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Review Article

Immunoliposomes: A review on functionalization strategies and targets for drug delivery

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ABSTRACT

Nanoparticles, especially liposomes, have gained prominence in the field of drug delivery for the treatment of human diseases, particularly cancer; they provide several advantages, including controlled drug release, protection of the drug against degradation, improved pharmacokinetics, long circulation, and passive targeting to tumors and inflammatory sites due to the enhanced permeability and retention effect. The functionalization of liposomes with monoclonal antibodies or antibody fragments to generate immunoliposomes has emerged as a promising strategy for targeted delivery to and uptake by cells overexpressing the antigens to these antibodies, with a consequent reduction in side effects. In this review, we address functionalization strategies for the non-covalent and covalent attachment of monoclonal antibodies and their fragments to liposomal surfaces. The main reaction occurs between the sulfhydryl groups of thiolated antibodies and maleimide-containing liposomes. Furthermore, we explore the main targeting possibilities with these ligands for the treatment of a variety of pathologies, including HER2- and EGFR-positive cancers, inflammatory and cardiovascular diseases, infectious diseases, and autoimmune and neurodegenerative diseases, which have not previously been reviewed together. Overall, many studies have shown selective delivery of immunoliposomes to target cells, with promising *in vivo* results, particularly for cancer treatment. Although clinical trials have been conducted, immunoliposomes have not yet received clinical approval. However, immunoliposomes are promising formulations that are expected to become available for therapeutic use after clinical trials prove their safety and efficacy, and after scaling issues are resolved.

Contents

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1. Introduction

Nanoparticle drug delivery systems have potential for the treatment of a variety of diseases, and their use in medicine is increasing rapidly [1,2]. Approximately 40% of small molecule drugs for cancer treatment, for instance, have low aqueous solubility, so the development of drug delivery systems capable of encapsulating these drugs, enhancing their aqueous solubility, and delivering them to target sites is highly desirable [3]. Within this context, liposomes, which are lipid vesicles containing an aqueous core enclosed by a biocompatible lipid membrane, can be useful to encapsulate both hydrophilic and lipophilic drugs [4,5]. These systems are able to selectively deliver the drug to a target site, improve its pharmacokinetics and pharmacological effects, and avoid local irritation and drug toxicity [5]. Due to their success, many examples of liposomes are currently available in the clinic. The first liposomal drug formulation approved by the Food and Drug Administration (FDA) was Doxil® in 1995 [6,7]. Since then a variety of liposomal drug delivery products have been developed and are now available on the market, such as DaunoXome®, DepoCyt®, Myocet®, Lipodox®, and Marqibo® [8].

Different targeting strategies can be employed with these versatile nanocarriers to optimize selective delivery. For instance, cancer tissues and inflammatory sites have a leaky vasculature that enhances the accumulation of liposomes compared to free drug, in a phenomenon named the enhanced permeability and retention (EPR) effect, which results in passive targeting of liposomes with a mean diameter of 100–200 nm [1,4,9,10]. Additionally, liposome surfaces can be coated with polyethylene glycol (PEG), which reduces interactions with blood components and binding to plasma proteins, thereby preventing interactions with opsonins and reducing capture by the reticular endothelial system. This mechanism is related to shielding the liposome surface, hydrophilicity, and repulsion between the coated liposome and blood components, resulting in longer circulation time [11].

Active targeting is another possible approach, which is accomplished by coupling targeting ligands to the surface of liposomes, such as monoclonal antibodies or their fragments (fragment antigen-binding (Fab) and single-chain variable fragment (scFv)), proteins, peptides, or carbohydrates. Thus, liposomes can be selectively taken up by cells that overexpress the receptor for the moiety, which has the potential to improve therapeutic outcomes by increasing efficacy and reducing off-target toxicity [1,12]. Among the different moieties that can be covalently or non-covalently attached to the liposome surface, antibodies and antibody fragments are the most widely employed, producing immunoliposomes [13].

