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Targeted Multimodal Liposomes for Nano-delivery and Imaging: An Avenger for Drug Resistance and Cancer

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Abstract: Understanding the cellular target structure and thereby proposing the best delivery system to achieve sustained release of drugs has always been a significant area of focus in biomedical research for translational benefits. Specific targeting of the receptors expressed on the target cell represents an effective strategy for increasing the pharmacological efficacy of the administered drug. Liposomes offer enhanced conveyance as a potential carrier of biomacromolecules such as anti-cancer proteins, drugs and siRNA for targeting tumour cell death. Commonly used liposomal constructs for various therapies are Doxil, Myocet, DepoCyt and Abraxanes. However, recent strategy of using multifunctional liposomes for the sustained release of drugs with increased plasma residence time and monoclonal antibody-based targeting of tumours coupled with imaging modalities have attracted enormous scientific attention. The ability of liposomes coated with specific ligands such as Apo-E derived RGD R9 and Tat peptide, to reverse the conceptualisation of drug resistance and cross the blood brain barrier, provides promising future for their use as an efficient drug delivery system. By outlining the recent advancements and innovations in the established concept of liposomal drug delivery, this review will focus on the multifunctional liposomes as an emerging novel lipid based drug delivery system.

Keywords: Liposomes, multidrug resistance, immunoliposomes and gene transfer.

1. INTRODUCTION

Research on lipids has increased rapidly in the past few decades due to significant advancements in biochemistry, metabolomics and systemic biology. Lipids belong to a diverse group of organic molecules that are characterised by their solubility in non-polar solvents and water. These biological entities constitute the cell membrane for two main functions; storage and regulation of gene expression [1]. Storage lipids are the primary component of a fat tissue constituting about 99% of the volume. On the other hand, membrane lipids consist of two non-polar acyl groups and one polar head group [2]. The cell membrane is constituted of lipid bilayer and series of proteins. The mechanistic working of the membrane relies on the role of lipids for its non-permeable nature and proteins for information transduction from the outer environment to internal organelles [3]. Looking back in evolution, the first proposed form of life was able to produce the simple lipid vesicle with phospholipids [4]. In highly evolved eukaryotes, bilayer is made of phospholipids, sphingomyelin, and cholesterol. Within the phospholipid groups, phosphatidyl serine; phosphatidyl choline; phosphatidyl ethanolamine and phosphatidylglycerol determine specific biological functions [5]. Therefore, nanoformulations can be synthesised to mimic these natural constructs and one such example is liposomes. Liposomes are spherical phospholipid bilayer constructs which close upon themselves to form a vesicle of colloidal dimension. This sequesters the hydrophilic end of the phospholipid molecule to face outwards making them water soluble while hydrophobic tails interact with bilayers. This chemical nature of liposomes can be exploited to possess uniform particle size distribution within the range of 20nm-10μm. The physico-chemcial characteristics of liposomes include bilayer phase behaviour, permeability, lamellarity, colloidal nature and charge density. Liposomes are known for their high solubilising nature for various degrees of compounds. In addition to the above mentioned characteristics, they also interact with lipid bilayer of cells [6]. To account for this property, liposomes are used as drug delivery vehicles to carry vaccines [7], enzymes [8], non-enzyme proteins [9], anticancer proteins [10] and immuno-stimulatory molecules [11]. They can be made bio-

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logically functional through the addition of antibodies, aptamers [12] protein receptors, ligands and other biosensor molecules for targeted therapies. Multifunctional liposomes with theragnostic property are in focus for the establishment of safe and efficient tumour therapy.

2. TYPES OF LIPOSOMES

Copolymers are the building blocks of vesicles and have property to self-assemble when dispersed in solvents like water and tetrahydrofuran (THF) [13]. Depending on the phospholipid – water interface, liposomes are categorised into three types. Multilamellar vesicles (MLV) – this is considered to be the simplest construct obtained by mechanical dispersion of dry lipids in water. Small unilamellar vesicles (SUV) are generated by systemically mixing two similar block copolymer [poly (butylenes oxide)-co-poly (ethylene oxide) designated PB_n PE_m in water. Conventionally, SUV are prepared from MLV using sonication procedure at 4°C for about 3-4 weeks or by subjecting them to freeze thaw cycles to induce fusion forming large unilamellar vesicles (LUV) [14]. Recent advances in this field combined the use of lamellae-forming non-ionic block copolymers PB_{10} PE_{10} with micelle-forming $PB_{10}PE_{18}$ in an aqueous solution. The self-assembled polysomes are then passed through filters with small pores to form nanoscaled liposomes. Presence of PE_{18} tail prevents the aggregation of the formed vesicles resulting in stable structures [13]. In order to improve the drug carrying capacity of the liposomes, MLVs are covalently crosslinked with adjacent lipid head groups stacked in concentric circles to form interbilayer-crosslinked multilamellar vesicles (ICMVs). The resulting constructs retain the drug payload and releases it in a controlled manner in serum for over 30 days [15]. In order to boost the immune response, the immuno-stimulatory ligand B7.1 which facilitates T cell proliferation (CD80) [16] or monophosphoryl lipid A, (MPLA) can be used. ICMVs elicit a 10-fold increase in antibody titre when compared to the MLVs. To translate the above strategy in terms of research, Moon JJ *et al.* synthesised ICMVs by using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), anionic 1.2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG) and anionic maleimide-headgroup lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl) butyramide (MPB) in 4:1:5 molar ratio. Mg^{2+} cations were added to cause fusion between vesicles. Polar head groups in concentric circles were linked covalently by dithiothreitol (DTT) to form cation salt bridges between lipid layers [15]. The above nano-carrier can be used for encapsulating TLR agonists, drugs, lipophilic and hydrophilic drugs (Fig. **1**).

3. DELIVERY OF THERAPEUTIC CONSTRUCTS

Two main divergent groups of gene carrier system have been developed. Firstly, viral vectors like adenovirus and retrovirus are widely used for their high transaction efficiency both *in vitro* and *in vivo*. Adenoviruses are known for their high level of gene delivery and expression [17]. Despite their high transfection efficiency, they are not recommended for therapies due to their de-limiting factors such as immunogenicity, random integration into the host chromosome and a limited amount of DNA/siRNA packaging. On the other hand, non-viral vectors offer many advantages. Polycation lipids are used to compact negatively charged DNA molecules to form polyplexes [18, 19]. These polyplexes exhibit efficient interaction with the cell membrane but fail in gaining high transfection efficiency. Though non-viral vectors offer low efficiency of gene transfer, they do not elicit an immune response and can carry a larger amount of DNA [20]. Chimeric vectors have also been constructed combining non-viral vectors and viral components. DNA loaded liposomes are fused with ultraviolet inactivated haemagglutinating virus to form 400-500nm in diameter particles that can efficiently transfer intact oligonucleotides to the nucleus [21]. Liposome based vectors are also employed for gene transfer in mesenchymal stem cells (MSC). MSC holds greater importance in several gene and cell based therapies. Liposomal vectors carrying DNA are utilised to enhance therapeutic efficacy of MSCs by directing the stable fusion of the constructs into genomic DNA. Transfected MSCs were shown to possess higher viabilities and recoveries with their inherent multipotency [22-24]. These collective aspects of non-viral methods attracted researchers to comply on the use of liposomal-based drug delivery.

