1

REVIEW ARTICLE

Immunoliposomes: Synthesis, Structure, and Their Potential as Drug Delivery Carriers

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Abstract: Immunoliposomes have emerged as attractive drug targeting vehicles for cancer treatment. This review presents the recent advances in the design of immunoliposomes encapsulating a variety of chemotherapeutic agents. We provided an overview of different routes that can be used to conjugate antibodies to the surfaces of liposomes, as well as several examples of stimuli-responsive immunoliposome systems and their therapeutic potential for cancer treatment.

1. INTRODUCTION

Drug targeting is an emerging field in the pharmaceutical industry. Targeted drug delivery involves the use of pharmacologically active moieties to target selected receptor upregulated targets in therapeutic concentrations, all the while restricting their uptake to normal cells, thus minimizing their toxic effects and maximizing their therapeutic index.

In conventional drug delivery systems, such as tablets, capsules, solutions, *etc.*, the systemic drug distribution is nonspecific. In order to get the desired effect at the targeted site, the drug has to overcome many obstacles, including crossing other biological barriers and healthy tissues, which will inadvertently alter its therapeutic effect. Targeted drug delivery has emerged as an alternative platform capable of overcoming the drawbacks of systemic drug delivery, including the lack of specific drug affinity toward a pathological site, the requirement of high dose drug administration, the premature metabolism of the drug, the reduced bioavailability and non-specific toxicity [1].

In targeted therapy, drug delivery systems are able to control the rate and frequency of the carrier's accumulation at the diseased location, meaning that the drug concentration at the pathogenic site will be considerably high while minimizing its pronounced negative side effects on non-targeted healthy tissues. Hence, the main objective of targeted therapy is to maximize the desired pharmacological response and allow higher specificity in drug targeting [2]. For example, in tumor-targeted drug delivery systems, the main focus is on conjugating a suitable moiety corresponding to the specific antigens present on the surface of tumor cells, along with developing improved drug formulations, thereby ensuring the maximum availability of the drug at the targeted site with its minimal loss in the blood circulation. After reaching the desired diseased tissue, anti-neoplastic agents will have the ability to selectively destroy the tumor cells without adversely affecting the surrounding healthy cells. In this review, we attempt to describe the major areas related to drug targeting with a particular focus on antibodies when conjugated to nano-drug carriers.

1.1. Properties of An Ideal Drug Carrier

The extensive research into targeted drug delivery has led to the development of several types of carriers including, polymeric micelles, liposomes, micro-nanoparticles, capsules, dendrimers, metal organ frameworks (MOFs) and several others. A drug delivery vehicle must be non-toxic, biocompatible, non-immunogenic, biodegradable, and must avoid recognition by the host's immune system [3, 4]. The most commonly used strategy to "hide" nanocarriers from the reticuloendothelial system (RES) and opsonizing proteins involves the modification of the carrier surface with polymers, such as polyethylene glycol (PEG). PEGylated liposomes have shown improved blood circulation, minimal toxicity, and improved passive accumulation at the tumor site [5].

Furthermore, this combination of the drug and ligand should be stable in plasma, interstitial fluid and other biofluids. The carrier must be selectively recognised by the target cells and maintain the specificity of the surface ligands [4]. These biologically targeted carriers should be able to cross

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Table 1.   Commonly used targeting ligands.

Type	Ligand	Target	Advantages		Limitations		Status
Proteins	Transferrin	Transferrin receptor	1.	High specificity.	1.	High production cost.	Phase I and II clinical trails [14]
					2.	Large size.	
Antibodies	Herceptin, CD19	HER2, CD19 antigen	1.	High specificity.	1.	Large size.	Phase II and III clinical trials of Trastuzumab-DM1 [15]
			2.	High affinity and strong binding.	2.	Production cost.	
					3.	Binding site barrier effect.	
					4.	Potential immunogenicity.	
Peptides	RGD	$\alpha_v\beta_3$ integrins	1.	Easy fabrication.	1.	Cleavable by peptidase.	Phase I and II clinical trials of RGD PET tracer in humans [16]
			2.	Small size.	2.	Reduced circulation half-time.	
			3.	High affinity.			
Aptamers	Pegaptanib	VEGF receptor	1.	High specificity.	1.	High cost.	Approved as Macugen® (Pegaptanib Sodium) Injection [17]
			2.	Small size.	2.	Cleavable by nuclease.	
			3.	Possible to develop for any target.			
Polysaccharides	Galactose, Hyaluronic acid, Lactose	Asialoglycoprotein receptors (ASGPr), CD44	1.	Low production cost.	1.	Reduced circulation time.	Not approved yet.
			2.	Low molecular weight.	2.	Receptors overexpressed in liver tissue.	
			3.	Simple chemistry.			
			4.	Can be used as polymer backbone of nanoparticles.			
Small molecules	Folate	Folate receptor	1.	Small size.	1.	Targets are also expressed in healthy tissue.	Phase II and III clinical trials [18]
			2.	Low cost.	2.	Reduced circulation time.	

biological barriers and, in the case of cancer therapy, tumor vasculature. In addition, the delivery system itself should be pharmacologically inactive with minimal cytotoxicity outside the diseased area; as well as readily metabolized and cleared from the circulatory system once it has delivered its payload. More importantly, after its recognition and subsequent internalization, the carrier should release the drug inside the target organs, tissues or cells in a controlled manner without altering the therapeutic effect of the drug [6].

The three primary approaches used for targeted drug delivery are: passive, active/ligand, and (externally or internally) triggered targeting.

Passive targeting is largely used for targeting solid tumors. Cancer cells proliferate at an accelerated rate, and if the tumor grows too rapidly, becoming hypoxic and deprived of nutrients, the tumor cells can stimulate angiogenesis through the enhancement of the vascular endothelial growth factor (VEGF) and other growth factors. These poorly developed blood vessels then continue to rapidly proliferate, producing a severely irregular and aberrant vasculature with gaps between the endothelial cells. The leaky nature of the tumor microvasculature enables the extravasation of drug-loaded nano-carriers. Furthermore, the poor lymphatic drain-

age of this microenvironment results in the slow clearance and high accumulation of nanoparticles at the tumor site in a phenomenon known as the enhanced permeability and retention effect (EPR). Hence, macromolecules with molecular weights above 50 kDa, including nanoparticles, can selectively accumulate in the tumor interstitium [7-12].

