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Review

Formation strategies, mechanism of intracellular delivery and potential clinical applications of pH-sensitive liposomes

*Xin Liu, Guihua Huang**

School of Pharmaceutical Science, Shandong University, No. 44 Wenhua Xi Road, Ji'nan 250012, China

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ABSTRACT

pH-sensitive liposomes are designed to specifically triggered release the loaded drugs in response to the change of pH in the surrounding serum. So pH-sensitive liposomes can effectively deliver drug or gene fragments into the cytoplasm via the endocytotic pathway. Furthermore, pH-sensitive liposomes can be successfully used in clinical if they enable the encapsulated drugs to be targeted to pathological tissues (such as primary tumors, metastases, local ischemia, inflammation and infection) of the body in which pH is less than the normal physiological value. That's the reason why a growing amount of literatures described the development and applications of pH-sensitive liposomes to improve the therapeutic index of the encapsulated active ingredients. In this review, the commonly used pH-sensitive molecules for pH-sensitive liposome and the mechanisms of intracellular delivery of pH-sensitive liposomes were addressed. Besides, the potential clinical applications were fully discussed in detail with an expectation to contribute to the clinical research of pH-sensitive liposomes.

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1. Introduction

The design and synthesis of hundreds of new drug candidates with potential activity against a wide range of therapeutic targets in vitro have resulted from recent advances in biomedical science, high throughput screening and combinatorial chemistry. However, most of the new drugs spread out in the system with no specific targeting and sometimes with toxic side effects (*e.g.*, anemia, vomiting, diarrhea, nausea, decreased infection resistance, and hair loss, *etc.*) when they are intravenously administered [1-3]. Furthermore, they may be detected and absorbed by the reticuloendothelial system (RES) or mononuclear phagocyte system (MPS) prior to interacting with cell membranes when they were delivered into the bloodstream. As a result, only a small fraction of unaffected substance appears in the cell cytoplasm, which will further give rise to the failure to fully develop the potential in clinical.

* *Corresponding author*. Tel.: +86 0531 88382015; fax: +86 0531 88382548.
E-mail address: hgh2003@126.com (G. Huang).

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Fortunately, over the past few years, researchers have paid great attention to improving drug efficacy and decrease undesired side effects by developing new drug delivery systems. Therefore, establishing a superior delivery system that is able to encapsulate drug in a biocompatible carrier to deliver sufficient drug specifically to the site of disease may be one solution to those problems mentioned above. As a delivery system, liposomes have been one of the most common used and well-investigated to evade RES detection and achieve high therapeutic efficiency of a drug at a target site while at the same time reducing or avoiding toxic side effects [4–6].

Liposomes are typically spherical self-closed structures composed of curved self-assembled lipid bilayers with a size vary from 50 to 1000 nm. Since their discovery and recognition of the structure and basic properties, liposomes have been interesting for applications in a wide range of areas from medicine and cosmetics to food technology and ecology, as they can encapsulate hydrophilic solutes in their interior aqueous compartment and incorporate hydrophobic substances into the hydrophobic compartment of the phospholipid bilayer (Fig. 1). This kind of delivery system has the advantages of targeted [7], long circulation [8], low toxicity [9], sustained-release [10], no immunogenicity [11] and protecting the encapsulated drugs from the destructive action of the external media [12]. Among these advantages, the most common reason for applying liposomes to drug delivery is probably the improved pharmacokinetics profile. The predominance in drug delivery has enabled liposomes to be used as a therapeutic tool in tumor targeting, gene therapy, immunomodulation and genetic vaccination. The growing number of liposomal formulations in the market (*e.g.*, Doxil®, Daunoxome® and Myocet®, *etc.*) or currently under clinical evaluation (*e.g.*, Lipoplatin, liposomal cisplatin under Phase III clinical trials; Thermodox, liposomal doxorubicin under Phase I clinical trials) provide best proof of their enormous potential [13].

However, after intravenous administration, the conventional liposomes are still rapidly recognized and uptaken by the cells of the RES mainly in liver and spleen and removed from the circulation, which leads to short plasma half-lives [14]. Therefore their clinical potential has been largely limited. Besides, liposomes enter cells mainly via the endocytotic pathway and eventually reach the lysosome within the cell. In the lysosome, liposomes and their encapsulated drugs or gene that cannot escape the endosome are exposed to the risk of being degraded by lysosomal enzymes (Fig. 2), which will further significantly reduce the drug efficacy and the level of gene expression [15].

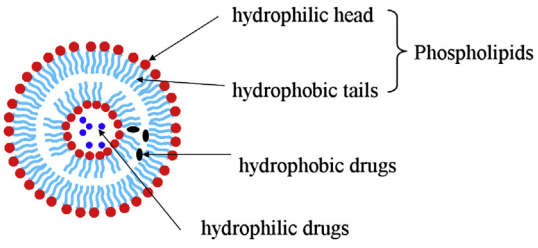

Fig. 1 – Structures of unilamellar liposome.