A monoclonal antibody is formed by 2 heavy (H) chains and 2 light (L) chains, which are further divided into constant and variable domains (CH/CL and VH/VL, respectively), stabilized by disulfide bridges. They are able to selectively bind to a specific part of an antigen; thus, they represent a growing class of medicines available for targeted therapies for a variety of diseases [14]. Commonly, the antibody is attached to the distal end of a PEG chain in PEGylated liposomes, using a variety of methods addressed herein [15]. The correct orientation of the PEG-terminal antibody facilitates antigen recognition more effectively than the random orientation of antibodies attached to the liposomal bilayer of PEGylated immunoliposomes [16].

Immunoliposomes have been studied and reviewed, with a focus on functionalization strategies, comparisons with antibody–drug conjugates and immunotoxins, and applications for cancer therapy [17–19]. However, this field has undergone rapid advancement in recent years, with many studies reporting new functionalization methods and targets in not only cancer therapy but other diseases, which have not been previously reviewed. In this paper, we will address the main aspects related to the development of immunoliposomes using a variety of antibodies or antibody fragments for the treatment of cancer, inflammatory and cardiovascular diseases, infectious pathologies, and autoimmune and degenerative diseases. In doing so, we will review and discuss the different functionalization strategies and targeting approaches used both *in vitro* and *in vivo*, with a special focus on preclinical findings. In addition, we will highlight the immunoliposomal formulations that are currently in clinical development.

2. Monoclonal antibodies and their fragments

To put the functionalization of liposomes with antibodies into perspective, it is important to provide a brief overview of antibody origin, structure, and function. Antibodies are freely circulating molecules that perform two main functions in the immune system. First, they recognize and bind antigens, which requires great antibody diversity, due to the huge variety of antigens. Second, antibodies play an important role in eliminating antigens [20]. Antibodies belong to the class called globulins because of their globular structure and are, thus, immunoglobulins.

Monoclonal antibodies bind to a specific epitope and are generated *via* hybridoma technology, in which a B cell clone is immortalized by fusion with myeloma cells [20]. The B cells are fused with an immortalized myeloma cell line, then cultured *in vitro* to eliminate non-hybridized cells. The first cell culture contains a mixture of antibodies; for this reason, a sequence of cell isolations is performed to acquire a final isolate culture that produces a unique type of monoclonal antibody [21,22].

The origin of each clinical monoclonal antibody can be identified by the suffix in its name; for instance, murine antibodies end with –omab, chimeric mouse–human antibodies (the entire antigen-specific variable domain of a mouse antibody is added onto the constant domain of a human antibody) end with –ximab, humanized antibodies (the murine hypervariable region is added onto a human antibody framework) end with –zumab, and human antibodies end with –umab [23].

The structure of an antibody determines several features of its participation in the immune response. These features are important for their specificity and biological activity. Antibodies are divided into 3 main parts: 2 Fab portions that are responsible for antigen recognition and specificity, and the fragment crystalline (Fc) portion that binds to receptors on target cells and enables complement fixation, which triggers effector functions that eliminate the antigen (Fig. 1a). The Fabs are formed by the connection of the L and H chains. Furthermore, both the L and H chains are connected by disulfide bonds, and each chain is comprised of domains. The first domain is highly variable (the V_L or V_H domain) in terms of amino acid sequence from one antibody to another. The second and subsequent domains have little variation of amino acids, being