In active drug targeting, different moieties including antibodies, antibody fragments, lipoproteins, hormones, mono-, oligo- and polysaccharides, charged molecules, and low molecular-weight ligands are used to modify the surface of the carrier. Active- (more correctly known as ligand-) targeting is mainly focused on the selective affinity of the targeting moieties to recognize and interact with the receptor structures expressed on the specific cell, tissue, or organ at the targeted site. The strong interactions between receptors and ligands eventually reduce the unwanted non-specific interactions and localization of the drug in peripheral tissues. For instance, the folate receptor is over-expressed in a variety of cancer types, including ovarian carcinomas, osteosarcomas and non-Hodgkin's lymphomas [13], carriers conjugated with folate ligands have an increased chance of being internalized, by binding to the folate receptors overexpressed on cancer cells compared to healthy cells. Table **1** provides a

Table 2.   Approved liposomal formulations.

Product Name	Active Drug	Cancer Type
Doxil/ Caelyx (PEG liposomes)	Doxorubicin	Refractory ovarian cancer, recurrent breast cancer, multiple myeloma
Myocet (non-PEG liposomes)	Doxorubicin	Recurrent breast cancer
Daunoxome	Daunorubicin	Breast and lung cancer
Onivyde®	Irinotecan	Metastatic pancreatic cancer
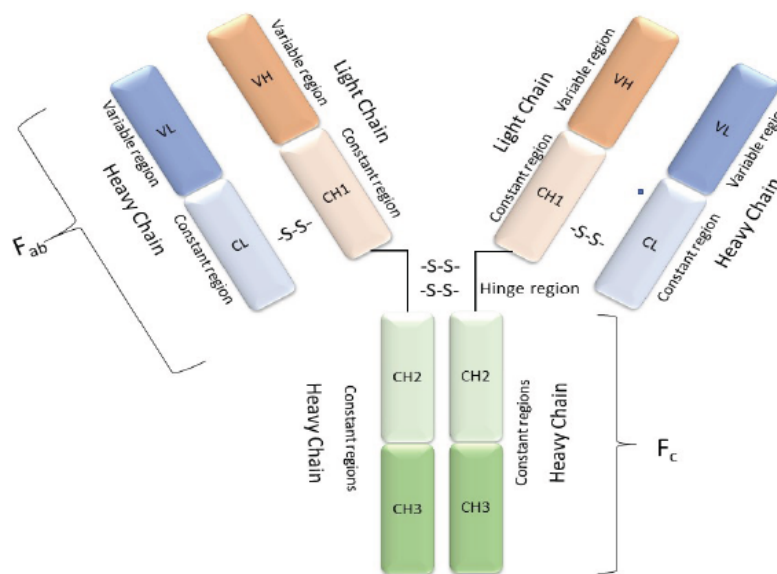
Fig. (1). Structure of human IgG antibody. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

summary of different ligands used for active-/ligand-targeting in liposomal drug delivery systems.

1.2. Liposomes

Among the different types of drug carriers, liposomes are the most commonly used in drug delivery (both in research and clinically). Liposomes offer several advantages over other delivery systems, including biocompatibility, a capacity for self-assembly, and the ability to sequester both hydrophilic and lipophilic drugs [19]. In addition, they possess a wide range of physical properties that can be modified to control their biological features, such as the ability to induce or inhibit the immune system, longer circulation times, improved loading efficiency, enhanced penetration, and target-specificity. Hence, over the past few decades, research on liposomal drug delivery has shown considerable promise. Moreover, some liposome-based products have achieved commercial success in gaining regulatory approvals. A number of liposome drug carriers are available in the market, with many more under clinical trials. Table **2** lists some liposomal formulations in clinical use or involved in clinical trials [20-26].

2. ANTIBODIES AS TARGETING LIGANDS

The primary type of proteins used as targeting ligands are antibodies and antibody fragments (Fab or scFv). Antibodies can recognize a vast array of antigens; they target specific antigens that are overexpressed on the surface of diseased cells compared to healthy cells. The majority of these antibodies target the extracellular domains (ECDs) of cell surface proteins. Another advantage of antibody conjugation to drug carriers is increased cellular uptake through endocytosis. Monoclonal antibodies like Immunoglobulins (IgG1, IgG2a, or IgG2b) are the most used in targeted drug delivery [27]. Five major classes of immunoglobulins have been discovered, IgA, IgD, IgE, IgG, and IgM. The significant difference between these classes is the components of their heavy chains, termed α (alpha), δ (delta), ε (epsilon), γ (gamma), and μ (micro), respectively. IgGs represent the dominant class of human immunoglobulins and can be further divided into four sub-types; IgG1, IgG2, IgG3, and IgG4 [28, 29]. Human IgG is a heterotetramer of two identical γ heavy chains and two identical light chains that are joined with a series of disulfide bonds. The heavy chain is made up of one variable region (VH) and three constant regions (CH1, CH2, and CH3). The light chain has one variable (VL) and one constant (CL) region. Within each VH and VL domains are three hypervariable regions, where sequence variability is concentrated, and loops are formed (refer Fig. **1**).

These hypervariable regions, also called complementary determining regions (CDRs), because the antigen-binding site is complementary to the structure of the epitope and are primarily responsible for antigen recognition. On each vari-
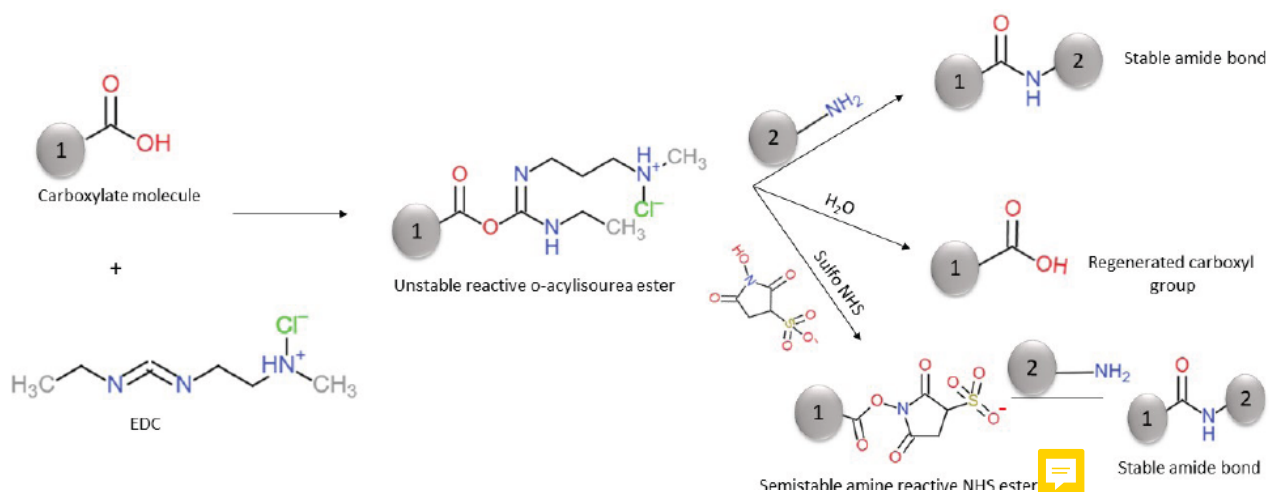
Fig. (2). EDC reacts with a carboxyl group on the linker lipid denoted by molecule 1, forming an amine-reactive O-acylisourea intermediate. This intermediate may react with an amine on molecule 2, yielding a conjugate of the two molecules joined by a stable amide bond. However, the intermediate can also hydrolyze, making it unstable and short-lived in aqueous solution. The addition of Sulfo-NHS stabilizes the intermediate by converting it to an amine-reactive Sulfo-NHS ester, thus increasing the efficiency of EDC-mediated coupling reactions (adapted from [27]). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

able chain, there are three complementarity determining regions which bestow vast variability to the specific antigen-binding capacity. VH and VL, together with CH1 and CL are known as the Fab (fragment antigen-binding), while the remainder of the molecule is known as the Fc region (fragment crystallizable), which confers biological activity and half-life. Recombinant antibody fragments include scFvs, which are comprised of the VH and VL segments joined using a peptide linker [30].

