an inert gas to form a thin lipid film. If desired, the film may be redissolved in a suitable solvent, such as tertiary butanol, and then lyophilized to form a more homogeneous lipid mixture which is in a more easily hydrated powder-like form. This film is covered with an aqueous buffered solution and allowed to hydrate, typically over a 15-60 minute period with agitation. The size distribution of the resulting multilamellar vesicles can be shifted toward smaller sizes by hydrating the lipids under more vigorous agitation conditions or by adding solubilizing detergents such as deoxycholate.

Preparation of unilamellar vesicles:

Unilamellar vesicles are generally prepared by sonication or extrusion. Sonication is generally preformed with a tip sonifier, such as a Branson tip sonifier, in an ice bath. Typically, the suspension is subjected to several sonication cycles. Extrusion can be carried out by biomembrane extruders, such as the Lipex Biomembrane Extruder. Defined pore size in the

extrusion filters can generate unilamellar liposomal vesicles of specific sizes. The liposomes can also be formed by extrusion through an asymmetric ceramic filter, such as a Ceraflow Microfilter, commercially available from the Norton Company, Worcester Mass.

Pharmaceutical applications:

The fusogenic liposomes of the present invention can be used to deliver drugs, peptide, proteins, RNA, DNA or other bioactive molecules to the target cells of interest. In this embodiment, the compound or molecule to be delivered to the target cell can be encapsulated in the aqueous interior of the fusogenic liposome and subsequently introduced into the cytoplasma (initially) upon fusion of the liposome with the cell plasma membrane.

STABILITY AND STABILIZATION:

Liposomes stability which can be divided into colloidal, chemical and biological stability is one of the most important issues in liposome application. First the chemical stability of liposome constituents will be discussed, followed by colloidal and biological stability of liposomal dispersion.

Chemical stability :

Lipids, like most biomolecules, undergo different degradation processes and the most common degradation pathways are oxidation and hydrolysis. These processes will be discussed with the focus on phosphatidylcholine since it is the most commonly used lipid in pharmaceutical application.

Oxidation

In the case of PC-lipids, it is the hydrocarbon chains and especially the unsaturated ones that are subject to oxidation. Saturated chains can, however, be oxidized at high temperatures. The oxidation is a radical; reaction, which finally results in the cleavage of the hydrocarbon chains or in the case of two double bonds, the formation of cyclic peroxides. The initiation step, abstraction of a hydrogen atom from the lipid chain, occurs most commonly as a result of exposure to light or trace amounts of metal ion contamination. Polyunsaturated lipids are thus particularly prone to oxidative degradation. In the presence of oxygen, process develops further into formation of peroxides and cleavage of the hydrocarbon chain. The use of lipids with high purity can minimize oxidation of PC- lipids in liposomes, as can storage at low temperatures and protection from light and oxygen.

Hydrolysis

The four ester bonds in phospholipids may all be subjected to hydrolysis in water but the carboxyl esters are hydrolysed faster than the phosphate esters are hydrolysed faster than the

phosphate esters. During the hydrolysis, the hydrocarbon chains are pinched from the lipid backbone, producing fatty acids and lysophospholipids. The lysopholipid can be further hydrolysed into a glycerophospho compound and ultimately the hydrolysis produces glycerophosphoric acid. The hydrolysis rate of PC-lipids is both pH and temperature dependent. The rate of hydrolysis has "V-shaped" pH dependence, with a minimum at pH 6.5 and thus an increased rate at both higher and lower pH. The effect of the temperature can be described by 'Arhenius relation':

$$K = A \exp (Ea/RT)$$

When K is the hydrolysis rate, A is a frequency factor, Ea is the activation energy, RT is the thermal energy. The means that the rate is significantly slower at low temperatures. Hydroly- sis and oxidation of phospholipids liposomes occur, in vivo concomitant with their interaction with serum components. In addition, hydrolysis of phospholipids can be catalyzed by enzymes, phospholipases.