4. RECENT BREAKTHROUGHS IN CATIONIC AND ANIONIC LIPOSOMES

Liposomes are considered as non-viral methods in gene delivery techniques and also considered to be a therapeutically efficient system in the case of both benign and malignant tumours [25]. Depending on the net charge, they are classified as cationic, anionic, zwitterionic and non-ionic liposomes. Cationic lipids are amphiphilic molecules with one or more fatty acid side chains alkyl or acyl moiety and a linker with hydrophobic amino group. Positive charge on their polar head group aids in their interactions with negatively charged DNA to form lipoplexes. Previously, 3β-[N-[(N', N' – dimethylamino)ethane]-carbamoyl]cholesterol [DC-Chol], N-[1-(2,3-dimyristyloxy)propyl]-N, N-dimethyl-N-(2-hydroxyethyl) ammonium bromide and N, N, N-trimethyl-2-bis[(1-oxo-9-octadecenyl(oxy]-(Z, Z)-1 propanaminium methyl sulfate have been used to develop cationic liposomes [26]. Many cationic compounds have been formulated due to the advent of N-[1-(2, 3-dioleyloxy) propyl] - N, N, N-trimethylammonium chloride [DOTMA] and 1, 2-bis (oleyloxy)-3-(trimethylamino) propane [DOTAP] [27]. Cationic lipids are usually mixed with neutral lipids like dioleoylphosphatidylethanolamine [DOPE] to enhance the stability of liposomal structures at low pH in endosomal compartments [28]. Additionally, stabilizers like poly (L-lysine) and protamine decrease the size of the lipoplex complex and offer protection to the loaded DNA from nuclease activity [29]. Earlier studies were upon the use of DC-chol liposomes, but their use has become very limited because of their tendency to form large DNA/liposome complexes [30]. Recently, dioleolylphosphatidylcholine (DOPC), cholesterol, stearyl amine has gained importance in the field of intranasal delivery [31]. Some of the commercially available cationic liposomes like Lipofectamine 2000 and Oligofectamine significantly increased the efficiency of gene transfer. Though cationic liposomes offer numerous advantages, they are inactivated in serum. Additionally, they are cytotoxic to macrophages and monocyte-like U937 cells but not on

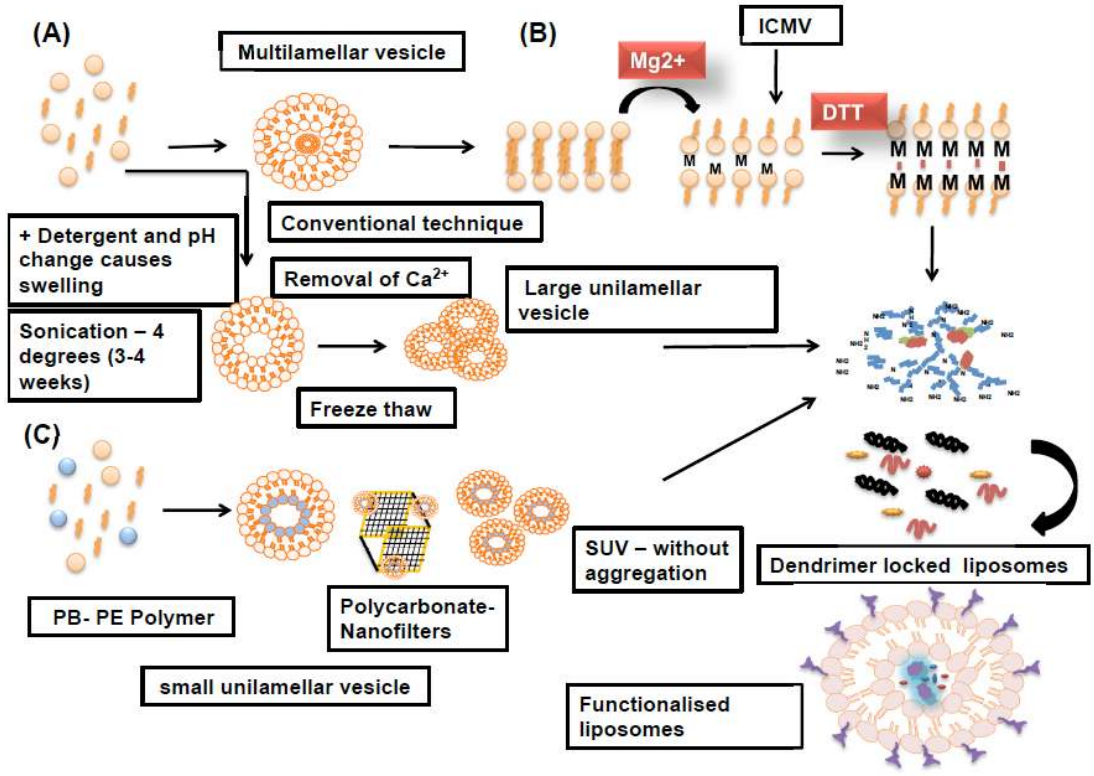

Fig. (1). Preparation of liposomes:

A) Phospholipids are the main constituents of biological membrane in which the extrinsic and intrinsic proteins are embedded. Among the various structure of phospholipid, liquid crystalline phase is the biologically most relevant one. Liposome vehicles are constructed in tune with nature's architecture for delivering the drug specifically. When lipids are suspended in water, they form MLVs with many layers of lipids. Conventionally, SUVs were prepared by sonicating the MLV constructs for about 3-4 weeks or by the addition of detergents. Further freeze thawing and removal of Ca2+ leads to the formation of LUVs.

B) In order to stabilise the reactive head groups of the lipids at mild conditions, ICMVs are constructed from MLVs via covalent crosslinking of fused reactive head groups with DTT. This stable formulation has higher half-life in serum (30days).

