Liposomal Drug Delivery: A Versatile Platform for Challenging Clinical Applications

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ABSTRACT - Liposomes are lipid based vesicular systems that offer novel platform for versatile drug delivery to target cell. Liposomes were first reported by Bangham and his co-workers in 1964 (1). Since then, liposomes have undergone extensive research with the prime aim to optimize encapsulation, stability, circulation time and target specific drug delivery. Manipulation of a liposome's lipid bilayer and surface decoration with selective ligands has transformed conventional liposomes into adaptable and multifunctional liposomes. Development of liposomes with target specificity provide the prospect of safe and effective therapy for challenging clinical applications. Bioresponsive liposomes offer the opportunity to release payload in response to tissue specific microenvironment. Incorporation of novel natural and synthetic materials has extended their application from stable formulations to controlled release targeted drug delivery systems. Integration and optimization of multiple features into one system revolutionized research in the field of cancer, gene therapy, immunotherapy and infectious diseases. After 50 years since the first publication, this review is aimed to highlight next generation of liposomes, their preparation methods and progress in clinical applications.

INTRODUCTION

Liposomes are lipid based vesicular systems in which a well-defined aqueous core is contained within a lipid bilayer. In May 1964, Alec D. Bangham and his colleagues reported spontaneously forming lipid vesicles and named them as "smectic mesophases" (1). These vesicle were named "Bangasomes" after the name of Bangham, the father of liposomes. In 1968, Weissmann coined a more descriptive term "liposomes", an analogy to the term lysosomes (2). Liposomes were initially used as membrane models in biological studies. However, the cell-like structure of liposomes persuaded scientist to investigate liposomes as a tool to smuggle potent drug molecules into human body. In 1971, first report was published on the liposomal encapsulation of a therapeutic agent (3). Liposomes can load hydrophilic drugs in aqueous core and increase penetration through lipophilic physiological membranes while lipophilic drugs are contained inside the lipid bilayer and increase their solubility in the aqueous body fluids (4). Liposome demonstrates better protection from external degradation caused by enzymes (5) and because they are prepared from natural materials or their synthetic derivatives, liposomes are biocompatible and biodegradable (6).

Conventional liposomes have some drawbacks. They are complex to produce, have less inherent stability and therefore, cannot be stored for a long time. Liposomes show rapid uptake by the reticulo-endothelial system thus decreasing their circulation half-life. Leakage of loaded drugs from liposomes resulted in less drug loading efficiency. New materials and techniques employed in liposomal research may be toxic and non-biocompatible (7). Recently, it has been reported that repeated low dose injections may lead to accelerated blood clearance of liposome (8).

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To overcome these drawbacks, liposomes have undergone extensive research on surface modification, size optimization and understanding the disposition mechanism.

The next generation of liposomes is characterized by high mechanical stability, ability to induce or inhibit the immune system, longer circulation times, improved loading efficiency, enhanced penetration and target specificity. Accordingly, liposomes have emerged as promising carriers for sensitive drugs and macro molecules. Indeed, progress in pharmacology has introduced a number of potent therapeutic agents that require drug carriers that are selective and bio-responsive. This challenge to control drug release has been a milestone in the development of next generation liposomes. The improvements in liposomes include long circulating or stealth liposomes that have been prepared by decorating liposome surface with hydrophilic polymers such as polyethylene glycol which reduces liposomes uptake by the reticulo-endothelial system (9) and toxicity of encapsulated drugs (10). Stealth liposomes have long circulation life and constitute an integral part of controlled release multifunctional liposomes. On the other hand, PEGylation or stealthing strongly inhibits cellular uptake of liposomes which limits its application in macrophage and tumor targeting. The problem has been addressed by the development of cleavable PEG systems that can detach PEG in response to target tissue microenvironment (11). A negative charged DNA can bind a positively charged lipid forming cationic liposomes with reduced size and increased circulation time (12). Targeted specificity is achieved by anchoring targeting ligands which bind to the desired receptors (13). The number of lamellae or crosslinking of lipid controls the drug release rate from liposomes (14). Different drugs can be loaded in different lamella that enables sequential release of drugs and simplifies the treatment regimen (15). Liposomal microarrays have been employed for the detection of proteins and peptides in different body fluids (16).

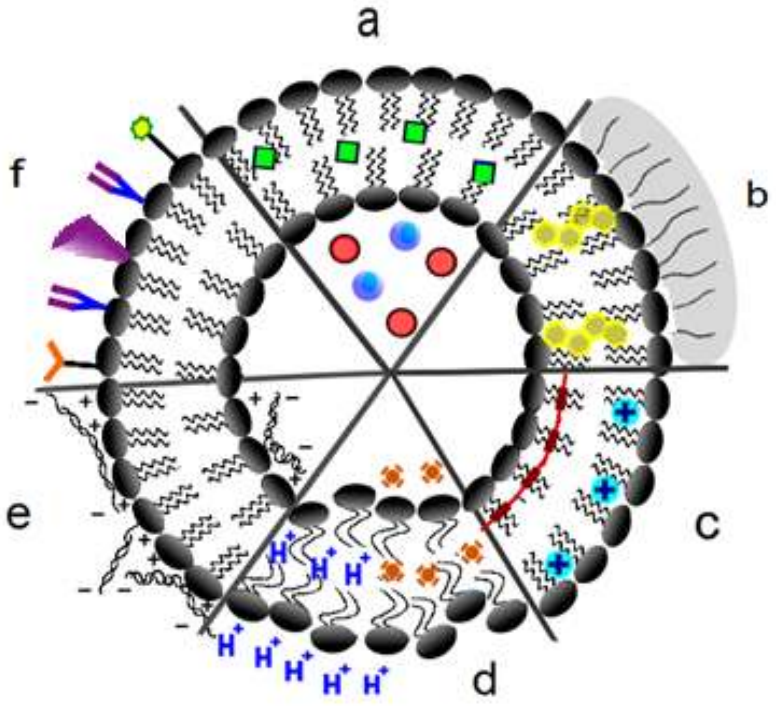

Figure 1: Multi-functionality of liposomes: (a) Encapsulation of hydrophilic drug (red) and gas bubbles (blue) into an aqueous core and entrapment of lipophilic drug (green) inside the bilayer, (b) Stabilization of lipid bilayer with cholesterol (yellow) and attachment of hydrophilic polymer layer on the bilayer surface, (c) Liposome lipid bilayer is strengthened and stabilized by polymerization (red) or by incorporation of multivalent cations (blue), (d) bio-responsive destabilization of lipid bilayer in acidic pH (blue) or increased temperature achieved by magnetic agents (Blue), (e) negatively charged DNA molecules are attached to positively charged lipid molecules, (f) attachment of immunogenic or targeting ligands on the liposome surface.

Chimeric liposomes have been prepared by the combination of phospholipids with low quantities of other biomaterials to control physiochemical and structural properties of liposomes (17). Different imaging techniques and *in silico* models have been devised to predict or monitor biodistribution as well as quantitative structure-property relationship of liposomes (18-20).

