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REVIEW ARTICLE

LIPOSOMAL TOXICITY: A'SEEM TO BE' CONCERN FOR FORMULATION TECHNOLOGISTS

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ABSTRACT

Liposomal drug delivery (LDD) has become a promising strategy & is continuing to be a big success for the Pharmaceutical Industry. Toxicity associated with LDD did not pin point intricately making it impossible to construe. Although the induction of Liposomal toxicity is mysterious & there has been a limited exploration, there was some evidence from animal studies which include haemolysis platelet aggregation, emboli, seizures & 'proton sponge effect'. The mechanism responsible for induction of immune responses is revealed by protein kinase-C mediated down regulation of TNF-α, NO, PGE-2. Reports at the molecular level has been proved that Liposomal efficiency/ toxicity profile was influenced by factors like molecular weight, ionic strength particle size & the amount of drug load into the liposome i.e., anticancer drug loaded excessively into liposome has lead to cardio toxicity, skin toxicity. Increased concentration of drug hampered the efficiency due to crystalline formation & altered rate kinetics of drug release. The present objective of the study is to signify the causes of toxicity & attempts to wipe out the harmful effects associated with LDD. The task of minimizing the toxic effects of LDD is challenging aspect for the pharmaceutical technologist & makes this novel drug therapy safe, effective & unblemished.

Key words*:* Cationic, Cellular toxicity, Phospholipids, Protonsponge effect

INTRODUCTION:

Liposomal drug delivery (LDD) is a rapidly expanding area of pharmaceutical research that holds the great promise for targeted drug delivering & has become a fertile topic of research currently.LDD seems to be attractive compared to conventional dosage forms because of the innovative ride taken up by the pharmaceutical technologist in the formulation, which is not going to be that easy as there are issues like stability, efficacy & toxicity have to be considered. So far several studies focused on efficacy & stability of the formulations which is of course important but today the toxicity profile has become a serious consequence which leads to unsparing effect on life. Despite a wealth of data is available on both the stability & efficacy of liposomes and the toxic nature & their physicochemical interaction with cellular components is still scarce. In the view of recent findings, apart from the pharmacological actions cationic liposome's have profound toxic effects like emboli, platelet aggregation, haemolysis, immune response, neurotoxicity etc, which might be due to interactions of liposome with cellular components. LDD for cancer therapy was found to be effective but the toxicity addressed is still an obstacle i.e. drugs like doxorubicin encapsulated in very high concentration in liposomes shown cardio toxicity & skin toxicity[1].

Causes of toxicity:

- **Liposomal components**

The composition of lipid formulations can be carefully tailored, because chronic administration of liposomes can induce toxicity dependent on composition. For instance, one frequently used lipid formulation deals with the incorporation of cationic lipids in liposomes to improve cellular uptake of the particles. Therefore, it is necessary to study the nonspecific effects associated with the particular composition and other properties of liposomes should be carefully considered during

formulation.

Although phospholipids themselves are generally non cytotoxic, cationic lipids, like 1, 2-dioleoyl-3-trimethyl ammonium propane (DOTAP) which is a well known cationic lipid in DNA transfection procedures, have been described to damage cellular physiology [2].The degree of haemolysis depended on the concentration and the type of phospholipids used in the formulation. Negatively charged DSPG (Distearoyl phosphatidyl glycerol)-liposomes induced a small but reversible platelet aggregation. These liposomes also affect the coagulation cascade, which shows prolonged coagulation times. Cationic liposomes may be useful in the treatment of a number of diseases, including DNA delivery for gene therapy. But have higher toxic effects than neutral & anionic because they are highly fusogenic & have highest transfection activity[3].

Example: DOPE: cholesterol: DC-C-14(3:3:4)

Table 1: Differences in toxicity between different Liposome types

Lipids	Urinary bladder carcinoma	Stomach infections	Colonadeno Carcinoma	Lung carcinoma	Rhabdomyo carcinoma
PC:Chol	++++	Unknown	+	+++	++++
PC:Chol	++	Unknown	+	++++	++++
DPPC:Chol	+++	Unknown	+++	++	+++
Card:PC:Chol:SA	+/-	+	++	++	+
PG:PC	++	+	+	++	++

PC: phosphotidyl choline; SA: stearylamine; DPPC: d ipalmitoylphosphatidylcholine

The correlation between the net Liposomal charge and toxicity is unambiguous. At the present, the mechanism(s) for these toxic effects are not Known, but thought to be mediated directly at the cell surface. Neutral DPPC: chol and PC: chol liposome were nontoxic, whereas neutral card: PC: chol: SA Liposomes were as toxic as the positive SA: PC: chol liposome. Stearyl amine liposomes with blood cells induced cell lysis [4].