2.1. Strategies to Conjugate Monoclonal Antibodies to Liposomes

Immunoliposomes are liposomes functionalized using monoclonal antibodies or their fragments (Fab) and scFvs. The antibodies are conjugated to the liposomes either through covalent bonding between the antibody, or its fragment, and the carrier lipid or by chemically modifying the antibodies in order to increase their hydrophobicity, resulting in a higher affinity towards the bilayers of the liposomal carriers. If the antibody is conjugated directly to the lipid bilayer of PEGylated liposomes, the bonding efficiency may be lower compared to the conjugation of Ab to the terminus of the PEG chain. Amines, carboxylate groups and sulfhydryl groups are the major functional groups in antibodies, as they are present abundantly in all proteins. In general, the conjugation methodology is based on three main reactions; a reaction between activated carboxyl groups and amino groups which yields an amide bond, a reaction between pyridyl dithiols and thiols which yields disulfide bonds, and a reaction between maleimide derivatives and thiols, which yields thioether bonds [31].

2.1.1. Conjugation by Amino Acid and Carboxylic Acid Residues

Amine groups are distributed throughout the antibody and are easily modified due to their steric accessibility. These amines can be used directly at the target site as an amine-reactive crosslinker (crosslinking reagents are mainly

used to target the nucleophilic primary amine of lysine exposed on the surface of the antibody) or after the pre-modification of its function; N-hydroxysuccinimide (NHS), 2-succinimido-1,1,3,3-tetra-methyluronium tetrafluoroborate (TSTU), and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) are the most commonly used chemicals for the activation of amines. In amine-based reactions, the most frequently used strategy is the covalent conjugation of the primary amine in the antibody or antibody fragment to exofacial carboxyl groups of preformed nanocarriers in the presence of carbodiimide-based cross-linking agents. Carboxylic acid groups located on the side chain of glutamic acid and aspartic acid residues, and at the carboxyl terminus, are mainly involved in the covalent linkage. Usually, this conjugation process is carried out as a two-step coupling procedure. The first step involves activating the free carboxyl group of the linker lipid incorporated in the nanocarrier with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide, which reacts with the carboxyl to form an amine reactive intermediate (O-acylisourea). The produced O-acylisourea can be easily displaced by the nucleophilic attack from the primary amino groups in the reaction mixture. However, this intermediate is unstable and hydrolyzes in aqueous solutions. In order to prevent the hydrolysis of the intermediate, sulfo-NHS (N-hydroxysulfosuccinimide) is added to EDC to produce a significantly more stable and more soluble active intermediate (NHS ester). In the second step, the antibodies covalently conjugate through the N-terminus to the lipids by the displacement of sulfo-NHS groups through the formation of an amide bond. The carbodiimide/sulfo-NHS coupling reactions are highly selective and highly efficient. Most importantly, the biological activity of the conjugated protein or peptide is preserved. This reaction is depicted in Fig. (**2**) [32-36].

The sugar moiety of liposomes can also be used in the non-covalent modification of liposomes (reaction depicted by Fig. **3**). The glycosphingolipids incorporated into the liposomal membrane can be used in this conjugation. The polysaccharides are first oxidized at the carbohydrate sites
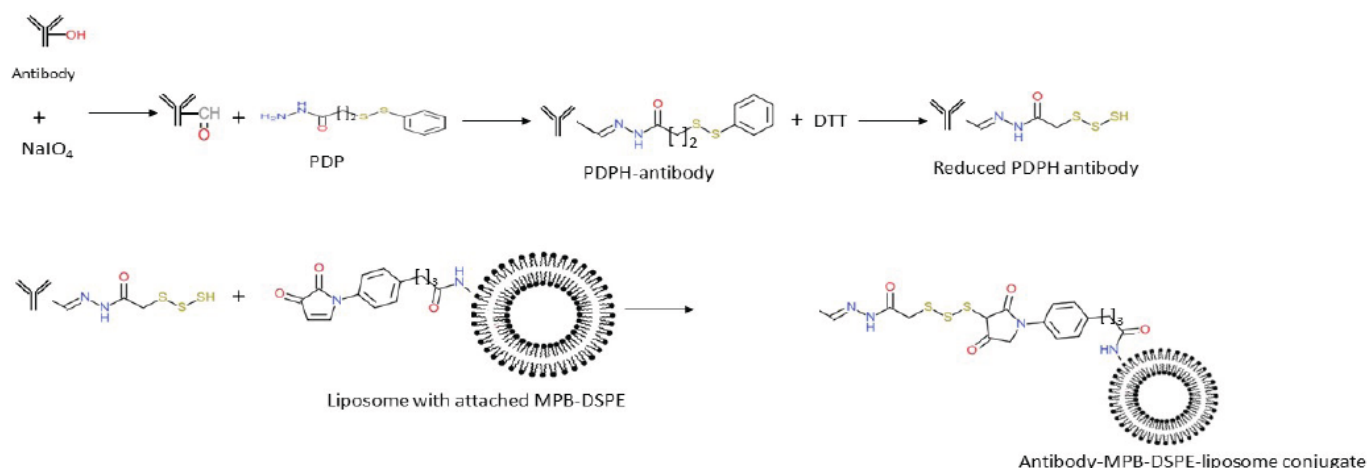
Fig. (3). Antibody conjugation to liposomes *via* hydroxyl groups associated with the carbohydrate in the Fc region of the antibody (adapted from [29]). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

with sodium meta-periodate to generate an aldehyde group. A cross-linking reagent, 3-(2-Pyridyldithio) propionyl hydrazide (PDPH), containing a hydrazide functional group can then be used to conjugate these aldehydes to the amine group of the antibody and, in turn, conjugate the antibody to the drug carrier [37]. The same reaction can also be utilized in a reverse scheme where the carbohydrate groups on antibodies can be oxidized to form aldehydes, and a subsequent Schiff base reaction can be carried out with the primary amine groups conjugated to the carrier [38-42]. Since the carbohydrate moiety is found predominantly on the crystallizable fragment (Fc) region of the antibody, conjugation can be achieved through the site-directed modification of the carbohydrate away from the antigen-binding site. Puertas *et al.* [43] showed that direct conjugation through antibody carbohydrate chains resulted in the highest retention of the antigen-binding activity over the more reactive amino group attachment [44].