Almost 50 years after the discovery of liposomes, the US FDA has approved 13 liposomes based products for human use (Table 1). In addition, a large number of liposomal products are in different phases of clinical trials (21,22). The next generation of liposomes are prepared conventionally either by modification of conventional preparation methods or special laboratory techniques. Liposomes are prepared by a lipid hydration, ethanol injection, freeze-thawing and reverse phase evaporation. In the lipid hydration method, lipid is dissolved in an organic solvent followed by drying to form a thin lipid film. This lipid film is hydrated using an aqueous solution of a drug and vortexed to form liposome system. In the ethanol injection method, lipid is dissolved in ethanol and its solution injected into an aqueous buffer containing the drug. In the freeze-thawing method, a lipid film is hydrated with an aqueous buffer solution and subjected to repeated cycles of freezing and thawing thus yielding a liposome system. In the reverse phase evaporation technique, a solution of a lipid and an organic solvent is added to an aqueous buffer solution to form a water in oil emulsion. This emulsion is subjected to evaporation under low pressure which results in the formation of a liposome system. Researchers have also designed in-house laboratory scale detection methods for the characterization and quality control of liposomes (23). However, the scale-up of these sophisticated laboratory methods to industrial production still needs to be addressed. Major issues in industrial manufacturing of liposomes include the presence of residual organic solvent, difficulty of controlling liposome size distribution and stability problems associated with sterilization processes (24).

ADVANCEMENT IN LIPOSOME TECHNOLOGY

In this section, attempts have been made to reviews the next generation of liposomes, their preparation methods and progress in clinical applications.

Archeosomes: Archeosomes are liposomes made up of polar lipids of archaebacteria. Archaebacteria are extremophiles which can live in extremes of environment. Their cell membrane consists of ether-lipids which differs from other eukaryotic and prokaryotic membrane lipids.

Table 1. Liposome based formulation available in market for clinical applications.

Liposome Product	Active Ingredient	Liposome Type	Indication
Ambisome	Amphotericin B	Liposome	Fungal Infections
Abelcet	Amphotericin B	Lipid Complex (not true liposomes)	Fungal Infections
Amphotec	Amphotericin B	Lipid Complex (not true liposomes)	Fungal Infections
DaunoXome	Daunorubicin	Liposome	Blood Cancers
Doxil	Doxorubicin	Stealth liposome	Kaposi's Sarcoma, Ovarian and Breast Cancer
Lipo-dox	Doxorubicin	Stealth liposome	Kaposi's Sarcoma, Ovarian and Breast Cancer
Marqibo	Vincristine	Nano-size liposome	Acute lymphoblastic leukaemia
Myocet	Doxorubicin	Liposome	Metastatic breast cancer
Visudyne	Verteporfin	Liposome	Photodynamic therapy
Depocyt	Cytarabine	Liposome	Neoplastic and lymphomatous meningitis
DepoDur	Morphine	Liposome	Pain management
Epaxal	Inactivated hepatitis A virus	Virosome	Hepatitis A
Inflexal V	Inactive hemaglutinine of Influenza virus	Virosome	Influenza

The incorporation of inherently stable archae lipids, archaeol (diether) lipids and caldarchaeol (tetraether) lipids makes archeosomes resistant to pH, temperature and oxidative stress (25-28). Tetraether lipids are bipolar unlike di-ether or other liposomal lipids which have one polar head (Figure 2). Additionally, ether lipids of bacterial origin are recognized as foreign substance by human immune system eliciting strong immunoadjuvant effects to the encapsulated vaccine.

Preparation: Polar lipids from archeobacterium are extracted by solvent extraction. High amount of detergent is added to the lipid followed by evaporation of the solvent. This lipid detergent mixture is dissolved in anaqueous buffer and detergent is removed by dialysis (29). Archeosomes can be prepared from these archeae lipids by lipid hydration method (30).

Applications: Archeosomes serve as carriers for vaccines to provide long circulation times. They can also act as adjutants for cell mediated immune responses (31-33). Tetraether archael lipidsare bipolar forming a stable archeosomes. Recent research has focused on manipulating synthetic analogues of archael ether lipids (34). The synthetic analogues facilitate the industrial production and purification of the finished product (35).

Cochelates:

Cochelates are cylindrical lipid bilayer assemblies prepared from preformed liposome in such a way that lipid bilayers of liposomes are stabilized with inorganic multivalent cations of zinc, calcium (36) and other organic multivalent cations (37). The multivalent cations act as bridging agent to hold lipid bilayers together which results in shrinkage of aqueous core (Figure 3).The unique solid structure of lipid bilayer is responsible for high mechanical strength and storage stability. The dried cochelates are stable at room temperature while cationic buffer solution of cochelate can be stored at 4°C for two years (38). In a similar study, multilamellar liposomes with covalently crosslinked lipid bilayers were reported for controlled release of anticancer drugs (10).

Preparation: Cochelates are prepared by two methods from preformed liposomes. Method 1: The solution of multivalent cations is added to the suspension of prepared liposomes followed by sonication. The milky suspension of liposomes is converted to colorless solution of nano cocheltes as sonication proceeds (37). Method 2 (Hydrogel method): Liposome are suspended in an aqueous two-phase polymer solution. Cations diffuse from one polymer phase to the other polymer phase containing liposomes which results in cochelates formations. The precipitate is washed with cationic buffer to remove the residual polymers (39).

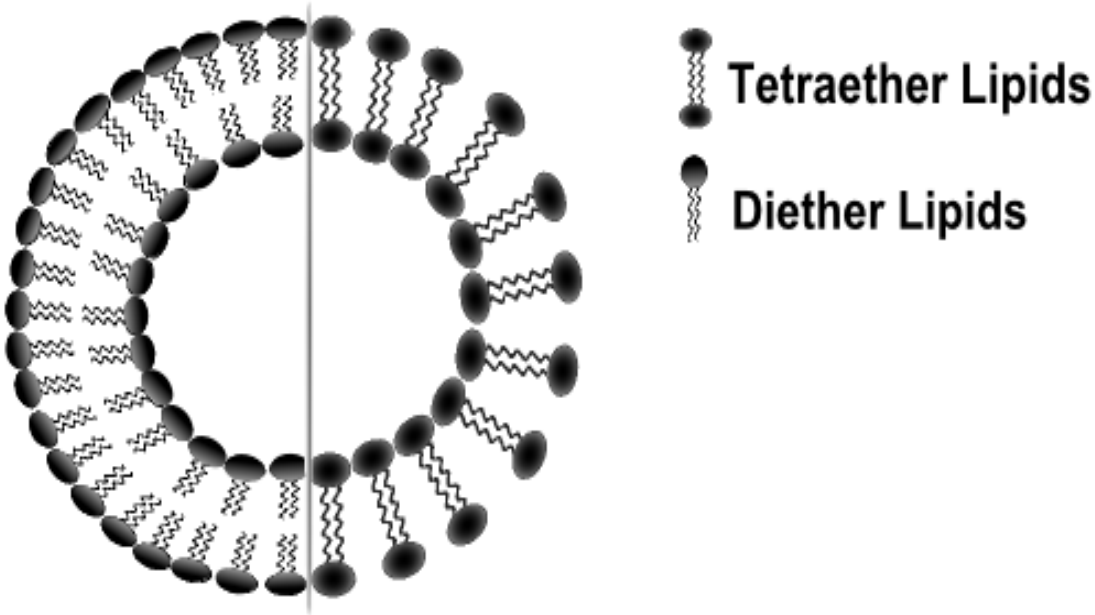

Figure 2: Archeosomes made from tetraether (bipolar) and diether lipids.