Lipid structure vs. toxicity

Cationic lipids used for LDD are composed of three basic domains: A positive charged head group often consists of primary, secondary, tertiary amines or quaternary ammonium salts, cytotoxic effect associated with the lipid formulation is mainly determined by the structure of its hydrophilic group. A linker joins the polar and non-polar regions, most of that in the lipids are ether, ester or amide bond. Although compounds with ether linker render better transfection efficiency, they are too stable to be biodegraded thus cause toxicity, finally a hydrophobic chain[5].

Example: The three basic domains of 1, 2-dioleoyloxy-3-trimethylammonium propane (DOTAP). The polar and hydrophobic domains of cationic lipids may have dramatic effects on both the transfection and toxicity levels.

Various studies found that toxicity of liposomes is associated with type of lipid used in formulation of liposomes & few are depicted as follows: There are two major types of hydrophobic moieties, namely aliphatic chains and cholesterol-based derivatives. In general, for aliphatic chains, single-tailed cationic lipids are more toxic and less efficient than their double-tailed counterparts.6- lauroxyhexyl ornithinate (LHON) with one tail was more efficient & of lower cytotoxicity compared with DOTAP[6].

Cationic amphiphiles containing steroid back bone were more potent inhibitors of PKC than their straight chain analogues; therefore they had higher toxicity profiles[7]. Cetyl trimethylammonium Bromide (CTAB) was more toxic and less efficient than DOTMA (dioleyl tri methyl ammonium)[8]. The obvious difference between cationic polymers and cationic lipids is that they do not contain a hydrophobic moiety and are completely soluble in water [9]. Compared with cationic liposomes, they have the absolute advantage of compressing DNA molecules to a relatively small size[10].This is most useful in gene transfer as small particle size may be favorable for improving transfection efficiency[11]. Modifications to these polymers such as molecular weight, geometry (linear vs. branched) and ligand attachment can be easily achieved, so that the out comes from such modifications can be extensively used for the formulation by conducting structure/function relationship studies.

For example, Polyethylenimine (PEI), a commercially available cationic polyamine is one of the most successful and widely used as gene delivery polymers. There are mainly two types of structure: Linear molecule and branched molecule. PEI is a gene carrier with high transfection efficiency and high cytotoxicity [12]. Many factors affect the efficiency/cytotoxicity profile of PEI such as molecular weight, degree of branching, ionic strength of the solution, zeta potential and particle size [13]. One study, as an exemplary, showed that low molecular weight (10 k Da), moderately branched polymer resulted in efficient delivery with low toxicity when compared with high molecular weight commercially available PEI. Cationic lipids are usually used in association with dioleyol phosphatidyl ethanolamine (DOPE) to deliver nucleic acids inside mammalian cells for gene therapy[14].

The rank order of toxicity profiles for some lipids was as follows:

DOPE/DDAB > DOPE/DOTAP > DOPE/DMTAP > DOPE/DPTAP > DOPE/DSTAP.

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DOPE :(Dioleoylphosphatidylethanolamine); DDAB (Dioleyl diacyl ammonium bromide)DOTAP :(Di oleyl tri methyl ammonium propane); DMTAP: (dimethyl di oleyo diacyltrimethylammonium propane); DPTAP (dipalmitoyl- diacyltrimethyammonium propane); DSTAP :(distearoyl-diacyltrimethyammonium propane);[2].

Three Novel galactosylated cholesterol derivatives, such as cholesten-5-yloxy- N-(4-((1-imino-c-β-D-thiogalactosyl-ethyl) amino) butyl) formamide (Gal-C4-Chol) and its ethyl formamide and hexyl formamide analogues (Gal-C2-Chol, Gal-C6-Chol) were synthesized by Liposome/DNA complexes prepared with these lipids showed low cytotoxicity in human hepatoma Hep G2 c therefore different reagents have different degrees of toxicity to cells, and toxicity is cell-specific [15].