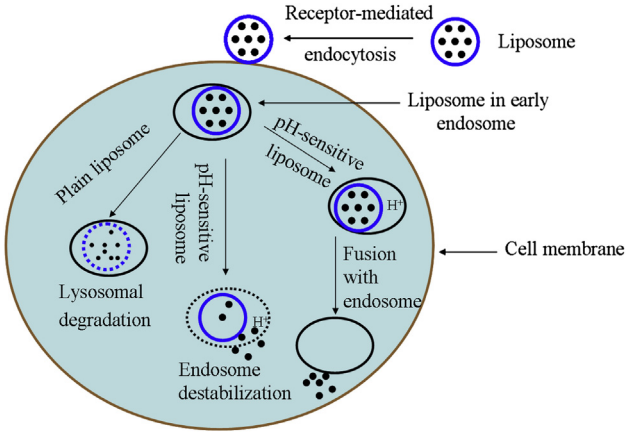

Fig. 2 – Intracellular delivery by pH-sensitive and plain liposome.

To solve this problem, many stimuli-sensitive liposomes have been and are being developed that avoid lysosomal degradation of loaded drugs and release their substance in one single burst as a result of destabilization of the liposome membrane caused by certain internal or external stimuli (such as changes of physiological pH, tissue specific enzymes, physiological temperature or electrolyte concentration, *etc.*) [16,17]. Among these trigger-release liposomes, pH-sensitive liposomes have been an attractive strategy to avoid lysosomal sequestration and degradation, which is a serious hurdle for intracellular delivery of drugs of low cellular permeation ability and enzymatic instability.

pH-sensitive liposomes can be prepared by simply adding pH-sensitive units to the liposome dispersion or by mixing pH-sensitive lipids and polymers during the preparation of vesicles [18]. Such liposomes stay intact at physiological pH but destabilize and acquire fusogenic properties under acidic conditions (pH7.4 ~ 5.3) of the target tissue, thus leading to efficiently release of their aqueous encapsulated contents into the cytoplasm [19]. Therefore a high local drug level at the target site is obtained due to their controlled release. Besides, they are stably internalized by cells mainly via an endocytic pathway, and they are destabilized at low pH (~5) in the endosome hence the drugs can be easily released into cytoplasm or actively targeted to lesions [20,21]. So the drugs or gene fragments that are encapsulated into pH-sensitive liposomes can effectively escape lysosomal sequestration and degradation. Therefore drug loaded pH-sensitive liposomes are believed to increase the efficiency of targeting drugs to desired cellular sites while effectively protecting them from being potentially degraded at the lysosomal level (Fig. 2) [22]. That's the reason why recent progress and the insight gained from clinical use of pH-sensitive liposomal formulations are highlighted.

2. pH-sensitive molecules for liposomes triggering

Various strategies for formulating pH-sensitive liposomes have been reported and developed in the past decades. pH-

sensitive liposomes mainly depend upon acid-induced destabilization of the vesicle bilayer structure. So this review divided pH-sensitive liposomes that are commonly used into the following four categories based on their components and the mechanism of triggering pH-sensitivity.

2.1. *Combine polymorphic lipids*

The typical polymorphic lipid used to prepare pH-sensitive liposomes is the unsaturated phosphatidylethanolamine (PE), such as diacetylenic-phosphatidyl-ethanolamine (DAPE), palmitoyl-oleoyl-phosphatidyl-ethanolamine (POPE) and dioleoyl-phosphatidyl-ethanolamine (DOPE) [23]. DOPE is usually combined with mildly acidic amphiphiles that act as stabilizers at neutral pH, such as oleic acid (OA), cholesteryl hemisuccinate (CHEMS) (Fig. 3) and palmitoyl homocysteine (PHC), to formulate pH-sensitive liposomes. Their carboxyl group was protonated in the acidic environment such as that found in the lumen of endosomes or lysosome. The three-dimensional volume of the hydrophilic side got small and lost its remedy to the phospholipid accordingly, which will result to membrane destabilization of pH-sensitive liposomes [24]. Then the encapsulated bioactive molecules were released from pH-sensitive liposomes into the cytoplasm.

2.2. *Contain "cage" lipid derivatives*

Most of this kind of liposome contains the derivatives of PE or annular lipid compositions with alkyl ether [25], such as N-citraconyl-dioleoyl-phosphatidyl- ethanolamine (C-DOPE) and N-citraconyl-dioleoyl-phosphatidylserine (C-DOPS). Liposomes that contain such compositions can reversibly exhibit the ability to form non-bilayer phase simply with the drug permeable membranes or with the fusion competent