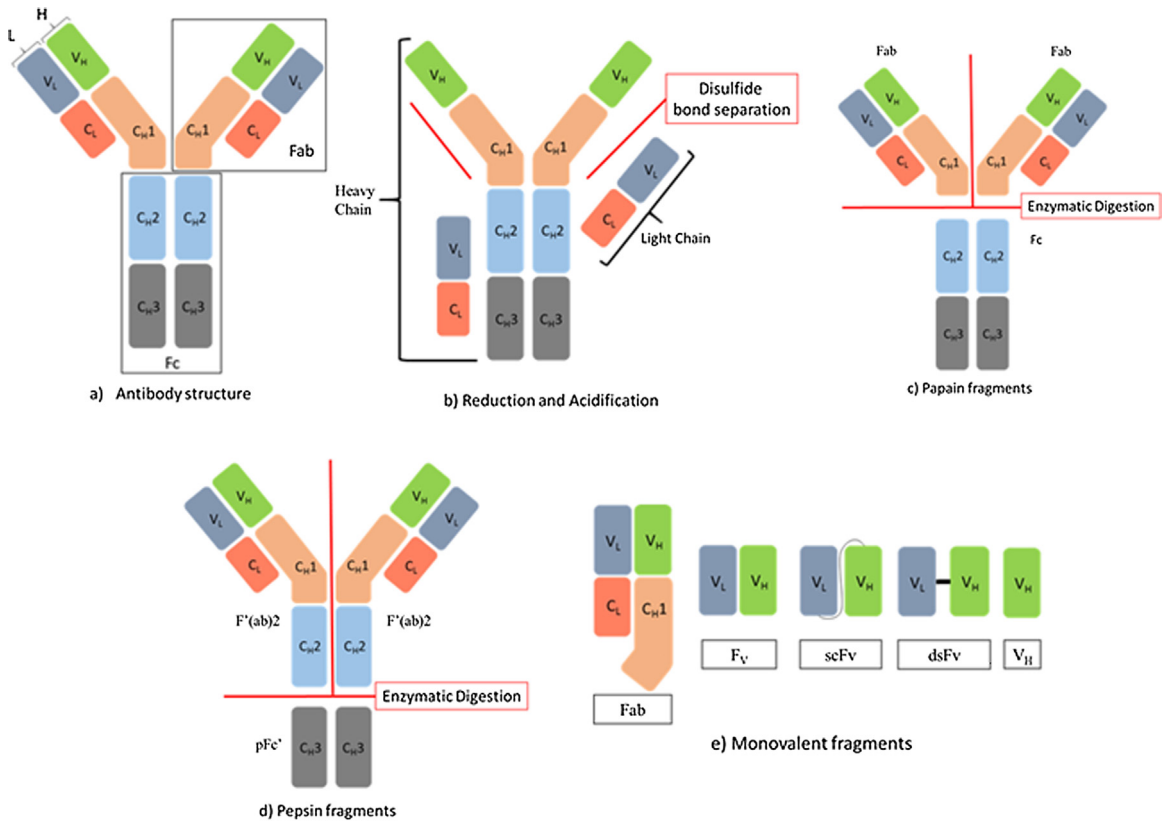

Fig. 1. (a) Layout of the antibody molecule. The structure consists of 4 peptide chains, 2 identical light chains (L), and 2 identical heavy chains (H). There are also 2 identical fragment antigen-binding regions constituted by a highly variable domain (V_L) and a constant domain (C_L), and the fragment crystalline constructed of 2 constant domains (C_H2 and C_H3). (b) Antibody reduction and acidification. (c) Enzymatic papain digestion. (d) Enzymatic pepsin digestion. (e) Monovalent antibody fragments.

designated C_L or CH1, CH2, and CH3 domains, as shown in Fig. 1 [20,23–28].

There are 5 classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM. The major differences between these classes of immunoglobulins are the components of their heavy chains, termed α (alpha), δ (delta), ε (epsilon), γ (gamma), and μ (micro), respectively. The light chains are designated by the Greek letters κ (kappa) and λ (lambda) [25]. Regarding the functionalization of liposomes with immunoglobulins, it is important to know that most studies use IgG as the main model. IgG is the predominant immunoglobulin in the blood, lymph fluid, cerebrospinal fluid, and peritoneal fluid. IgG is subdivided into 4 subclasses: IgG1, IgG2, IgG3, and IgG4. The differences between the subclasses influence their chemical properties and the resulting biological responses [27].

Liposomes are functionalized not only with whole antibodies, but also with isolated Fabs. The first fragmentations of antibodies resulted from enzymatic digestion, reduction, or changes in pH. As depicted in Fig. 1b, reduction and acidification can cause the separation of the L and H chains. Fabs and portions the of Fc can be isolated by papain enzymatic digestion (Fig. 1c). Moreover, antibodies can be further separated using pepsin enzymatic digestion of the C-terminal half of the Fc region. This results in the formation of 2 Fab regions attached to half of an Fc fragment, termed F(ab')2, and the fragment that corresponds to the C-terminal region of the Fc portion, termed pFc'(c) (Fig. 1d) [20,25]. As shown in Fig. 1e, other fragments include the variable domain, a single chain of the variable domain (scFv), a variable domain disulfide-stabilized, and the single variable heavy domain [21,26].

