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pH-Sensitive liposomes: mechanism of triggered release to drug and gene delivery prospects

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A growing amount of literature describes the development and applications of novel targeting and/or release triggering schemes to improve the therapeutic index of drugs encapsulated within liposomes. The composition of liposomal systems can be modified to facilitate site specific release in response to environmental conditions, namely at the gross anatomical level, at the cellular or sub-cellular level. They are designed in order to release their contents in response to acidic pH within the endosomal system while remaining stable in plasma thus improving the cytoplasmic delivery of various polar materials and macromolecules such as anti-tumor drugs, toxins, proteins and DNA. This review covers various aspect of research on pH-sensitive liposomes.

1. Introduction

Novel liposomal systems may incorporate some time-dependent or other specific inducible changes in the liposome membrane or its coating to produce "intelligent" systems that will change their properties (e.g. leakage rate, fusogenic activity and interaction with particular cells) upon a specific trigger following administration. In recent years, considerable attention has been given to liposomes as a carrier for site specific delivery in a specific cellular or subcellular compartment. Liposomes that are sensitive towards applied signal or environmental stimuli are being investigated as a future tool for targeted drug delivery. The triggering of the release with the help of physical modulation or manipulation of the internal or external environment of the lipid constructs has been discussed under various titles, viz. signal sensitive, stimuli sensitive or physical targeting approaches. Signal sensitive liposomes belong to a class of ligand appended liposomal constructs which are responsive to bio-signals and environmentally modulated by physical means to program and monitor selective release of contents. In addition to influencing the *in vivo* distribution of the liposomes, different membrane compositions can alter the manner in which the liposomal contents is presented and released within the biological environment. The compositions can be manipulated to facilitate site specific release in response to environmental conditions, namely at the gross anatomical level, at the cellular or sub-cellular level. Mechanisms that can be exploited for targeting to each of these levels include pH and temperature-sensitivity, enzymatic triggering, magnetic and light-sensitive liposomes. Many of the approaches have, in fact, been explored for unloading liposomes upon application of an external stimulus. This review explores various facets and motifs associated with pH-sensitive liposomes.

2. pH-Sensitive liposomes

pH-Sensitive liposomes have been developed to improve the efficiency of the cytoplasmic delivery of various polar materials and macromolecules such as anti-tumor drugs, toxins, proteins and DNA. The effectiveness of the liposomes as a delivery system for these compounds has been illustrated by many studies. pH-Sensitive liposomes are designed with an aim to circumvent the problems associated with plain liposomes (e.g., lysosomal enzymatic degradation), and to deliver their contents directly to the cytoplasm. The use of pH-sensitive liposomes as drug delivery system was suggested by the observations that pathological tissues (tumors, metastases, inflammation and infection) have an ambient pH that is considerably lower than the normal tissue. Therefore, the pH could judicially be used for site specific delivery of bioactive molecules.

3. Underlying mechanism(s) for pH-triggered release

Induction of the well known lamellar to hexagonal phase transition (L_{α}-H_{II} phase transition) for phosphatidylethanolamine (PE) lipids upon protonation of amine group (or analogous, pH-sensitive co-surfactant) is the most common functional mechanism employed in case of pH triggered release of liposomes (Fig. 1). The lipid composition of pH-sensitive liposomes, where the commonly used lipid is dioleoyl PE (DOPE), determines the membrane stability at neutral pH and the sensitivity of the membrane to destabilization so that it is fusogenic at acidic pH of the endosomal system/target site. The majority of the pH-sensitive liposomes reported in the literature have used PE bilayers that are stabilized at non-acidic pH by the addition of some lipid constituents or co-surfactant that contain a carboxylic acid group. In pH-sensitive liposomes, the negative charge of carboxylic acid increases the size of the lipid head group at pH greater than the pKa of carboxylic acid, thereby stabilizing the bilayer at pH of formulation and storage (7.4). However, at an acidic pH (as encoun-

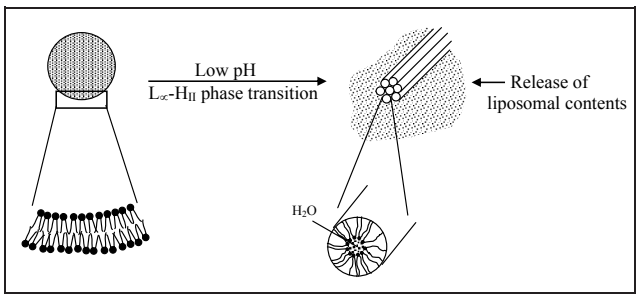

Fig. 1: Lamellar-hexagonal phase transition of pH-sensitive liposomes induced by low pH

tered in endosomes), uncharged and reduced charged species fail to stabilize the PE-rich bilayers. In the event of pH change, pH-sensitive liposomes are converted into a form, which prefers to adopt an inverted hexagonal phase rather than the conventional bilayer sheet of the lipoidal membrane. Typically, the co-surfactant is added at concentrations sufficient to stabilize the lamellar phase of PE at 37 °C and pH ~7.4. Acidification of these systems either *in vivo*, within endosomal compartments (pH 4.5~6.5), or at sites of low pH (e.g., pH ~6.2-6.9 in tumor interstitial fluid and sites of inflammation) leads to destabilization of the PE : co-surfactant liposomes followed by release of its encapsulated contents [1]. These liposomes have extensive application in drug delivery of bioactive materials to cells as they provide a means for enabling the contained contents to escape the lysosome and the subsequent enzymatic degradation. The first step in the intracellular processing of liposomes is the uptake into an endosome, which has an intravesicular pH of 5–5.5. If liposomes can be induced to become fusogenic upon exposure to this pH, then they may release their contents into the cytoplasm of the cell before they are broken down and degraded within the lysosome. Mechanisms for pH-sensitive liposomes which are largely based on phosphatidylcholine and supported trigger components are quite distinct, individual in nature and at times more than one mechanisms may operate to make them pH-responsive.

4. pH-Sensitive lipids used for liposome triggering

4.1. pH-Triggering of phosphatidylethanolamine (PE)-based liposomes

Delivery with pH-sensitive liposomes is mainly based on acid-triggered events within an acidic endosome following endocytosis. Most pH-sensitive liposomes have been prepared using unsaturated PE as the phospholipid component stabilized by some lipid components/co-surfactant systems. Unsaturated PE has a strong tendency to form nonbilayer structures at neutral pH and a great ability to support fusion [2]. It does not form liposomes by itself, but liposomes can be prepared by adding another component to PE. Oleic acid (OA), a negatively charged amphiphile with a pKa of 7.3 ± 0.3 is widely used for this purpose [3], which allows PE to form vesicles at physiologically relevant pH range (pH 7.4). Lowering the pH of the system neutralizes the surface charge and allows the close approach of vesicles and subsequently results in fusion of the vesicles [4]. It has been reported that pH-sensitive liposomes composed of PE and OA rapidly aggregate and become leaky in the presence of plasma, primarily due to rapid extraction of OA from the liposomal membrane by albumin [5]. Other stabilizers, tritiable amphiphiles with carboxylic group(s) are commonly used for preparation of pH-sensitive liposomes. They include palmitoyl homocysteine [6, 7], fatty acids [4, 8], fatty acyl amino acids [6], *N*-succinyl PE and acid conjugates of PE [9], cholesteryl hemisuccinate (CHEMS) [10] are reported as the co-surfactants which have been utilized to stabilize the PE lamellar phase. *Trans*-PE has been used to confer fusogenic behavior and pH responsiveness to the vesicles. The stabilizing ability of amphiphiles decreases under acidic conditions, resulting in the destabilization and fusion of the liposomes.

Connor et al. [6] were among the first to report the pH-triggered liposomal systems composed of DOPE and *N*-palmitoyl-L-homocysteine (8 : 2 molar ratio) that fused upon acidification to pH 5 to liberate the entrapped contents. Substitution of DOPC for DOPE eliminated membrane fusogenicity, whereas inclusion of 40% cholesterol reduced content leakage but maintained the pH-induced membrane fusion. In a significant development, palmitoyl derivative of PE, 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) was exploited for pH-responsive triggered drug release. Though it was difficult to form stable liposomes with POPE near neutral pH in isotonic buffer, but when mixed with single chain amphiphile, tocopherol hemisuccinate, stable liposomes near physiological pH could be engineered [11]. These liposomes were found to have pH-sensitivity classically dependent on the lipid composition of the vesicles.

Membrane fusion and content release from DOPE : CHEMS liposomes have been shown by Szoka [12–14].These pH-responsive modules were shown to involve a hexagonal H_{II} phase formed by conversion of membrane-stabilizing, anionic cholesterol derivative at neutral pH to a membrane-destabilizing uncharged form upon protonation of the succinyl moiety at low pH (<pH 6.0). Recently, Hafez and Cullis [15] showed that CHEMS itself exhibits pH-sensitive polymorphism. This is evident from the fusogenic properties of large unilamellar vesicles (LUV) composed of CHEMS as seen in freeze-fractured electron microscopy. Below pH 4.3, LUVs composed of CHEMS undergo fusion as monitored by lipid mixing assays and freeze-fracture electron micrographs which reveal the characteristic striated signature of H_{II} (parallel) phase lipid.