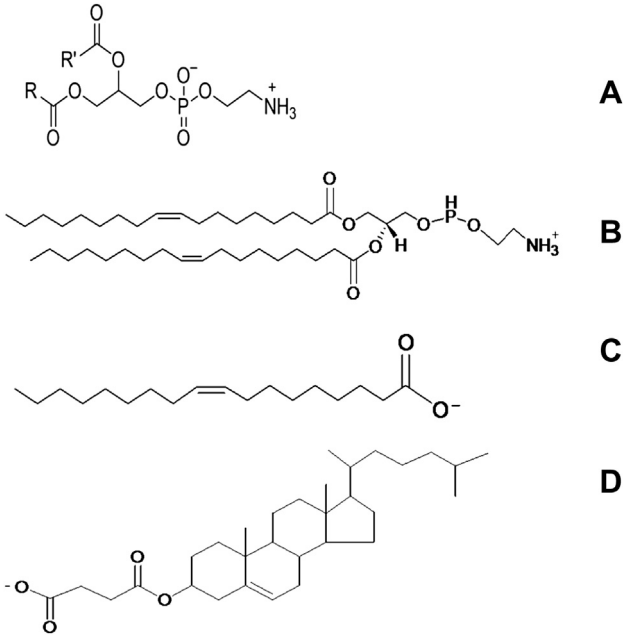

Fig. 3 – Chemical structures of the commonly used lipids for the construction of pH-sensitive liposome: A. PE, B. DOPE, C. OA, D. CHEMS.

included. The process mentioned above was performed by reversibly covalent modifying of a nucleophilic functionality on the head group of the lipid or cleaving the alkyl group of the liposome in the blood circulation to expose the long-chain of fatty acids that can undermine the stability of the biofilm [26] and thus increased permeability to entrapped drugs. So Daryl and David [27] indicate that the pH-sensitive liposomes that contain N-acyl-phosphatidylethanolamine can not only release their encapsulated contents in the environment of low pH values, but also promote the fusion with the cell membrane. Finally, poly(ethylene-glycol)-N-distearolyphosphatidyl-ethanolamine (PEG-DSPE), a new synthesized conjugate with a liable linkage, is another potentially very important "caged" lipid. The addition of PEG to the surface of liposomes reduced the RES uptake and simultaneously prolonged the duration of liposomes in the circulatory system [28].

2.3. *Composed of synthetic fusogenic peptides/proteins*

By inserting the pH-sensitive peptide/proteins, such as GALA, the N-terminus of hemaglutinin (INF peptides from influenza) or the listeriolysin O into the phospholipid double-fusion peptide or protein, thus a kind of novel pH-sensitive liposomes is developed. The peptide or protein is inactive when such liposomes are in the neutral pH environment. While in the acidic environment, the conformation of the fusion peptide or protein changed, which will promote the fusion between liposomal membrane and cell membrane [29]. Then the pH-sensitive liposomes release the encapsulated contents eventually. With membrane destabilization inducing by either full proteins or peptides, liposomal or lipid-based delivery systems can be triggered in response to pH. For example, GALA is a 30 amino acid synthetic peptide with a repeat unit of glutamic acid-alanine-leucine-alanine (EALA) [30]. EALA undergoes a pH-dependent conformational change from random coil at pH 7.5 to an amphipathic helix at pH 5 and induces leakage of contents from large unilamellar PC vesicles when in an α-helical conformation acids. Leakage of water-soluble fluorescent contents markers was shown to be rapid below pH 6 and maximal around pH 5, while being relatively slow at neutral pH [31]. Provoda *et al* [32] encapsulated Gelonin (a type I plant toxin) inside pH-sensitive liposomes with listeriolysin O, the pore-forming protein that mediates escape of the intracellular pathogen Listeria monocytogenes from the endosome into the cytosol. By direct intratumor injection into subcutaneous solid tumors of B16 melanoma in a mouse model, the results showed that this kind of pH-sensitive liposomes can improve the efficiency in curtailing tumor growth rates.

2.4. *Constructed with pH-sensitive polymers*

In recent years, growing scientific attention in the formulation of liposomal preparations has resulted from synthetic polymers. These polymers reported for the design of pH-sensitive liposomes are based on poly (alkyl acrylic acid)s, succinylated PEG, and N-isopropylacrylamide (NIPAM) copolymers [33]. Polymers exhibited one interesting feature that they can be tailored to participate actively in the release of drugs upon an

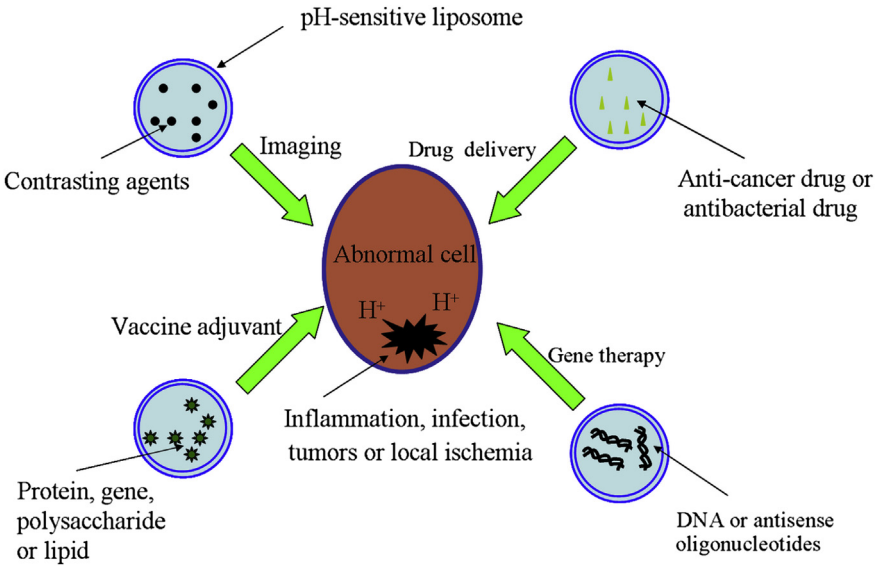

Fig. 4 – Possible clinical applications of pH-sensitive liposome.