3. Strategies to conjugate monoclonal antibodies or their fragments to liposomes

A variety of strategies have been reported for the preparation of long-circulating liposomes functionalized with antibodies or their fragments. Most strategies involve conjugation of the antibodies to the distal ends of PEG chains on the PEG-lipid components of the liposomes, on which the inert methoxy group at the end of the PEG chain has been replaced with chemical groups useful for antibody conjugation [15]. Different methods have been developed for this purpose, including non-covalent and covalent, which are more commonly reported, approaches. Antibody derivatization and conjugation have been thoroughly reviewed [17]. Herein, we will briefly focus on the most frequently described methods and highlight some of the characterization results.

For covalent attachment, it must be taken into consideration that the antibody molecule contains functional chemical groups, such as amines and carboxylates, which are prone to modification for targeting purposes. Furthermore, the sulfhydryl group plays a key role as a targeting group, and has been extensively reported. Nonetheless, this group occurs infrequently in antibody molecules and must be generated by either the reduction of disulfide groups or through appropriate thiolation agents [17]. Thiolated antibodies containing sulfhydryl groups will then react with antibodies containing a chemically reactive molecule, like a lipid-containing maleimide, forming a thioether linkage (Fig. 2) [29–32]. On the other hand, sulfhydryl groups may undergo oxidation with consequent disulfide crosslink formation, which can be prevented by oxygen removal from buffers and the addition of ethylenediamine tetraacetic acid (EDTA) to chelate the metal ions that are able to catalyze these reactions [17].

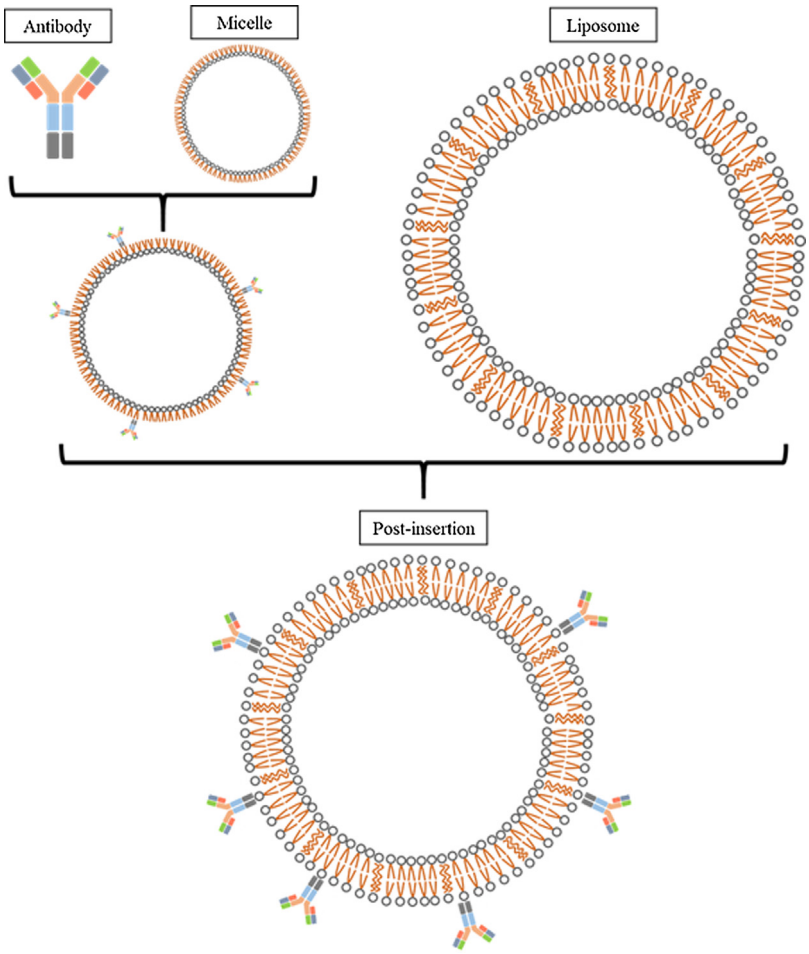

Fig. 2. Reaction between a thiolated antibody and sulfhydryl-containing liposome for immunoliposome formation.