The influences of liposomal phospholipid composition on the activity of incorporated immunomodulatory drugs have so far been assumed to be relatively unimportant because of the presumed inert nature of phospholipids towards the immune system[16]. There is, however, ample evidence of intrinsic immunomodulatory activity for several phospholipids. For example, phosphatidyl serine has been shown to stimulate macrophage growth [17] and to inhibit the production of IL-2 and IL-2 receptor expression in T-lymphocytes [18] while phosphatidylethanolamine can stimulate the synthesis of prostaglandin E2 by macrophages [19].

One of the significant factors was observed; that is the charge, which is negatively correlated to the sensitivity. Positive charge was defined as high level which means positively charged liposomes reduces the sensitivity value and consequently is more toxic than negatively charged liposomes. It has been reported that polycations such as polylysine, polyhistidine and polymyxin B cause aggregation, fusion and lysis of the membranes. The liposomes bound with these polycations would act as the membrane-damaging agents. The destabilization of the endosomic membrane and subsequently release of cationic lipid into the cytoplasm is necessary for the induction of toxicity which clearly confers that liposomes must be internalized to induce toxicity [20]. The liposome protects the drug from metabolism & reduces the extent of drug accumulation in healthy tissues [21] there by low level of adverse effects. However, Drugs loaded in liposomes in higher concentration hampers the drug efficacy because of reduce concentration of drug at tumor site & altered rate kinetics of drug. Molecular mechanism reveals that higher concentration of drugs in liposomes forms larger size vesicles that reduce the membrane stability of the cells due to electronic interactions [22].

One such case of drug formulation is LDOX (Liposomal doxorubicin), LDOX is a powerful mutagen, embriotoxic & induces long term sterility. The evaluation of reproductive toxicity was restricted to fertility studies, since there were data on the embryo toxic and teratogenic nature of doxorubicin HCl, as well as on its ability to induce long-term or permanent sterility, also involve in shrinking of cells by vacuolizing the cytoplasm. A high dose of cationic lipids increase electrostatic interactions with plasma membrane results in cytotoxicity & is inadequate for therapeutic application. Data on the haemolytic potential of LDOX submitted showed that they caused haemolysis of human RBCs or coagulation or precipitation of human serum or plasma. One of the most significant toxicities for LDOX is a condition consisting of dermal lesions referred to as H-F syndrome (Hand and Foot syndrome)[23,24]. This same condition was previously described in patients receiving long continuous infusions of 5- fluorouracil, DOX.

Table 2: Various toxicities associated with free doxorubicin and LDOX

Free doxorubicin HCL	**LDOX (liposomal formulation)**
a) Cardiac toxicity	Reduced or not observed
b) Myelosuppression	Reduced (to a greater extent with SSLs)
c) Mucositis	Slightly increased with Doxil
d) Alopecia	Reduced or not observed
e) Severe local tissue necrosis after drug extravasations	Observed with Doxil or with continuous infusion of free DOX
f) nausea & vomiting	Reduced or not observed
g) H-F syndrome	Observed with Doxil or with continuous infusion of free DOX

The toxicities represented in table 2 are thought to be due to the inability of liposomes to cross the endothelial cell barrier in the tissues and the low bioavailability of the free drug due to its encapsulation in liposomes. Indeed, in tissue sections of cardiac muscle, liposomes are found exclusively in the blood vessels and not in the muscle fibers suggesting that most of the drug is not bioavailable in the myocardium. Positively charged liposomes like stearylamine were reported to enhance the permeability of biomembranes and damage the cells. DC-6-14 liposomes can interact with plasma proteins and blood cells, leading to a gradual Liposomal lysis. However hemolytic activity of DC-6-14 liposomes was weaker than that of stearylamine-containing liposomes. Cationic lipids have longer times, escape from liver uptake & show specific receptor site of action. Shrinking of cells reduced no of mitoses & vacuolization of the cytoplasm are the unidentifiable effects, where as DC-6-14 Liposomes don't circulate accumulate in lung after administration leads to liver uptake.