Colloidal stability

The colloidal stability of a liposomal dispersion is determined by the interliposome interactions, which depend on the balance between attractive and repulsive forces. An increased repulsive contribution gives rise to an enhanced colloidal stability. Steric repulsion is often is often used for stabilizing liposomes both in vitro and in vivo. Polymer-coated liposomes are often used to create sterically stabilized liposomes. Stabilization can be produced in two different ways, by grafting or adsorption of the polymer to the liposomal surface. The grafting method is the most commonly used and normally the stabilization is achieved by incorporation of so called PEG^2 – lipids, poly (ethylene glycol) – phospholipids. When two polymers – covered surfaces approach each other they experience a repulsive force as soon as the outer polymer segments start to overlap. In addition, the difference in chemical potential between the water in the bulk and in the interaction region induced an osmotic repulsive force.

Biological stability:

The use of sterically stabilized liposomes dose not merely increase the colloidal stability of dispersion but it also promotes its biological stability. In the blood stream the liposomes will interact with lipoproteins and opsonins. The former interaction involves lipid exchange which eventually leads to breakdown of the liposome. Opsonisation, or adsorption of market macromolecules, such as immunoglobulins, is a part of the body's own defense mechanism. The marked invaders are taken up by macrophages (which belong to the reticuloendothelial system, RES) specialized in eliminating foreign particles from the circulation. Thus, the majority of

conventional liposomes will have a circulation time of only a few minutes. To prolong their circulation time, markers must prevent from reaching the liposomal surface. Sterically stabilized liposomes with their barrier of long polymer chains will protect the surface from interaction with both lipoproteins and RES marker molecules, thus prolonging the circulation time from minutes to days. A long circulation time is necessary for an efficient site specific in vivo delivery.

Alternative Stabilizers

PEG-lipid are the most commonly used stabilizer for liposomal drug delivery systems. The lifetime of the PEG – lipid in the membrane, which is crucial for the circulation time, depends on the length of the hydrocarbon chains. To minimize the loss of polymer from the lipid membrane, it is necessary to use lipids with long hydrocarbon chains. Triblock copolymers, adsorbed or incorporated, constitute an interesting alternative to PEG – lipids as steric stabilizers of liposomes. Pluronics is a collective name for a large group of triblock copolymers with a hydrophobic middle block (poly propylene oxide, PPO) and hydrophilic end blocks (Poly ethylene oxide, PEO)

Stabilization of PE – liposomes

Although PC-based liposomes are the most commonly used for pharmaceutical applications, PE-liposomes and in particular so called pH sensitive PE – liposome have been proposed as a promising alternative. The rationale for developing such liposomes is the failure of the conventional PC-liposomes to release all their entrapped substances rapidly at a special site. Dioleoylphosphatidyl – ethanolamine (DOPE), one of the most studies PE – lipids, forms an inverted hecagonal phase, above 10-15^{0}C at near neutral or acidic pH 96). However, at high pH (pH>9) the preferred phase is the lamellar phase which can be dispersed as liposomes. The stability of DOPE liposomes can be significantly improved by the incorporation of molecules that increase the spontaneous curvature of the lipid film. In this way DOPE liposomes are stabilized by addition of PEG lipids and the amount of PEG – lipid needed in the membrane, for an effective steric stabilization depends on the size of the PEG head group. Other types of stabilizers are block copolymers. Again the stability studies could be broadly covered under two main sections in vitro and in vivo.

Stability In-Vitro studies:

It covers the stability aspects prior to the administration of the formulation and with regard to the stability of the constitutive lipids (Diane J. Burgess, 2001).

Purpose of *In-Vitro* test

- Quality control and safety evaluation

- Batch to batch
- Manufacturing process change
- Sustention of label claims
- Evaluation of potential does dumping
- Assessment of *in-vivo* stability

- "Real time" vs. accelerated/stress test
- *In vitro – vivo* correlation

Current method of *in-vitro* testing of liposome systems

- Membrane diffusion technique
- Sample and separate technique
- In situ technique
- Continuous flow technique.

Liposome performance – *In-vitro* release and stability

- Separation of liposomes from dissolution media complicated testing
- Current USP methods designed for oral and transdermal route
- In vitro tests need to take into account the expected *in-vivo* performance of liposomes
- Release test for a targeted liposome – would need to show
- Liposomes is stable until uptake at the site
- Liposome release drug at the site
- Release test for an immediate release liposome would need to show
- Drug is released immediately in condition mimicking human plasma (Diane J Burgess. 2001).