C) Due to the advent in polymer technology, uniform SUVs are prepared from PB-PE polymer by subjecting them into polycarbonate nanofilters. All three types of liposomal constructs are used for the encapsulation of drug, siRNA and dendrimers to form functionalized liposomes.

non-phagocytic T lymphocytes. This is due to the toxicity and down regulation of immuno-modulators like nitric oxide (NO) and tumour necrosis factor – α (TNF-α) respectively by cationic lipids and DOPE. As individual components, DOPE and cationic lipids are non-toxic to macrophages, but the addition of DOPE to DOTAP and other cationic lipids increases the toxicity profile. Macrophages non-specifically internalize larger amounts of particles like liposomes due to high phagocytic activity. Hence lower level of toxicity is observed with non-phagocytic cells when compared to peritoneal macrophages. However, incorporation of DNA and other cargo molecules marginally reduced the toxicity of cationic lipids but failed in non-specific down regulation of NO and TNF-α [32]. To overcome this toxicity, anionic liposomes are used. Though DNA compaction with anionic lipids is not so simple due to the repulsive forces between phosphate group of DNA and anionic head groups, divalent cations like Ca^{2+}, Ba^{2+}, Mg^{2+} and Mn^{2+} can be used to facilitate their assembly by negating the mutual repulsion [27]. Among the divalent cations, Ca^{2+} has higher transfection efficiency. It is mediated via accommodation of larger (DNA) charge per unit area in the Ca^{2+} atom. In addition to this, it also enhances the uptake of lipoplexes via endocytosis. The most frequently used anionic lipid is DOPG in combination with DOPE. A study conducted by Patil SD *et al.* showed that though anionic liposomes were able to deliver the DNA cargo with similar efficiency as that of cationic liposomes, (Lipofectamine) the toxicity profile of anionic liposomes were significantly less when compared to cationic liposomes [33]. Despite these encouraging aspects in anionic liposomes, they lack stability when they encounter plasma lipoproteins due to the absence of cholesterol in the construct. With the overall knowledge about anionic liposomes, they can be used for studies involving non-receptor mediated endocytosis. On the other hand, neutral lipids such as DOPE and DOPC have been extensively used for imparting higher transfection efficiency. The possible reason for the efficiency could be due to electrostatic interactions between DNA and neutral lipids at low pH. When used in combination with cationic lipid DOTAP, salt bridges are formed between the positive charged head groups of cationic lipid and a phosphate group of DOPE groups. This association enhances the affinity of DOPE to negatively charged DNA thus helping in the package of DNA helices of interest [34, 35]. In spite of

various debates on the use of cationic liposomes, they are considered to be the most efficient delivery system.

5. ENCAPSULATION OF DRUGS INTO LIPOSOMES

Liposomes represent a highly flexible group of delivery vehicle amenable for modification and encapsulation. A vast array of therapeutic compounds can be encapsulated within the liposomes. Maximum encapsulation is desired for its use as a delivery vehicle. The lipid bilayer is almost 8 - 10 nm thick and guards the cell against water, gases, sugars and proteins. Hence, exogenous hydrophilic substances have to be encapsulated inside the liposome for easy transport across the cell membrane. A very simple method of encapsulation is thin lipid film method. Lipids with random orientation are dissolved in water to form small degree of bilayer. On the addition of water with drug/antibiotics, lipid bilayers swell to form invaginations for efficient loading. These structures form the basis of MLVs with passive encapsulation of water-soluble drugs [36]. Another method to encapsulate the drug is through freeze thawing technique. During the formation of LUVs from SUVs, distance between the lipid bilayer increases allowing the drug to penetrate via the transient pores. A significant setback relates to the limited encapsulation efficiency with only 15-20% entrapment into the liposomal core [37]. Another technique previously used was ethanol injection. In this method lipid ethanol solution is forcefully injected into the drug solution to from vesicles with diameter dependent on the concentration of the lipid. Untrapped drug or protein is separated by a crossflow-ultradiafilteration unit. Various factors including the rate at which lipid encounters the drug, pressure, ethanol concentration and low encapsulation profile have diverted the researchers to find an alternative high throughput technique to encapsulate the drug efficiently [38]. Reverse phase evaporation technique and pH gradient method are highly preferable method. The pH gradient loading of drug includes the use of basic solution like HEPES/NaCl buffer (pH7.5) to replace intra-liposomal solution of 300 mM citrate buffer or 300 mM sucrose/20 mM/15 mM EDTA for $MnSO_4$ and $MnCl_2$ (pH3.5) [39]. The ammonium sulfate method is used for encapsulating anthracycline drugs. In this case, liposomes are prepared in ammonium sulfate salt with pH around 5.5 and subsequently subjected for ammonium sulfate exchange through counter buffers with pH 7.4 to cause pH gradient. For every molecule of ammonia that is exchanged with its counter buffer, one proton is left to create pH gradient. This mechanism of salting out causes the gelation and flocculation of the encapsulating drug thus improving its efficiency [40]. Topotecan (TPT), a topoisomerase I inhibitor known for its use in cancer therapy has been successfully encapsulated within liposomes with 90% efficiency [41]. Encapsulation efficiency also depends on the hydrophilic and hydrophobic nature of the drug. Hydrophilic drugs can be encapsulated within the aqueous component and lipophilic drugs within the bilayer. One of the favoured means of trapping drugs within liposomes is the microencapsulation vesicle technique. This strategy utilises phospholipids dissolved in organic solvent (chloroform) and water to form water/oil emulsion. Organic solvent is evaporated to get liposomal suspension. Drugs like flurbiprofen, ibuprofen, ketoprofen and amitriptyline are added during the oil phase whereas, 5-fluorouracil and diclofenac sodium can be added in the water phase [42]. A versatile approach for loading drug that are fundamental in nature is through the ionophore-pH gradient method. Addition of ionophores such as nigericin or A23187 creates transmembrane gradients of Mg2+ and K+ for the transport of the drug into the liposomes. Ionophore addition shows strong dependence of retention on the drug to lipid ratio or intra-vesicular drug concentration improving the loading capacity. Biodistribution of three commonly used anticancer drugs - vincristine, vinblastine and vinorelbine had shown to increase when delivered in a liposomal carrier prepared using ionophore technique [43].

6. LIPOSOMES: AN AVENGER IN DRUG DELIVERY

Liposomes have a decade long clinical presence as a nano drug delivery vehicle. Their hybrid constructs present considerable opportunities for combinatorial therapeutic and imaging modalities. The versatile nature of liposomes lies in their structural integrity which can efficiently carry drug into the cell. Currently used liposomal constructs for various therapies include Doxil/Caelyx [44-46], Myocet [47], DepoCyt [48], DaunoXome [49], ONCO-TCS [50], GemLip [51], Abraxane [52, 53] and Navelbine IV [54]. Other common drugs like curcumin [55], resveratrol [56] and oxiplatin [57] are also used in combination with liposomes. To obtain the maximum benefit from liposomal delivery, certain modifications and covalent conjugations are needed. Polyethylene glycol moiety; construction of liposomes for triggered release and addition of antibodies for target specific delivery has been discussed in this review.