Furthermore, it was reported that the use of cationic liposomes to target DNA to the gastrointestinal tract is inappropriate & also Cationic DOPE: DOTAP liposomes are extremely toxic to CD1 (mice) on the administration of a single dose, provoking a profound and lethal hypothermia. Hence, Cationic lipids with heterocyclic rings shown low le vel of toxicity & would be worth for in vivo delivery of drugs. Tertiary analogues of DOTAP (dioleoyl trimethylammonium propane) DODAP(dioleoyl-diacyltrimethylammonium propane) did not induce inflammation but histological assays revealed mild toxicities in muscle cells.di-c-14 amidine up regulated CD80,CD86 membrane expression of cytokines &interferes with cell signaling path way. The uptake of liposomes in cells involves binding of the liposomes to the cell surface followed by endocytosis.

Different cells vary with their ability to take up liposomes and thereby by their sensitivity towards the Liposomal formulation. It seems possible that at least some of the toxic effects of liposomes are mediated directly at the cell surface, especially for cells which are not strongly endocytotic. The usage of neutral lipids allows one to decrease toxicity and attain higher transfection levels in vivo which may be determined by their special structures. For instance, DOPE can facilitate membrane fusion and aid the destabilization of the plasma lemma or endosome.

Mechanism:

The mechanism of induction of toxicity has a limited exploration. Further data from the research have yet to be elucidated. Lipids are not just carriers for the drug delivery but do modify cellular path ways & stimulate anti inflammatory responses especially cationic lipids[25]. Ex: poly cationic sphingolipid shown strong humoral & cellular responses (production of inter lueikins-4) cytotoxic activity.