Stability in vitro mainly covers the chemical stability of the constitutive lipids under the various accelerated or long term storage conditions.

Long Term and Accelerated stability study

- Exposure of the product to high temperature and large gravitational force.
- For liposomes, higher temperature may dramatically alter the nature of the interfacial film.
- Studies at $45^0 – 50^0$ c (long term and heat cool cycling) are quite justified.
- If a liposomal dispersion is partially frozen and then thawed, ice crystals nucleate and grow. If the crystal grows to a size greater than the void spaces, they lead to instability.

Stability In-Vivo studies:

The stability of different liposomal constituents was tested under gastrointestinal conditions.

- Low pH and for the gastric environment.
- Bile salts and lipases from the intestine.

Liposomal entrapment may protect some labile drugs (like insulin) form luminal enzymatic or proteolytic activities when physical structure of the bilayered system is retained in harsh G.I. environment.

***In-Vivo* factors affecting Drug release**

❖ Delivery system independent

- Barriers to drug diffusion: fluid viscosity
- Tissue barriers (e.g. connective tissue)
- Drug partitioning at the site
- Available volume at the site
- Motion at site

❖ Delivery system dependent

- Enzymatic degradation of delivery system
- Protein adsorption
- Phagocytosis
- Inflammatory response

Marketed Products

Name of the product	Drug	Manufacturing Company
Ambisome	Amphotericin B	Fuzisawa Health care
Daunoxome	Daunorubicin Gilead Sciences	Gilead Sciences
Doxil	Doxorubicin	J&J ALZA
Depocyt	Cytarabine	
Decadron – LA Sterile suspension	Dexamethasone Acetate	Merck
Myoset	Doxorubicin	*Elan(Approved in Europe)*

(Source: Burgess,2001; Ansel *et al.* 2005.)

Approved lipid complex product:

Product	Drug	Year
Ambelcet	Amphotericin B	1995
Amphotec	Amphotericin	1997 (Burgess, 2001)

THERAPEUTIC APPLICATIONS OF LIPOSOMES:

Liposomes are used for the following range of therapeutics and pharmaceutical application.

Liposomal Anticancer drugs

The toxic side effects associated with the administration of anticancer makes these drugs ideal candidates for drug targeting. Anticancer agents have been encapsulated within liposomes in an effort to target such agents to tumors. The use of liposomes was hampered by the realization that liposomes are rapidly cleared from the circulation and largely taken up the liver macrophages (Gregoniadis,1972). The reduced liver and spleen uptake of stealth liposomes, as polyoxyethylene liposomes is due to reduced coating (opsonisation) of these liposomes aggregation of stealth liposomes in the blood is also responsible for the increased circulation time (Alexander, 1993). Liposomes size also affects biodistribution and a size of between 70 and 200 nm is necessary to achieve prolonged circulation times with stealth liposomes (Harashima *et al,* 1995).

Liposomes as Adjuvants

Liposomes have been firmly established as immunoadjuvants (enhancers of the immune-adjuvants (enhancers of the immunological response) potentiating both cell mediated and humoral immunity (Gregoniadis *et al,* 1996). Liposomal immunoadjuvants act by slowly releasing encapsulated antigen on intramuscular injection and also by passively accumulating within lymph nodes (Gregoriadis *et al,* 1997). Liposomal vaccines can been made by associating microbes, soluble antigens or DNA (Betageri *et al,* 1993) with liposomes. Liposomal vaccines may be stored dried at refrigeration temperature for up to 12 months and still retain their adjuvanticity.

Liposomal anti – infective agents

The use of amphotericin B, a polyene antibiotic, in the treatment of systematic fungal infec- tions by binding to sterols with extensive renal toxicity (Bennet *et al,* 1995). Amphotericin B acts mainly by binding to sterols such as ergosterol in membranes of sensitive fungi thus increasing membrane permeability (Bennet *et al,* 1996). Liposomal amphotericin B (Ambisome), the first liposomal preparation is used for the treatment of systemic fungal infections. The ability of liposomes to be taken up by macrophages and to concentrate in the liver and spleen undoubtedly makes them ideal for the treatment of diseases of the liver and spleen. Liposomes may be targeted to the lung by coating vesicles with O-stearoyl amylopectin and polyoxythylene. (Del P. et al 1334).