In an attempt to prepare sterically stabilized pH-sensitive liposomes, liposomes composed of CHEMS and DOPE were prepared using poly (ethylene glycol) derivative of PE as ancillary lipid [10]. These CHEMS/DOPE/PEG-PE liposomes were found useful for the intracellular delivery of highly negatively charged molecules such as gene, antisense oligonucleotide and ribozymes. pH-sensitive liposomes composed of DOPE and CHEMS (3 : 2 molar ratio) bearing the *N*-acetylglucosamine derivative of bovine serum albumin (*N*-Ac-BSA) could reportedly negotiate the receptor mediated delivery of contents into chicken hepatoma (LMH) cells expressing the *N*-Ac-BSA-binding asialoglycoprotein receptor [16]. It was suggested that the prerequisites for receptor-mediated delivery to LMH cells were both the pH-sensitivity of the module and the presence of N-Ac-BSA on the liposomal surface as a ligand. Stabilization of PE lamellar phase has also been achieved by incorporation of another cholesterol derivative, 2,3-seco-5α-cholestan-2,3-dioic acid (SCDA) in a well-characterized dielaido-PE (DEPE) liposomal system [17]. Acidification of DEPE:SCDA mixtures (2–6 mol% SCDA, pH 6.0) led to membrane fusion. The liposome "bursting" phenomena were also observed upon acidification, suggesting that a significant proportion (~65%) of the contents are lost through an "osmotic shock" caused leakage rather than the pH-induced lamellar to hexagonal phase transition.

Various pH-sensitive OA stabilized PE-liposomes are reported in the literature [4, 18–20]. Since the OA co-surfactant undergoes protonation over the pH range where leakage of contents is observed, its primary role is to stabilize the liposomes at physiological pH by providing a source of anionic charge that causes electrostatic repulsion between the membrane surfaces. Neutralization of the net negative surface upon protonation allows close approximation and contact of the membrane surfaces. An accelerating effect on the membrane fusion process was also observed in the presence of 2 mM Ca^{2+}, presumably due to divalent-cation which could have promoted binding of

Ca^{2+} to the OA component of the fusing liposomes. Fusogenic liposomes comprised of OA and transesterified PE (TEPE) in a 3 : 7 molar ratio have been shown to be destabilized at pH 6.5 in 150 mM NaCl, resulting in the release of calcein [4]. Liposomes comprised of 2 : 2 : 1 DOPE : Chol : OA also exhibit pH-dependent release of encapsulated materials. pH-dependent cellular uptake of calcein, diphtheria toxin A fragment [18] and 1-β-D-arabinofuranosylcytosine (ara-C) [21] targeted to L-929 cells, chloramphenicol acetyltransferase (CAT) gene plasmids targeted with anti-$H2K^K$ antibodies [22], and TK gene expression in Ltk cell cultures [19] have been demonstrated. The relevance of OA-stabilized PE-systems for *in vivo* delivery applications is impeded by the fact that albumin rapidly extracts the OA from these liposomal membranes, thereby leading to the destabilization of the lamellar phase upon exposure to serum or plasma.

In an array of experiments, various succinic acid derivatives were explored as co-surfactants to stabilize the PE based lamellar phases. Content release from pH-sensitive small unilamellar liposomes (~100 nm) containing N-succinoyl-dioleoyl-phosphatidyl-ethanolamine (COPE) was reported to be strongly dependent on the liposomal compositions. Pure COPE liposomes were stable at low pH (~4.0), however they released their contents at pH 7.4, presumably due to lipid packing defects and electrostatically induced bilayer destabilization at higher pH. This observation was checked when COPE was mixed with DOPE (3 : 7), such that the liposomes were observed to be more permeable at pH 4.0 than 7.4. Involvement of the DOPE H_{II} phase upon protonation of the COPE surfactant was implicated as the probable mechanism, as the substitution of egg phosphatidyl choline (EPC), DOPC, and TEPE for DOPE could significantly reduce the pH-sensitivity of the system. Similarly, SUVs composed of DOPE and a double chain amphipathic, 1,2-diacyl-3-succinyl glycerol (DASG) [23] and 1,2-dipalmitoyl-3-succinylglycerol (DPSG) [24] were reported to be pH-sensitive and relatively stable in serum/plasma. Their stability was further improved with the incorporation of GM_1 ganglioside [24]. Tari et al. [25] have compared the effect of protons and divalent cations such as Ca^{2+} and Mg^{2+} with bilayer liposomes composed of DPSG or DASG or 1,2 dioleoyl-3-succinylglycerol (DOSG). These cations augmented the membrane fusion and destabilization events in the endosomes by different mechanisms but the liposome destabilization was due to lateral phase separation as well as the formation of non-bilayer structures.

Wu et al. [26] reported SUVs of DOPE/sulfatide (70 : 30 mol/mol) as pH-sensitive and stable vesicular system in plasma at the physiological pH. The mechanistic evaluation for pH-sensitivity of the system revealed that hydration and partial dehydration of the sulfated head-group was critical in stabilization at physiological pH and destabilization at an acidic pH of DOPE/sulfatide based vesicles.

Liposomes composed of a mixture of dipalmitoyl-phosphatidylcholine/cholesterol/ GM_1 ganglioside/biotinyl-phosphatidyl-ethanolamine (100 : 20 : 6:0.25 molar ratio) showed a sharp sensitivity to temperature (lipid phase separation) and pH changes (reversion of L_α phase to H_{II} hexagonal phase). The maximum release of the entrapped molecules was recorded when both the stimuli, i.e. pH and temperature were implemented simultaneously, the former occurs naturally in pathological conditions, such as tumor, metastasis or inflamed sites, while the latter was artificially induced. Biotin-DPPE was added to this formulation to impart an ability to bind to specific ligands *in situ* and thus their pH/temperature sensitivity might be combined to negotiate eventual binding of carrier(s) to avidinated targets for improved clinical effectiveness [27].

Recently, Simoes et al. [28] described some molecular mechanisms by which pH-sensitive liposomes bypass the cytoplasmic and endosomal membranes to deliver their aqueous contents into the cytoplasm. Various liposome formulations were evaluated for their efficacy to mediate intracellular delivery of encapsulated material, including a novel sterically stabilized pH-sensitive formulation (DOPE : CHEMS : DSPE-PEG(2000)(6 : 4 : 0.3)). The results indicated that the efficacy of interaction of pH-sensitive liposomes, both plain and sterically stabilized, with cells was strongly determined by the inclusion of DOPE in their composition, independent of the type of the amphiphilic stabilizer used. Among different formulations studied, DOPE : CHEMS liposomes exhibited the maximum cell association. Moreover, the results with cells pretreated with metabolic inhibitors or lysosomotropic agents indicated that DOPE-containing liposomes were internalized essentially by endocytosis and acidification of the endosomes is not an absolute mechanism involved in the destabilization of the liposomes within the cell.

4.2. pH-Triggering of phosphatidylcholine (PC)-based liposomes

Although the phosphatidylcholine-based pH-sensitive liposomes described above are, in general, less active than their phosphatidylethanolamine counterparts, these systems exhibit greater plasma stability. Rather than attempting to develop pH-responsive liposome compositions from PE lipids that are intrinsically unstable as lamellar phase under physiological conditions, the simpler and potentially more fruitful approach is to develop stimulants for destabilizing plasma stable PC liposomes. The pH-induced triggers that have been studied are:

- Membrane phase separation/domain formation leading to an increase in membrane permeability;
- Changes in membrane solubility resulting from protonation of synthetic amphiphile peptides bound to the surface of PC liposomes;
- pH-sensitive, amphipathic peptide or polymer mediated liposome membrane fusion;
- Lamellar-to-micellar phase transition resulting from degradation of the plasminogen vinyl ether linkage of semi-synthetic palmitoyl plasmenylcholine (PlasPPC);
- Polymer precipitation/electrostatic adsorption on PC liposome surface; and
- pH and temperature-dependent polymer phase separation.

The first report of pH-sensitive liposomes describes the triggered release of carboxyfluorescein (CF) from dipalmitoyl, diheptadecanoyl, or distearoyl phosphatidylcholine (DPPC, DHPC, and DSPC) liposomes containing *N*-palmitoyl-L-homocysteine (NPLH) intercalated in their bilayers as a minor component [30]. At 37 °C, the extent of CF release was 15% at pH 7.4, compared with 67% release at pH 6.0. The authors proposed the reversible cyclization of homocysteine (negatively charged at physiological pH) to the uncharged thiolactone as the basis for pH-dependent release via conversion from the "fatty acid-like" homocysteine anion to the uncharged thiolactone, which they hypothesized was responsible for membrane destabilization in this system. Phase separation of PC : anionic surfactant liposomes have been demonstrated. Table 1 summarizes the studies carried with various pH-sensitive liposomes.