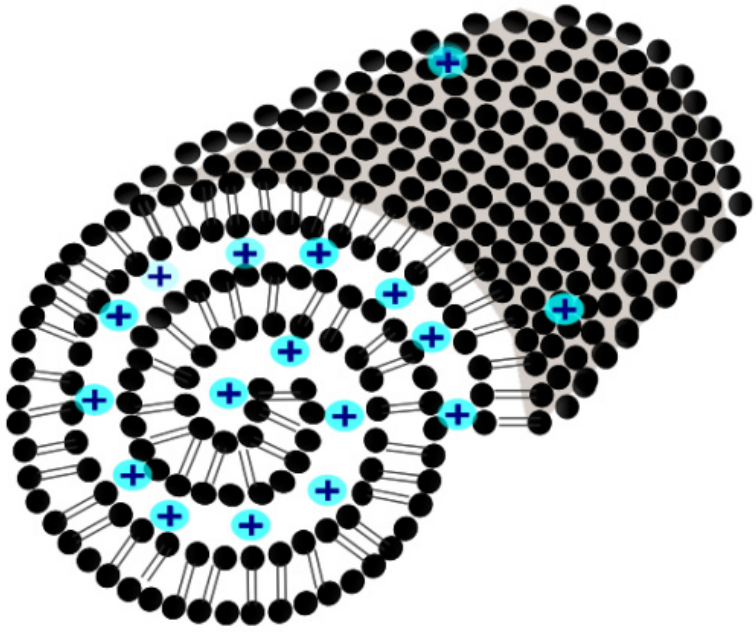

Figure 3: Incorporation of cations into lipid bilayer to improve stability.

Applications: Cochelates have been used for delivery of DNA and protein subunits. For mucosal and parenteral vaccine delivery, cochelates with virus antigen proteins are preferred over liposomes containing virus protein due to stronger immune response and longer circulation time (40). Oral cochelated Amphotericin B (CAMB) is 10 times more effective compared to conventional liposomes (41). Cochelates provide prolonged action of cationic antimicrobial peptides (AMPs) and alsocombat antibiotic resistance (42). In addition, presence of calcium cations in liposomes has been found to increase the fusion of fluid liposomes with bacterial cells thus enhancing the bactericidal activity in resistant strains (43).

Cubosomes:

Cubosomes are self-assembling cubic crystals of certain detergents with an intersecting network of water channels. Cubosomes do not fit properly under the strict definition of liposomes because they lack a well-defined aqueous core, but have attracted a vast research interest in lipid based drug delivery systems in the last decade (44,45).

Preparation: Cubosomes are honeycomb like structures that are formed by dispersing amphiphilic lipids in the aqueous solution and a lipid bilayer is formed in three dimensional structures (46,47). Cubosomes have been developed by a fragmentation method and solvent precursor dilution method. In the fragmentation method, the lipids are heated above melting point in a glass vial and water (25% w/w of lipid) is gently layered on to the surface of the lipid. This system is incubated for three days to allow the formation of cubosomes. Addition of stabilizer and a homogenization step may be required to get desired formulation (48). In the solvent precursor dilution method, lipid and stabilizer are first dissolved in ethanol. This modified lipid mixture is than dissolved completely in chloroform as lipid and solubilizer are soluble in it. Chloroform is evaporated under a stream of nitrogen (N_2) and 10uL aqueous drug solution is added to the lipid mixture. The liquid precursors are dispersed in deionized water with continuous stirring for 10 minutes (49).

Applications: Cubosomes have been used for oral, parenteral, topically and ocular administration. Dexamethasone cubosomes for ophthalmic use are four to five times more effective than eye drops. Cubosomes have also demonstrated superior peptide delivery properties over liposomes, transferosomes and ethosomes (50). Recent research has demonstrated their successful application in the treatment of cancer (51,52). In addition, cubosomes are used for delivery of

vaccines (48) and diagnostics and separation techniques (53). Although the reports of therapeutic applications of cubosomes are increasing, there is negligible data on the cytotoxicity of cubosomes. Further studies are required on cubosomes toxicity to justify their application in medical field (54).

Ethosomes:

Ethosomes are transdermal liposomes containing hydroalcoholic core for enhanced penetration through the stratum corneum and other skin layers (Figure 4). The presence of ethanol in core increases fluidity and penetration of ethosomes bilayer without affecting its stability. Ethosomes enhance transdermal delivery of drug by enhanced permeation in terms of depth and deposition behavior (55). Ethosomes are more effective in nanometer range and provide a noninvasive mean for enhancing skin permeation (56-60).

Preparation: Hot and cold methods have been employed for ethosomes preparation. In the hot method, phospholipids are dispersed in water at 40ºC. Drug is dissolved in ethanol and propylene glycol and this mixture is added into the phospholipids solution using magnetic stirring. In the cold method, phospholipids and drug are dissolved in ethanol and heated to 30ºC. This mixture is added to the water maintained at 30ºC. The ethosomes suspension is sonicated and extruded to produce homogeneous ethosomes (60,61).

Applications: Ethosomes serves as carriers for transcutaneous immunization (62) and gene delivery (63). These are also employed for anti-inflammatory (64,65), anti-psoriatic (66) and anti-microbial therapeutics (67). Recent research has focused on binary ethosomes that contain polyols (such as propylene glycol) in addition to alcohol and water inside the ethosome core. Ethosomes and binary ethosomes have demonstrated superior penetration efficiency over conventional liposomes, transferosomes and other specialized drug delivery systems (68-71).

Exosomes:

Exosomes are phospholipid vesicles released by normal and tumor cells. In normal cells, they control cell-to-cell communication by paracrine signals to induce protein synthesis and alter the behavior and proliferation of surrounding cells. In tumor cells, they help in maintaining a tumor specific environment by paracrine signaling for promoting angiogenesis and lymphocyte apoptosis. These tumor derived exosomes have been isolated from patients and loaded with anticancer agents for individualized therapy of cancer. Their surface complexity and non-specificity hindered their use in drug delivery systems and trigger the development of exosome-mimetic, liposomes with functional components of exosomes (72-74). The endogenous origin of exosomes is advantageous over synthetic nanocarriers due to excellent biocompatibility and targeting potential (75).

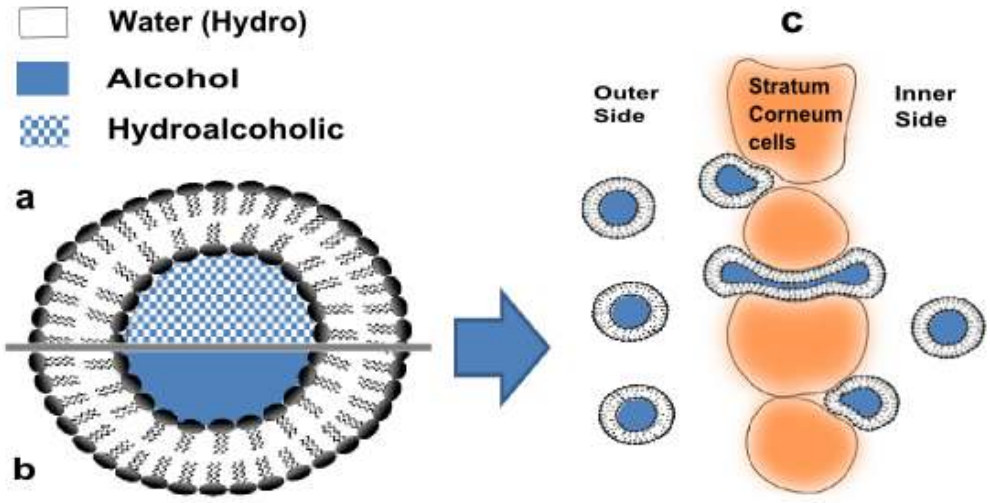

Figure 4: (a) Ethosomes with hydro-alcoholic (water and alcohol) core (b) Ethosomes with alcoholic core. (c) Passage of the ethosomes from outside the skin, through very small pores, into the inner site of the stratum corneum.