The thiolation of antibodies employing Traut's reagent (2-iminothiolane) is one of the most popular strategies employed to date. The cyclic group 2-imidothioester can react with primary amines, thereby opening the ring structure and generating a free sulfhydryl [33]. A variety of papers have reported the use of Traut's reagent for thiolation for the further conjugation of several monoclonal antibodies, including trastuzumab [29,32,34,35], cetuximab [36,37], and others [38–41], onto liposomal surfaces through the distal reactive maleimide terminus of the PEGylated-phospholipid maleimide group. Overall, authors have reported high functionalization efficiencies, as shown by protein analysis of immunoliposome fractions, and the maintenance of the primary integrity of antibodies, as shown by polyacrylamide electrophoresis [32,35,37]. Fragments of monoclonal antibodies have been loaded onto liposomes using the same strategies. For instance, Gao and co-workers reported anti-epidermal growth factor receptor (EGFR) and anti-HER2 tyrosine kinase receptor (also known as ErbB2, c-erbB2 or HER2/neu) antibody Fab' conjugation to liposomes based on a thioether bond. They characterized the conjugates by the quantification of Fab' on the surface of the liposomes and evaluated the fragment integrity through electrophoresis [42–44]. Other reports of Fab' liposome functionalization involved targeting the amyloid-beta (Aβ) complex, for which the intact binding affinity was demonstrated. Additionally, the presence of 1 thiol group per antibody was confirmed, and electrophoresis under non-reducing conditions analysis demonstrated that intact antibody molecules were bound to the liposomes [45].

Alternative molecules have been reported for antibody thiolation prior to covalent linkage with liposomes through maleimide-containing lipids. Many studies employed 3-(2-pyridyldithiolpropionic acid)-*N*-hydroxysuccinimide ester (SPDP) to achieve this purpose. Some studies employed the lipid N-4-(*p*-Maleimidylphenylbutyryl) dipalmitoyl-L-cY-phosphatidylethanolamine (MPB-PE) for antibody linkage [31,46,47]. Alternatively, other lipids have been used, such as *N*-(6-maleimidocaproyloxy)-dipalmitoyl phosphatidylethanolethanolamine and poly(ethylene glycol)-α-distearoyl phosphatidylethanolamine-maleimide [48,49]. Interestingly, Zalba et al. prepared oxaliplatin-loaded liposomes targeted against EGFR for the treatment of colorectal cancer. For this purpose, the authors compared the functionalization of liposomes with the whole cetuximab antibody or the Fab' portion, which were both confirmed by electrophoresis. Moreover, the coupling efficiency was similar for both the monoclonal antibody and the fragment, with a linear increase between 5 and 30 μg of protein per μmol of lipid followed by a plateau in the range of 30–40 μg of protein per μmol of lipid [50]. Another popular thiolation reagent is N-succinimidyl S-acetylthioacetate (SATA), which enables antibody reaction with MPB-PE or succinimidyl 4-(N-malemidomethylcyclohexane)-1-carboxylate [51–54]. In another study, assessment of SATA modification using 10×–100× molar excess relative to the antibody concentration indicated that sulfhydryl group generation, coupling efficiency, the amount of antibody per μmol lipid, and the antibody number per liposome were higher when a higher concentration of SATA was used;

however, this strategy inhibited cell targeting, probably because of massive crosslinking of the NH_2 groups near the antigen recognition sites of the antibodies [30].