Zellmer et al. [31] reported fusogenic liposomes composed of diacylphosphatidyl-choline and fatty acid mixtures that were sensitive towards phase transition temperatures (42–46 °C) of the constitutive lipids and pH below 5.5 at which fully protonated fatty acid exists. This study suggested that the direct transition into a non-bilayer state is not essential in the inter-membrane fusion for the temperature and pH-controlled fusogenic behavior. An alternative mechanism for pH-triggered release of liposomal contents has been developed that is based upon the putative fusogenic peptide sequences known for several membrane coated viruses [32]. Various reports have suggested that liposomes could be induced to fuse at acidic pH, in the presence of various synthetic and amphipathic peptides, 20-amino acid peptide [33] and 30-amino acid amphipathic peptide [34]. At low pH the amphipathic peptide undergoes a pH-dependent aperiodic to α-helical transition at pH 5.7, which subsequently interacts with the bilayer membrane to promote liposomal aggregation, fusion and lipid mixing. The fusion is evident and confirmed using light scattering, fluorescence energy transfer and content leakage experiments using a lipid : peptide ratio of 100 : 1 [34]. Similar mechanisms based on interaction between α- or β-helix to PC monolayers at acidic pH were proposed

Table 1: Studies performed with pH-sensitive liposomes

Engineered constructs (Encapsulated moiety)	Purpose/ result	Ref.
TEPE/OA vesicles (calcein)	Study of fusogenicity and pH-dependent calcein release	4
Tocopherol hemisuccinate (THS)/ 1-palmitoyl-2-oleoyl-PE (POPE)/ Chol based vesicles	Novel pH-sensitive liposomes composed of unsaturated PE and tocopherol hemisuccinate	11
DOPE/CHEMS vesicles (fluorescent dye)	Demonstration of membrane fusion and content release mechanism from pH-sensitive liposomes	12, 13
DEPE/SCAA vesicles (fluorescent dye)	Study of pH-dependent membrane fusion and content release	17
PE/OA/Chol vesicles (fluorescent dye)	Characterization of OA for pH-sensitivity, plasma stability and Ca^{2+} induced fusogenicity of liposomes	18–20
DOPE/ DSPG vesicles (^{125}I labeled tyramine inulin)	pH-sensitive, plasma stable liposomes with relatively prolonged residence in circulation	25
DOSG/DSPG/DASG based vesicles (calcein)	Interaction of liposomal bilayer composed of 1,2-diacyl-3-succinyl-glycerol with protons and divalent cations	26
DOPE/sulfatide vesicles (calcein)	Stability and pH-sensitivity of sulfatide containing PE vesicles and their fusogenic properties in presence of Ca^{++}	27, 88
DPPC, DHPC or DSPC/NLPH vesicles (CF)	Demonstration of pH-dependent CF release	30
EPC/NIPA sterically stabilized vesicles (pyranine or doxorubicin)	Effect of NIPA on pH-triggering of sterically stabilized liposomes	44–46
SUVs composed of PC/N-stearoyl cysteamine/Chol (5,6 CF)	pH-sensitivity and plasma stability of liposomes containing N-stearoylcysteamine	47
DOPE/OA/Chol vesicles (15-mer oligonucleotide ON)	A physicochemical study of the interaction between DOPE and ON in quasi-anhydrous samples and in excess water for development of pH-sensitive liposomes as carrier for oligonucleotides	67, 68
PE/diacylsuccinyl glycerol vesicles (calcein, diphtheria toxin A)	Diacylsuccinyl glycerol as novel stabilizers of bilayers at physiological pH and trigger pH-sensitive release of content	87
DOPE/CHEMS vesicles (FITC-labeled BSA)	A rational strategy to target macromolecules to the nucleus using pH-sensitive liposomes in conjunction with the nuclear localization signal (NLS) via the endocytic pathway	89
Cationic liposomes/DIP (dodecyl-2-(1′-imidazolyl) propionate- pH sensitive surfactant)	Characterization of DIP to enhance cationic liposomes mediated gene delivery	90
Biodegradable pH-sensitive surfactant (BPS) vesicles	Effect of biodegradable pH-sensitive surfactants on membrane fusion and rupture in liposomes	91
Cationic liposomes/BPS	Characterization of BPS in liposome-mediated nucleic acid cellular uptake and distribution	92
DOPE/CHEMS vesicles (calcein or HPTS fluorescence marker)	Biophysical studies and intracellular destabilization in cultured macrophages of pH-sensitive liposomes	93
DPPC vesicles complexed with Poly(malic acid) (PMLA) (calcein)	Effects of complexation between liposomes and poly(malic acid) on aggregation and leakage behaviour (novel pH-sensitive liposomes with improved release characteristics)	94
Vesicles composed of mixture of anionic lipid CHEMS and cationic lipid DODAC (*N,N*-dioleoyl-*N*, *N*-dimethyl ammonium chloride); vesicles composed of mixture of cationic lipid DC-Chol (3α-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride)/acidic lipid DOPA (dioleoylphosphatidic acid)	Investigation of pH-dependent fusion properties of large unilamellar vesicles (LUVs) composed of binary mixtures of anionic and cationic lipids.	95
DOPE/POD (poly(ethylene glycol)-diortho ester-distearoyl glycerol conjugate) vesicles (ANTS)	Steric stabilization of fusogenic liposomes by a low pH-sensitive PEG-diortho ester-lipid conjugate	96

for the pH-responsive, amphipathic peptide mediated membrane fusion events. Fusogenic properties of poly (ethylene glycol) become operationally pH-sensitive on incorporation of carboxylic groups. The liposomes made from such molecules were found to be pH-sensitive [35, 36]. These PEG derivatives bearing a carboxylic group termed as succinylated poly (glycidol)s were incorporated with PC forming liposomal constituents. In this study, pH-sensitive fusogenic liposomes were prepared using modification of PC with a fusogen (succinylated poly-glycidol). The fusion efficiency of these liposomes was enhanced under weakly acidic conditions [35]. These succinylated poly (glycidol)-modified liposomes were reported to provide intracellular delivery with the transfer of water-soluble marker (calcein) in to the cytoplasm following fusion with the endosomal membrane [36].

Stimuli sensitive pH-responsive liposomes are classically designed systems responsive to bio-signals, i.e., physiological pH, substrate or light. Tirell et al. [37, 38] first designed and reported the release of entrapped marker (calcein) from PC liposomes by electrostatic adsorption of hydrophobic electrolytes. The basis of this approach has been the environmentally sensitive association of hydrophobic polyelectrolytes to the liposomal membrane where the latter are integrated into the liposomal membrane. Adsorptive aggregation leads to reorientation in lipid bilayers followed by mixed micelle formation thus allowing release of liposomal contents. In particular, poly(2-ethyl) acrylic acid (PEAA) is adsorbed strongly on the liposomal surface at low pH (pH < 7), but only weakly in basic solutions. Liposomes suspended in dilute aqueous solutions of PEAA are stable above pH 7 but release their contents rapidly upon acidification. The pH-sensitive adsorption leads to important changes in the membrane structure accompanied with the loss of the barrier properties of the membrane. The incorporation of glucose-oxidase into suspending medium renders the liposomal membrane sensitive to glucose concentration. The pH-responsiveness of the liposomal suspension similarly can be tuned finely via copolymerization of 2-ethylacrylic acids, so that the release may be triggered essentially at the desired pH. A photochemical variant of this process has also been reported [39] using 3,3′-dicarboxydiphenyliodonium irradiated at 254 nm to generate a low pH environment *in situ*, thereby triggering PEAA adsorption and content release. However, detailed investigations revealed that the release behavior of liposomes showed a concentration dependence on PEAA. At low polymer concentration, channel-like behavior was observed for small ions, whereas membrane disruption via micellar solubilization occurred at higher PEAA concentration [40]. Fig. 2 illustrates various ligands that confer responsiveness to the liposomal systems towards bio-signals.

Ferraretto et al. [28] have combined the triggered release approaches based upon pH and temperature and engineered biotinylated liposomes, sensitive to both tempera-