Preparation: Exosomes secreting cells are immortalized *in vitro* and exosomes are separated by differential centrifugation. Therapeutic agents are loaded into prepared exosomes by standard transfection techniques such as electroporation or lipofection. Electroporation is a physical method in which successive electric impulses are applied on exosomes which increase their permeability and entrapment of DNA molecules. Colloidal stability of exosomes after electroporation should be given keen consideration which is function of composition of media, exosomes concentration and charge applied (76). Lipofection is a chemical techniques in which positively charged lipids bind negatively charged DNA molecules (77).

Applications: Exosomes are employed for the delivery of genes because of their intrinsic ability to cross biological barriers and their non-immunogenic nature (78). Suppression of the immune system by cancerous exosomes has been assumed to treat autoimmune diseases (77). Some researchers are thus focusing on targeted cancer vaccination and individualizing therapy with patient derived exosomes (79,80).

Gas containing liposomes:

These liposomes are modified to encapsulate a gas bubble that contains gas up to 80% of their inner capacity (81,82). Liposomes loaded with chemical agents such as ammonium chloride produce gas bubbles and cause the release of loaded therapeutic moiety (83). Targeted gas containing liposomes are currently being investigated for deep tissue tumor diagnosis (84).

Preparation: Gas containing liposomes are prepared by the film hydration and freeze thawing method. The process is carried out in the presence of gas to be encapsulated under high pressure (84).

Applications: Targeted gaseous liposomes are employed for tumor tissue detection. These liposomes oscillate under low frequency ultrasound thereby producing a characteristic echo which is different from the echo of surrounding tissues (81). Therapeutic drug molecules loaded to gas containing liposomes release the drug at a controlled rate by applying specific ultrasound frequency outside the body region (82). Nitric oxide containing liposomes have been used to induce relaxation of the vascular smooth muscles (83). Xenon gas is reported to be an effective and non-toxic neuroprotectant agent when encapsulated in gaseous liposomes (85).

Immunoliposomes:

Surface ligands such as monoclonal antibodies when attached to the liposomes are effectively targeted to different body tissues (86). Immunoliposomes selectively bind to a receptor of target cell and become internalized, thereby increasingthe bioavailability of therapeutics. These Immunoliposomes confine the action of very toxic and/or potent pharmacological agents at target tissues and safeguard other body tissues from unnecessary exposure (86-88). Development of the immunoliposomes in the 1980s provided a milestone in the evolution of advanced multifunctional liposomes (Figure 5).

Preparation: Antibodies or antibody fragments are conjugated on liposome surface by mixing isolated antibody fragments with already prepared cationic liposomes. These immune-cationic liposomes are then mixed with therapeutic drug solution to form a drug-loaded immunoliposomes (89). Recently, folate conjugated immunoliposomes have been prepared by one step microfluidic synthesis. In this method, folate conjugated phospholipid are passed through thermoplastic microfluidic device in a controlled laminar flow. This method can produce immunoliposomes in nano size range (90).

Applications: Various monoclonal antibodies produced against different cancerous tissues have been used in cancer chemotherapy by formulating immunoliposomes. Multifunctional immuoliposomes have long circulation time and can carry more than one therapeutic and diagnostic agents (91). Immunoliposomes have successfully been targeted to tumor cells (92-95), vascular endothelium (96) and infarcted heart tissue (97). Immunoliposome are meant to improve safety profile of potent drugs by limiting drug exposure to target sites but recently reported results of Shmeeda et al., (2013) found that liposomal zoledronic acid was almost 50 times more toxic than free zoledronic acid solution.

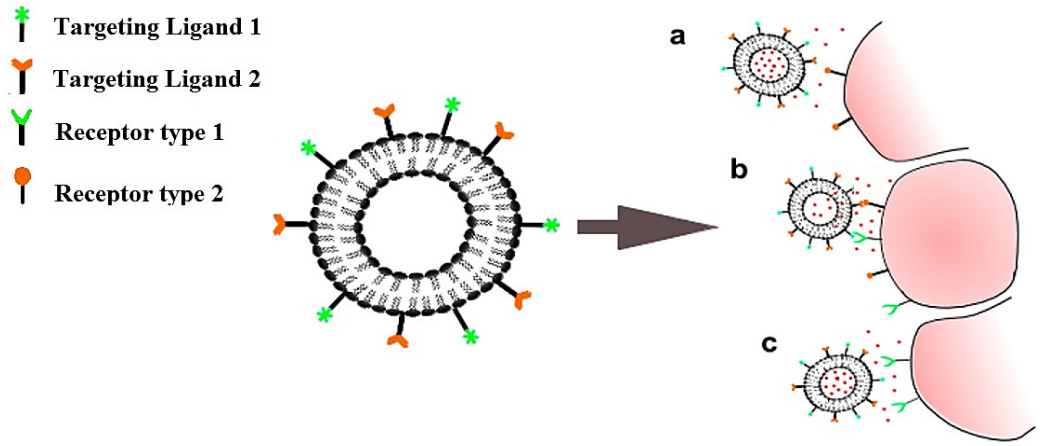

Figure 5: Bi-specific targeting by attachment of two different ligands; Immunoliposomes attached to receptors for targeting both ligands simultaneously. Immunoliposomes can bind and release drugs at tumor cells expressing any one receptor type (a) and (c) or both receptors simultaneously.

Toxicity was found to be dose dependent and attributed to unexpectedly high macrophages activation (98). However, this enhanced toxicity might underlie entirely different mechanism because liposomal drugs, such as cisplatin, have been found to act differently than free drug at molecular level (99). Current research is focused on developing bispecific ligands for dual targeting of tumor cells with more specificity (100). Moreover, liposomes displaying antigenic proteins and glycan ligands for the inhibitory co-receptor CD22 have been reported to elicit only antigen specific immune response and prevent B cell mediated harmful immune responses (101). Harmful immune and inflammatory response to immunostimulatory agents can also be prevented by localized immunotherapy. One such strategy is the intratumoral injection of controlled size liposomes which allows dissemination of liposomal drugs into tumor tissue and tumor draining lymph nodes but prevents entry into systemic circulation due to large size of liposomes (102).

Immunosomes and Virosomes:

Immunosomes are prepared by attaching viral glycoproteins on liposome lipid bilayer. Immunosomes are nano-sized globular particles having uniform glycoprotein attachments (50-60 nm) (103-105). Immunosomes are non-toxic vaccine carriers with strong immuno-adjuvant properties to stimulate or inhibit the humoral as well as cell mediated immunity. Research on immunosomes has led to the discovery of virosomes. Virosomes are substituted viral coats of lipids, glycoproteins, haemagglutinin and other antigenic components. Virosomes lack viral nucleic material and fail to replicate (106). Immunosomes differ from virosomes in their composition. Immunosomes are a type of liposomes that contain only certain isolated antigens of a virus while virosomes are complete virus shells with all viral lipids, glycoprotein and antigenic determinants (Figure 6). Usually, these terms are used interchangeably but the term virosome is preferred over immunosome to avoid any confusion with immunoliposomes. The surface fastened viral glycoproteins provide an indistinguishable structure to immunosome and virosome and is responsible for their immunogenic properties similar to the parent virus (107).

Preparation: Using the detergent solubilizing method, immunosomes are prepared by mixing viral surface proteins with liposomes. Virus hemagglutinin is prepared by solubilizing the virus cell with a 10 nM HEPES saline solution followed by centrifugation that separates the virus hemagglutinin. Its purified rosette is mixed with liposomes and incubated at 37^{o}C for 30 min (105,108). Stability of immunoliposomes is also dependent upon the interaction between phospholipid head groups and viral proteins. Immunosomes consisting of binary lipid mixtures are more stable as compared to liposomes composed of single lipid type (109).