Active Targeting of Liposomes

Immunoliposomes

The production of stealth liposomes, which are not rapidly cleared by the liver and spleen made the active targeting of liposomes a real possibility. Various antibodies have thus been conjugated

to the surface of stealth liposomes to produce immunoliposomes for active targeting. Stealth liposomes bearing an antibody that specifically binds intracellular myosin in ischemic or necrotic cardiomyocytes targets infracted areas in rabbits (Torchilin et al, 1994). Attachment of antibodies like OX26 transferrin antibody (Huwyler et al, 1997) and a lung endothelial cell antibody of stealth liposomes (Maruyama K. 1997). Limitation to the clinical use of immunoliposomes include a failure to demonstrate an advantage in experiment al cancer chemotherapy.

Ligand bearing liposomes

An alternative targeting strategy that has a wider application utilizes surface moieties that are up regulated in certain disease as targets. These include the folate receptor over expressed in ovarian carcinoma. Liposomes bearing specific ligands such as folate may be used to target intergrins and selections (Fpnsse'e et al, 1998). Targeting with small ligands appears more likely to succeed than the use of antibodies since these ligands are easier to handle and manufacture.

Liposomes in Gene Delivery

Recombinant – DNA technology and studies of gene function and gene therapy all depend on the successful delivery of nucleic acids (genetic materials) into cells in vitro and in vivo. A variety of physical (e.g., electroporation microinjection) chemical (Calcium phosphate precipitation, poly-lysine conjugates) and biological (e.g. virus) method have been developed for transferring genes into cells. The most widely used type of vehicles for gene delivery are –viral (eg.Adenovirus, retrovirus and non – viral (e.g. Liposomes, lipid based systems). Among the non-viral vector system, liposomes and lipid complexes, especially engineered liposomes such as pH sensitive liposomes, cationic liposomes, fusogenic liposomes and lipopolyplex have extensively been investigated for their gene delivery potential. (Vyas and Khar). The cationic liposomes deliver the content through membrane fusion thus avoid lysos- omal and nucleolus degradation of DNA. It is an efficient means of gene delivery (Vyas and Khar). The pH sensitive liposomes have been reported as plasmid expression vectors for the cytosolic delivery of DNA. These liposomes were found to mediate the efficient transfection of DNA into a variety of cells in culture as fusogenicity was offered by both the constitutive lipids (Rahman *et al*,1990)

Other liposomes formulation

Other interesting liposomes based multivesicular formulation has recently been reported. This sustained formulation, developed by Depotech, has a particle size, of 1-5 mm and is formed from phospholipids, cholesterol and the oil tripalmitolein (Elena *et.al*) The system effectively controls the release of insulin like growth factor I.

Other Therapeutic Applications

1. Liposomes as artificial blood surrogates
2. Liposomes as radiopharmaceutical and radiodiagonostic carriers.
3. Liposomes in cosmetics and dermatology
4. Liposomes in enzyme immobilization and bioreactor technology.

EVALUATION OF LIPOSOMES

Liposome drug delivery systems are being developed for a variety of drugs. An eight step method to scale up the liposome process from laboratory scale to large scale process is known. A pH gradient across liposome bilayers was established in order to load a model drug (orciprenaline sulphate) into liposomes vesicles, which yields 80-86% encapsulation. Finally drug loaded liposomes were lyophilized using lactose. Five month stability data for the liposomes is reported. Liposomes prepared by pH gradient method show high encapsulation efficiency (Sriram, 1997). Feasibility studies were undertaken in order to explore the use of size exlusion chromatography, as a large scale process. Chromatographic conditions were optimized to scale up the process to larger batch size. Washing of liposomes with normal saline and subsequent separation of drug loaded liposomes is also a viable separation technique for scale up activities (Sriram, 1997)

Different techniques are used in the evaluation of liposomes like

High performance liquid chromatography assay

A Beckman Gold HPLC system with a # 168 detector and Pinnacle octyl amine (c8) column) (5 um, 150 X 4.6 mm) were used for all analyze. (Ran *et al*, 2003)