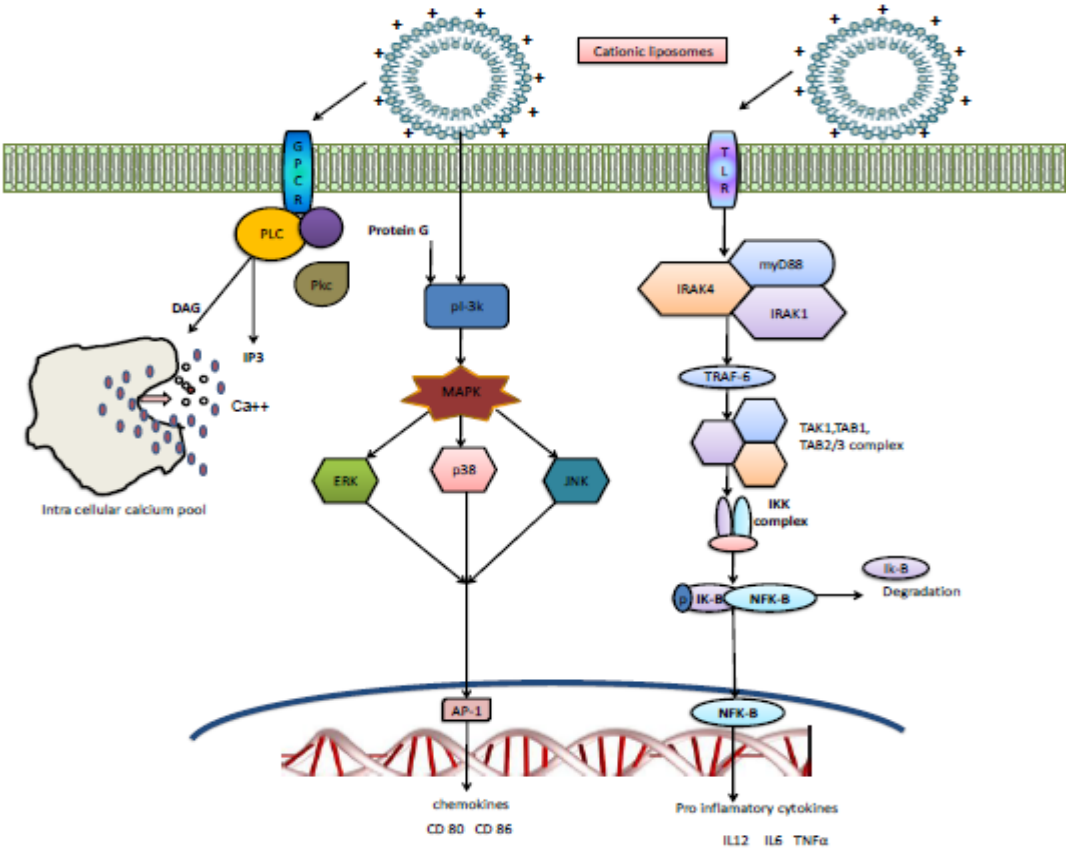

Figure 1: Representation of Mechanism of Cationic liposomes or components recognize G-protein couple receptor and activates MAPK (Mitogen activated protein kinases through PI-3 kinase and induce the expression of chemokines in dendritic cells, via Ap-1(Activator protein). In general signalling pathways of MAPKs transduce extracellular stimuli to altered gene expression resulting in cell proliferation, differentiation and apoptosis mediated by ERK (Extracellular Signal Regulated Kinase), JNK (C Jun amino-terminal kinase) and P38 kinase. Cationic liposomes activate phospholipase C, thereby increase the intracellular calcium and IP-3(Inositol triphosphate) levels and stimulate the MAPK's, or directly gets inserted into the plasma membrane and can alter the membrane proteins function. Liposomes containing amide and amine groups can stimulate the dendritic cells through their interaction with TLR (Toll like receptor) and activates a series of proteins i.e., MYD88 (Myeloid differentiation protein) IRAK (Interleukin-1 receptor associated kinase), TRAF (Tumor necrosis factor associated kinase) and IKK(Ik-B kinase) which are involved in the synthesis of nuclear transcription factor NF-kB(Nuclear factor kappa light chain enhancer of activated B cells) that involves in production inflammatory mediators like Interleukins, TNf-α etc further gets recruited by macrophages at the site of tissue damage and ultimately worsens the damaged tissue.

Proton sponge effect: Amine groups present in lipids gets protonated by the addition of protons pumped into lysosomes together with chloride ions to maintain charge neutrality results in higher ionic strength of lysosomal matrix followed by osmotic swelling & rupture of lysosomes. The release of lysosomal enzymes & acidic contents leads to cell death [26].