6.1. PEGylated Liposomes

Liposomes reach the target site of target either by active or passive targeting. Active targeting exploits the overexpression of surface receptors in the cancer cells. By coupling the liposomes with targeting ligands, particulate drugs can be delivered on to the local site. Passive targeting focuses on the use of non-targeting nanoparticles that have the ability to accumulate in the interstitial spaces of tumour cells mainly through enhanced permeability and retention effect [EPR]. Passive or physiologic targeting of tumours depends on the long circulating liposomes which have the ability to dodge and resist the uptake by reticuloendothelial cells [58]. When liposomes are delivered intravenously, they interact with both high-density lipoproteins and opsonins. Some of the opsonising proteins like complement; immunoglobulins enhance the interaction of liposomes with phagocytic cells like macrophages and dendritic cells [59]. The rate of clearance from the blood stream depends on the ability of opsonins to bind to the liposomes. It was reported that opsonins bind to fluid liposomes more avidly than rigid lipid bilayers [6]. Additionally, the clearance also depends on size and charge of the liposomes [60]. Mode of delivery plays a vital role in biodistribution of liposomes. Intravenous delivery results in the accumulation of liposomes in liver, spleen and bone marrow. When administered subcutaneously or intramuscularly only a small proportion of liposomes accumulated in the above sites. Most of the liposomes were arrested at the site of injection and attacked by immune cells [6]. To circumvent this and to increase the half-life of liposomes, polyethylene glycol is used. This PEGlyated or STEALTH

liposomes circulate in the blood stream as stable constructs which can extravasate in the target tissue. They behave as biologically inert molecules with slow release of entrapped drug thereby increasing its bioavailability [60]. The main thrust to develop PEGylated liposomes is to increase the bioavailability of the encapsulated drug to the target tissue after local administration. The use of PEGylated liposomes also increases the pharmacokinetics of the therapeutic agent in various cancer models with target accumulation in mitochondria and caveolae [61]. Unlike other approaches, PEGylation does not involve the covalent attachment of stabilising agents in the carrier vehicle. PEGylated liposomal doxorubicin (DOXIL^{R}) is approved worldwide for treating advanced breast cancer, ovarian cancer, AIDS related Kaposi's sarcoma, hepatocellular carcinoma and multiple myeloma. In the U.S., PEGylated liposomal doxorubicin is used for the treatment of metastatic ovarian cancer that is refractive to conventional therapies such as paclitaxel- and platinum-based chemotherapies. Combinatorial treatment with vincristine and reduced dose of dexamethasone had encouraging results in a phase II study. Conventionally, doxorubicin is administered at a concentration of 60-90mg/m^2 every 3 weeks, while liposomal delivery of the same drug accounts for 50mg/m^2 administration every 4 weeks [62, 63]. Thus PEGylated liposomes serve as a better vehicle of particulate drugs *in vitro* for improved quality of life in the ovarian cancer patients. An additional benefit of PEGylated liposomes is that, they are used to treat inherited bleeding disorder haemophilia. Prophylactic treatments for haemophilia have always been hampered by poor patient compliance. In addition to this, half-life of free FVIII [precursor for factor X] in serum is only about 10-12 hours. A long lasting active form of FVIII would provide protection against bleeding. Therefore, PEGylated liposome composed of 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) and 1, 2 distearoyl-sn-glycero-3-phosphatidylethanolamine-N-methoxy (polyethyleneglycol)-2000 (DSPE-PEG2000) is used. PEG formulations of FVIII and FVIIa increased the survival rate and decreased the clotting time in haemophilia patients [64].

6.2. Stimuli Sensitive Liposomes

A strategy to construct liposomes to sense the external stimulus for effective drug release intrinsically may improve the biodistribution and intracellular fate of the drug. Stimulus such as pH, temperature and redox microenvironment can trigger the release of drug from liposomal constructs. Precisely, acidic environment in solid tumours represents pH value of about 6.5 to the administered drug. The pH in cellular compartments like endosome and lysosomal vesicles are around 5.5 and 4.5 respectively imposing lower values than cytosolic pH. To surpass the lysosomal degradation, pH sensitive liposomal constructs are used to deliver the cargo into cytosolic environment. As a proof of concept, liposomal formulation is destabilised in the endosomal compartment promoting the release of contents thus, preventing the further degradation at lysosomal levels. The mechanism of pH triggered release depends on four factors; I) neutralisation of negative lipids in bilayer by protonation forming hexagonal phase, II) protonation of negative polymers and eventual absorption to the bilayer leading to destabilisation of the construct by the formation of pore and fusion, III) hydrolysis of lipid bilayer and IV) ionisation of neutral lipids to their positive and surface active conjugate acids [65, 66]. The first generation of pH sensitive liposomes were composed of phosphatidyl choline and N-palmitoyl homocysteine [67]. One of the interesting aspects of pH sensitive liposomes is their ability to mimic *Listeria monocytogenes* pathology. The bacterium utilises listeriolysin O (LLO) to escape the degradation from endosome and lysosomal pathway facilitating the entry into the cytosol. A targeting strategy based on the use of LLO along with desired drug in a liposomal formulation enhanced the protection of drug from plasma by increasing the pharmacokinetics of the drug administered. Gelonin, a type I plant toxin is known for its antitumour activity and acts by inactivating the 28S ribosomal subunit. They belong to type I family of toxins with characteristic absence of B chain which can facilitate their entry into the cytosol. Hence, their bioavailability as a drug was of a major concern. Co-encapsulation of LLO with gelonin in a pH sensitive liposome effectively delivered the toxin into the cytosol of the target cell [68]. On the other hand, temperature can also be used in initiating drug release from the liposome constructs. These thermosensitive liposomes undergo temperature-dependent phase transitions from gel to the liquid phase and become permeable at elevated temperatures, resulting in gradual release of the desired drug to the target cell [69]. Heating the tumour tissue to temperatures of around 43°C [mild hyperthermia (HT)] has been in practice for efficacious treatment in combination with chemotherapy. HT increases vascular permeation in solid tumours for accumulation of liposomes in the target area for controlled release of the cargo. To increase the half-life and membrane permeability, PEG and lysolipids or oligoglycerol are used. A lysolipid formulation "Thermodox" is currently used in combination with HT in patients with loco-regional breast carcinoma of the chest wall, and with radio frequency ablation in cases of metastatic liver cancer. First HT is given to improve tumour vascular permeability which can last for four hours, and upon the considerable accumulation of liposomes second HT is administered to trigger drug release from tumour localised liposomes [69, 70]. Doxorubicin has been widely encapsulated in thermosensitive liposomes. Doxorubicin encapsulated in lysolecithin containing thermosensitive liposomes can release the contents within 20 seconds at 42°C thereby increasing the bioavailability of the drug on the tumour site [71]. To improve the imaging capabilities of thermosensitive liposomes, dextran coated iron oxide nanoparticles are used. Iron oxide can serve as a heating source in an alternative magnetic field. This multivalent nanovehicle has the potential to combine targeted delivery and diagnosis with controlled drug release property [72]. Magnetic forced driven transfection has been used as a new modality for delivering siRNA into target cells. Magnetofection using N, N'-dioleylglutamide (DG) with cationic lipids and iron oxide nanoparticles substantially increases the cellular delivery of siRNA when compared to the commercially available Lipofectamine 2000 [73]. Introduction of plasmid DNA for IL-10 gene expression in the magentoliposomes aids the inhibition of plasminogen activator-1 responsible for vascular inflammation in cancer and atherosclerosis. This strategy has found significant application in transfecting highly sensitive HUVEC cells [74].