Optical microscope observation

An optical microscope (Leica Microsystems) was used to observe the particle size and shapes before particle size reduction. For Particle size reduction, Particle diameters above 0.3 µm were determine by using a calibrated eyepiece scale. Photograph were taken using a SPOT camera (Ran *et al*, 2003)

Particle size reduction

A laboratory homogenizer (Emulsifles-C5) is used to reduce the particle size of the liposomes (Ran *et al*, 2003)

Transmission electron microscope observation

It is used to observe the size and lamellarity of liposomes after size reduction by homogeniz-ation. Liposome suspensions were stained by 2% phosphotungstic acid/sodium hydroxide (pH 6.2) and dried on carbon – coated grids for observation (Ran *et al*, 2003)

Particle size measurements

The Zetasizer 1000 is used to measure the final particle size of liposomes in the range of 1 to 1000nm.

Drug Encapsulation Efficiency determination

$$EE = \frac{Cp}{Cp + Cs} x100\%$$

Where, Cp = Drug conc. in the pellet.

LITERATURE REVIEW:

Novel mucosal insulin delivery systems based on fusogenic liposomes.

Fusogenic liposomes (FLs) are unique delivery vehicles capable of introducing their contents directly into the cytoplasm with the aid of envelope glycoproteins of Sendai virus (SeV). The FLs containing insulin were prepared by fusing insulin-loaded liposomes with inactivated SeV particles and were administered directly into the ideal, the colonic, and the rectal loops (10 IU/kg). The FL successfully enhanced the insulin absorption and induced a significant hypoglycemic effect following the colonic and the rectal administration without detectable mucosal damage (Goto et al).

Report

FL is a useful carrier for improving the absorption of poorly absorbable drugs, such as insulin, via the intestinal tract.

A phase I clinical trial and pharmacokinetic evaluation of liposome encapsulated doxorubicin. (Rahman et al,1990)

14 cancer patients were treated with liposome encapsulated doxorubicin (LED) at doses of 30,45,60 and 90 mg/m2. Nausea and vomiting, phlebitis and stomatitis were minimal or absent at each dose, but dose – limiting granulocytopenia occurred at 90 mg/m2 and all cases at 90mg/m2. The plasma elimination of LED out to 24 hours was analyzed in terms of a two compartment model. These values are significantly higher than those expected for free doxorubicin. Urinary excretion of LED was approximately 10% after 24 hours.

Report

LED was well tolerated and produced only moderate nausea and vomiting and little stomatitis at myelosuppressive doses. The study also suggested that LED produces less venous sclerosis than free doxorubicin.

Phospholipid liposomes in antiatherosclerosis division of cardiology (Liu et al, 1992)

The effects of multiple intravenous injections of phospholipids liposomes in diet induced atherosclerotic rabbits are described. Elevated serum cholesterol and excessive phospholipids

removal through enhanced reverse cholesterol transport mechanisms were observed. RBC deformability caused by hyperlipidemia and blood viscosity decreased. Substantial shrinkage of atherosclerosis deposits in the aorta was also observed.

Treatment of Antigen induced arthritis in Rabbits with liposome-entrapped methotrexate injected intra-articularly. (Foong et al, 1993)

The determine the efficacy of treatment of antigen induced arthritis in rabbits with liposome entrapped methotrexate, rabbits with a bilateral antigen induced arthritis were injected intra articularly in one joint with methotrexate as the free drug or entrapped in liposomes.

Effect of collagen on liposome permeability (Pajean & Herbage, 1993)

The effects of collagen on the permeability and stability of various liposomes were studied.

In liposomes containing tocopherol, peroxidation was inhabited and to antioxidant effect of collagen was seen. However collagen decreased permeability by 20%. In liposomes containing cholesterol, antioxidant effect of colleges was seen. However collagen decreased permeability by 20%. In liposomes containing cholesterol, antioxidant effect collagen and its effect on permeability were similar to those in liposomes containing of 100% lecithin. The stability effect of collagen on permeability was completely suppressed in positive vesicles, but was 2 – fold greater in negative than in neutral liposomes.

Report

It was concluded that collagen improved the stability of liposomes containing α - tocopherol and cholesterol.

Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse phase evaporation. (Szoka and Papahad jopoules, 1978).