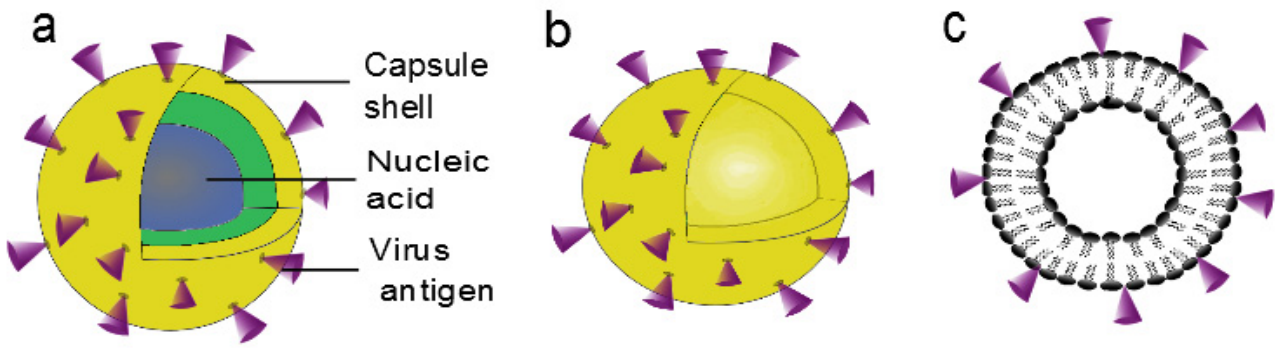

Figure 6. (a) The whole influenza virus is converted into a virosome (b) The virosome retains only the outer shell and associated antigens. The immunosome is prepared by attaching virus glycoproteins antigens on the liposome surface (c).

Application: Immunosomes and virsomes are vaccine carriers with strong adjuvant propertiesagainst AIDS, influenza, hepatitis and rabies viruses in mice. These carrier vaccines have also proved to be effective against these viral strains in ex-vivo and animal studies (110-114). The vaccines Epaxal (Hepatitis A) and Inflexal (Influenza) are already available for clinical use (Table 1).

Immune stimulating complexes (ISCOMs)

ISCOMs are vaccine adjuvants which induce a wide range of antibodies by humoral activation as well as strong cell mediated responses against antigen. Major structural components of ISCOMs are cholesterol, phospholipids and quiallaja saponins (Quil A). They contain amphiphilic antigens like membrane proteins and in aqueous solution can be stored for years at cool temperature (115).

Preparation: ISCOMs are prepared by ether injection method. Phospholipids and cholesterol are dissolved in ether and injected into aqueous solution containing Quil A. An optimal ratio (5:3:2) of Phospholipids, Quil A and cholesterol and injection rate ensures homogenous preparation (116).

Applications: Immune stimulating complexes produce a wide range of antibodies by humoral and cell mediated responses making them a carrier of choice for vaccines against intracellular and chronic infections (117). Posintro™ denotes a new generation of ISCOMs with modified surface charge for enhanced penetration through the skin. Incorporation of positively charged cholesterol derivatives and DC-cholesterol reduces overall negative charge of the lipid bilayer. Thus, Posintro™ may follow the intracellular pathway of absorption by disrupting the stratum corneum (118).

Lipoplexes

Lipoplexes are formed when cationic lipids are coupled with a negatively charge of DNA molecules. Lipoplexes are considered toxic due to the presence of cationic lipids. Toxicity can be reduced by using compact plasmid DNA (pDNA). When an elongated DNA is transformed into a compact form, its overall negative charge decreases and lesser amount of cationic lipid is needed (119-122). Effective lipoplex formulations require optimization of size and structure of the self-assembled vesicles (123). Major disadvantage of lipoplexes is acute inflammatory response after administration. The sequential administration of plasmid DNA and lipoplexes as repeated doses has been found to minimize the acute inflammation and other lipoplexes associated toxicities (124).

Preparation: *p*DNA is isolated from purified plasmid using suitable techniques. Cationic liposomes are prepared by lipid hydration and sonicated until a clear translucent solution is formed. The *p*DNA solution is added to cationic liposomes and mixed gently. The obtained lipoplexes suspensions is stored at room temperature for 15–30 min before use (125).

Applications: Lipoplexes are used for viral DNA transfection avoiding virus associated immunogenicity and oncogenecity. Lipoplexes are

easier to produce compared to other viral carrier preparations (126-127). Incorporation of co-lipids such as dioleyl phosphatidylethanolamine (DOPE), cholesterol and some fatty acids have been reported to increase gene transfection rates. Co-lipids make lipoplexes more rigid and increase their size which facilitates their fusion with cell membranes and phagocytosis. Lipoplexes may also act as immune-adjuvant when admixed with protein antigens. However, their adjuvant activity is primarily due to enhanced uptake of proteins by antigen presenting cells as compared to negatively charged liposomes that promote activation and maturation of antigen presenting cells (128). Recent research has focused on making lipoplexes more plasma friendly, optimizing morphological features which can control cellular uptake and intracellular disposition (129-130). The manipulation of co-lipids has led to improved transfection efficiency (131-132) and targeted delivery (133).

Magnetic liposomes:

These are the type of advanced liposomes which contain magnetic or paramagnetic entities such as gadolinium (Gd^{3+}) and Iron (Fe^{3+}/Fe^{2+}), and are used for diagnostic imaging and targeted therapeutic delivery. Lipid-magnetite complexes are incorporated on the liposome surface (134) or in the lipid bilayer or aqueous core (135) (Figure 9). Magnetic liposomes also act as a contrast agent in MRI imaging (136).

Preparation: Magnetic liposomes are prepared by a lipid hydration method. In this method, the lipid is dissolved in an organic solvent and dried to form a thin lipid film. A magnetizing agent is dissolved in water to form an aqueous magnetizing solution. The thin layer of lipid in the round bottom flask is hydrated with the aqueous magnetizing solution to form magnetic liposomes (137).

Applications: Magnetic liposomes provide "clickable" release of therapeutic agents by applying an alternating magnetic field. Magnetic liposomes provide permeability and controllable release (138-139). Magnetic liposomes are decorated with various surface ligands, such as polyethylene glycol, as targeting ligands (140), and are bi-functional which means they can carry fluorescent probe and MRI contrast agents for imaging as well as functional DNA delivery to the same cell (141). Recent research has reported super magnetic liposomes to treat cancer tissues and their metastasis without involving surgery or chemotherapeutic agents (142). When targeted super magnetic liposomes are subjected to an alternating magnetic field, they produce local hypothermia (about 45^{o}C) and cause tumor tissue necrosis (143).

Niosomes:

Niosomes are unilamellar or multilamellar vesicles composed of non-ionic surfactants (Figure 10). In niosomes, phospholipids bilayer is replaced by nonionic surfactants, making niosomes more stable and producing a longer shelf life. Niosomes can be administered through oral, intramuscular, intravenous and transdermal routes of administrations (144-147).

Preparation: Niosomes can be prepared by conventional lipid hydration and ether injection method with slight modifications depending on noisome application. Size homogeneity can be achieved by using an extrusion method (148-150).