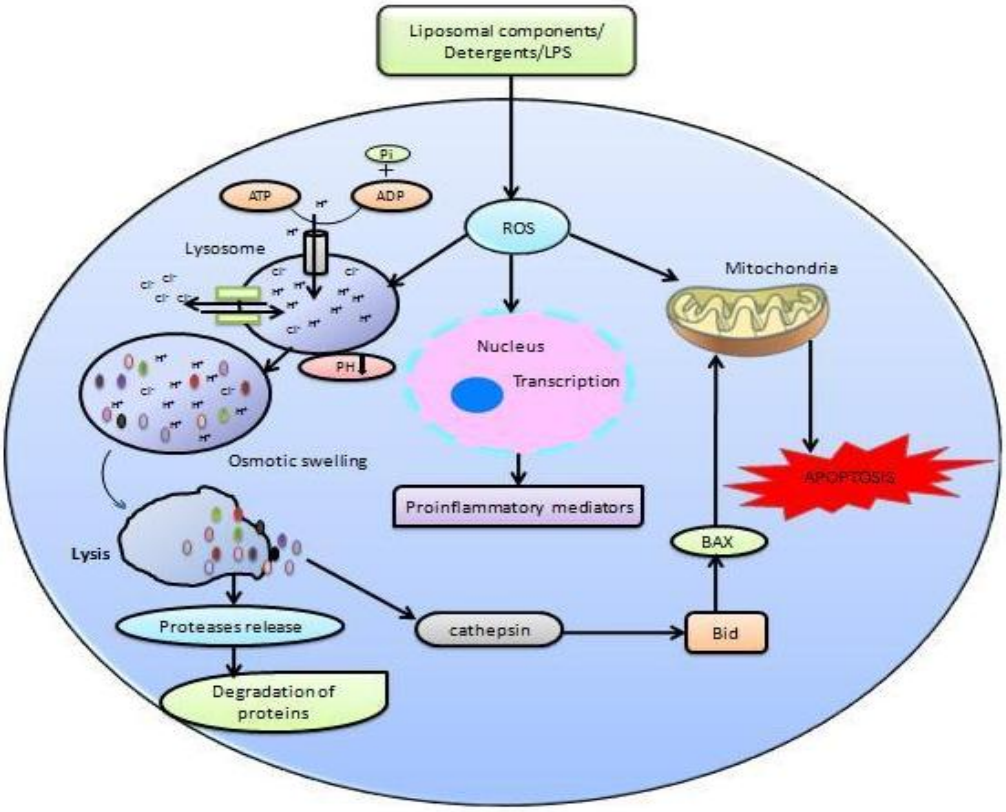

Figure 2: Cationic (for example PEI-coated) particles bind with high affinity to lipid groups on the surface membrane and are endocytosed into the cell. When a liposomal component interacts with cell membrane due to oxidative stress it generates Reactive Oxygen Species (ROS) which in turn has effects on the cell organelles. Once these cationic particles enter into an acidifying lysosomal compartment, the unsaturated amino groups are capable of sequestering protons that are supplied by the v-ATPase (proton pump) on the lysosomes. This process keeps the pump functioning and leads to the retention of one Cl^- ion and one water molecule per proton. Subsequent lysosomal swelling and rupture leads to spillage of the lysosomal content into the cytoplasm. Cathepsins are the components of lysosomes which can amplify the apoptotic signals by degradation of antiapoptotic factors like Bcl (B-Cell Lymphoma) and promoting the cleavage of Bid. Cleaved Bid gets translocated into mitochondria via bax which is a proapoptotic factor. As a consequence procaspases activated into caspases in mitochondria leads to cell death. ROS alters the transcription in the nucleus and produces proinflammatory mediators which have profound effect on cells.

The toxicity and the immunomodulatory activity of different cationic lipids toward macrophages and T-lymphocytes have been evaluated. Some of the lipids, such as derivatives of cholesterol, are protein kinase C (PKC) inhibitors, which may be associated with their own profile of toxicity i.e., Cationic amphiphiles containing steroid backbones were more potent inhibitors of PKC than their straight-chain analogues, therefore they had higher toxicity too. Cationic amphillic compounds can inhibit PK-C activity results in down regulation of NO, PGE-2, TNF-α. The fact that cationic liposomes are highly toxic toward macrophages but not toxic toward T lymphocytes was explained by the enhanced relative phagocytic activity of macrophages compared to T cells. Toxicity and down regulation of NO and TNF- α was related to the presence of cationic lipid and was enhanced by DOPE (Dioleoylphosphatidylethanolamine) & Dc-Chol (dimethyl aminoethanecarboyl– cholesterol).

Cationic liposomes down regulated NO, TNF-α and PGE2 synthesis which has been proved from the experimental studies like incubation of macrophages with DOPE: DOTAP and DC: Chol resulted in reduction of macrophages. The mechanism by which liposomes results in loss of cell integrity and cell death might be the same, the initial adherence of the formulation to the cell surface which may be of greater importance for the toxicity in cells that are less phagocytic in nature. The toxicity toward macrophages indicates that the cationic liposomes must be internalized to induce toxicity. The replacement of DOPE by non- fusogenic DPPC lipids also abolished this toxicity, indicating that the destabilisation of the endosomic membrane and subsequently release of cationic lipid into the cytoplasm is necessary for the induction of toxicity.

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Reported adverse effects associated with the use of cationic lipids or Cationic liposomes [2]

In vitro toxicities	In vivo toxicities
Induction of chromosome aberrations in human culture cells	Induction of eyes inflammation in rabbits when instilled intra-ocularly
Induction of haemolysis	Affects immune response to antigens in mice, dependence upon type of antigen, dose and time of administration
Inhibition of the respiratory burst of neutrophils	Neurotoxic after i.c. injection; produces epileptic seizure and death in mice
Enhanced superoxide production by neutrophils	Activation of complement via the alternative pathway
Inhibition of PKC activity	Toxic when administered intra-articularly into knee joints
Toxic for non-phagocytic cells	Induction of acute pulmonary inflammation reaction
Down-regulation of the production of IgG and IgM by human peripheral blood mononuclear cells	Induction of emboli after i.v. injection
Aggregation of cationic liposomes by albumin, IgG or salivary glycosaminoglycan	Highly toxic when administered orally; provoking a profound and lethal hypothermia
Highly toxic for phagocytic cells	Down-regulation of NO, TNF-a and PGE2 synthesis
Down-regulation of NO, TNF-a and PGE2 synthesis	Strong anti- inflammatory activity in carrageenan and in sheep red blood cell challenge inflammatory models
Inhibition of DNase activity	Induction of anti-single strand DNA antibodies
Inhibition of DNase activity	Induction of anti-single strand DNA antibodies

Formulation factors behind the toxicity:

When formulating liposomes the choice of main lipid, the choice of charged component, and the amount of charged component in the liposomes are important issues and have to be considered to obtain Liposomal formulations with minimum toxicity. When formulating positively charged liposomes a low amount of charged lipid is favorable. DPPC (dipalmitoylphosphatidylcholine) seems to be the best choice as the main lipid.