Some other chemical groups have shown utility for thiolation for further reaction with PEGylated lipids conjugated with maleimide [55–57]. Gagné et al. tested 2-mercaptoethylamine-HCl for reducing anti-HLA-DR Fab' portions, in order to minimize the immunogenicity associated with the Fc portion of the whole antibody, for the delivery of indinavir for HIV therapy. The anchor lipid for conjugation was distearoylphosphatidylethanolamine[poly-(ethyleneglycol)2000] maleimide. The liposomes bearing the Fab' portions were 2.3-fold less immunogenic than the liposomes with the whole IgG [58]. Using a different strategy, Shin et al. conjugated an anti-CD133 antibody to liposomes; unlike the previous reports discussed herein, they reacted the antibody with 4-(maleimidophenyl) butyrate and conjugated it to the lipid, which was first thiolated with dithiothreitol [59].

Thiolated antibodies can also react with groups other than maleimide. For example, vinylsulfone groups at the distal PEG termini on the surface of liposomes (such as PEG-vinylsulfone-*N*-hydroxy-succinimidyl ester) can react with the free thiol groups on monoclonal antibodies [60]. Rather than reacting the thiolated antibody with maleimide-containing liposomes through a thioether bond, other studies have reported different chemical reactions. For instance, Bendas et al. functionalized liposomes through *N*-glutaryl PE as a membrane anchor for preparing the liposomes through an amide bond. For protein linkage, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide was incubated with liposomes, followed by gel permeation chromatography. Then, antibody was added, followed by incubation, then separation of free antibody by gel permeation chromatography. Interestingly, the authors compared this protocol to functionalization *via* thiolated antibody reaction with maleimide, and found that they obtained better results with the amide reaction approach [61]. Another study reported functionalization using a carboxyl group. Monoclonal antibodies were conjugated to *N*-glutaryl-phosphatidylethanolamine in the presence of octylglucoside by using N-hydroxysulfosuccinimide as a carboxyl-activating reagent [62]. Another approach involves the formation of a covalent bond between periodate-oxidized antibody molecules with liposomes containing hydrazide-derivatized distearoyl phosphatidylethanolamine, through hydrazone linkage formation [63,64]. Finally, another covalent approach involves lipids containing the nitrile group, which does not require prior antibody activation or derivatization [65–67].

Additionally, covalent chemical bonds between liposomes and antibodies can be formed with a post-insertion approach (Fig. 3). In this case, liposomes are first prepared in parallel to micelles covalently bound to antibodies, which are then incubated together to facilitate antibody transfer to liposomes. The authors argued that the post-insertion technique is a simple, flexible, and effective method for preparing targeted liposomal drugs for clinical applications [68]. Using this approach, different antibodies or fragments have been attached to liposomes, particularly through reactions with maleimide. Examples include immunoliposomes targeted to soluble *Leishmania* antigens, EFGR for glioma, endoglin (CD105) and fibroblast activation protein (FAP), and HER2 for breast cancer, among others [34,36,69,70].

The orientation of the covalently attached antibody on the liposome surface influences its association with the tumor cells. Antibodies can be attached on conventional liposomes in a random orientation, on pegylated liposomes also in a random orientation or at the terminal end of the PEG-chain in a site-specific manner, rendering the antibody molecules with their antigen-binding site exposed and protruding from the liposome. It has been previously shown that the attachment of the CC52 antibody at the terminal end of the PEG-chain of immunoliposomes strongly enhanced the association with CC531 cells even at a relatively low antibody density [16]. Furthermore, the spacer length for antibody attachment can potentially influence the coupling efficiency. Fleiner et al., 2001 compared ethylene glycol, tetraethylene glycol, PEG 400, PEG 1000, dodecyl as thiol-reactive coupling lipids. They found that polar spacers (tetraethylene glycol) achieved a higher coupling efficiency than a nonpolar spacer with approximately the same length (dodecyl) and the best results were obtained using coupling lipids with a long polar spacer (PEG 1000). The authors argued that a long and polar spacer (*e.g.*, PEG) might be more suitable to present the thiol-reactive maleimide group at the liposome surface [71]. Finally, a long PEG chain has the potential to enhance the targeting effect, because the nanoparticle can better associate with the cells [72].