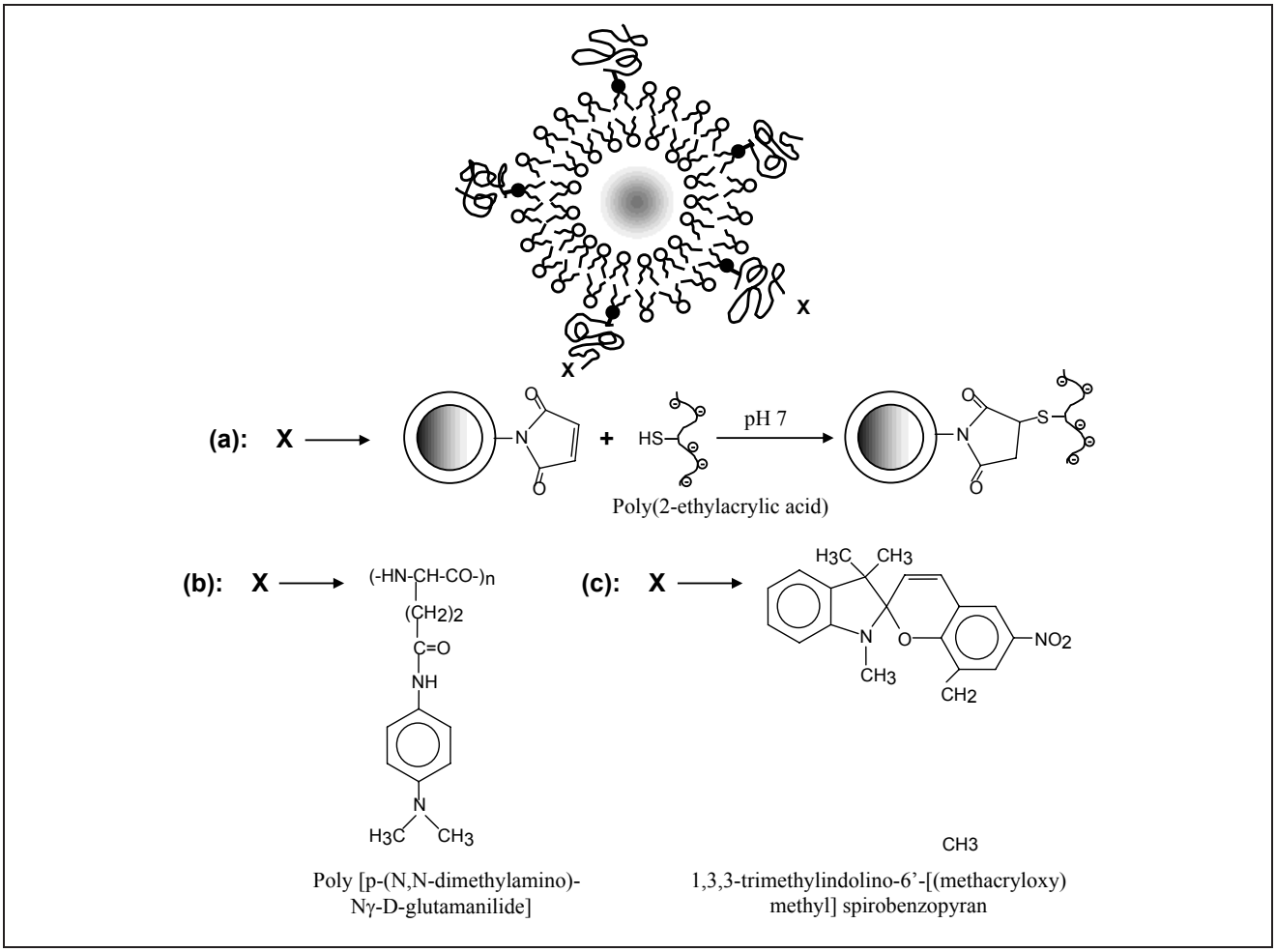

Fig. 2: Signal responsive liposomes
Liposomal constructs appended with signal sensitive modules (X)
X = pH-sensitive (a) and Photosensitive liposomes (b & c)

ture and pH (pHT liposomes). Liposomes modified with copolymers of *N*-isopropylacrylamide were reported to be temperature sensitive modules as described earlier [41–43]. However, some scientists have recently reported that copolymers of *N*-isopropylacrylamide (NIPA) could trigger pH-sensitivity to stable liposomes. At low pH (pH 5.5–4.9, the values corresponding to those of endosomes/lysosomes) the phase transition of the polymer, EPC-sterically stabilized liposomes quickly released a significant part of both a highly water-soluble fluorescent marker (pyranine), and an amphipathic anticancer drug doxorubicin [44–46]. Small unilamellar vesicles composed of EPC and N-stearoylcysteine (9 : 1 molar ratio) were reported to demonstrate pH-sensitivity as evidenced from the release of fluorescent marker on moderate acidification of the medium from 7.4 to 6.8 [47]. These liposomes have been proposed as potential tool for specific pH-triggered delivery of drug components to pathological sites such as tumors, inflamed and ischemic areas.

5. pH-Sensitive immunoliposomes

pH-Sensitive liposomes could be developed to release their contents in response to an acid environment of endosomal system especially following receptor mediated endocytosis of the immunoresponsive colloidal carrier. They undergo transient destabilization at a mildly acidic pH such as of endosomes and thus could selectively deliver the contents intracellularly. Incorporation of the anti-target monoclonal antibodies (MAbs) in pH-sensitive liposomes associated with surface, direct them to respective target where under low or specific pH environment drug is delivered [7, 21]. The vesicles based on DOPE and OA (8 : 2 molar ratio) with a palmitoyl derivatized monoclonal antibody (anti-$H2K^K$) incorporated into the lipid layer represent for such systems. These pH-sensitive immunoliposomes when evaluated *in vitro* could specifically deliver the entrapped dye (calcein) [7] and cytotoxic drugs (Ara-C and methotrexate) into the cytoplasm of cells [21]. Connor et al. [5] demonstrated the feasibility of employing these liposomes as a therapeutic delivery modality *in vivo*. pH-sensitive immunoliposomes mediated delivery and subsequent expression of exogenous genes in target cells have also been reported [48]. The researchers have also compared pH-sensitive immunoliposomes to both, non pH-sensitive immunoliposomes and pH-sensitive liposomes for their efficiency in transfecting the HSV-TK gene into the mouse lymphoma cells *in vitro* [19]. Immunoliposomes were shown to adsorb DNA non-specifically on their surface and to transfer it directly into the cytoplasm, without being presented to lysosomes for degradation (Fig. 3). pH-Sensitive immunoliposomes guided by MAbs towards a surface antigen on mouse L cells, were observed to fuse with endosomal membrane in response to acidic pH of the endosome. Specific gene delivery via this mode was found to express TK (thymidine kinase) genes at a statistically significant level as compared to pH-sensitive plain liposomes (without immunological targeting ligand). The ability of pH-sensitive immunoliposomes to deliver synthetic antisense oligonucleotides (oligos) into human myeloid and lymphoid leukemia cells has been examined [49]. The cellular uptake of an 18mer anti-myb oligonucleotide encapsulated in liposomes was 3–5 fold higher than that of 32P-oligos alone.

Selective targeting of immunoliposomal doxorubicin against human multiple myeloma (MM) *in vitro* and *ex vivo* was reported [50]. *In vitro* binding studies using the

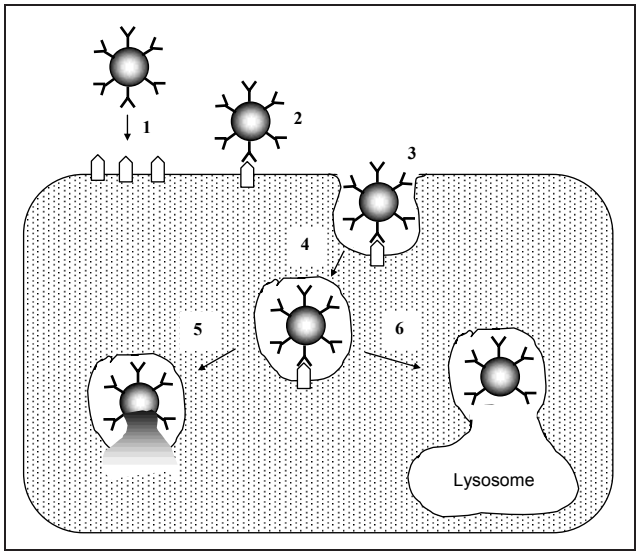

Fig. 3: pH-Sensitive cytosolic delivery of contents. 1. pH Sensitive immunoliposomes bind with receptors via its MoAb component. 2, 3 and 4 indicates receptor-mediated internalization. 5 indicates cytosolic drug release at endosomal pH (4.5) and 6 indicates the fate of the system without the pH sensitive components (fusion of endosomes with lysosomes)

circulating malignant CD19(+)MM cell line ARH77 demonstrated that CD19-directed immunoliposomes (SIL[anti-CD19]) specifically get attached to these cells. With the pH-sensitive fluorophore, 1-hydroxypyrene-3,6,8-trisulfonic acid, binding of SIL[anti-CD19]to CD19 antigens suggested receptor-mediated internalization of the antibody-antigen complexes into endosomes. Improved therapeutic efficacy of doxorubicin loaded sterically stabilized anti-idiotype immunoliposomes was reported in a murine B-cell lymphoma model. pH-sensitive fluorescent dye (HPTS) was used to estimate cell binding and internalization ability of these immunoliposomes by a fluorescence assay technique [51].

6. pH-Sensitive liposomes for gene delivery

6.1. pH-Sensitive liposomes for gene therapy

Somatic gene therapy has emerged as a new approach for the treatment of a variety of genetic and acquired diseases. Gene therapy methods are designed to introduce genetic material into patient's cells to cause these cells to produce a therapeutic protein. Two major methods have been proposed for *in vivo* gene delivery: Viral mediated and non-viral mediated gene transfer. Due to their natural ability to infect cells efficiently, several viruses, such as retrovirus, adenovirus, adeno-associated virus and herpes virus, have been investigated for *in vivo* viral mediated gene delivery, but there is a potential risk of generating an infectious, replication-competent virus during the production or use of viral vectors for gene transfection [52].

Non viral 'gene medicines' have emerged as a promising technology for effecting *in vivo* gene transfer and control of gene functions. Liposomes based gene transfection systems have been promoted as a means of achieving the transfection efficiency of viral constructs without the associated risks. The earliest attempts at liposomes based *in vivo* gene transfer involved conventional entrapment in negatively charged vesicles, and transfection efficiencies were accordingly low. Improvements arose when increased insights into the mechanisms of uptake prompted the development of pH-sensitive liposomes, which could exploit

the cellular endocytic mechanisms. Such liposomes are intended to fuse with lipid membranes in the acidic environment of the endosomes, thus facilitating the endosomal release of encapsulated gene expression system in the cytoplasm of transfected cells. Fig. 4 shows the proposed mechanism of cellular uptake of liposomes associated gene medicines [52].