external stimulation. In solution, such copolymer can interact with the lipid bilayer, which promotes the fusion between liposomes and endsomal membrane [34]. Roux *et al* found that NIPAM and its derivatives can endow the liposomes with Specific pH-sensitivity [35]. Such polymers can be attached to the surface of drug/DNA-loaded liposomes, which contributes to endosomal destabilization and cytoplasmic escape. Zignani *et al* [36] demonstrated rapid and pH-sensitive release of a highly water-soluble fluorescent aqueous content marker, pyranine, from egg phosphatidylcholine liposomes following incorporation of N-isopropylacrylamide (NIPA) copolymers in liposomal membranes. Yuba *et al* [37] modified of liposomes with hyperbranched poly (glycidol) (HPG) derivative, which is used as a new type of pH-sensitive polymer. Results demonstrated that the backbone structure of pH-sensitive polymers had great impact on their pH-sensitivity and interaction with liposomal and cellular membranes.

All four classes offer unique advantages and disadvantages that may vary in potential depending on the desired application purpose and methods.

3. Mechanisms of intracellular delivery mediated by pH-sensitive liposomes

Generally, as is discussed in the Section 2, the commonly used pH-sensitive lipid to design pH-sensitive liposomes is PE and its derivatives, such as DOPE. PE contains a minimally hydrated and small head group that occupies a lower volume as compared with the respective hydrocarbon chains, exhibiting a cone shape, which hinders the formation of a lamellar phase. Besides, PE can effectively assemble into non-bilayer structures in an inverted hexagonal phase when dispersed in pure form. The PE bilayers can also be stabilized in the lamellar phase by the additions of some lipid constituents or co-surfactant that contains a carboxylic acid group [38]. By incorporating these acid-titratable charged amphiphilic molecules, the intermolecular interaction of the PE headgroups can be reduced by the electrostatic repulsion of charged groups, thus the pH-sensitive liposomes that are stable at physiological pH and temperature can be constructed.

It is reported that pH-sensitive liposomes that can successfully circumvent the endosome are internalized by cells more effectively than non-pH-sensitive liposomes [39]. This phenomenon can be explained by their high affinity to adhere to cell membranes due to the poor hydration head group of PE-containing liposomes resulting in aggregation. Following receptor-mediated endocytosis, liposomes will be retained in the early endosomes (internal pH 6.5), which will mature into late endosomes (internal pH 5.5 ~ 6.5) independents of the internalization process. So the compounds that cannot escape the endosome (internal pH 4.5 ~ 6.5) and accordingly end up in the lysosome (internal pH 5.0 or lower) are exposed to the risk of being destructed by the lysosomal enzymes. This process will result in a limited delivery of therapeutic agents to the intracellular targets. However, as the pH decreases, the carboxylic group of the amphiphiles reduces their stabilizing effect, thus pH-sensitive liposomes can convert from conventional bilayer sheet of the lipoidal membrane to inverted hexagonal phase. The pH-sensitive liposomes composed of PE and co-surfactant were destabilized as a result of the acidification of either in endosomal compartments or pathological tissues, such as tumor interstitial fluid or inflamed tissue. And the loaded drug can be released into the cytosol due to the subsequent destabilization of the endosomal membrane. Besides, the encapsulated compounds can be released directly into the cytoplasm due to the fusion between pH-sensitive liposomes and the endosomal membrane (Fig. 2). This function of pH-sensitive liposomes enabled the loaded bioactive materials to evade the degradation at the lysosomal level and therefore increase entry to the cytosolic or nuclear targets [40]. The concrete mechanisms for different kind of pH-sensitive liposomes may vary depending on the phosphatidylcholine

and the trigger components. This review didn't discuss them in detail any more.

4. Applications of the pH-sensitive liposomes

After being endocytosed in the intact form, pH-sensitive liposomes fuse with the endovacuolar membrane on the condition of lower pH value inside the endosome and destabilize it, thus releasing their content into the cytoplasm. So pH-sensitive liposomes can be appropriately designed to release their encapsulated contents, especially the biological macromolecules, such as drugs [41], enzymes [42], antibodies [43] and antisense oligonucleotide (ODN) [44], plasmids [45], proteins and peptides [46], into cytoplasm before reaching the lysosome to ensure the activity of drugs. Besides, inflammation, infection, some tumors and local ischemia all will lead to abnormal acidification of the pathological tissues, so pH-sensitive liposome in the pH range of 6.5 ~ 7.4, as a delivery carrier, has great clinical value. That's why different applications of pH-sensitive liposomes were envisaged, including for the transport and specific delivery of potent drugs (for cancer, pulmonary and infectious diseases), vaccination (as immunological adjuvants), imaging (carrying contrasting agents) and as well as nucleic acids which is aiming at gene therapy applications (Fig. 4) [47].