6.3. Immunoliposomes

Immunoliposomes also known as "guided missiles" can actively target the tissues through directed guidance provided by grafted molecules on their surface. This molecular fingerprint can either be an antibody itself or an antibody fragment. In order to compromise RES system, liposomes are engrafted with hydrophilic [PEG] or glycolipids [ganglioside: GM1]. The tagged molecules may increase the circulation time since they surpass hydrophobic interactions with immune cells. Taking advantage of PEGylated liposomes, tumours can be targeted via conjugation with antitumour antibodies. Tumours over-express antigens on their cell surface even in primary sites or during metastasis. Some of the antigens are shed in the blood stream and become soluble posing difficulties in immunotherapies. Despite this difficulty, monoclonal antibodies [mAbs] have been successfully used to develop immunoliposomes for targeted therapy. One such example of mAb used in immunoliposomes is herceptin. Herceptin is a therapeutic antibody used for treating metastatic breast cancer by specifically targeting HER-2/neu protein. Engineered antibodies or chimeric mAbs do not elicit host immune response when Fc fragment of the Ab is removed. Use of Abs enhances the cellular internalisation and efficiency of drug delivery presumably by enhancing the tumour cells for endocytosis. Use of immunoliposomes constructed with the Fab portion of human anti-HER-2 fragment and doxorubicin, resulted in targeted distribution within the tumour bypassing passive accumulation in the interstitial space of the tumour cells [75]. Of late, research on the cell adhesion molecule for targeted therapy has gained enormous attention in the field of tumour immunology. Intercellular adhesion molecule (ICAM-1), a glycoprotein of the immunoglobulin superfamily plays an important role in extravasation of tumour and in the inflammatory process mediating leukocyte binding to endothelium [76]. We carried out the elicitation of CTLs through using gene transfer of xenogenic ICAM-1 into EL-4 lymphoma. In combination with 5, 6-dimethylxanthenone-4-acetic acid (DMXAA), ICAM-1 therapy was capable of suppressing the tumour size with increased cytotoxic T lymphocytes (CTL) and natural killer (NK) cell activation [77]. This study is of importance since the administered ICAM-1 gene-mediated the antitumour immunity by overruling tumour mediated immunosuppression [78]. The strategy of delivering ICAM-1 gene through immunoliposomes for targeting endothelial cells in tumour could open a new window in future therapeutics. In many inflammatory disorders such as rheumatoid arthritis, ICAM-1 remains the main target due to elicitation by cytokines such as TNF-α and interleukin-1 (IL-1). Increased expression of ICAM-1 is found in the synovial tissue due to the migration of leukocytes. Therefore, potential targeting mechanism by immunoliposomes to deliver bioactive compounds to the site of inflammation has been considered in several studies. A very recent study on the (1, 2-distearoyl-sn-glycero-2-phosphocholine) [DSPC] and 1-[8-[4(p-maleimidophenyl) butaroylamino]-3, 6-dioxaloctyl]-2,3-distearyl-glyceryl-dl-ether [DSGE] lipids in conjugation with thiolated anti-ICAM (CD54) mAb was able to deliver the drug directly into the inflammatory site [77]. However, endocytic uptake of multivalent anti-ICAM conjugates and anti - ICAM coated nanoparticles were reported emphasising their utility in the successful delivery system [80]. In addition to ICAM-1, vascular cell adhesion factor (VCAM-1) is also over-expressed in tumour vessels. Anti VCAM-1 immunoliposomes display specific targeting of endothelial cells under static conditions with increased accumulation in target tumour vessels within 30 minutes to 24 hours. Very few anti VCAM-1 immunoliposomes migrate to other organs and are co-localised with macrophages. Immunoliposomes were prepared from soy PC/cholesterol/cyanur-PEG2000/DiO with trace amounts of cholesteryloleylether with M/K-271 mAb for VCAM (Fig. **2**). Monoclonal antibodies have been tagged with the above mentioned liposomes with special molecule cyanour-PEG2000-PE for conjugating the mAb to terminal ends of PEG [81]. With the advent of mAb against a range of angiogenic and cellular markers, this targeting strategy can aid in developing complete antitumour formulations. Expression of other biomarkers such as CD74 and glycoproteins in tumour microenvironment is also looked upon in order to achieve targeted therapy. CD74 is a type II transmembrane protein expressed on B cells and has recently been regarded as a target for antibody-mediated therapy. In relation to chronic lymphocytic leukemia, (CLL) CD74 is up-regulated with enhanced NF-kB and B cell proliferation. Milatuzumab drug when conjugated with cross-linking antibody against CD74 induced the cytotoxicity *in vitro*. Furthermore, incorporating milatuzumab in liposomes increased the drug bioavailability and cytotoxicity when compared to the free drug indicating that targeting through CD74 enhanced the activity of the drug on lymphoma cells [82]. Glycoproteins expressed on the tumour cells are a potential diagnostic marker as well as a target in cancer therapy. Over expression of mucin [MUC-4], a major cell surface glycoprotein in highly metastatic models of adenocarcinoma is due to up-regulation of retinoic acid via TGF-β2. Over- expression and cell altering properties of MUC-4 makes it a prognostic marker for targeting via gemcitabine loaded immunoliposomes with anti-MUC4 mAb (8G7). Lipid formulations used for immunoliposome targeting MUC-4 include DSPC, DOTAP, cholesterol, DSPE-PEG2000, DOPE-PEG2000-biotin. In order to increase the affinity of antibody to liposome, biotin conjugated mAb is added to neutravidin bound liposome. This strategy holds promise for cells expressing mucin glycoprotein during all stages of development [83]. Inadequate oxygen supply to the tumour tissues results in hypoxic microenvironment rendering them refractive to chemotherapeutic drugs. Activation of downstream signalling pathways by hypoxia correlates with increased tumourigenesis in a variety of organs. Mitogen-activated protein kinase [MAPK], phosphoinositide 3 – kinase [P13K] and beta-catenin pathways are activated by Ron receptor and hypoxia inducible factor – 1 (HIF-1) resulting in tumour progression. Humanised antibody against RON generated with F(ab)2 fragment and conjugated with PEGylated doxorubicin loaded immunoliposomes are commonly used. Increased cytotoxic efficacy of anti-RON-DOX-immunoliposome [Zt/g4 and Zt/c1] is attributed to antibody mediated RON activation, followed by endocytosis of RON-immunoliposome [84]. For imaging purposes, both gadolinium (MRI contrast agent) and quantum dots can be incorporated in the immunoliposomal constructs to facilitate multimodal targeting system through liposomes.