Large unilamellar and oligolamellar vesicles are formed when an aqueous buffer is introduced into a mixture of phospholipids and organic solvent and the organic solvent is subsequently removed by evaporation under reduced pressure. These vesicles can be made from various lipids or mixtures of lipid and have aqueous volume to lipid ratios that are 30 times higher than sonicated preparations and 4 times higher than multiamellar vesicles. A substantial fraction of the aqueous phase (up to 65% at low salt concentration) is entrapped within the vesicles, encapsulating even large macro molecular assemblies with high efficiency.

Report

This relatively simple technique has unique advantage for encapsulating valuable water – soluble materials such as drugs, proteins, nucleic acids and biochemical reagents.

Liposome Bilayers Nature's double (Alexander, 1993)

The use liposomes in cosmetics and a description of their physicochemical properties, which allows them to interact with the skin without disrupting its organization, are discussed, including the encapsulation of active ingredients, liposomes available for use in cosmetics application and currently marketed products.

Report

Special care is required in the handling of products containing liposomes.

Drug Delivery using Antibody – Liposomes conjugates (Betageri et.al 1993)

Liposomes bearing surface attached antibody were prepared to delivery didanosine triphosphate, the active metabolite of didnosite and evaluated in vitro for antibody uptake by human monocyte/ macrophage.

Report

Result indicated that uptake increased with time for all samples except for free didnosine triphosphate. It was concluded that didanosine delivery can be increased by antibody – liposome conjugates. (Betargeri et al, 1993)

Chromatographic Analysis and pharmacokinetics of liposome encapsulated doxorubicin in non-small cell lung cancer patients.

A sensitive and specific quantitative assay for total doxorubicin concentration in plasma containing liposome encapsulated doxorubicin hydrochloride using HPLC is presented and evaluated on 12 patients (age> 18 yrs). Blood samples were collected frequently up to 72 hours after drug administration and analyzed for pharmacokinetic parameters.

Report

Result indicated that the data were best described by a 3 compartment model. The plasma conc. time course for total doxorubicin suggested that the disposition of the liposomal formulation was determined more by the pharmacokinetics of the liposome than of the encapsulated drug.

Fusogenic liposome delivers encapsulated nanoparticles for cytosolic controlled gene release. (Kunisawa et.al)

Therapeutic agents based on DNA or RNA oligonucleotides require a regulation of their kinetics in cytoplasm to maintain an optimal concentration during the treatment period. In this study, we establish a protocol for the encapsulation of nanoparticles into liposome, which is further fused with ultra violet-inactivated Sendai virus to compose fusogenic liposomes.

Report

When nanoparticles were encapsulated in conventional liposomes, endocytosis-mediated uptake of nanoparticles was observed. In contrast, numerous amounts of nanoparticles were delivered

into the cytoplasm without any cytotoxicity when the particles were encapsulated in fusogenic liposomes. And they also had shown high ability to deliver nanoparticles containing DNA oligonucleotides into cytoplasm, which indicates that this combinatorial nanotechnology using fusogenic liposome and nanoparticle is a valuable system for regulating the intracellular pharmacokinetics of gene-based drugs.

Development of novel fusogenic vesosomes for transcutaneous immunization.

Transcutaneous immunization (TCI) is a novel vaccination strategy based on the application of antigen together with an adjuvant onto hydrated bare skin. In the present study, novel fusogenic vesicular carrier constructs, i.e. vesosomes were developed and evaluated for topical delivery of vaccines using tetanus toxoid (TTx) as a model antigen. Prepared vesosomes were examined for in process antigen stability and long-term storage stability studies. The immune stimulating activity of these vesicles was studied by measuring the serum anti-tetanus toxoid IgG titer and isotype ratio IgG2a/IgG1 following topical immunization in three different protocols and results were compared with the alum adsorbed tetanus toxoid given intramuscularly and topically administered plain tetanus toxoid solution, plain liposomes and cationic fusogenic liposomes.

Report

Serum IgG titers after three consecutive topical administrations were significantly better (*P < 0.05) than single administration of TTx antigen with vesosomal systems, suggesting an effective stimulation of serum immune response. (Mishra et al)

Efficacy of Liposome – Encapsulated butylaminobenzoate for topical anesthesia of the skin.(Veenbass et al, 1993).