4.1. *Drug delivery*

It is reported that pH-sensitive liposomes are stable at physiological pH (pH 7.4) but undergo destabilization, and acquire fusogenic properties under acidic conditions, thus leading to the release of their aqueous contents. Therefore, in theory, pH-sensitive liposomes can prolong the circulation time and improve the efficiency of drug delivery. In practical, pH-sensitive liposomes have been reported to have possible clinical implications for delivering drugs to target sites such as primary tumor and inflammation sites where the pH could be less than physiological.

4.1.1. *Anti-tumor therapy*

The systemic chemotherapy is almost impossible to achieve therapeutic levels of a drug at the solid tumor without injuring the healthy organs and tissues [48]. In addition, several drawbacks, such as low bioavailability of the chemotoxin, low drug concentrations at the tumor site, lack of specificity and drug-resistant also provide obstacle to its clinical applications.

Although the nanocarriers less than 200 nm are able to be passively targeted to tumor tissue due to the enhanced permeation and retention (EPR) effect [49]. However, one of the drawbacks of the conventional drug delivery system is the fast elimination from the blood and capture by the cells of the RES, primarily in the liver.

It is reported that the extracellular environment of solid tumors is acidic with a pH ranging from 5.7 to 7.8 compared with the pH 7.4 of the blood and normal tissue [50]. pH-sensitive liposomes can be induced to undergo a pH-induced fusion of liposomal membranes with endosomal membranes or destabilization of the endosomal membrane, thus releasing contents into cytoplasm. Since most liposomes are internalized by endocytosis, pH-sensitive liposomes undergo destabilization at this step and thus prevent degradation at the lysosomal level, which can promote cytosolic delivery of the intact contents [51]. In recent years, as a drug carrier, the research and application of pH-sensitive liposomes in the treatment of cancer develop rapidly.

At present, DOPE may be the most commonly used lipid for pH-sensitive liposomes. In general, PEG served as a stabilizer of DOPE containing pH-sensitive liposomes for triggered release of the loaded anti-cancer drugs. Ishida *et al* prepared doxorubicin encapsulated pH-sensitive liposomes with the mixture of DOPE/HSPC/CHEMS/CHOL/mPEG2000-DSPE at a molar ratio of 4:2:2:2:0.3 and DOPE/HSPC/CHEMS/CHOL at a molar ratio of 4:2:2:2 in the hydration way [52]. As a result, pH-sensitive liposomes increased intracellular drug release rates within acidic compartment, resulting in a further increase in the therapeutic efficacy of B lymphoma. Besides, the cisplatin loaded pH-sensitive liposomes with DOPE/CHEMS/DSPE-PEG were prepared to cure the small cell lung cancer [53]. Compared with free cisplatin, this formulation has a better stability in blood and its cytotoxicity is significantly enhanced. Furthermore, it's effective for the cells that are tolerance to cisplatin. So the addition of lipids with covalently attached PEG in liposomes was shown to avoid the rapid identification and elimination of the MPS, which may contribute to fully take advantage of the superiority of pH-sensitive liposomes.

pH-sensitive liposome modified with monoclonal antibody could be directed to target to the lesions with a low pH environment. The therapeutic efficacy of the anti-cancer drug entrapped in pH-sensitive liposomes can be improved by the monoclonal antibody that can direct the pH-sensitive liposomes to the cell surface receptors. Kim *et al* developed gemcitabine pH-sensitive liposome (DOPE and CHEMS) with epidermal growth factor receptor (EGFR) antibody attached and used A549 cells and BALB/c-nu/nu mouse tumor model for testing. The results showed that treatment of pH-sensitive immunoliposomes encapsulating gemcitabine resulted in an increased apoptosis of tumor cells, leading to tumor growth inhibition [54]. Simard and Leroux formulated pH-sensitive immunoliposomes by including a terminally alkylated copolymer of NIPAM in the liposome bilayer and by coupling the anti-CD33 monoclonal antibody to target leukemic cells. Finally, the pH-sensitive ILs-CD33 formulation exhibited the highest cytotoxicity against HL-60 cells [55].

Last but not the least, nucleic acid, plasmid DNA or antisense oligonucleotides mediated by pH-sensitive liposome can be delivered in the treatment of cancer, as well. The detailed significance of pH-sensitive liposomes will be discussed in the section of gene therapy.