Application: Niosome have been used for the treatment of leishmaniasis (151), cancer therapy (152,153), immune induction (154) and as carrier for diagnostic agents (155). Research has been focused on providing controlled release of antimicrobial agents, anti-inflammatory drugs, peptides and various macromolecules (156-160). Cationic niosomes have been prepared by the combination of cationic lipids and non-ionic surfactants in niosomes (161). Recently, novel multicomponent drug delivery systems have been prepared in which preformed niosomes were loaded into liquid crystal gel. Both components of this system were prepared from the same mixture anionic and non-ionic surfactant for enhanced drug permeation after topical application (162).

pH sensitive liposomes:

In viral infections, mild acidic conditions are responsible for fusion of virus envelopes with cell membranes. This observation led to the development of pH sensitive liposomes which release loaded therapeutics at acidic pH. Serum albumin and protein fragments have been reported to enhance liposome fusion at a lower pH of 6.5 (163-166). However, pH sensitive liposomes provide bioresponsive delivery to tissues. Lower pH values destabilize the lipid bilayer and drug is

released at tumors, infections and inflammation (167-169). (Figure 9). Phosphatidylethanolamines and a stabilizing amphiphiles are used to stabilize liposomes at acidic pH (170-172). During endocytosis, the pH sensitive liposomes are exposed to the acidic environment of endosomes and may release their content inside the cells.

Preparation: Freeze thawing and reverse phase evaporation methods are used for the preparation of pH sensitive liposomes. Lipids, co-lipids and PEGare dissolved in an organic solvent. In freeze thawing, the lipid suspension is frozen followed by thawing in a water bath. After ten freeze-thawing cycles, pH sensitive liposomes are formed (173). In the Reverse Phase Evaporation method, a lipid film is hydrated with another organic solvent followed by adding an aqueous saline solution of therapeutics. The emulsion is vortexed and the organic solvent is evaporated to form pH sensitive liposomes (174).

Applications: pH sensitive fusogenic polymers have been subject of extensive research for the development of pH sensitive liposomes (175,176). Hydrophobic derivatives of natural polymers have been conjugated with phospholipids molecules. Such liposomes demonstrate excellent pH responsiveness *in vitro* and *in vivo* (177,178). Recent advancements have led to the development of 'fliposomes' which are more stable and show instant release in seconds (179). Fliposomes contain flipids, the lipids that undergo conformational switch at lower pH and destabilize the liposome bilayer (180). They are used for delivery of gemcitabine (169) and cisplastin (181). Research has been focused on the development of stealth and targeted pH sensitive liposomes (182). In addition, understanding the polymer backbone structure and resulting hydrophobic behavior can provide very helpful insights into membrane interactions and fusogenic activity of these liposomes (183,184). Juan *et al* (2012) have reported a pH sensitive liposome using NH_4-HCO_3 for the release of doxorubicin. Within the target cell, CO_2 is liberated from NH_4-HCO_3 which lowers pH and enhance drug release (185).

Polymerized liposomes:

Leakages of therapeutics from the conventional liposomes which are toxic to the normal tissues poses a serious challenge to clinical applications. This problem can be solved by polymerization of the lipids in the liposome bilayer. These polymerized liposomes are completely stable, provide modified release and enhance the half-life of therapeutic agents (186-188). Polymerized liposomes should be differentiated from capsosomes. Capsosomes are not true liposomes but contain a polymeric capsule with multiple sub-compartments composed of liposomes (Figure 7), (189).

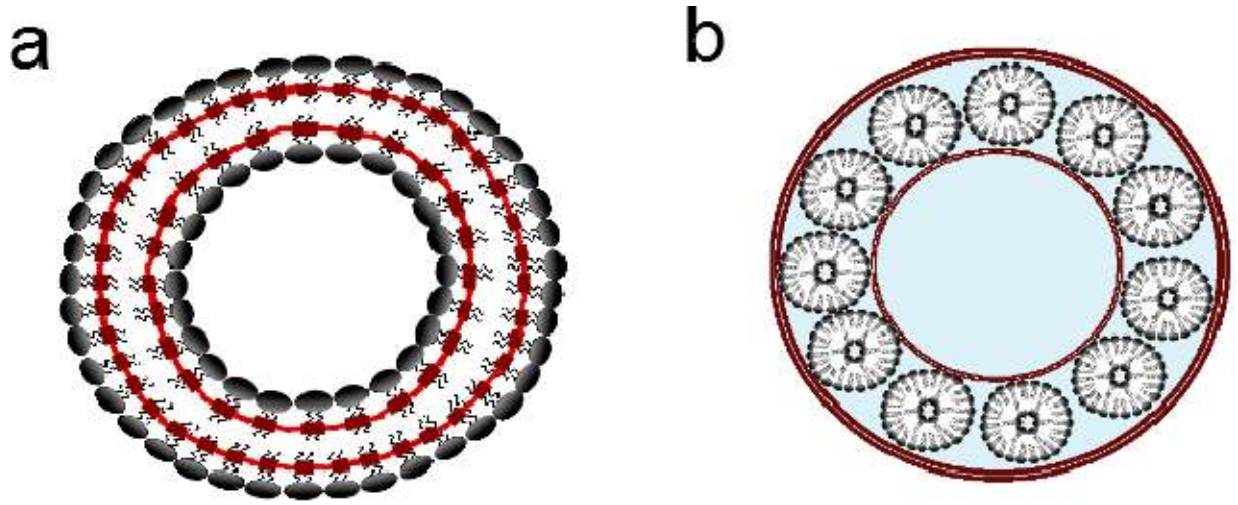

Figure 7: (a) A representation of polymerization of lipid molecules in liposome bilayer to increase the mechanical strength of lipid bilayer, (b) Capsosomes (not a liposome type), a polymer capsule containing numerous liposomes.

Preparation: Polymerized liposomes are prepared by film hydration method. Cholesterol is chemically combined with polymerizable monomer. Phospholipids and the monomeric cholesterol are mixed in chloroform and dried in a rotary evaporator to form a thin film. The lipid film is hydrated with an aqueous buffer and sonicated for 30 minutes resulting in liposome formation. Polymerization of the lipid bilayer may be carried out by thermal, radiation or redox reaction. In thermal polymerization, the liposomes are heated to 60-70^{0}C in the presence of free radical donor such as Azobis-isobutyronitde which initiates a polymerization reaction (190). In the radiation polymerization, liposomes are subjected to ultra violet (UV) radiations for 1-2 hours resulting in lipid polymerization (187). In the redox polymerization, redox initiator system consisting of ammonium per-sulphate and sodium meta-bi-sulphite are added to liposome formulations and heated in the presence of $N_2$to initiate polymerization. Residual polymeric monomers, reactants and un-entrapped drug are removed by dialysis (191).

Applications: Polymerized liposomes can be used for parenteral, oral and mucosal delivery of various diagnostic (191-193) and therapeutic agents. These are employed as vaccines and for the delivery of allergens, carbohydrates and antimicrobial agents (194-196). Polymerized liposomes are prepared in nanometer size range with multifunctional variants for controllable release responsive to pH and magnetic fields (197-200). In a recent study, liposomes were functionalized with surface adsorption of chitosan followed by polymerization of lipid bilayer.

Proliposomes:

Proliposomes are dry and free flowing particles consisting of phospholipids coatings on water soluble powder particles. These lipid particles yield homogenous suspension of liposomes when dissolved in an aqueous solution. Proliposomes solve the problem associated with sterilization of liposomes as they have a high storage stability and provide simple large-scale production (201-203). Recently, liquid proliposomes have been reported that spontaneously form liposomes upon hydration (204) (Sun et al., 2013).

Preparation: Proliposomes are prepared by film deposition on the carrier method (Figure 8). Dried powder is placed in a round bottom flask and maintained at 30-40^{o}C. Drug and phospholipids are dissolved in an organic solvent. This organic solution is added in aliquots to a continuously rotating bed of dry powder. Complete drying of organic solvent occurs for each aliquot addition. The resulting proliposomes can be lyophilized or desiccated overnight to yield a fine free flowing powder (205). Proliposomes can also be prepared by fluid bed coating, spray drying and freeze-thawing depending upon the type of drug and its application (206).