Finally, antibodies can be non-covalently linked to liposomes, although this strategy is less common. The reaction between biotin and neutravidin or streptavidin is particularly useful. A recent paper demonstrated that cellular uptake of immunoliposomes is more efficient if the antibody is conjugated through the streptavidin–biotin complex instead of the maleimide group, through PEG-biotin and PEG-maleimide, respectively, for brain-targeted delivery. The authors argued that the cause is likely related to the higher availability of antibody on liposomal surfaces provided by the biotin–streptavidin complex, which could be related to the length and conformation of PEG on the liposome surface [73].

4. Monoclonal antibody- and antibody fragment-mediated targeted delivery

4.1. Vascular targeting: cardiovascular diseases and cancer

Nanoparticles, such as liposomes, are naturally prone to vascular targeting due to the EPR effect in the leaky vasculature of cancers and inflamed areas [74]. In cancer blood vessels, there are tight cell–cell junctions with openings of up to 400–600 nm between endothelial cells, which enable passive targeting to take place [75]. Besides the leakiness of the tumor blood vessels, there are tumor-specific endothelial cell changes that can be exploited for the development of actively targeted nanoparticles [76]. In order to be useful for endothelial-targeted delivery, an endothelial determinant must be present on the lumen of the endothelium, making it accessible to carriers circulating in the blood stream [77]. Several endothelium-expressed molecules have been proposed as targeting options to the vascular wall, including vascular endothelial growth factor receptors (VEGF-R) in cancer-associated angiogenesis and leukocyte adhesion molecules in diseases associated with inflammation [78]. Other targets include platelet endothelial cell adhesion molecule 1 (PECAM-1), intercellular adhesion molecule 1 (ICAM-1), transmembrane glycoproteins constitutively expressed on endothelial cells, integrins (specifically $\alpha v\beta 3$ and $\alpha v\beta 5$), selectins, and the family of tumor endothelial markers (especially TM) [77].

In response to inflammation, endothelial cells express cell adhesion molecules for the recruitment of leukocytes to inflammatory sites. Among these receptors, the carbohydrate-binding selectins initiate and regulate the adhesion cascade [79]. Selectin-directed antibodies have been evaluated as a targeting moiety for liposomes, and target sensitivity was evaluated [61,80]. Promising results have been reported by Spragg and coworkers, who demonstrated that E-selectin-targeted immunoliposomes for doxorubicin (dox) delivery mediated marked cytotoxicity when incubated with activated human umbilical vein endothelial cells (HUVECs) that express E-selectin, but not when incubated with non-activated HUVECs [52]. Additionally, cellular uptake of E-selectin-targeted immunoliposomes by activated endothelial cells was attributed to

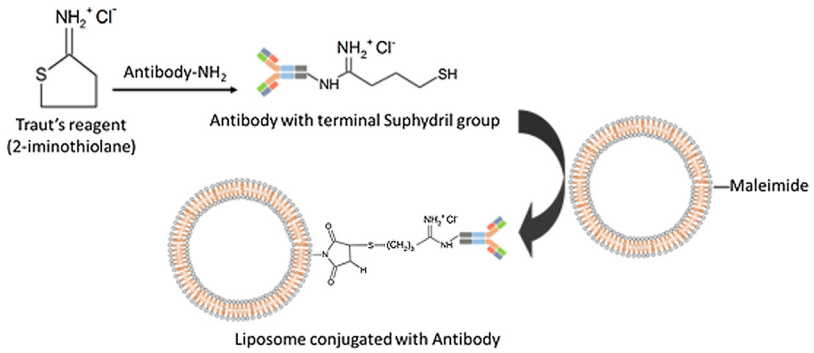

Fig. 3. Immunoliposome formation through the post-insertion method.

the endocytic pathway [65]. Using a different approach, administration of P-selectin-targeted immunoliposomes containing vascular endothelial growth factor (VEGF) for delivery to the affected areas in myocardial infarction resulted in increased anatomical and functional vessels, with a substantial improvement in cardiac function in animals [81].