2.1.2. Conjugation by Thiol Group

The sulfhydryl group plays a key role as a targeting group; it exists as a cysteine bridge in proteins and reagents such as Traut's reagent (2-iminothiolane), succinimidyl (acetylthio)acetate (SATA), and sulfosuccinimidyl 6-[3-(2-pyridyldithio)propionamido]hexanoate (Sulfo-LC-SPDP). These reagents are used as thiolate cross-linkers to enable the introduction of multiple sulfhydryl groups *via* reactive amino groups [45-48]. Sulfhydryl groups contain 14 interchain disulfide bonds in the hinge region and 12 intrachain disulfide bonds associated with 12 individual domains of the IGg. Out of these disulfide bonds, the interchain disulfide bonds are more susceptible to reduction than intrachain disulfide bonds and often present potential conjugation sites [49, 50].

Generally, the reduction of SATA and SPDP *via* hydroxyl amine, the decrease of Sulfo-LC-SPDP *via* DTT, or the hydrolysis of Traut's reagent generates up to eight thiol groups which become available for conjugating drug molecules. However, the thiolation of antibodies by Traut's reagent (2-iminothiolane) is the most popular approach used by researchers. In this method, the cyclic thioimidate reacts with primary amines (-NH$_2$), thereby opening the ring structure to introduce the sulfhydryl (-SH) groups while maintaining

charge properties similar to the original amine group. Once an amine on a protein is modified with 2-iminothiolane, the terminal thiol can be recyclized by attacking the amidine carbon. This can then rearrange into an iminothiolane derivative, which effectively ties up the thiol. Since there is a chance of losing substantial amounts of the available thiol in the recyclization process, the conjugation should be carried out immediately to avoid a significant loss of activity [51, 52].

Most of the sulfhydryl groups are highly reactive and can be easily oxidized to form disulfide crosslinks. For example, when SPDP is used for thiolation, the NHS ester end of SPDP reacts with amine groups to form an amide linkage, while the 2-pyridyldithiol group at the other end can react with sulfhydryl residues to form a disulfide linkage [53]. Since this linkage is highly reactive, the reactions need to be carried out in an oxygen-free environment and in the presence of ethylenediamine tetra-acetic acid (EDTA) to reduce the disulfide group and release the pyridine-2-thione leaving group forming the more stable and less-reactive disulfide group. The terminal (–SH) group can then be used to conjugate with any crosslinking agent [54].

Thiolation using the crosslinking agent SATA is another approach that has garnered considerable attention among researchers (reaction depicted by Fig. **4**). The active NHS ester end of SATA reacts with amino groups in proteins to form a stable amide linkage. The introduced sulfhydryl group will be in a protected state and yield a free sulfhydryl group upon incubation with hydroxylamine. Since the conjugation of thiolated antibodies to liposomes requires the liposomes to incorporate thiol derivatives, the sulfhydryl-reactive groups containing chemicals like maleimide [55], iodoacetyl group [56] or 2-pyridyldithiol groups [57] can be used to link the thiol group to the lipid and thus act as an anchor in the antibody-drug carrier conjugation.

Finally, the use of maleimides to conjugate antibodies to drug carriers is one of the most commonly used methods. The thiolated antibodies are conjugated to the maleimide terminus of the maleimide group, which is attached to the distal end of PEG-conjugated lipids. However, maleimide-based antibody-drug conjugates have recently been found to
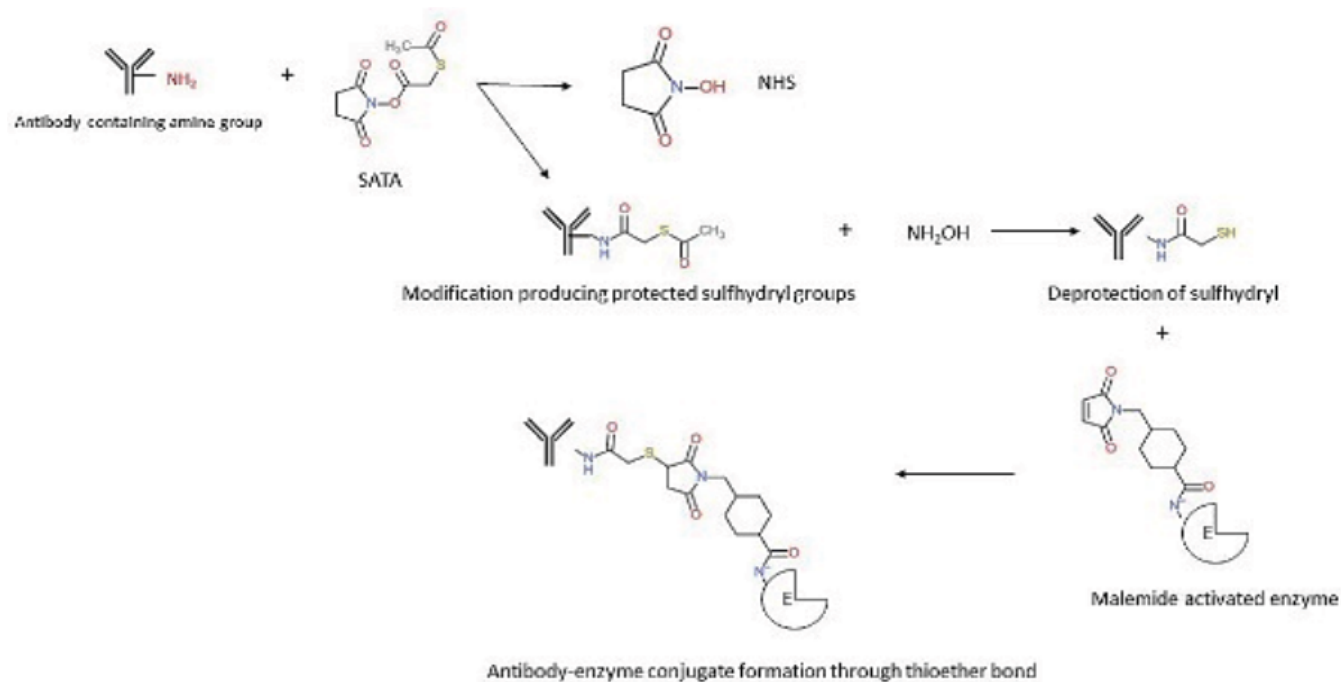
Fig. (4). Antibody-enzyme conjugation through thioether bond (adapted from (28)). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