In earlier approaches anionic, pH-sensitive liposomes have been developed for the delivery of DNA, however, the negative charge of these vesicles prevents them from being efficiently taken up and interaction with cells, thus limiting their utility for transfection [19]. Holmberg et al. [53] used pH-sensitive immunoliposomes in conjugation with antibodies specific to glial cells (gliasomes) to deliver plasmid DNA. Two different monoclonal antibodies from different subclasses, IgM and IgG, were examined for the transfection specificity against cultured C6 glioma (specific target cell type) using gliasomes. Authors claimed that gliasomes were more effective in their transfection efficiency than the cationic lipid complex i.e. lipofectin (DOTMA/DOPE, 1 : 1) *(DOTMA: N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride)* and transfectase (DDAB/DOPE, 1 : 3) *(DDAB: dimethyl-dioctadecyl ammonium bromide)*. Results of these studies indicate a three-fold increase in percent transfection when IgM subclass ligands were used and an approximately two-fold increase in percent transfection with IgG subclass ligand. Gliasomes were further tested for their transfection specificity by the addition of an excess of antibodies to the cell culture in order to presaturate specific receptors on C6 glioma cells. Administration of pH-sensitive immunoliposomes to these glioma cells pre-saturated with excessive antibodies, showed a net reduction in transfection.

pH-Sensitive cationic liposomes were found to mediate efficient transfection of DNA into a variety of cells in culture as fusogenicity was offered by both the constitutive lipids, i.e., pH-sensitive and cationic lipids [54]. pH-sensitive cationic lipids containing an amine with a pKa within the physiologic range of 4.5–8.0 were synthesized and incorporated into liposomes (cationic lipid : DOPE in 1 : 1 molar ratio). Acid conditions promoted the DNA binding, DNA incorporation and DNA induced fusion by these cationic, pH-sensitive liposomes. The transfection ability of the pH-sensitive cationic lipid-DOPE bilayer liposomes was assessed in NIH 3T3 cells and compared with DOTMA/DOPE based cationic liposomes. It was found to be 2–5 times greater than that of DOTMA/DOPE [54]. Transfection efficiency in cultured cells by these liposomes was dependent on endosomal acidification in a manner akin to acid-induced endosomal release of viruses and hence been named as synthetic virus like vectors. Several mechanisms have been proposed to implicate acid-induced fusogenicity of cationic lipid/DOPE liposomes. With a decreased pH, as would occur in the endosomal compartment, the polar head group of the cationic lipid DPIm (4-(2,3-bis-palmitoyloxy-propyl)-1-methyl-1*H*-imidazole) would become more positive. This would increase the effective size of DPIm's head group due to electrostatic repulsion. DPIm's increased positive charge would also increase its interaction with the negatively charged DNA and with other anionic components of the endosomal membrane. These pH-dependent changes cause dislocations in the liposomal membrane that could lead to membrane fusion.

Lee and Huang [55] developed a lipidic gene transfer vector, LPDII, where DNA was first complexed to polylysine and then entrapped into folate-guided pH sensitive anionic liposomes composed of DOPE/CHEM/folate-PEG-DOPE via charge induced interaction. LDPII transfection of KB cells, a cell line with tumor marker folate receptors, was affected by both the lipid to DNA ratio and the lipid composition. At low lipid to DNA ratios (e.g. 4 and 6), LDPII particles were positively charged; however, transfection and cellular uptake levels were independent of the folate receptor and did not require pH-sensitive lipid composition. Meanwhile, transfection and uptake of negatively charged LDPII particles, i.e. those with high lipid to DNA ratios (e.g. 10 and 12), were folate receptor-dependent and required a pH-sensitive lipid composition. The transfection activity of LDPII was lost when the inverted cone shaped DOPE was replaced by DOPC. The potential of this novel gene transfer vector in gene therapy for tumor-specific delivery has been documented and suggested.

Efficient non-viral vectors for gene therapy have been developed [56, 57]. Association of transferrin or pH-sensitive peptide GALA (a 30 mer peptide of the sequence WEAALAEALAEALAEHLAEALAEALEALAA) with cationic liposomes composed of 1,2-dioleoyl-3-(trimethyl ammonium) propane and its equimolar mixture with DOPE. In the cases where the liposomes/DNA complex was negatively charged, the luciferase expression drastically increased from b CMV lue gene. The percentage of cells transfected was also increased about 10-fold [56]. They also reported transfection of human macrophages by lipoplexes mediated through transferrin and pH-sensitive peptide GALA. Commonly used synthetic gene delivery vectors have not been successful in transfecting these non-dividing cells. A combination strategy involving cationic liposomes to condense and carry DNA, transferrin to facilitate cellular uptake and the pH-sensitive peptide GALA to promote endosome destabilization, resulted in significant expression of a luciferase gene. The system exhibited a net negative charge, which may obviate a limitation of cytotoxicity and the expected lack of immunogenicity of

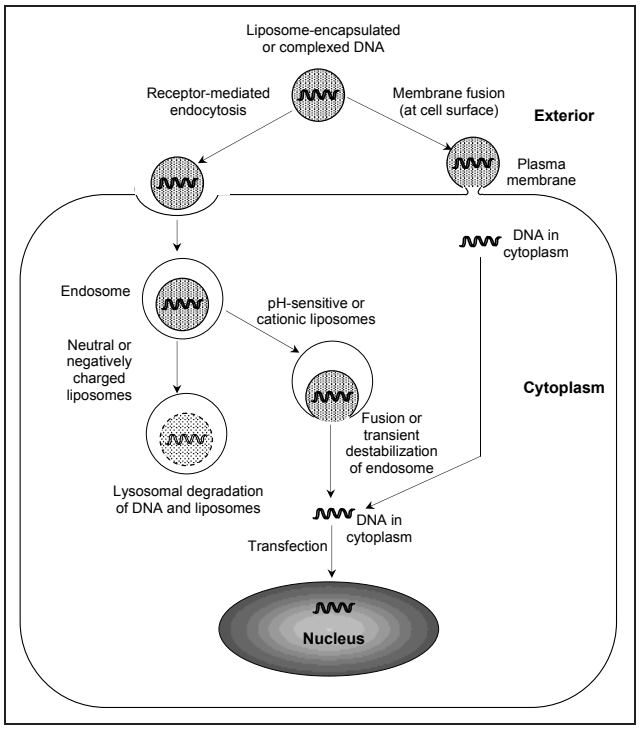

Fig. 4: Proposed mechanisms of cellular uptake of liposomes associated DNA

these complexes may render them useful for gene delivery to macrophage *in vivo* [57].

A novel folate targeted pH-sensitive, anionic liposomal vector for efficient cytoplasmic delivery of DNA into folate receptor bearing cells has been recently reported [58]. *N*-Citraconyl-dioleoyl phosphatidyl ethanolamine (C-DOPE), a derivative of DOPE that hydrolyses rapidly at pH 5 (pH of endosomal compartment) to yield DOPE, was synthesized and incorporated with DOPE and folate-PEG-DOPE into liposomes. The resulting liposomes were stable at extracellular pH (approximately pH 7.4) but fusogenic at pH 5. Transfection of cultured cancer cells with pH-sensitive liposomal vectors was more efficient than transfection with DOPE-CHEM based vectors.

Fusogenic pH-sensitive succinylated poly(glycidol)-modified liposomes were developed [35, 36]. Recently, complexation of these pH-sensitive, fusogenic liposome with a lipoplex consisting of 3β-(*N*-(*N*′,*N*′-dimethylaminoethane) carbamoyl) cholesterol, DOPE and plasmid DNA has been described that proved to be as efficient gene delivery systems [59]. These complexes were termed SucPG-complexes and prepared with a positively or negatively charged surface by mixing the lipoplex with varying amounts of the SucPG-modified liposomes. The positively charged SucPG-complexes either bearing or not bearing a cell-specific ligand, transferrin, could transfect HeLa cells efficiently. The negatively charged SucPG complexes bearing transferrin exhibited high transfection ability against HeLa and K562 cells, indicating that gene delivery was achieved through their binding to the cellular receptors. These transferrin-appended, negatively charged complexes retained the high transfection ability in the presence of serum. The complexes may be useful as nonviral vectors *in vivo* [59].

In the recent studies efficacy of lacZ gene transfer into the L929 cell line and localized [^{14}C]-DNA delivery in male NMRI mice using new pH-sensitive liposomes, containing PC and glycyrrhizin (PC/GL) or alpha-tocopherol ester of succinic acid (PC/TSA) has been described [60]. The reporter gene (pQE-LacZ plasmid) was transferred into L929 cells using corresponding lipoplexes, 0.5% of cells being transfected. Tissue distribution of Gasserian ganglion neurinoma cell [^{14}C]-DNA fragments and corresponding PC/GL and PC/TSA lipoplexes, were examined 24 h following intraperitoneal administration. The [^{14}C]-DNA itself was not detected in any organs after 1.5 h. The use of PC/GL or PC/TSA lipoplexes considerably changed the biodistribution of [^{14}C]-DNA in mice tissues. The maximal content of [^{14}C]-DNA for both types of lipoplexes was recorded in the intestine (50%) and the spleen (30%). The [^{14}C]-DNA content was equal to 4 and 10% in liver and kidneys in the case of PC/GL-lipoplexes, and 15 and 6%, for PC/TSA, respectively.

6.2. pH-Sensitive liposomes in genetic immunization

Genetic vaccines are the most recent version of present immunization strategies and are considered as third generation vaccines. The concept entails the direct injection of antigen-encoding plasmid DNA which, following its uptake by cells, finds its way to the nucleus where it transfects the cells episomally. After antigen is recognized as foreign protein by the host, it is then subjected to pathways similar to those operate for the antigens of internalized pathogens but without their disadvantages. These vaccines express antigen over a prolonged period thus eliminate the need of booster and more so avoid possible virulence, that is otherwise frequently encountered with attenuated vaccines [61, 62].