Liquid proliposomes were prepared by dissolving hydrophobic drug, phospholipid, sodium deoxycholate, poloxamer and polyethylene glycol in ethanol. This light yellowish liquid was filled in hard gelatin capsule and dried. These liquid proliposomes will yield liposomes when manually mixed with distilled water (204).

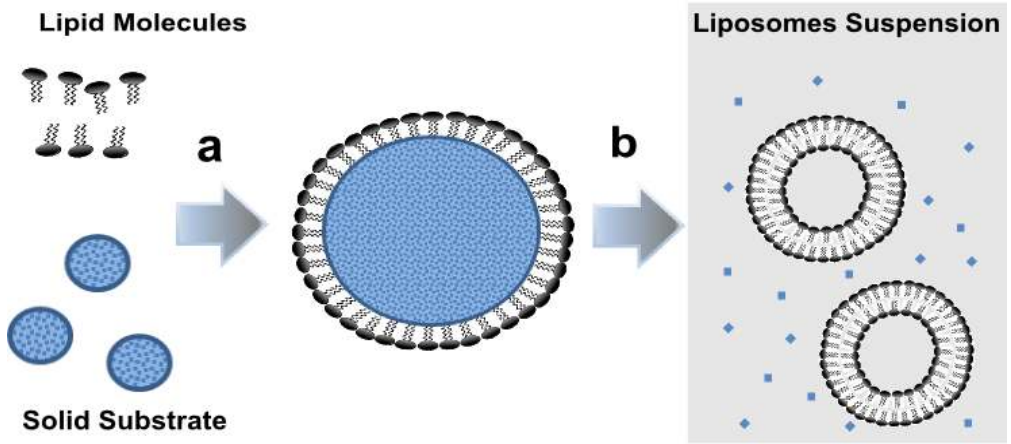

Figure 8: Synthesis of proliposomes. (a) A lipid is mixed with a solid substrate which forms lipid coated solid particles. (b) Upon hydration, solid substrate is dissolved and lipids arrange to form liposomes.

Applications: The administration of dry proliposomes via inhalation provide enhanced bioavailability and sustained therapeutic effects (207,208). Proliposomes are reconstituted into a prescribed solvent to yield a liposome system. Effervescent proliposomes provide carbon dioxide upon dissolution (209). Proliposomes show great flexibility in the route of administration including reconstituted parenteral, oral, topical, intranasal administration and as dry powder aerosol (210). Proliposomes have successfully been used for enhancing bioavailability of anticancer drugs and other therapeutic agents (211-213).

Temperature sensitive liposomes:

Temperature sensitive lipids are stable at body temperature and undergo phase transition from gel to liquid at higher temperatures and provide a successful mean for targeted delivery (214). Temperature sensitive liposomes circulate throughout the body but when they reach an area of significant hyperthermia, they release therapeutics due to increased fluidity of the lipid bilayer (Figure 9).

Preparation: Temperature sensitive liposomes are prepared with slight modifications of the reverse phase evaporation and lipid hydration method. In both methods, temperature sensitive lipids are mixed in an organic solvent followed by evaporation of the solvent in a rotary evaporator. In the hand shake method, the lipid film is hydrated with an aqueous solution containing a therapeutic agent and a surfactants. The system is heated above their phase transition temperature to yield temperature sensitive liposomes (215). In the reverse evaporation method, the lipid film is re-dissolved in an organic solvent followed by the addition of an aqueous saline solution containing the therapeutic moiety. The mixture is heated to 60^{o}C and vortexed for 30 seconds. The organic solvent is removed by rotary evaporation to yield temperature sensitive liposomes (216).

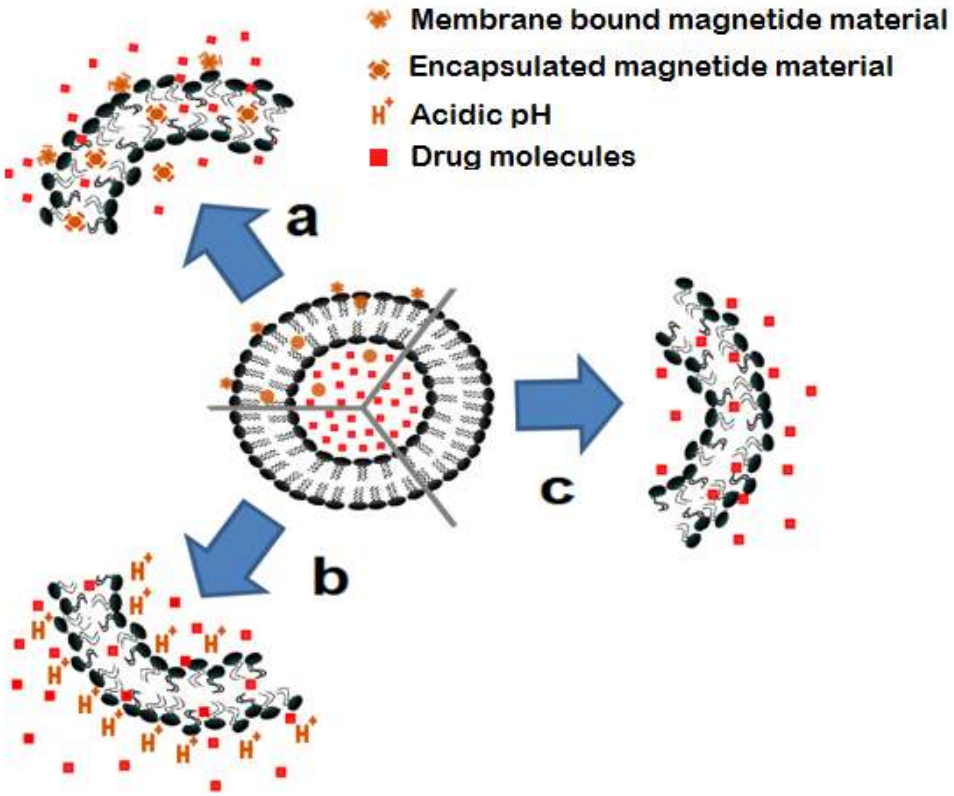

Figure 9. A representation of clickable drug release from magnetic, pH sensitive and temperature sensitive liposomes. (a) Magnetite materials (MRI contrast agents) are loaded in the bilayer and core, or attached to the lipid molecules. Under an externally applied alternating magnetic field, liposomes become leaky and release the loaded drug. (b) pH sensitive liposomes contains pH sensitive lipids in their bilayer which lose their geometric conformation at acidic pH and release the drug. (c) Temperature sensitive liposomes are activated by local or artificially applied hyperthermia and drug release is achieved.

Applications: Traditional temperature sensitive liposomes (TTSL) have been used to target tumor cells as a result of relatively high temperature compared to normal body tissues (217,218). TTSLs require a temperature of 42^{o}C to 45^{o}C for effective therapeutic release, which is impossible in *In-vivo* conditions normally. This problem has been solved by the development of low temperature sensitive liposomes (LTSL) which are stable at 37^{o}C and show complete release of therapeutics at 39^{o}C (219,220). Another attempt is the development of new temperature sensitive liposomes (NTSL) (221) to control the release of therapeutic agents maintaining mild external hyperthermia generated by focused ultrasound (222), radiofrequency waves (223) and alternating magnetic field (224,225). Recently, two steps hyperthermia has been reported to enhance antitumor activity of the encapsulated drugs. First hyperthermia (41°C) induced hyperpermeable tumor vasculature which persisted for 8 hours while second hyperthermia (42°C or above) induced drug release from temperature sensitive liposomes (226).