be unstable in the circulatory system [58]. Succinimide or maleimide hydrolysis is a promising method to bypass this problem; because once hydrolyzed, the antibody-drug conjugates are no longer subject to the elimination reactions of maleimides through retro-Michael reactions which improve the stability, exposure, and efficacy of maleimide-based antibody-drug conjugates [58-60]. Several studies have investigated the effectiveness of the thiolation method to synthesize immunoliposomes. Huwyler *et al.* [61] developed immunoliposomes carrying [3H]daunomycin and mediated by the thiolation of OX26 monoclonal antibodies. Streptavidin [62], trastuzumab, cetuximab [63], and several other monoclonal antibodies have been used in the preparation of immunoliposomes by the thiolation method. Ojima *et al.* [64] used another approach whereby taxoid−antibody immunoconjugates were synthesized by forming a new disulfide bond between the drugs and the antibodies. In this study, taxoid bearing a free thiol group was conjugated to the pyridyldithio groups of the modified anti-EGFR antibodies through a disulfide−thiol exchange reaction. These conjugates were shown to possess remarkable target-specific antitumor activity against EGFR-expressing A431 tumor xenografts in immune-deficient mice. Later Nunes *et al.* [65], developed a potent and highly stable antibody-drug conjugate (ADC) through the reduction and re-bridging of the sulfide by TCEP. The antibodies conjugated to the liposomes either through the conjugation of the polar head group of the phospholipid component or through the conjugation of antibodies to the distal end of the PEG chains on the PEG-lipid components of the liposomes. To enhance the antigen-binding capacity, it is preferable that the antigen-binding sites (the two Fab domains) of the antibody be oriented upward and away from the surface of the nanocarrier. In order to increase the efficiency of immunoliposomes, Zalipsky *et al.* [66] incorporated maleimidophenyl butyrate-PE (MPB-PE) to PEGylated

liposomes. However, the synthesized immunoliposomes resisted the remote drug loading of the anticancer drug doxorubicin (DOX), and an increased rate of drug leakage was observed. Later, Torchilin *et al.* [67] utilized a different approach to extend the circulation time of immunoliposomes. The proposed method relied on N-glutaryl-PE (NGPE) for the preparation of IgG conjugates with PEG-grafted liposomes, but the high molecular weight mPEG chains grafted onto the surface next to IgG residues inhibited their ability to interact with the intended target antigens. To the group concluded that replacing the inert methoxy group at the end of the PEG chain with functional groups like amide, sec-amine, chlorotriazine, succinate *etc.*, will increase the conjugation rate of liposomes to biologically active ligands [39, 43, 57].

2.2. Strategies to Conjugate Antibody Fragments to Liposomes

Currently, the use of antibody fragments is preferable to the use of whole antibodies as a targeting moiety. The main advantage of this strategy is avoiding the risk of the inactivation of the antibody during surface functionalization [68]. The large sizes of the full-length antibodies limit the number of antibody molecules that can be accommodated/conjugated on the surface of the carrier molecules, and its complicated structure (with heavy and light chains) tends to affect its site-specific conjugation to nanoparticles. Consequently, significant research is currently focused on the use of smaller functional antigen-binding fragments of antibodies, the advantages of this include: avoiding the possibility of initiating an immune response, higher surface loading due to the reduction in crowding with minimal perturbation to the shape, size, and the functionality of both the nanoparticle and the antibody fragment itself, and the efficient delivery of the drug at the targeted site.

There are different techniques to generate antibody fragments; for instance, the treatment of antibodies with proteolytic enzymes can produce antigen-binding fragments like Fab, Fab′, F(ab′)₂. The Fab fragments comprise the constant and variable domains of immunoglobulins, linked by a single disulfide bond present at the c-terminus. The most commonly used scFvs, are readily generated using recombinant antibody technology. The scFv fragments contain the entire antigen-binding site of an antibody; they are formed by connecting the variable heavy and light chain domains with a short peptide linker [69-72].

The modification of the antibody fragments at the amine, carboxyl and the thiol group has been attempted using the same methods used in the modification of antibody conjugates. The amino acids of the lysine, cysteine, and glutamic/aspartic acid residues are the most common sites for modification. Initially, lysine was a popular target for modification as it could readily be conjugated, but the abundance of this amino acid on the surface of many proteins resulted in random functionalization and a heterogeneous mixture of antibody fragment products post-conjugation. With respect to cysteine, one or more additional cysteine residues are attached to the C-terminus of scFv fragments. This allows for site-directed conjugation with the reactive sulfhydryl groups located opposite to the antigen-binding sites and is therefore similar to the conjugation of Fab' fragments.

The conjugation of scFv' fragments does not interfere with target cell recognition, as the tumor penetration capacity appears to be higher than that of Fab fragments. Currently, site-selective methods which exploit the natural structure of antibody fragments, such as the hinge thiols of Fab′ fragments, or utilize amino acids incorporated through site-directed mutagenesis, have been successfully employed for the production of homogenous conjugates. Pastorino *et al.* [73] showed that Fab' type II immunoliposomes have approximately a two-fold reduced immunogenicity and longer circulation times when compared with IgG type II immunoliposomes. In another study, Qian *et al.* [74] successfully used scFvs to target CD44v6 (a transmembrane glycoprotein overexpressed on pancreatic adenocarcinoma cells) using arsenic loaded polymeric nanoparticles. Moreover, Hung *et al.* [75] conjugated scFvs to the surface of iron oxide nanoparticles pre-loaded with β-cyclodextrin encapsulated docetaxel to create drug-loaded, endoglin targeted SPIONs.

3. STIMULI-RESPONSIVE IMMUNOLIPOSOMES

Generally, there are two ways by which drugs can enter the targeted cells: through the selective uptake of liposomes by endocytosis (pinocytosis or receptor mediated endocytosis) or the release of the liposome payload near the targeted cells. Therefore, developing immunoliposomes capable of releasing the encapsulated drug in response to a stimulus provides a novel strategy in the assembly of smart multifunctional immunoliposomes. Generally, liposomal carrier systems triggered release is based on the principle of membrane destabilization from local defects within bilayer membranes to effect release of liposome-entrapped drugs. The unique features of the tumor microenvironement can serve as an internal trigger, such as reduced pH, temperature and altered

enzymes levels, or the stimulus can be externally applied, such as magnetic fields, light, heat or ultrasound [76].