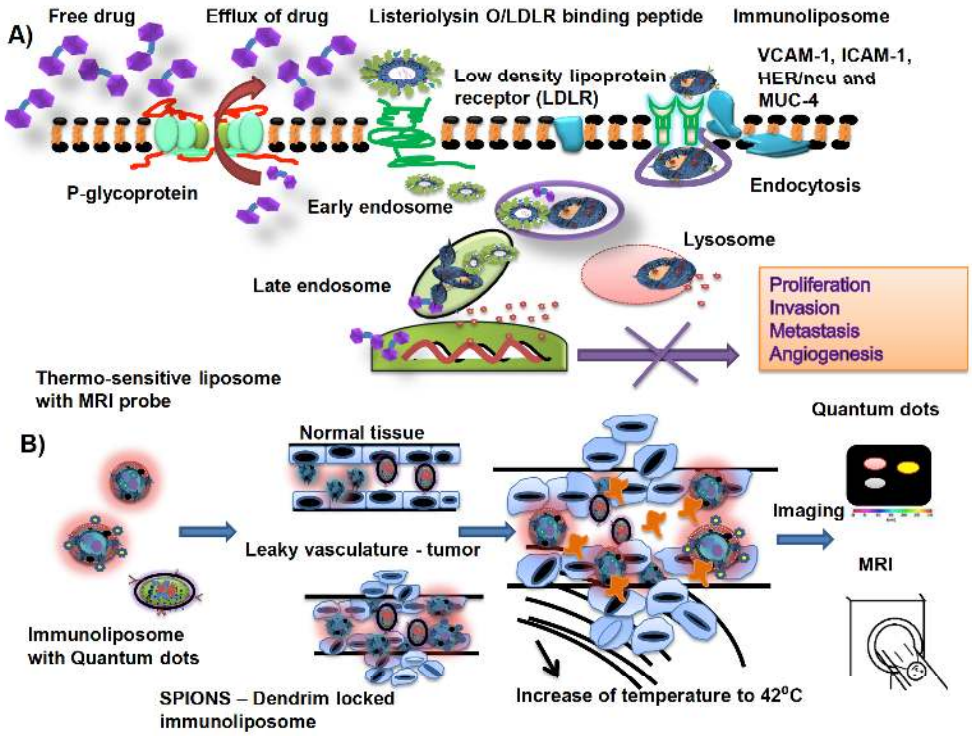

Fig. (2). Multimodal capabilities of liposomes

A) Tumours can evolve resistance to powerful candidate of drugs over a time due to the drug efflux mechanism. P-gp expression forms the baseline for this uncontrolled mechanism. Most of the administered drugs are pumped out by energy-dependent transporters eventually insensitivity to anticancer drugs. Since tumours cells express LDLR, liposomes are coupled to the listeriolysin O/ lipoproteins for efficient drug delivery preventing the entry via transporters for drug resistance. Upon endocytosis, they directly release the cargo into the cytosol escaping lysosomal degradation. Immunoliposomes constructed against VCAM-1/ICAM-1/HER/neu and MUC- 4 antigens have greater specificity with the related tumour. Targeted delivery is achieved through this guided missile which leads to decreased metastasis, proliferation, metastasis and angiogenesis.

B) Theragnostic liposomes have attracted many researchers to comply on this strategy. Stimuli sensitive liposomes with gadolinium (MRI contrast agent) immunoliposomes with quantum dots and SPIONS based dendrimers locked immunoliposomes are used for better imaging strategy. Leaky vasculature in tumour milieu is utilised for the accumulation and entry of these nanoparticles. Local hyperthermia (42-43°C) results in the release of drug from the nanoparticles. Bio distribution of the drug can be monitored using MRI or quantum dot imaging systems.

7. LIPOSOMES FOR REVERTING DRUG RESISTANCE

Although there have been remarkable breakthroughs in cancer therapy like chemotherapy and targeted drug delivery, several impediments such as drug transport, detoxification of glutathione conjugation, topoisomerases, DNA repair, Ras mutation and dysfunction in cell cycle - lead to drug resistance in several tumour models. A well characterised biomarker involved in multidrug resistance (MDR) is P-glycoprotein (P-gp) belonging to ATP-binding cassette i.e. ABC-transporter family [85]. The most frequently observed resistance for doxorubicin in cancer cells is due to the active efflux of the drug through P-gp. Use of P-gp inhibitors like verapamil, cyclosporine A and PSC 833 have limiting factors such as poor specificity and high toxicity. In order to circumvent the drug resistance, liposomal formulations are preferred since the free component of the drug is not available to cancer cells for up-regulating P-gp levels. When doxorubicin is encapsulated in an anionic liposomal construct [Lipodox], it interacts with one of the drug-binding sites of P-gp and inhibits the efflux activity [86]. Drug resistance is also correlated to the higher content of cholesterol in the plasma membrane and increased expression of low-density lipoprotein receptor (LDLR). The candidate biomarker LDLR is utilised in targeted delivery of drug through liposomal formulations. Drug-loaded liposomes with recombinant LDLR binding peptide enhanced the cellular uptake of the nanoparticles through LDLR-mediated endocytosis circumventing P-gp mediated uptake. The efficacy of the above strategy can be enhanced by the use of simvastatin, which up-regulates LDLR levels and suppresses P-gp activity. Simvastatin reduces the endogenous level of cholesterol synthesis in drug resistant cell lines to impair the ATPase pump function [89]. Neutral lipids such as PC and PE are P-gp substrates that compete with drugs for P-gp binding. PC/PE constituent liposomes increase the cellular uptake with enhanced cytotoxicity [84]. A second widely-used liposomal formulation for MDR condition is Daunoxome (DNX), which is a combination of daunorubicin (DNR) with distearoylphosphatidylcholine and cholesterol. Both DNX and free DNR were effective in non-MDR cases of tumour