To investigate the suitability of liposomes as drug delivery system for local anesthetics on the skin, the local anesthetics efficacy of NAT liposome encapsulated butylaminobenzoate (butamben) was tested by performing a pin-prick technique.

Report

It was concluded that suitable combination of a local anesthetic and liposome of certain phospholipids composition needs to be developed.

Liposome mediated depletion of macrophases: mechanism of action, preparation of liposomes and applications. (Rooijen & Sanders ,1994).

Selective depletions of macrophages from tissues in vivo can be used to investigate weather these cells are playing a role in defined biological processes. A macrophage ‘suicide’ technique has been developed, using the liposomes mediated intracellular delivery of

dichloromethylenebiphosphonate (C_{12}MBP or clordronate), the method is specific with respect to phagocytic cells of the mononuclear phagocyte system (MPS).

Report

The preparation of C_{12} MBP – liposomes has been described in detail. Furthermore the mechanism of action of the new approach and its applicabilities are discussed (Rooijen *et al*, 1994)

Preparation of tissue type plasminogen activator (t-PA) containing liposomes: entrapment efficiency and ultracentrifugation damage. (Heermans et al, 1995).

A method for the efficient entrapment of active alteplase (tissue type plasminogen activator; t-PA; Actilyse) concentration, different buffer pH and liposomes size was determined in the presence and absence of polysorbarte 80 (tween-80)

Report

Alteplase entrapment strongly dependent on experimental conditions and varied from 30 to 90%. Ultracentrifugation used for removal of non entrapped alteplase, was shown to have a damaging effect on the liposomes leading to alteplase loss. (Heermans *et al*, 1995)

***In-vivo* disposition characteristics of plasmid DNA complexed with cationic liposomes.** (Mahato et al, 1995)

To control disposition and hence gene expression, the disposition characteristic of plasmid deoxyribonucleic acid (DNA) complexed with the cationic liposomes lipofectin and lipofect ACE after i.v. injection in mice via the tail vein were studied. Following administration of radiolabelled plasmid deoxyribonucleic acid, radioactivity was rapidly eliminated from plasma and approximately 60% of dose was taken up by the liver in 1.5 minutes. With lipofect ACE complexes radioactivity elimination was equally rapid.

Report

For, lipofection samples, radioactivity was initially accumulated in both liver (55%) and the lung (29%). But lung accumulation was not sustained beyond 5 minutes after administration (Mahato, R.I.; *et al*; 1995)

Fusogenic liposome delivers encapsulated nanoparticles for cytosolic controlled gene release (Jun Kunisawa & Takashi Masuda *et al*, 2005)

Therapeutic agents based on DNA or RNA oligonucleotides (e.g., antisense DNA oligonucl-eotide, small interfering RNA) require a regulation of their kinetics in cytoplasm to maintain an optimal concentration during the treatment period. In this respect, delivery of functional nanoparticles containing these drugs into cytoplasm has been thought to have a potential for the

cytosolic controlled gene release. In this study, we establish a protocol for the encapsulation of nanoparticles into liposome, which is further fused with ultra violet-inactivated Sendai virus to compose fusogenic liposomes. When nanoparticles were encapsulated in conventional liposomes, endocytosis-mediated uptake of nanoparticles was observed. In contrast, numerous amounts of nanoparticles were delivered into the cytoplasm without any cytotoxicity when the particles were encapsulated in fusogenic liposomes. Additionally, fusogenic liposome showed a high ability to deliver nanoparticles containing DNA oligonucleotides into cytoplasm.

Report - These results indicate that this combinatorial nanotechnology using fusogenic liposome and nanoparticle is a valuable system for regulating the intracellular pharmacokinetics of gene-based drugs.

CONCLUSION

In this review article, we have studied the preparation, classification, drug loading and release characteristics, stability, marketed products, evaluation of liposomes. Also we covered recent scientific developments of liposomes including research articles and review articles. Although liposome has some disadvantages like their tendency to be taken up by reticuloendothelial system and the slow release of the drug when the liposomes are taken up by phagocytes through endocytosis, fusion, surface adsorption or lipid exchange. But it can safely be said that liposomes are the most widely studied modern drug delivery system. While effective anticancer formulations have been developed after years of painstaking research, development in the field of gene delivery are predicted to be the next growth area.

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