4.1.2. *Anti-infection therapy*

Intracellular infection by bacterial is difficult to manage clinically and is often refractory to conventional chemotherapeutic treatment strategies due to poor penetration of drug into cells [56]. As one of the drug carriers mentioned above, liposomes have achieved their greatest success against facultative and obligate intracellular pathogens in the treatment of infectious diseases, most notably those with a tendency to infect the MPS. Furthermore, liposomes have shown

a particular validity in the treatment of infections by intracellular bacteria [57]. In case the infectious focus is located outside the MPS, conventional liposomes are of limited value. Therefore, research has been aimed at decreasing the MPS uptake of liposomes and consequently increasing their circulation time and targeted distribution.

pH-sensitive liposomes can be triggered to release their contents and fuse with the biomembrane in response to acidic environment of the infected and inflamed tissues [58]. So the dissociation of the drug from pH-sensitive liposomes and their rapid accumulation in the target organs (the liver and spleen) makes pH-sensitive liposomes an ideal candidate for in vivo evaluation in an antifungal as well as antibacterial efficacy model. Lutwyche *et al* [59] encapsulated gentamicin into pH-sensitive liposomes composed of DOPE-N-succinyl-DOPE and DOPE-N-glutaryl-DOPE (70:30; mol:mol) to treat with murine macrophage-like J774A.1 cells those were infected with bacteria. As a result, gentamicin encapsulated in lipid vesicle that undergo pH-dependent lipid mixing and fusion confered to this membrane-impermeative antibiotic a significant improvement in therapeutic activity against intracellular bacterial infections. Nasti *et al* [60] evaluated the efficacy of pH-sensitive liposomes of nystatin against *Cryptococcus neoformans* infection in a murine model. As a result, pH-sensitive liposomes of nystatin showed better efficacy compared with its free or egg-PC liposome form against *C. neoformans* infection in BALB/c mice. So the enhanced anti-cryptococcal efficacy of the pH-sensitive nystatin liposomes can be attributed to the pH-dependent release of the drug in the low pH environment of lysosomes. Nicolosi *et al* [61] have exploited the fusogenic properties of DOPE/DPPC/CHEMS unilamellar vesicles with the purpose of releasing the antibiotic not inside cells but specifically in the narrow area of periplasmic space of Gram-negative bacteria. As a result, the outer membrane barrier can be bypassed and antibiotic can operate its molecular activity at the level of the cell wall. The enhanced efficacy observed for encapsulated antibiotics in pH-sensitive liposomes may due to targeted delivery of lipid carriers to the infected area.

4.2. *Gene therapy*

Somatic gene therapy has emerged as a new approach for the treatment of a variety of genetic and acquired diseases [62]. The key to success for any gene therapy strategy is to design a vector that is able to serve as a safe and efficient gene delivery vehicle. At present, the common carrier for the study and clinical application of gene therapy includes viral vector and non-viral vector. The viral vector has the natural ability to infect cells efficiently, but there is a potential risk of generating an infectious, replication-competent virus during the production or use of viral vectors for gene transfection [63]. While the non-viral vector has no immunogenicity and it is easily prepared, so it has higher safety in vivo [64].

Liposomes based gene vector has been promoted as a means of achieving the transfection efficiency of viral vector without the associated risks. In recent years, with the advent of cationic liposome [65] and active targeting technology [66], liposome technology has been widely applied in the transfer of antisense ODNs for its virtues of high transfection efficiency, protection for the entrapped and potential of chemical modification. Besides, they are noninfectious, non-immunogenic and simple and easy to produce in large scale [67].

However, non-viral vector, like cationic lipids/liposomes, also showed certain drawbacks, such as nonspecificity and cytotoxic reactions [68]. Besides, the efficiencies of gene transfection meditated by conventional liposomes were accordingly low. But pH-sensitive liposomes can release the loaded gene expression system in the cytoplasm before entering the lysosome by fusion with the biofilm due to the lipid bilayers of the basic structure of their biofilm. That's why pH-sensitive liposomes can transfect gene into cytoplasm more efficiently and avoids lysosomal degradation to some extent [69]. So the pH-sensitive liposomes may be a promising non-viral vector for gene therapy.

Fattal Yuba *et al* [70] transfected of a murine DC2.4 cells with pH-sensitive fusogenic liposomes that comprise polymers based on poly (glycidol) with carboxyl group. The results indicated these complexes with pH-Sensitive fusogenic liposomes exhibited higher transfection activity toward DC2.4 cells than some commercial reagents and hence may be useful as a gene vector for DCs. However, the transfection efficiency of gene delivery directly mediated by pH-sensitive liposomes was less than the cationic liposomes owning to the negative charge. So the pH-sensitive cationic liposomes are expected to be an excellent gene carrier. Rosa *et al* [71] precondensed plasmid DNA with an arginine-based cationic surfactant, arginine-N-lauroyl amide dihydrochloride (ALA), which was incorporated the blood protein transferrin (Tf) into two cationic liposomal formulations. One composed of a mixture of dioleoyl trimethylammonio propane and cholesterol (DOTAP:Chol) and the other pH-sensitive formulation constituted of DOTAP, Chol, DOPE and CHEMS. The results demonstrated complexes based on the pH-sensitive liposomal formulations present better transfection profiles.