The effective immunization using naked DNA (which is generally administered intramuscularly) suffers various bio-barriers and impediments. The uptake of DNA by myocytes (muscle cells) is minimal and involves only a minor fraction of these cells. This necessitates larger dose administration often into regenerating muscles. It is also likely that some of the injected DNA suffers deoxyribonuclease attack. Moreover, although myocytes carry MHC-I molecules, they are not professional antigen presenting cells (APC) as they lack vital costimulatory molecules. It is thought that responses to genetic vaccines are, at least in part, the result of transfer of antigenic material from myocytes to professional APC [63].

It has been already proposed that APC are a preferred alternative to muscle cells as targets for DNA vaccine up-

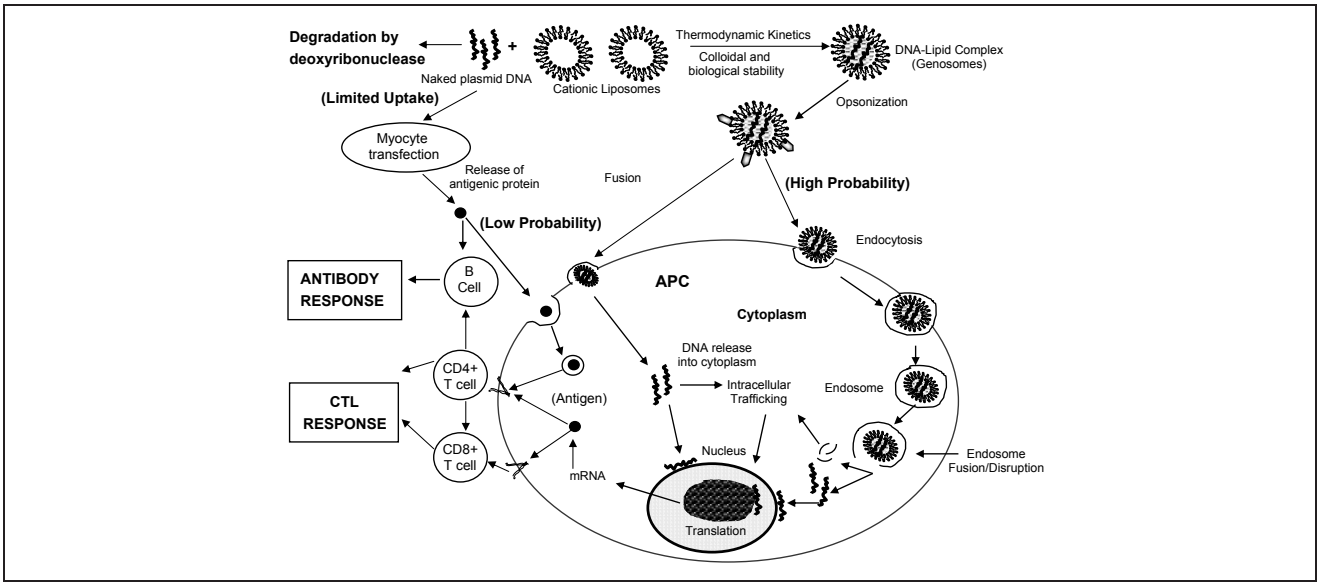

Fig. 5: Schematic representation of proposed mechanism of DNA immunization via endocytic pathway. Naked DNA is taken up by a small no. of myocytes after i.m. injection, which are then transfected episomally. The produced antigen is released from the cells to interact with APC and thus induce immunity. In contrast, liposomal DNA interacts with APC directly and induces better immune response. It also protects DNA from degradation by deoxyribonuclease attack.

take and expression [64]. Administration of antigen-encoding plasmid DNA via liposomes could circumvent the need of muscle involvement and facilitate its uptake by APC, for instance those infiltrating the site of injection or in the lymphatics, at the same time protecting DNA from nuclease attack [65]. Transfection of APC with liposome-entrapped DNA could be promoted by the judicial choice of vesicle surface charge and lipid composition or by the co-entrapment of other adjuvants together with the plasmid DNA. Cationic lipids have been used to reduce the net negative charge on DNA plasmid-based gene expression systems in attempts to reduce charge-charge repulsion at the surface of biological membranes. Such lipids form stable complexes with DNA with high transfection efficiency. The pathway to transfection of cationic or pH-sensitive liposomes is thought to commence with endocytosis or membrane fusion. This is followed by destabilization of the endosomal membrane whereupon (either fusion of cationic liposome membrane with anionic endosomal membrane or destabilization of pH-sensitive vesicle membrane in low endosome pH environment), DNA is released into the cytosol. Fig. 5 explains the proposed mechanism of cationic pH-sensitive liposomes based immunization.

6.3. pH-Sensitive liposomes in antisense oligonucleotide therapy

Antisense oligonucleotides are nucleic acid fragments that block the synthesis of specific proteins at either the DNA (transcription) or RNA (translation) levels. The antisense oligonucleotide strand will base pair in a complementary fashion with the sense (message containing) strands of DNA or RNA, blocking their ability to orchestrate protein synthesis [66]. Three mechanisms, namely, triplex helix formation, translation arrest via antisense-mRNA hybrid formation and Rnase H based destruction of mRNA translation are described in the literature (Fig. 6). Liposomal delivery of these agents avoids the limitations associated with the other modes of administration, i.e. degradation by nucleases, increased toxicity and undesirable interaction with proteins.

15-mer oligonucleotide (ON) encapsulated DOPE/OA/Chol vesicles were prepared and physicochemical interaction between DOPE and ON in quasi-anhydrous samples and in excess water for the development of pH-sensitive liposomes has been described [67, 68]. The application of pH-sensitive liposomes composed of DOPE/CHEMS (3 : 2 mol/mol) in delivery of antisense oligodeoxynucleotides (asODN) into NG 108–15 neuroblastoma and glioma cells has been discussed [69]. The uptake of fluorescent asODN (free or entrapped in liposomes) by NG 108-15 cells was monitored by fluorescence-activated cell sorting and confocal microscopy. Delivery of asODN was significantly improved when antisense molecules were entrapped in liposomes as compared to free (nonliposomal) asODN. Lubrich and coworkers [70] reported DOPE/CHEMS vesicles encapsulated oligonucleotide of 20 bases, complementary to a region of the sodium/myoinositol co-transporter (SMIT) mRNA for enhanced delivery of oligonucleotide into astrocytoma cells and primary astrocyte cultures. The uptake efficiency and activity of transferred antisense oligonucleotides with regard to substrate (inositol) uptake were investigated and it was found to be inhibited by astrocytes.

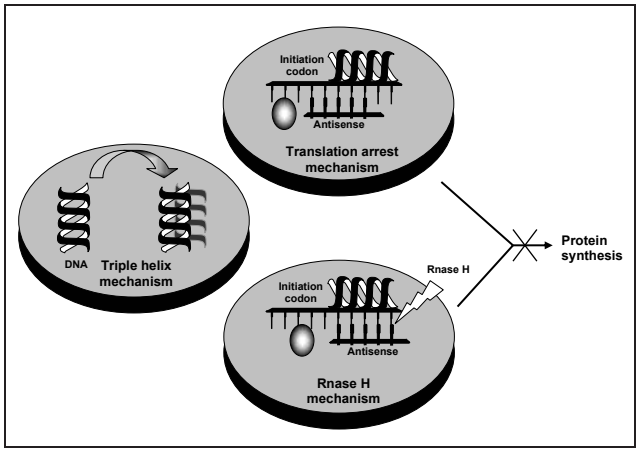

Fig. 6: Antisense oligonucleotide therapy

Ponnappa et al. [71] described a method of encapsulation in pH-sensitive liposomes and specific delivery of an antisense phosphorothioate oligonucleotide (TJU-2755) against TNF-α. Male Sprague-Dawley rats were administered (i.v.) with liposome-encapsulated TJU-2755 and 48h postinjection, the animals were administered a single dose of lipopolysaccharide and were sacrificed 90 min later. The tumor necrosis factor-α (TNF-α) produced by *ex vivo* incubated excised liver and the levels of plasma TNF-α were determined. After a single administration of liposome-encapsulated antisense TJU-2755, a 30% reduction in TNF-α produced by liver slices was observed. Two daily doses of the antisense oligonucleotide effectively inhibited TNF-α production by 50%. This was associated with a 65 to 70% reduction in plasma levels of TNF-α, as compared to controls. These results indicated that oligonucleotide TJU-2755 encapsulated in pH-sensitive liposomes can be used to reduce endotoxin-mediated production of TNF-α in macrophages *in vivo* and thus may be of value in attenuating or preventing macrophage-mediated liver injury.

Recently Duzgunes et al. [72] reported that an antisense oligodeoxynucleotide against the HIV-1 Rev response element, a ribozyme complementary to the HIV-1 5′-LTR, and the reverse transcriptase inhibitors 9-(2-phosphonylmethoxyethyl) adenine (PMEA) and (R)-9-(2-phosphonylmethoxypropyl)-adenine (PMPA) inhibited virus replication in monocyte-derived macrophages more effectively when delivered in pH-sensitive liposomes compared to the free drugs. Table 2 summarizes various pH-sensitive liposomes engineered for gene and oligonucleotides delivery.