Transferosomes:

These are ultra-deformable and stable advance liposomes which can cross the pores of the stratum corneum. The degree of deformability of transferosomes is directly related to their bioavailability. Transferosomes are prepared from different ratios of phosphatidylcholine and surfactants. Surfactants act as edge-activators and incorporate deformability into transferosomes (Figure 10). By changing the type and ratio of surfactant, one can optimize the vesicle morphology, size and therapeutic loading (227,228). The addition of permeation enhancers into transferosomes further improves therapeutic delivery through the skin (229).

Preparation: Transferosomes are prepared by a film hydration method. Lipids and surfactants are dissolved in an organic solvent. The organic solvent is evaporated using a rotary evaporator to form a lipid film. This film is hydrated with an aqueous buffer and sonicated for 5 minutes to obtain transferosomes (230).

Applications: Transferosomes have been used to deliver macromolecules which are difficult to be transported by other dosage forms. Transulin is transferosomal insulin complex which shows *in vivo* delivery similar to subcutaneous insulin and lacks the problem associated with non-compliance of injections (231). Recent research has shown that corticosteroids might be ideal candidate for transferosomal delivery (232-234).

Different types of next generation liposomes or their combination present exciting solutions for diagnostic as well as therapeutic applications. Table 2 summarizes challenging disease conditions that have been broadly investigated with liposomes based therapy.

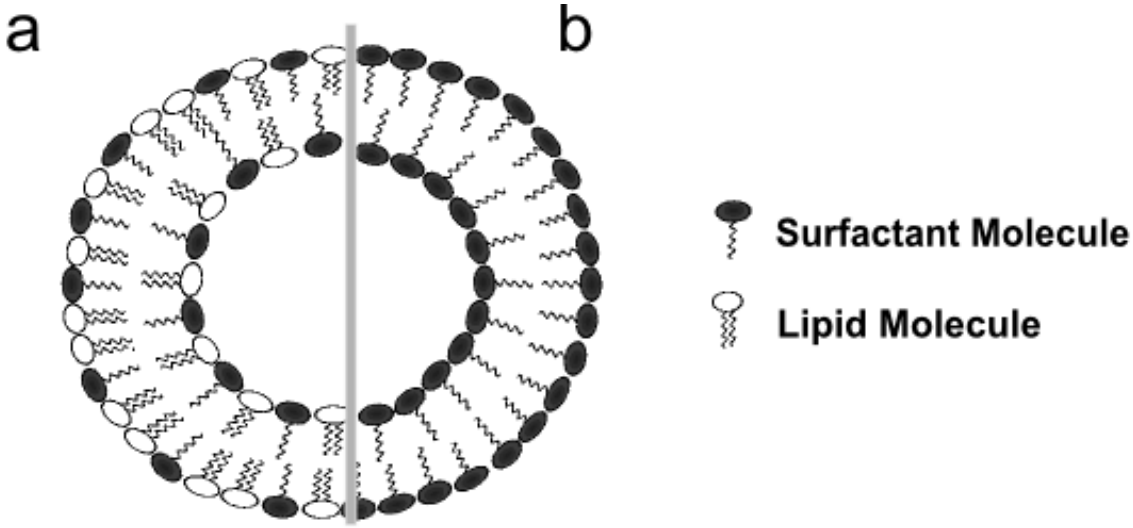

Figure 10. (a) Represents transferosomes formed by mixing surfactants and lipids (b) represents Niosomes containing bilayer of surfactant molecules only.

Table 2. Clinical diseases and the liposomes drug delivery

Therapeutic Application	Type of liposome	Novelty in Drug Delivery
Cancer Chemotherapy	Cochelates	Cation-stabilized liposomes prolong circulation time of anticancer drugs
	Niosomes	Improve stability of encapsulated anticancer drug and final product due to bilayer of stable non-ionic surfactants
	pHsensitive liposome	Release of drugs at tumor sites in acidic enviorments.
	Polymerized Liposomes	Polymerization of lipid bilayer
	Temperature sensitive liposomes	Lipid bilayer destabilizes at elevated temperature of tumors
	Immunoliposomes	Tumor targeted delivery of chemotherapeutic agents via ligends
	Magnetic Liposomes	Drug loaded magnetic liposomes can be localized by external magnetic field to provide clickable drug release
Vaccine	Archeosome	Archaeal bacteria contains ether lipids which are stable and act as self-adjuvants for vaccines
	Cocheltaes	Cations stabilized liposomes provide long circulation time and used to encapsulated protein antigens
	ISCOMs	Quillaja Saponins act as self-adjuvant for vaccines
	Polymerized Liposome	Polymerization of lipid bilayer
	Transferosomes	Increased penetration of vaccines in transcutaneous immunization
	Immunosomes and Virosomes	Virus antigens on the surface provide adjuvant property and help in adapting virus like entrance into cells
	Exosomes	Their free movement in tumor microenvironment provides efficient delivery of anticancer drugs and vaccines to cancer cells.
Gene Therapy	Lipoplexes	Bilayer of positively charged lipids encapsulate and binds negative charged DNA on its surface
	Exosomes	Intrinsic ability to cross biological barriers and enter cells effective delivery of DNA through transfection
Antimicrobials	Niosomes	Improved the stability of encapsulated antimicrobial drugs and final product due to stable bilayer of non-ionic surfactants
	Polymerized Liposome	Polymerization of the lipid bilayer protects the encapsulated drugs from degradation
	Ethosomes	Anti-inflammatory and antifungal drugs for skin and deep tissuesdelivery.
Sensitive and macromolecules	Polymerized Liposome	Polymerization of the lipid bilayer protects sensitive molecules from degradation
	Gas containing liposomes	Encapsulate novel therapeutic gasses
	Proliposomes	Sensitive molecules are stored in dry state to improve stability and withstand storage conditions
	Cubosomes	More resistant to degrading factors and provide oral, parenteral, ocular and topical delivery of sensitive molecules

Topical drug delivery	Transferosomes	Surfactants in lipid bilayer provide flexibility and improve penetration through skin. Increased skin penetration of insulin and used for transcutaneous immunization.
	Ethosomes	Transcutaneous immunization and topical delivery of anti-inflammatory drugs
Diagnostic Techniques	Magnetic liposomes	Paramagnetic agents loaded on liposomes act as contrast agent for MRI
	Immunoliposomes	Ligands such monoclonal antibodies are attached to liposome surface to target cancerous tissues expressing tumor specific receptors. This confines chemotherapeutics release to tumor tissues.
	Gas containing liposomes	Contain gas bubbles that echo ultrasound waves

CONCLUSION

Liposomes have emerged as one of the most studied novel drug delivery system in the last two decades. Liposomes have revolutionized medical research for cancer chemotherapy, gene therapy, vaccines, antimicrobial and diagnostic agents. The US FDA has approved thirteen liposome formulations for clinical use and many other have been tested in clinical trials. Highly potent chemotherapeutic agents encapsulated in stealth liposomes offer long circulation time. Immunoliposomes with various targeting ligands deliver the payload specifically to target tissues and safeguard other normal tissues from toxic effects. Liposome encapsulated drugs have shown much high efficacy as compared to other conventional drug delivery systems. The main advantage of modern liposomes lies in their sustained action, enhanced bioavailability, high cellular uptake and targeted delivery of therapeutic agents. However, industrial scale up of sophisticated laboratory preparation methods is still a challenge to be addressed.

Keywords: liposomes, cancer treatment, drug targeting, micelle systems, drug delivery, clinical applications.

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