Although, compared with the non-pH-sensitive immunoliposomes, pH-sensitive immunoliposomes have much higher capacity to mediate cytosolic delivery of the encapsulated therapeutic molecules due to endosomal escape [72]. Their stability in the presence of plasma proteins and the stability of obtaining a sustained-release of the therapeutic agent still put obstacles to their applications in gene therapy. PEG modification may be a very interesting strategy to solve the problems mentioned above. C-DOPE, a derivative of DOPE that hydrolyzes rapidly at pH 5 to yield DOPE, was synthesized by Low *et al* and incorporated with DOPE and folate-PEG-DOPE into liposomes. The resulting pH-sensitive liposomes were stable at neutral pH and had a higher transfection efficiency compared with DOPE-cholesterol hemisuccinate based vectors [73].

4.3. *Vaccine delivery adjuvant*

Since 1974, Allison *et al* [74] firstly reported that liposomes can be used as immunological adjuvant, thorough study has been made on the implications as a vaccine carrier and adjuvant of liposomes.

From disposition studies of liposome in vivo, it is reported that large liposomes are efficiently taken up by macrophages

of RES in blood and tissues, including the liver and spleen (the main immune organs), which contributes to delivery the antigen to antigen-presenting cells or other immune cells. Furthermore, liposomes have the function of immunological adjuvants without the side effect of common adjuvant [75]. A liposomal vaccine against hepatitis A successfully developed by the Swiss Serum Institute (Bern, Switzerland) provided the best proof [76].

However, conventional liposomes are endocytosed on contact with antigen-presenting cells and degraded, coupled with the entrapped molecules, inside the endosome via endosome-lysosome pathway. Whereas, pH-sensitive liposomes release liposomal antigen into the cytoplasm after endocytosis because of their fusion capacity with the endosomal membrane at low pH (range from 5.5–6.5 in the early and late endosome compartment). Then they were transported to the endoplasmic reticulum where they combined with class I molecules. So pH-sensitive liposomes can deliver the encapsulated material more safely and efficiently than conventional liposomes, which suggests pH-sensitive liposomes may be a superior vaccine delivery adjuvant [77].

pH-sensitive liposomes have been used as a non-viral adjuvants with bacterial, viral, protozoan, tumor and other antigens. Vyas *et al* [78] prepared and characterized the Carboxyl-terminal 19 kDa fragment of merozoite surface protein-1 of Plasmodium falciparum (PfMSP-1_{19}) encapsulated pH-sensitive liposomal formulations using oleyl alcohol (OAlc) in combination with EPC as the membrane destabilizing components. The results demonstrated pH-sensitive liposomes showed excellent immuno-adjuvant action and enhanced the immunogenicity of a soluble malaria antigen. So the present study of pH-sensitive liposomes might open new ways for the feasibility for the development of blood stage malaria vaccine.

Besides, the presentation of CTL-peptide antigen mediated by pH-sensitive liposomes occurs in lymph nodes. Lee *et al* [79] investigated the antigen delivery route by pH-sensitive liposomes in vivo using fluorescein isothiocyanate (FITC)-conjugated H-$2K^b$ cytotoxic T lymphocyte (CTL) epitope as a model system. The pH-sensitive liposomal formulations showed significant effects on the generation and activation of antigen specific CTLs, indicating that the formulations might be used as a potential peptide adjuvant for priming and boosting against target antigens. The results suggest that pH-sensitive liposomes, as a strong peptide adjuvant, may be useful for peptide delivery for the development of therapeutic or prophylactic vaccines. Furthermore, stronger cellular immune responses can be induced by ovalbumin-loaded pH-sensitive liposomes from nasal cavities of mice. Yuba *et al* [80] developed ovalbumin-encapsulated pH-sensitive liposomes modified with poly (glycidol) derivatives such as succinylated poly (glycidol) and 3-methylglutarylated poly (glycidol). Such pH-sensitive liposomes were applied to DC2.4 cells, a murine dendritic cell line, to investigate the potential of this fumulation as a carrier of antigen proteins for induction of cellular immunity. The results indicated that the ability of the polymer-modified pH-sensitive liposomes to activate cellular immunity and the feasibility to develop efficient vaccines for immunotherapy.

Liposomes have been reported to promote immune responses to DNA vaccines by facilitating uptake of the plasmid by antigen-presenting cells [81]. Akita *et al* [82] reported on a mechanism-based development of a siRNA delivery system that was optimized for endsomal fusion by modifying on a lipid mixture with a pH-dependent fusogenic peptide (GALA). Furthermore, they applied this system to deliver siRNA to primary mouse bone marrow-derived dendritic cells. The results demonstrated that siRNA loaded in this system efficiently suppressed endogenous gene expression and consequently enhanced dendritic cell-based cancer vaccine in vivo.

4.4. *MRI contrast agents*

Magnetic resonance imaging (MRI) technique has become one of the most important diagnosis tools available in medicine. A majority of MRI contrast agents in clinical used today are based on paramagnetic gadolinium complexes that shorten the relaxation times of free water protons [83]. The contrast agents in combination with MRI have been effective tools to get a perspective of inflammation, infarct, tumor, atherosclerotic plaques, live stem-cell tracking, brain perfusion and many other applications [84].