Liposomal dose: High lipid doses bind to endogenous lipids results in the interactions may result in toxicity i.e., of two types[27,28]. Immediate due to interaction with the plasma proteins majorly with albumin and delayed due to clusters of RBC adhere to cell surface & destabilize the plasma membrane.

Drug loading: Drugs loaded in liposomes in higher concentration hampers the drug efficacy because of reduced concentration of drug at tumor site & altered rate kinetics of drug. LDOX (Liposomal doxorubicin) is a power full mutagen, embryo toxic & induces long term sterility, also involve in shrinking of cells & vacuolizes the cytoplasm.

Liposomal size: Larger vesicles containing higher amount of drugs reduces the membrane stability of the cells due to electronic interactions which leads to toxicity. It was also proven that SUV were more toxic than MLV. Diameter does not influence toxic profile significantly.

Method of sterilization: Liposomes composed of unsaturated phospholipids revealed toxic effects on the cell line, and their toxic effects were increased after gamma irradiation. Liposomes composed of saturated phospholipids did not show any toxic effects, and form safety & drug delivery point of view, gamma irradiation is an appropriate sterilization method for them.

Few recent findings to conquer problems associated with LDD:

Solutions proposed to surpass the toxicities by making use of biodegradable polymers, saturated oleic lipid chains, complexation of lipids with DNA adjusting physical properties, dose, and composition of carriers. It has been reported that modifying the molecular structure of lipids and other components made their utilization in a safe manner. Biomaterials because of their non-toxic and enzymatically biodegradable properties, like Carboxymethyl chitin (CM-chitin) has been used to stabilize liposomes composed of phosphatidylcholine (PC) ,CM-chitin will bound to the surface of the liposomes, thereby strengthening the liposomes and increasing their stability[29]. Neutralizing the charge of cationic lipid & certain alterations in the molecular structure of lipids has made their utilization more feasible. Cationic lipids with heterocyclic rings show low level of toxicity & would be worth for in vivo drug delivery. The replacement of DOPE by DPPC reduced cationic liposomes toxicity toward macrophages and the use of DPPE-PEG2000 abolished toxicity by blocking liposomal endocytosis [30]. It was suggested that i.v injected DC-6-14 aggregates with blood cells & these aggregates were trapped in lung capillaries temporarily as the liposomes left the lung, aggregates breakage might occur. Interaction between cells & liposomes might be relatively weak as to induce degradation of aggregates in lung capillaries. Then blood cells & liposomes were redistributed gradually to blood stream & to liver. Glycolipid liposomes were suggested to be adsorbed on blood cell surface & escape liver uptake. Tertiary analogues of DOTAP (dioleyl-trimethylammonium propane) & DODAP (dioleyl diacyl ammonium propane) did not induce inflammation. Di-c-14 amidine up regulated CD 80, CD86 membrane expression of cytokines & interferes with cell signaling path way. Galactosylated cholesterol derivatives were used in

Liposomal / DNAcomplexes showed significantly less cytotoxicity compared to the commonly used cationic liposomes [31].

Detoxification of Liposomal doxorubicin (LDOX) by double filtration in-*vitro* plasmapheresis Liposomes composed of unsaturated phospholipids revealed toxic effects on the cell line, the liposomes became more negatively charged after irradiation due to the presence of charged degradation products by undergoing peroxidation. Liposomes composed of saturated phospholipids did not show any toxic effects, and from safety and drug delivery point of views, gamma irradiation is an appropriate sterilization method for them.

CONCLUSION:

It is apparent that potential non specific effects associated with particular composition & other properties of liposomes should be carefully assessed. This problem is likely to intensify the risk factor involved in formulation. Though much has been said about LDD things have so far not been turned out as expected, if they could be carefully handled by technologist, which might further attain higher success rate & better results so that LDD becomes appreciable in drug targeting. Cationic liposomes for DNA therapy should be considered in order to avoid these dose-limiting and often fatal adverse effects associated. Hence, it is note worthy that pharmaceutical technologist should pay keen attention & inculcate the appropriate selection of lipid components which may open new avenues in attempts to design LDD for targeting. This could be required not only for minimizing toxic effects but also adequate for therapeutic effect.

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