7. Therapeutic applications

Cytoplasmic delivery of bioactive molecules is an important goal of the targeted therapy. This is particularly the case for macromolecules such as drugs, enzymes, antibodies, DNA and antisense oligonucleotides as they do not easily penetrate the cellular membrane and their final cellular destination is lysosomal compartment. Diphtheria toxin, radiolabeled albumin, pH-sensitive fluorescent dyes [14], calcein and chloroquine [7] have also been selectively targeted using pH-sensitive liposomes. Dioctadecyl amido glycyl spermine (DOGS) immunoliposomes that entrap diphtheria toxin A chain were suggested as a model for cytoplasmic delivery [14]. Fusogenic, pH-sensitive liposomes have an apparent potential of cytoplasmic delivery. Reddy et al. [73] have suggestively presented pH-sensitive liposomes as an efficient means of sensitizing target cells to class-I restricted cytotoxic T-lymphocytes (CTL) recognition of a soluble protein. They further optimized the antigen presenting system and demonstrated that ovalbumin (OVA) containing liposomes get destabilized at low

Table 2: Engineered pH-sensitive liposomes for gene and oligonucleotide delivery

Engineered constructs (Genetic moiety)	Purpose/ result	Ref.
Complex of sucPG-modified pH-sensitive liposome and lipoplex (3β-(*N*-(*N*′,*N*′-dimethylamino-ethane) carbamoyl) cholesterol/ DOPE/plasmid DNA	Development of novel nonviral gene delivery vectors *in vivo*	35, 36, 59
Lipofectin/transfectase vesicles conjugated with antibodies specific to glial cells (gliasomes) (β-galactosidase plasmid DNA)	Enhanced transfection efficiency and specificity to glioma cells	53, 97
DPIm (4-(2,3-bis-palmitoyloxy-propyl)-1-methyl-1*H*-imidazole)/DOPE (DNA)	pH-Sensitive cationic liposomes for efficient transfer (vectorization) of DNA into a variety of cells in culture	54
DOPE/CHEMS/Folate-PEG-DOPE vesicles (polylysine-condensed DNA)	Folate-targeted anionic liposomes entrapped polylysine-condensed DNA for tumor cell-specific gene transfer	55
DOPE/1,2-dioleoyl-3-(trimethyl ammonium) propane liposomes complexed with pH-sensitive peptide GALA and/or transferrin (lipoplex) (DNA)	Efficient non-viral vectors for gene delivery to macrophages *in vivo* with reduced cytotoxicity and immunogenicity	56, 57
DOPE/C-DOPE/Folate-PEG-DOPE vesicles (polylysine-condensed DNA)	Folate-targeted anionic pH-sensitive liposomal vector for specific gene transfer to cultured cancer cells more efficiently than conventional DOPE/CHEM based vectors	58
PC/GL or PC/TSA vesicles (pQE-LacZ plasmid DNA)	Efficient gene transfer into the L929 cell line and enhanced local [^{14}C]-DNA delivery in male NMRI mice	60
DOPE/OA/Chol vesicles (15-mer oligonucleotide ON)	A physicochemical study of the interaction between DOPE and ON in quasi-anhydrous samples and in excess water for development of pH-sensitive liposomes as carrier for oligonucleotides	67, 68
DOPE/CHEMS vesicles bearing antisense oligodeoxy-nucleotides (ODN)	pH-Sensitive liposomes for delivery of antisense oligodeoxynucleotides to neuroblastoma cells (NG 108-15 neuroblastoma and glioma cells)	69
DOPE/CHEMS vesicles (oligonucleotide of 20 bases, complementary to a region of the sodium/myo-inositol co-transporter (SMIT) mRNA)	Enhanced delivery of oligonucleotide into astrocytoma cells and primary astrocyte cultures and investigation of the uptake efficiency and activity of transferred antisense oligonucleotides with regard to substrate uptake (inhibition of inositol uptake in astrocytes)	70
pH-Sensitive vesicles (antisense phosphorothioate oligonucleotide (TJU-2755) encapsulated)	*In vivo* delivery of antisense oligonucleotides in pH-sensitive liposomes for inhibition of lipopoly-saccharide-induced production of tumor necrosis factor-alpha (TNF-α) in rats	71
DOPE/CHEMS vesicles (DNA encoding luciferase or β-galactosidase)	Delivery of plasmid DNA into mammalian cell lines	75
pH-Sensitive vesicles (oligonucleotide)	Oligonucleotide encapsulated in pH-sensitive liposomes are efficient towards Fried retrovirus	76

pH and sensitize target cells (mouse thymoma cells) to lysis by class-I MHC restricted OVA-specific CTL [74]. The approach appears to be suitable to measure CTL against less available soluble antigens such as viral proteins. The pH-sensitive liposomes have also been reported as plasmid expression vectors for the delivery of DNA [22, 53, 75] and intracellular trafficking of antisense oligonucleotides [76, 77]. Transfection efficiency in cultured cells largely depends on endosomal acidification in a manner akin to acid induced endosomal release of virus.

Briscoe et al. [78] reported the delivery of superoxide dimutase (SOD) to pulmonary epithelium via pH-sensitive liposomes for respiratory insufficiency, which when treated with oxygen supplementation or exposure to diverse pulmonary toxins can cause lung damage as a result of increased oxygen radical production. Enzyme SOD may disable and attenuate this pathological process, however, the intracellular delivery and antioxidant action of SOD is impeded as it locks in due to its inability to cross-cellular membranes. The delivery of SOD to lung cells was accomplished using pH-sensitive liposomes prepared with DOPE and DOSG (1-oleoyl-2-oleoyl-sn-glycero-3-succinate) and were added to cultured fetal rat lung distal epithelial (FRLE) cells. These cells express a high affinity receptor for surfactant protein A (SP-A), which was utilized for targeting liposomes to cells, after incorporating SP-A. After incubation of pH-sensitive liposomes borne SOD with cultured FRLE cells, the cell associated SOD activity was recorded to be increased 5.1 fold i.e. from 7.8 ± 2.5 to 40.1 ± 3.3 U SOD/mg cell protein. Incorporation of SP-A into liposomes increased the delivery of liposomal SOD to cells by 6.2 fold.

Duzgunes et al. [79, 80] reported intracellular delivery of novel antiviral agents including antisense oligodeoxynucleotides, ribozymes and therapeutic genes against human immunodeficiency virus type-I (HIV-I) using liposomes. The EC_{50} of the reverse transcriptase inhibitor 9-(2-phosphonyl methoxyethyl) adenine (PMEA) was reduced when delivered to HIV infected macrophages using pH-sensitive liposomes. A 15-mer antisense oligodeoxynucleotide inhibits HIV-I replication in macrophages when delivered in pH-sensitive liposomes, which was, however, ineffective in its free form. The same oligodeoxynucleotide when encapsulated in sterically stabilized pH-sensitive liposomes

Table 3: pH-Sensitive liposomes with their constitution and therapeutic purpose

Engineered constructs (Therapeutic moiety)	Purpose/result	Ref.
DOPE/Palmitoyl homocysteine vesicles coupled with palmitoyl anti-H2kk (calcein)	Efficient cytoplasmic delivery of fluorescent dye by pH-sensitive immunoliposomes	7
DOPE/CHEMS vesicles (Diphtheria toxin A chain)	Efficiency of cytoplasmic delivery to eukaryotic cell lines	14
DOPE/CHEMS/PEG-PE (calcein)	Intracellular delivery of aqueous contents and prolonged circulation *in vitro* by sterically stabilized pH-sensitive liposomes	10
DOPE/CHEMS vesicles (*N*-acetyl glucosamine derivative of BSA)	pH-Sensitive liposomes for receptor mediated delivery to chicken hepatoma (LMH) cells, expressing N-Acetyl glucosamine BSA binding asialoglycoprotein receptor	16
DPPC/Chol/GM1 GM1/biotinoyl-DPPE vesicles (CF)	pH-Sensitive biotinylated liposomes as tools for anti-cancer drug delivery	28
PC/succinylated poly (glycidol) vesicles (calcein)	Cytoplasmic delivery of liposomes modified with a pH-sensitive poly (ethylene glycol) derivative	35, 36
pH-Sensitive vesicles (ovalbumin)	Improved loading within pH-sensitive liposomes and enhanced delivery of soluble proteins to the class I restricted cytotoxic T lymphocytes (CTL)	73, 74, 100
DOPE/DOSG vesicles anchored with SP-A (SOD)	Targeted delivery of SOD to pulmonary alveolar epithelial cells for treatment of respiratory insufficiency	78
pH-Sensitive vesicles (hantaan nucleocapsid protein, M6 or human papilloma virus, E7)	pH-Sensitive liposomes as an effective peptide delivery system for CTL mediated tumor therapy	81
DOPE-*N*-succinyl-DOPE/DOPE-*N*-glutaryl-DOPE vesicles (gentamycin)	Intracellular delivery and antibacterial activity of gentamycin encapsulated in pH-sensitive liposomes	82
PE-N-succinyl-DOPE vesicles (gentamycin)	Intracellular delivery and antibacterial activity of gentamycin encapsulated in pH-sensitive liposomes	83
DOPE/OA/DSPE-PEG and DOPE/DPSG/DSPE-PEG vesicles (Methotrexate)	Serum-stable and long-circulating pH-sensitive liposomes as a targeted drug delivery system	85
DOPE/CHEMS vesicles (FITC-labeled BSA)	A rational strategy to target macromolecules to the nucleus using pH-sensitive liposomes in conjunction with the nuclear localization signal (NLS) via the endocytic pathway	89
DOPE based pH-sensitive vesicles (staurosporin)	Staurosporin encapsulated into pH-sensitive liposomes reduces TNF production and increase survival in rat endotoxin shock	98
PE/CHEMS vesicles (9-acetoxy-tetra-n-propyl porphylene, ATPPn)	Photodynamic therapy of human bladder carcinoma cells *in vitro* with pH-sensitive liposomes as carriers for ATTPn	99
pH-Sensitive vesicles (antiviral agents: PMEA, ribozymes, oligo-deoxynucleotide	Intracellular delivery of novel macromolecular drugs against HIV-I	101
Phosphatidylethanolamine-beta-oleoyl-gamma-palmitoyl (POPE)/CHEMS/monophosphoryl lipid A (MPL) (V3 peptide)	Immunogenicity of synthetic HIV-1 V3 loop peptides by MPL adjuvanted pH-sensitive liposomes	102
pH-Sensitive vesicles (SOD)	Delivery of superoxide dismutase for prevention of nitric oxide-dependent motor neuron death induced by trophic factor withdrawal	103