Table 1. Commercial and Conventional Liposomes Used in Drug Delivery

Liposomes	Trade Name	Assignee	Cancer Therapy	Nanoparticle	*In vitro/ In vivo*	References
Doxorubicin HCL liposome	Doxil/Caelyx	Alza Corporation	Ovarian cancer Kaposi's sarcoma; Activation of complement; liver cancer	DMPC, DMPG, EPC - PEGylated liposome; RGDF peptide coupled to liposome; Liposome/SiO2 /Au	SMMC-7721	[38-40]
Doxorubicin	Myocet	Sopherion therapeutics	B cell non-Hodgkin lymphoma	Egg phophatidylcholine/cholesterol	Patients	[41]
Cytarabine liposome	DepoCyt	Enzon Pharmaceuticals	neoplasticmeningitis	Cholesterol/triolein/dOPC/DPPG	Patients	[42]
Liposomal daunorubicin	Dauno Xome	Gilead Science	Relapsed meningeal acute myeloid leukemia	DOPC/Cholesterol	Patients	[43]
Vincristine	ONCO-TCS	INEX	Non-Hodgkin's lymphoma	DOPC/cholesterol	Patients	[44]
Gemcitabine	GemLip	-	Pancreatic cancer	Hydrogenated egg PC/cholesterol	BxPC-3 and PSN-1	[45]
Paclitaxel	Abraxane	Abraxis Bioscience	Breast cancer and ovarian cancer – multidrug resistance	Albumin; liposome – EPC, CHOL, DOTAP	SKOV- 3; SKOV-3TR	[46, 47]
Vinorelbine	Navelbine IV	Pierre Fabre Medicament	Squamous carcinoma, non-small cell lung cancer and advanced breast cancer	Temperature sensitive liposome (DPPC, MPPC, DSPE-PEG2000, EPC, CHOL)	A431	[48]
Curcumin	-	-	Colorectal adenocarcinoma, hepatocellular carcinoma, lung carcinoma and cervical cancer	DOPC and DLPC - PEGlyated	CT26, HT 29, HepG2 A549 and HeLa	[49]
Resveratrol	-	-	Lung cancer	DQA-PEG2000-DSPE	A549 and drug resistant cell A549/cDDP	[50]
Oxaliplatin	-	-	Colorectal carcinoma	PEG- liposome	SW 480	[51]

but, DNX was found to be more effective in a MDR variant tumour condition emphasising the escape of the drug from pump action of P-gp [89]. For enhanced target specificity, arginine-glycine-aspartic acid peptide (RGD peptide) modified cationic liposomes represent an ideal vehicle. Sequential tailoring of doxorubicin and siRNA against P-gp with RGD peptide results in improved cytotoxicity with reversal of drug resistance [90]. Another crucial biomarker expressed in tumour cells is "transferrin (Tf) receptors" Tf is a non-heme iron binding glycoprotein with high affinity for Tf receptors. To surpass P-gp mediated intake of the drug, liposomal carriers targeting Tf receptor-mediated endocytosis will feature the reversing of drug resistance mechanism. The importance of Tf receptors in targeted drug delivery is due to their high turnover rate in tumour cells for iron metabolism. A study by Kobayashi T *et al.* emphasises on the use of egg-PC/cholesterol

Table 2. Targeted Liposomes for Tumour Therapy

S.NO	Type of Liposome	Composition of Lipids	Therapeutic Protein/ligand	Treatment/Cell Lines	References
1.	Immunoliposome	DSPC and DSGE	Anti-ICAM mAb	HUVEC	[73]
2.	PEG- Immunoliposome	Soy PC/cholesterol/cyanur-PEG2000/DiO and cholestery-loleylether	M/K-271 mAb for VCAM	Human Colo 677 xenograft tumours	[75]
3	PEG- Immunoliposome	HSPC:chol:mPEG2000-DSPE:Mal-PEG	Milatuzumab	Chronic lymphocytic leukemia	[76]
4.	Immunoliposome	DSPC, DOTAP, cholesterol, DSPE-PEG2000 and DOPE-PEG2000	Anti-MUC4 mAb	Glioblastoma [U-87MG]; pancreatic adenocarci-noma [HPAF-II and Ca-pan-1] and pancreatic ductal carcinoma [PANC-1].	[77]
5.	Immunoliposome	HSPC:chol:mPEG2000-DSPE	Anti-RON mAb – Zt/c1 and Zt/g4.	T-47D, MCF-7, SW620, SW837, HCC1937	[78]
6.	PEGlyated	DSPE-PEG2000-PC	FVIII and FVIIa	Haemophilia	[58]
7.	PEGylated	PEGylated liposomal doxoru-bicin	Cyclophosphamide and trastuzumab	Pilot study on humans for efficacy and cardiac safety	
8.	pH sensitive	PE and CHEMS	Gelonin/LLO	Murine melanoma	[62]
9.	PEGylated-Thermosensitive	PAA - lysolecithin	Doxorubicin	Squamous carcinoma	[65]
10.	Thermosensitive – iron oxide	DPPC and DSPC	Carboxyfluorescein	-	
11.	Magnetic-lipoplexes	BPEI-SPION	pDNA for IL-10	HUVEC and prostate cancer	[68]

or hydrogenated egg PC/cholesterol covalently coupled with Tf for the delivery of doxorubicin. Tf receptor targeted cells showed substantial uptake of liposomes with the reversal of drug efflux by P-gp [91]. Solid tumours that remain robust for various chemotherapeutics can also be targeted via folate receptors. Folate receptors can be tethered via folate-hapten conjugates coupled with methotrexate for enhancing the immunity against tumour. A commonly used copolymer for folate targeting is poly (lactide-co-glycolide)-D-a-tocopheryl polyethylene glycol succinate and D-alpha-tocopheryl polyethylene glycol succinate – COOH. Yet another interesting approach for tumour targeting circumventing drug resistance is use of cell surface glycans expressed on the plasma membrane. Lectins represent the second generation of bioadhesive molecules capable of recognising cell mucosal surface receptors. They bind specifically to the carbohydrate moiety expressed in the tumour environment [92]. A recent study on statin (3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor) reduces the efflux activity in Pgp/ABCB1 (ATP-binding cassette) and BCRP/ABCG2 (breast cancer resistance protein) transporters and increases the expression of LDLR in glioblastoma. Statin, in combination with LDL receptor targeted liposome encapsulating doxorubicin was effective in delivering the drug which is otherwise non-permeable and does not cross the blood brain barrier. This nanoformulation elicited nitric oxide synthesis by statins and decreased the activity of MDR transporters [93]. Other biomarkers such as vascular endothelial growth factor receptor, integrin receptor, cell adhesion molecule receptor and prostate specific membrane antigen can be employed for targeted delivery [94]. To combine the tumour sensitisation and drug resistance; targeting of P-gp and hypoxia in solid tumour is an essential factor. Administration of anti-hypoxia inducible factor (anti-HIF) compounds and toll-like receptor agonists which regulates HIF signalling can be efficiently delivered through liposomes through Tf conjugation (unpublished data by our group) [95, 96].