3.1. pH-responsive Immunoliposomes

The standard extracellular physiological pH in healthy body tissues and blood is around 7.4; however, in tumors, the extracellular pH values are usually around 6.5 [77, 78]. This phenomenon can be explained by the Warburg effect; which states that cancer cells favor the anaerobic glycolytic pathway, even in the presence of sufficient oxygen, rather than oxidative phosphorylation for cellular energy generation. A side effect of opting for the glycolytic path is the generation of lactic acid, ergo the acidic nature of tumor microenvironments [79]. This pH difference between neoplastic and normal tissues has stimulated researchers to develop pH-responsive liposomes for anticancer therapeutics. These liposomes are stable at physiological pH but the alteration of pH at tumor sites causes instabilities in the lipid bilayer resulting in the liposomes releasing their contents [80, 81].

Apte *et al.* [82] conducted an experiment in which they made multifunctional PEGylated DOX liposomes by conjugating the cell-penetrating peptide, TATp to PEG 1000-PE, the pH-sensitive polymer, PEG 2000-Hz-PE, with a pH-sensitive hydrazone (Hz) bond was used to shield the peptide in the body and expose it only at the acidic tumor cell surface, as well as the anti-nucleosome monoclonal antibody 2C5. The uptake and cytotoxicity of these multifunctional immunoliposomes were tested in both *in vitro* and *in vivo* settings. The results showed an enhanced cytotoxicity in drug-resistant cells, compared with non-modified liposomes. Additionally, in comparison with the non-modified liposomes, after the intravenous injection of these multifunctional immunoliposomes into mice with tumor xenografts, a significant reduction in tumor growth and an enhanced therapeutic efficacy of the drug was observed. Li *et al.* [83] evaluated the modification of 1,5-Dihexadecyl N,N-diglutamyl-lysyl-L-glutamate (GGLG) liposomes with a Fab′ fragment of an ErbB2 antibody to the terminus of PEG-lipid (Fab′-GGLG liposomes). The conjugation of Fab′ fragments did not affect the antibody activity, drug encapsulation efficiency, liposome stability, and pH-sensitivity. However, the binding affinity of Fab′-GGLG liposomes to human breast cancer cells with high ErbB2 expression (HCC1954) was increased by 10-fold in comparison to GGLG liposomes.

3.2. Enzyme-responsive Immunoliposomes

Certain pathological conditions, such as infections, inflammations and cancer cause an elevation in the concentration of several extracellular and intracellular enzymes. This aberration can be used to trigger the release of the liposomal payload. The most common examples of up-regulated enzymes in cancerous tissues are secreted phospholipase A2 (sPLA₂) and proteases, namely cathepsin B [84]. The upregulation of sPLA₂ has been associated with prostate, breast, and pancreatic cancers. sPLA₂ is a calcium ion-dependent esterase. It hydrolyzes phospholipids at the *sn*2-fatty acyl ester position, producing free fatty acids and lysophospholipids. Therefore, the incorporation of sPLA₂ into liposomes releases their payloads by disrupting the integrity of the lipid bilayer. The first developed secretory phospholi-

pase A$_2$ (sPLA$_2$) responsive liposomal cisplatin formulation (LiPlaCis®) is currently undergoing clinical evaluation. Østrem *et al.* [85] tested the therapeutic efficiency of sPLA$_2$ responsive liposomes in *in vitro* and *in vivo* settings. The *in vitro* release studies of oxaliplatin from spLA$_2$ sensitive liposomes revealed enzyme-specific drug release, as well as efficient growth inhibition compared to that of conventional liposomes. However, in the *in vivo* experiment, three days after the treatment, all mice having received the sPLA$_2$ sensitive liposome formulation were euthanized due to severe systemic toxicity. Similarly, Pourhassan *et al.* [86] conducted a study to evaluate the therapeutic potential and safety of LiPlaCis® *in vivo*. Nude mice bearing sPLA$_2$-deficient FaDu squamous carcinoma and sPLA$_2$-expressing Colo205 colorectal adenocarcinoma were injected intravenously with the liposomal formulation. The results of the experiment showed that tumor growth was decreased, but there was a weak response to sPLA$_2$-sensitive liposomes compared to non-sensitive liposomes. Also, the mice did not show a statistically significant, more prolonged survival. Overall, these results indicated that although liposomal carriers can improve the antitumor efficacy of anticancer drugs, some issues still need to be addressed regarding the therapeutic window and safety of sPLA$_2$-triggered formulations.

The second enzyme of interest is Cathepsin B, a lysosomal protease of the papain family. This enzyme is overexpressed in several tumor tissues, including brain, breast, colon, prostate, and lung cancers. The enzyme enhances extracellular matrix degradation through interaction with cystatins and annexin II tetramer [84, 87]. Matarrese *et al.* [88] investigated the effects of cathepsin B inhibitors (*e.g.* CA-074 and CA-074Me) in modulating the invasiveness of metastatic melanoma cells. Their *in vitro* studies showed that the inhibition of cathepsin B significantly inhibited cell invasiveness and metastasis. Moreover, *in vivo* results showed that the cathepsin B inhibitor CA-074 significantly reduced human melanoma growth.

Other studies focusing on enzyme-sensitive immunoliposomes include the work of Fonseca *et al.* [89] who compared the cell-associated enzymatic activity of immunoenzymosomes (tumor-targeted immunoliposomes bearing enzymes on their surface) with that of the corresponding antibody-enzyme conjugates. Immunoenzymosomes were able to induce a 15-fold increase in cell-associated enzymatic activity compared to that obtained by the corresponding antibody-enzyme conjugate. In addition, immunoenzymosomes were able to inhibit cell growth in the tumor cells to which immunoenzymosomes were bound, as well as a large number of neighboring cells.

3.3. Redox-responsive Immunoliposomes

Redox responsive liposomes have gained ~~considerably~~ attention in cancer and gene therapy research. In biological systems, different redox conditions exist between intracellular and extracellular compartments. Redox-responsive polymers contain reducible disulfide bonds that remain intact while the drug carrier is circulating in the oxidizing extracellular environment but are readily cleaved when the carrier is introduced into the intracellular reducing environment, triggering the cytosolic release of the drugs [90]. This reducibil-

ity of disulfide bonds can be exploited to design redox-sensitive liposomes for intracellular delivery of drugs or functional genes in targeted tumors and other tissues. A good example of such a system are liposomes responsive to the levels of Glutathione (GSH). GSH is a reducing agent abundant in cells, particularly the cytosol and the nucleus. The intracellular levels of GSH are considerably higher than those in the extracellular environment. Similarly, the concentration of GSH in tumor tissues, and the cytosol of tumor cells, is at least four times higher than that in normal tissues, making tumors a reducing environment. This high redox potential difference can break the reducible disulfide bonds, destabilize the liposomal system, and release its payload [78, 81].

Goldenbogen *et al.* [91] conducted an experiment in which the uptake of antibody-conjugated reduction-sensitive-DOX-liposomes was tested against a breast cancer cell line overexpressing the HER2 receptor. Tumor cells treated with reduction-sensitive liposomes with antibodies showed specific uptake and higher release than all other combinations.