was highly effective with prolonged circulation profile *in vivo*. A ribozyme complementary to HIV-I 5′-LTR delivered in pH-sensitive liposomes inhibited virus production by 90%, while free ribozyme caused only a marginal inhibition. These carriers not only facilitate cytoplasmic delivery but also protect the drugs from nuclease digestion.

The effectiveness of pH-sensitive liposomes as an effective vehicle for peptide delivery for cytotoxic T lymphocytes (CTL) mediated tumor therapy has been described [81]. CTL recognizes tumor-associated antigen and CTL response is triggered on cytosolic delivery of antigen. The immunization of mice with CTL epitope peptides from Hantaan nucleocapsid protein (M6) or human papilloma virus E7 encapsulated in pH-sensitive liposomes induced effective antigen-specific CTL responses. The CTL responses induced by M6 peptide encapsulated in pH-sensitive liposomes blocked the formation of tumor mass from Hantaan NP transfected B16 melanoma cells in C57BL/6 mice and delayed the growth of preinoculated melanoma cells. During the blockade of the tumor growth, the CTL response was maintained for at least approximately 6 weeks, with concurrent systemic secretion of Th1 type cytokines such as interleukin-2 (IL-2) and interferon-γ (IFN-γ).

Antibacterial efficacy of gentamycin encapsulated in pH-sensitive liposomes against *Salmonella enterica serovar typhimurium* intracellular infection *in vitro* and *in vivo* was reported [82, 83]. Cell membranes are relatively impermeable to the antibiotic gentamycin, a factor that, along with the toxicity of gentamycin, precludes its use against many important intracellular bacterial infections. The pharmacokinetics and biodistribution of the free and PE-based liposomal gentamycin (DOPE-*N*-succinyl-DOPE/DOPE-*N*-glutaryl-DOPE mixture [82] and PE/*N*-succinyldioleoyl-PE lipid mixture [83]) were examined in mice bearing sys-

temic *Salmonella enterica* serovar *typhimurium* infection. Encapsulation of gentamycin in pH-sensitive liposomes significantly increased the concentrations of the drug in plasma compared to free gentamycin. Furthermore, the levels of accumulation of drug in the infected liver and spleen were increased 153- and 437-fold, respectively. The increased accumulation of gentamycin in the liver and spleen affected by liposomal delivery was associated with a 104-fold greater antibacterial activity than that associated with free gentamycin in a murine salmonellosis model. These pH-sensitive liposomal antibiotic carriers with enhanced *in vitro* activity could be used to improve both *in vivo* intracellular drug delivery and biological activity.

PEG stabilized DOPE containing pH-sensitive liposomes for targeted delivery and triggered release of doxorubicin (DXR) could negotiate an enhanced cytotoxicity against human B lymphoma cells [84]. Vesicles were stabilized using a cleavable lipid derivative of polyethylene glycol (PEG) in which the PEG was attached via a lipid anchor through a disulfide linkage (mPEG-S-S-DSPE). pH-Sensitive liposomes, targeted to the CD19 epitope on B-lymphoma cells, showed enhanced DXR delivery into the nuclei of the target cells with an increased cytotoxicity compared to non-pH-sensitive liposomes. Pharmacokinetic studies suggested that mPEG-S-S-DSPE was rapidly cleaved during circulation. In a murine model of B-cell lymphoma, the therapeutic efficacy of an anti-CD19-targeted pH-sensitive formulation was measured superior to that of a stable long-circulating formulation of targeted liposomes. The results suggested that targeted pH-sensitive formulations of drugs may be able to increase the therapeutic efficacy of entrapped drugs.

Serum-stable and long-circulating pH-sensitive liposomes for drug delivery have been reported recently [85]. pH-Sensitive liposomes were prepared with DOPE/OA and DOPE and 1,2-dipalmitoylsuccinylglycerol (DOPE/DPSG). The inclusion of polyethylene glycol-derived phosphatidylethanolamine (DSPE-PEG) enhanced the serum stability of both DOPE/OA and DOPE/DPSG liposomes, but also shifted the pH-response curve to more acidic regions and reduced the maximum percentage leakage. The impact of DSPE-PEG, however, was much lower in the DOPE/DPSG liposomes than in the DOPE/OA liposomes. In tumour tissue homogenates, where the pH is lower than normal healthy tissues, the pH-sensitive DOPE/DPSG liposomes released the entrapped markers rapidly, in comparison with pH-insensitive dipalmitoylphosphatidylcholine/cholesterol/DSPE-PEG liposomes. Moreover, the release rate was not affected by the content of DSPE-PEG. The blood circulation time of methotrexate incorporated in DOPE/DPSG liposomes was significantly prolonged with increasing content of DSPE-PEG. Taken together, the liposomes composed of DOPE, DPSG and DSPE-PEG (up to 5%) were pH sensitive, plasma stable and had a long circulation time in the blood. The complete destabilization of the liposomes at tumor tissues suggested the usefulness of such system for the targeted delivery of anticancer drugs. Various pH-sensitive engineered liposomes with their therapeutic potentials are summarized and recorded in Table 3.

8. pH-Sensitive liposomes as MRI contrast agent

Lokling et al. [86] described the feasibility of pH-sensitive paramagnetic liposomes as MRI contrast agents. A low molecular weight gadolinium (Gd) chelate (GdDTPA-BMA) was encapsulated in pH-sensitive liposomes. The *in vitro* relaxometric properties of the liposomal Gd chelate were shown to be a function of the pH in the liposomal dispersion and the membrane composition. Only a minor pH-dependency of the T(1) relaxivity (r(1)) was observed for liposomal GdDTPA-BMA composed of the unsaturated lipids DOPE and OA. On the other hand, the r(1) of GdDTPA-BMA bearing DPPE/PA based liposomes demonstrated a strong pH-dependency. At physiological pH and above, the r(1) of this system was significantly low compared to that of non-liposomal Gd chelate, which was explained by an exchange limited relaxation process. Lowering the pH below physiological value, however, gave a sharp and 6–7 fold increase in r(1), due to liposome destabilisation and subsequent leakage of entrapped GdDTPA-BMA. The pH-sensitivity of the DPPE/PA liposome system was confirmed *in vitro* employing magnetic resonance imaging (MRI) phantom study.

9. Conclusions

The pH-sensitive liposomes are promising drug carrier systems for the delivery of biomolecules into living cells. These carriers have shown promising results for enhanced cytoplasmic delivery of macromolecular drugs including DNA, ribozymes, enzymes (SOD, GOD), peptides, antisense oligonucleotides, therapeutic genes, protein toxins as well as small molecules of therapeutic interest like antibiotics (gentamycin), antiparasitic (chloroquine), anticancer (methotrexate, Ara-C and doxorubicin) and some fluorescent markers (calcein, CF etc.). Studies suggest that the pH of the lumen of the endocytic vesicles is mildly acidic. Thus it is possible for the pH-sensitive liposomes to remain stable in plasma and destabilize in the low pH environment of pathological tissues. pH-sensitive cationic liposomes mediate an efficient transfection of DNA into a variety of cells as fusogenicity is offered by both the constitutive lipids, i.e. pH-sensitive and cationic lipids. These conditions promote DNA binding, DNA incorporation and DNA induced fusion by cationic pH-sensitive liposomes. They have been proved as effective delivery vehicle for genetic vaccine and other genetic medicines for non-viral gene therapy. For construction of pH-sensitive liposomes, which are effective *in vivo* and stable until an acidified environment is encountered certain factors are of paramount importance. These include lipid type, lipid composition, lipid amount and vesicle size. These liposomes have not been successfully used in animals due to the poor stability of liposomes in buffer and in serum and the rapid uptake by the RES system. Combination of pH-sensitive liposomes with other release mechanisms, e.g. temperature-sensitive or magnoresponsive liposomes may be promising tool in drug delivery to tumors, which is under investigation. pH-Sensitive liposomes are also suggested for future therapy especially in the treatment of endotoxin shock, as a tool for photodynamic therapy, as an antigen presenting system, and for intracellular cytoplasmic delivery of biomolecules.

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