8. LIPOSOMES FOR ENHANCEMENT OF IMMUNITY

As the roles of both antigen-presenting cells and mononuclear phagocytic system (MPS) have been well studied, strategy to target these cell types holds importance therapeutically. The initial impact of inflammation in diseases including asthma, atherosclerosis, cancer and pathogenic diseases such as human immunodeficiency virus (HIV) is targeted via MPS. Though targeting MPS poses difficulties, they represent a highly attractive group for improved therapeutic ef-

fect. Liposomes have been widely investigated as a delivery system for phagocyte targeted therapies due to their low immunogenicity, biocompatibility, cell specificity and drug protection capabilities. Upon infection, the host responds by branching into two defence mechanism: humoral and cellular immune response. Antibodies are produced against the pathogen to neutralise them and T cells are activated for presenting the antigen to major histocompatibility complex (MHC) groups. The very basic immunology defines the presentation of extracellular antigens via MHC class II to $CD4^+$T cells, whereas intracellular antigens are processed by MHC class I via $CD8^+$T cells. For generating antitumour response, cytotoxic T lymphocytes (CTL) play a crucial role. To induce CTL responses, antigen must be processed by MHC class I pathway in APCs via cross-presentation. Our study on co-stimulatory molecules like B7-H3, B7-1 and ICAM-1 showed that they stimulated antitumour activity via $CD8^+$Tcells accompanied by CTL activity [78, 97-99]. Conjugation of antigens in liposomes induces effective antiviral $CD8^+$T cell responses against major viruses such as HIV, hepatitis C virus (HCV) and SARS coronavirus. An alternative to attenuated, heat inactivated, or subunit vaccines are the use of immunodominant CTL epitope, which is more effective to elicit $CD8^+$T cell response against these viruses. Surface coupled CTL epitopes on liposomes along with CpG, a ligand of TLR 9 induced the proliferation of $CD8^+$T cells [100]. Taken together, surface linked liposomal antigens can be applicable for CTL based vaccines against viruses that evade humoral immunity by altering their surface proteins and robust cancer cells. In the research point of view, liposomes can also be used to create macrophage depleted organ or tissue in an animal by employing clodronate liposomes. At the therapeutic level, application of the same is effective in autoimmune diseases, neurological disorders and in gene therapy. Clodronate (dichloromethylene diphosphonate) is a first generation anti-osteoporotic drug recently used in liposome mediated macrophage suicidal approach. Intravenous and subcutaneous injection of clodronate liposome depletes kupffer cells (largest population of macrophages in the body) and macrophages in draining lymph nodes [101]. Atopic keratoconjuctivitis (AKC) is a severe case of ocular blinding caused due to homing of immune reactive cells. Macrophage infiltration in conjunctiva responsible for AKC was efficiently suppressed by systemic (intravenous) administration of clodronate liposomes [102]. Thus, liposomes can also be used to create localised immunosuppression and tolerance induction.

9. CONCLUSION

One of the most prolific areas in the study of drug delivery is focused on liposomes, due to their inherent ability to harness the cells to provide specific targeting and enhanced distribution of the drug. First generation liposomes enabled easy modifications in their structural skeleton yet did not alter the biological properties of the cell upon intravenous administration (which is the most widely used route in medical applications). Therefore, optimistic goals of using PEGylated, stimuli sensitive and immunoliposomes yielded very encouraging results both *in vitro* and *in vivo* in many tumour models. Liposomes can also be used to deliver drugs into the lung through liposome aerosol. Oral applications of liposomes are at an infacy and rather limited because of the liposomicidal environment in stomach and the duodenum.

FUTURE PERSPECTIVES

Despite major advances in drug delivery, liposomes have been a preferred vehicle due to controlled release of poorly soluble anticancer drugs and many bio-macromolecules. For effective delivery of the desired targets, new strategies are followed like synthesis of asymmetric liposomal nanoformulations using the inverted emulsion technique. Cationic lipids form the core so that the liposomes can accommodate larger negatively charged cargoes (siRNA/miRNA/aptamers/locked nucleic acid). Catioinc liposome DOTAP can be used to deliver siRNA against cathepsin S to hematopoietic stem cells aiding in their differentiation into dendritic cells for enhancing immunity. Liposomal delivery of microRNA-7 in epidermal growth factor receptor over expressing lung cancer cells can be used to decrease the chemotherapeutic resistance to tyrosine kinase inhibitors significantly. Aptamer conjugated cisplatin-encapsulated multifunctional liposomes enabled cancer cell specific targeting and also increased the plasma residence time of aptamers. Use of neutral or anionic lipid nanoformulation on the other hand helps in escaping MPS that readily eliminates positively charged particles in the body. When these stable nanoformulations are administered into the system, cancer cells internalize them via endocytic pathways after enhanced permeability and retention (EPR) effect. To circumvent the lysosomal entrapment, hemolysin containing liposomes or pH sensitive lipids (cholesteryl hemisuccinate – CHEMS) can be employed. The former escapes the lysosome by hemolysin's lipid bilayer poration and disruption capabilities and the later becomes unstable at low pH. Hence the drug can be specifically given to the cytosol or nucleus. Further use of antibody conjugation to the above construct will enable targeted therapy with lowering multidrug resistance in cancers.

EXECUTIVE SUMMARY

- Liposomes are lipid based closed self-assembled structures that can be employed to improve the pharmacokinetic profile of a drug and can also be used to reduce the side effects of antitumour drugs.
- Multidrug resistance - A major phenomenon occurs due to efflux of free chemotherapeutic drugs by primary transporter proteins like P-gp, and ATP-binding cassette transporters.
- Anionic liposomes with doxorubicin interact with one of the binding sites of P-gp and decrease the efflux activity.
- Increased expression of LDLR due to multidrug resistance phenomenon can be utilised to deliver the nanoformulations through these receptors.
- Immunoliposomes exhibit specific cytotoxicity against antigen over-expressing cancer cells. Antigen and antibody reactions are much preferred than any other interactions enabling specificity.
- Liposomes can also act as an adjuvant and hence they can be used to target dendritic cells to improve antitumour responses.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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PATIENT CONSENT

Declared none.

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