3.4. Temperature-responsive Immunoliposomes

Inflamed pathological sites and tumors are characterized by higher temperatures compared to healthy tissues. The difference in temperature between the tumor site and normal tissues can act as an internal trigger for functionalized drug carriers. Another temperature-responsive strategy utilizes the fact that hyperthermia is associated with increased tumor permeability to enhance drug uptake. In this technique, the temperature of the tumor site is manipulated externally in such a way to incite increased blood flow and vascular pore size in the area, which in turn results in the improved internalization of the drug-loaded liposomes. In general, thermosensitive nanocarriers are designed to retain their payloads around the physiological temperature of 37°C, and release their payloads rapidly when the temperature is increased above 40-45°C. Temperature-sensitive liposomes are usually prepared using thermo-sensitive lipids or polymers with a low critical solution temperature (the temperature below which the components of a mixture are miscible for all compositions). The most commonly used thermo-sensitive lipid is dipalmitoylphosphatidylcholine (DPPC), and its polymeric equivalent poly N-isopropylacrylamide (PNIPAM). The commercial anticancer liposomal formulation Thermodox® (Celsion, Lawrenceville, NJ, USA) is an example of temperature-sensitive liposomes. This formulation is in Phase III clinical trials for the treatment of hepatocellular carcinoma and Phase II trials for breast cancer and colorectal liver metastases [77, 81, 92].

The first heat-sensitive immunoliposome formulation was described by Sullivan *et al.* [93]. The cellular uptake of uridine encapsulating immunoliposomes was enhanced upon heating, with the maximal release and largest accumulation of uridine in target cells observed when the cell-liposome mixture was heated to 41℃. Gaber *et al.* [94] synthesized DOX-thermosensitive liposomes conjugated to Fab fragments of HER2/neu. The cellular uptake of HER2-immunoliposomes by breast cancer (SK-BR-3) cells was shown to be eight times higher than that of conventional liposomes. The toxicity of targeted thermosensitive liposomes was similar to

that of free DOX; however, heating the cells, after incubation with liposomes, to 42°C did not enhance the cytotoxicity of targeted immunoliposomes. Wang *et al.* [95] investigated the use of hyperthermia as a triggering mechanism. Tumor-homing peptide Cys-Arg-Glu-Lys-Ala (CREKA)-conjugated Lysolipid-thermosensitive immunoliposomes containing DOX (DOX-LTSL-CREKA) were synthesized and used to target clotted plasma proteins in the tumor vessels. The MCF-7/ADR cell line was used to investigate the *in vitro* cytotoxicity of DOX-LTSL-CREKA, with and without heating at 43°C, in comparison with free DOX. The cellular DOX level for DOX-LTSL-CREKA in the unheated treatment group, the heated before incubation treatment group, and the heated after incubation treatment group was about 1.7-, 3.1-, and 2.1-fold higher than that in the corresponding sterically stabilized DOX only liposomes. Moreover, in *in vivo* settings, the groups given DOX-LTSL-CREKA, with and without heating, showed significant inhibition of tumor growth compared with the DOX-LTSL and free DOX treatment groups.

3.5. Magnetic-responsive Immunoliposomes

Magnetic targeting is an alternative approach to induce cancer-specific hyperthermia, which in turn induces the release of the payload from the drug delivery system. Superparamagnetic iron oxide nanoparticles(SPIONs), have been extensively studied for simultaneous imaging and stimuli-responsive drug delivery. Su *et al.* [96] investigated the use of polymeric liposomes (PEG/RGD-MPLs), RGD peptide grafted OQPGA, and magnetic nanoparticles as a multifunctional platform for targeted drug delivery. Compared with conventional magnetic liposomes (MCLs), PEG/RGD-MPLs exhibited satisfactory size and zeta- potential stability and decreased magnetic nanoparticles leakage. *In vitro* results suggested that the PEG/RGD-MPLs exhibited more drug cellular uptake than non-RGD and non-magnetic nanoparticles in MCF-7 cells. Cytotoxicity assays revealed that PEG/RGD-MPLs showed lower *in vitro* cytotoxicity in human gastric GES-1 cells.

3.6. Light-responsive Immunoliposomes

The use of light irradiation for the activation/deactivation of biochemical processes has long been recognized as one of the most valuable tools in the biomedical field. Light irradiation has been used extensively in biomedical research because it is non-invasive nature and its parameters, *e.g.* intensity, wavelength and exposure duration, can be readily manipulated. Visible light, ultraviolet (UV) and near- infrared (NIR) light have extensive clinical applications, however light in the NIR region is the most desirable form of light for tumor targeting since it penetrates deep into the tissues and is less damaging to the biological system than UV light. For the above-mentioned reasons, photodynamic therapy has become a well-established treatment tool of superficial tumors where photosensitizing agents, such as chlorines, which generate radical oxygen species are used to eradicate malignant tumors. Various light-sensitive lipids/polymers are being used in drug delivery applications. Whether the carriers are made out of lipids or polymeric materials, to be light-responsive, they must contain a chromophore in their architecture. Chromophores are moieties that undergo structural

and conformational changes, *e.g.*, photoisomerization, photodimerization or photocleavage, upon exposure to light, with subsequent disruption of the liposome and release of the drug [78, 81, 90].

Li *et al.* [97] developed a human epidermal growth factor receptor-2 (HER2) antibody-conjugated drug delivery system, and used near-infrared (NIR) light to release the contents of the liposomes. The combination of targeted liposomes with NIR irradiation increased the accumulation in the tumors with the positive expression of HER2 by two-fold; this increased accumulation leads to significant antitumor activity *in vivo* with the tumor inhibition efficiency up to 92.7%.

4. CHALLENGES FACING THE CLINICAL TRANS-LATION OF IMMUNOLIPOSOMES

Despite the substantial preclinical research in the field of stimuli-responsive immunoliposomes for the treatment of cancer, there has been limited progression towards clinical application. Several important factors exist that may be responsible for this lack of clinical development of immunoliposomes in general, and stimuli-sensitive immunoliposomes in particular past the preclinical stage.

The unique physiology of solid tumors entails that they have increased vascular permeability and reduced lymphatic drainage, which leads to the enhanced permeability and retention (EPR) effect. Drug delivery, using both targeted and nontargeted liposomal formulations, relies on the EPR effect. This effect, however, is highly variable across different tumor types, and even regions of the tumor, as the permeability of blood vessels may vary throughout a single tumor. Moreover, the results obtained from preclinical trials using animal models may not be representative of clinical tumors in several key aspects [98].

Other important aspects include the shape, size, and surface characteristics of the synthesized immunoliposomes play an important role in the pharmacokinetics and biodistribution of these targeted vehicles. Developing an increased understanding of the interactions between the immunoliposomes and serum proteins in humans, as well as the fate of these targeted drug delivery vehicles is important in advancing their clinical translation [98, 99].