Most of these MRI contrast agents are complexes of gadolinium (Gd III) as this kind of ion has a high magnetic moment and a long electronic relaxation time [85]. They can effectively pass through the damaged blood-brain barrier [86] and can be quickly excreted by renal. So their enhancement effect is not proportional with the concentration. What's worse, these contrast agents are all toxic and non-specific, even if their distribution in the body is far from homogeneous.

As a new contrast agent, liposomes have received growing attention because of relatively long circulation time in blood, the ability of development, easily controlled properties and good pharmacological characteristics. But the contrast agent of conventional liposomes is easily ruptured and absorbed by RES, which may reduce the contrast effect. Finally, MRI contrast agents are presented that react to variables in their environment, such as magnetism and pH [87]. This concept of pH-mediated drug release could be investigated in MRI of tumors, infection and local ischemia. Paramagnetic pH-sensitive liposomes accumulated in the acidic environment within the pathological tissues could be triggered to structural rearrangements and thus release the encapsulated contrast agents into the cytoplasm. So if properly designed, these pH-sensitive liposomes would exhibit a function as "off-on" switches and markedly increased contrast effect.

Terreno *et al* assessed the in vitro potential of several paramagnetic complexes loaded pH-sensitive liposomes formulated with the fusogenic phospholipid POPE and the membrane stabilizer D-α-tocopherol-hemisuccinate, as imaging tools for visualizing drug delivery and release processes by MRI. It was found that the resulted pH-sensitive liposomal formulation has the potential for visualizing drug delivery and release processes by in vivo MRI [88].

Besides, the basic properties of pH-sensitive liposomes loaded with MRI agents were investigated by Lokling *et al* in a series of papers. Lokling *et al* [89] encapsulated a low-molecular-weight Gd-chelate (GdDTPA-BMA) in pH-sensitive liposomes and studied the in vitro relaxometric properties. When the surrounded pH decreased below physiological value, the pH-sensitive paramagnetic contrast agent gave a sharp and 6-

7 fold increase in T_1 relaxivity due to liposome destabilization and subsequent leakage of entrapped GdDTPA-BMA.

Then the potential of a pH-sensitive liposomal MRI contrast agent for low pH in the tumor interstitium was further investigated. Lokling *et al* formulated DPPE/DPSG pH-sensitive liposomal GdDTPA-BMA and investigated its stability in blood at physiological pH and pH-sensitivity. The potential of this system for monitoring pH was demonstrated in an in vitro MRI phantom study. The MRI study indicated that the DPPE/DPSG system has the potential value as a probe for mapping pH [90]. The ideal MRI contrast agent will be focused on the neutral tissue- or organ-targeting materials with high relaxivity and specificity. So Lokling *et al* encapsulated gadofosveset, a low-molecular-weight Gd-chelate with high affinity for albumin, into pH-sensitive liposomes to study the biodistribution in healthy rats. The results demonstrated that this promising system showed in blood a markedly higher relaxometric response than the corresponding system with GdDTPA-BMA, due to release of gadofosveset at low pH and subsequent binding to albumin [91].

5. Conclusion and future prospects

It is believed that pH-sensitive liposomes can significantly increase cytoplasmic delivery of various fluorescent markers with various molecular sizes, ribozymes, enzymes, cytotoxic agents, proteins, RNA, and DNA to cells with considerable efficiency. However, so far, none of this kind of preparations is used in clinical due to their drawbacks. Because, a clinically viable pH-sensitive liposomal formulation requires several essential properties including efficient pH-triggered release, serum stability, and enough long circulation time in vivo. Additionally, after being injected into the body, pH-sensitive liposomes still can be recognized by the opsonin in the plasma and phagocytized by RES to some extent, which is an important limitation to the in vivo use and the main barrier of the delivery of drugs and gene to pathological organs (in addition to the liver, spleen). Furthermore, the physicochemical and biological stability issues, acid sensitivity and bioavailability, particle size control, batch to batch reproducibility and sterilization method are still to be overcome in order to satisfy the prerequisites of treating diseases in animals or humans.

While as novel responsive polymer compositions are continually being developed and the ability to prepare macromolecules with topological complexity is expanding, those problems mentioned above will be solved gradually. By that time, pH-sensitive liposomes would have been an attractive carrier for therapeutic drugs or macromolecules with intracellular targets. Furthermore, developing 'smart' multifunctional pharmaceutical nanocarriers by combinating of pH-sensitive liposomes with active targeting and other release mechanisms (such as enzyme-responsive [92], temperature-sensitive [93], light-sensitive [94], magnetic responsive [95] and ultrasound-responsive), and selecting appropriate pH-sensitive compositions, triggering signal and mechanism of action to be suitable for a specific application, pH-sensitive liposomes could be utilized in numerous medical treatments for enhanced efficiency in the foreseeable future.

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