With regard to imparting immunoliposomes with stimuli-sensitive properties, this approach can greatly improve the therapeutic benefits of the administered drug; however, the method itself is rather complex which adds new difficulties in predicting the behavior of the nanocarrier. Furthermore, additional capabilities mean additional procedural and purification steps which increases cost. Internal stimuli are particularly difficult to control as they vary from one bodily location to another, from tumor to tumor, as well as from one patient to another. External triggers, on the other hand, offer more flexibility in design; however, the biocompatibility of the external stimuli, level of tissue damage, depth of penetration, exposure time, and the availability of external source location may restrict their applications. Finally, *in vitro* and *in vivo* preparation and testing of ligand-directed and stimuli-responsive liposomes is performed using small volumes, mostly milliliter quantities. However, upscaling the produc-

Table 3. **Summary of studies invovling stimuli-responsive immunoliposomes.**

Author (year)	Payload	Target	Stimulus	Status	Tumor Model, Animal Strain	Reference
Biswas *et al.* (2011)	Carboxyfluorescein Con-A, avidin	Anti-2C5, 2G4 mAbs	pH	*In vitro*	Cervical carcinoma HeLa cells and breast cancer MCF-7 cells	[100]
Apte *et al.* (2014)	DOX	Anti-2C5 mAb, TAT	pH	*In vitro, in vivo*	Human ovarian cancer cells SKOV-3, nude mice	[82]
Koren *et al.* (2012)	DOX	TATp, anti-2C5 mAb	pH	*In vitro, in vivo*	B16-F10, HeLa and MCF-7, male Balb/C mice	[101]
Li *et al.* (2017)	DOX	Anti-ErB2 Fab'	pH	*In vitro, in vivo*	HCC1954 cells and MDA-MB-468 cells, female BALB/c nu/nu mice	[83]
Fonseca *et al.* (2003)	Glucuronide prodrug of doxorubicin (DOX-GA3)	Anti-Ep-CAM specific mono-clonal antibody (MAb) 323/A3	Enzyme	*In vitro*	Human ovarian carci-noma cells OVCAR-3	[89]
Østrem *et al.* (2017)	Oxaliplatin	-	Enzyme	*In vitro, in vivo*	$sPLA_2$ secreting mam-mary carcinoma cells MT-3, nude NMRI mice	[85]
Pourhassan *et al.* (2017)	Cisplatin, Calcein	-	Enzyme	*In vitro, in vivo*	HT-29 human colon carcinoma HT-29 and Colo205, nude NMRI mice	[86]
Matarrese *et al.* (2010)	Cathepsin inhibitors (CA-074, CA-074Me)	-	Enzyme	*In vitro, in vivo*	Human melanoma cells HLA-A2 1B6, M20, CD-1 male nude (nu/nu) mice	[88]
Vingerhoeds *et al.* (1996)	Daunorubicin-glucuronide	Anti-OV-TL3 F (ab') of mAb	Enzyme	*In vitro*	Human ovarian carci-noma SKOV3	[102]
Houba *et al.* (2001)	DOX-GA3	Anti-EpCAM antibody (323/A3)	Enzyme	*In vitro, in vivo*	Human ovarian cancer cell line Fma, nude mice	[103]
Goldenbogen *et al.* (2011)	DOX	Anti-p185HER2	Redox	*In vitro*	Breast cancer BT-474 cells	[91]
Sullivan *et al.* (1986)	Uridine	Anti-H2K^{k}mAb	Temperature	*In vitro*	Mouse lymphoma MDR4 cells	[93]
Smith *et al.* (2011)	DOX	Anti-HER2/neu affibody	Temperature	*In vitro*	Breast cancer cells SK-BR-3	[104]
Wang *et al.* (2015)	DOX	CREKA peptide	Temperature	*In vitro, in vivo*	Multidrug resistant hu-man breast cancer cells MCF-7/ADR	[95]
Shin *et al.* (2016)	Gemcitabine	Anti-HER2	Temperature	*In vitro*	Breast cancer cells, SK-BR-3	[105]
Gaber *et al.* (2000)	DOX	Anti-HER2/neu F (ab') of mAb	Temperature	*In vitro*	Breast cancer cells SK-BR-3	[94]

Table 3. contd…

Author (year)	Payload	Target	Stimulus	Status	Tumor Model, Animal Strain	Reference
Li *et al.* (2015)	DOX and hollow gold nanospheres (HAuNS)	Anti-HER2	Light (NIR)	*In vitro, in vivo*	Human ovarian carcinoma SKOV3, human breast carcinoma BT474 and SK-Br-3, and human lung adenocarcinoma A549 cells, nude mice	[97]
Khosroshahi *et al.* (2015)	Magneto-plasmonic nano shells	Anti-HER2 mAb	Light (laser)	*In vitro*	Human breast carcinoma BT-474 and human lung carcinoma Calu-6 cells	[106]
Li *et al.* (2015)	DOX	Anti-CD20	Light (UV)	*In vivo, ex vivo*	Non-Hodgkin's lymphoma cells	[107]
Su *et al.* (2012)	Epidoxorubicin and Fe_3O_4	RGD peptide	Magnetic	*In vitro*	Breast cancer MCF-7 cells and GES-1 cells	[96]
Kikumori *et al.* (2008)	Magnetite nanoparticles	Anti-HER2	Magnetic	*In vitro, in vivo*	BT474 human breast cancer cells and BT474 human breast cancer cells, Female Balb/c nu/nu mice	[108]

Abbreviations: DOX, Doxorubicin; NIR, Near-infrared; UV, Ultraviolet.

tion of liposomes to meet the quantities needed for clinical use can be challenging, since existing laboratory-based liposome production methods are generally not suitable to scale-up beyond the milliliter scale [76, 98, 99].

Considering the above-mentioned issues, the clinical translation of multifunctional drug delivery systems represents a new area in the field of drug delivery which, if the requirements are met, could become an important part of the personalized therapy/medicine in upcoming years.

CONCLUSION

The functionalization of liposomes with monoclonal antibodies or antibody fragments to generate immunoliposomes has emerged as a promising strategy for targeted delivery. In this review, we addressed the different functionalization strategies that can be used to conjugate monoclonal antibodies and their fragments to liposomal surfaces. Moreover, we provided an overview of the work conducted by several research groups focusing on stimuli-responsive immunoliposomes. The findings of these research groups showed promise, particularly in the *in vivo* settings using cancer xenograft models. Consequently, immunoliposomes are promising formulations that may be available in clinics after clinical trials prove their safety and efficacy, and after scaling issues are resolved.

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The authors declare no conflict of interest, financial or otherwise.

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