Adhesion molecules, including endothelial leukocyte adhesion molecule-1 (ELAM), vascular cell adhesion molecule 1 (VCAM-1), and ICAM-1, play an important role in the inflammatory process, such as in atherosclerosis through the recruitment of circulatory monocytes [82,83]. For instance, anti-ICAM-1 liposomes have been reported to bind human bronchial epithelial cells (BEAS-2B) and HUVECs in a specific, dose-, and time-dependent manner [51]. Liposomes targeted with anti-ICAM-1 and anti-ELAM antibodies have been developed for vascular targeting with applications for the treatment of cardiovascular diseases [84]. Echogenic liposomes (responsive to ultrasound stimuli) targeted with anti-VCAM-1 antibody were evaluated as a tool for molecular imaging of atheroma; the authors found that pre-treatment with nitric oxide-loaded echogenic liposomes plus ultrasound improved the contrast effectiveness [31]. Interestingly, anti-VCAM-1 antibody conjugated to liposomes was able to catalyze blood coagulation reactions, with applications for controlling thrombogenesis [48]. Finally, VCAM-1 and E-selectin antibodies have been used in combination for vascular targeting in inflammation caused by IL-1α and TNF-α [85].

Considering the important role of angiogenesis in tumor growth, targeting the endothelium could serve as an approach for cancer treatment. Thus, VCAM-1 has been reported for tumor vasculature targeting by Gosk et al., who observed that anti-VCAM-1 targeted immunoliposome exhibited specific binding to activated endothelial cells and that immunoliposomes accumulated *in vivo* in tumor vessels [67]. Additionally, other targets are relevant in this process. For example, kinase insert domain-containing receptor VEGF is predominantly expressed on tumor vessels, representing a promising target for tumor angiogenesis inhibition [38]. Endoglin, a protein of the transforming growth factor receptor complex, has potential as a cancer vasculature target; dox-loaded immunoliposomes directed to endothelial cells, using a scFV fragment against endoglin, mediated enhanced cytotoxicity toward endothelial cells [86]. Finally, Rabenhold et al. developed immunoliposomes targeted with bispecific single-chain antibodies against endoglin and FAP, which resulted in strong interactions with a human fibrosarcoma cell line [70].

Finally, bevacizumab is an FDA-approved, recombinant, humanized monoclonal antibody for the treatment of metastatic colorectal cancer in combination with 5-fluorouracil (5-FU). It targets and inactivates all isoforms of VEGF, thereby inhibiting angiogenesis and tumor growth [87]. Within this context, cationic liposomes, which preferentially target the tumor vasculature, were functionalized with bevacizumab and had high cellular uptake into endothelial cells [88]. However, other bevacizumab immunoliposomes have not been further developed.

4.2. Cancer targeting: HER2

HER2 contributes to the regulation of epithelial cell proliferation and survival, and is a member of the HER family, which also includes the HER1 (EGFR), HER3, and HER4 receptors. HER2 is a type 1 transmembrane glycoprotein that contains 3 distinct regions: an N-terminal extracellular domain, a single α-helix transmembrane domain, and an intracellular tyrosine kinase domain. Although the HER2 receptor has no known ligand, it can heterodimerize with the other receptors, resulting in autophosphorylation of the tyrosine residues within the cytoplasmic domain, which initiates signal transduction *via* the PI3K/AKT and RAS/MAPK pathways [89,90]. Overexpression of HER2, found in approximately 20%–25% of cases of breast cancer, is an adverse prognostic indicator of survival, due to the key role of HER2 in this process. Besides the more aggressive phenotype, it is associated with a greater likelihood of lymph node involvement and increased resistance to endocrine therapy [91].

The recombinant, humanized monoclonal antibody trastuzumab (Herceptin®) was the first anti-HER2 agent that improved survival in metastatic patients [92]. Blocking HER2 signaling is a promising strategy to inhibit tumor growth. Trastuzumab is able to induce apoptosis in breast cancer cells through antibody-dependent cellular cytotoxicity. After binding to HER2 and reducing signaling, trastuzumab increases $p27^{Kip1}$ levels, promoting cell cycle arrest and apoptosis. However, not all HER2-positive patients respond to trastuzumab therapy, and HER2-overexpressing tumors can develop resistance to trastuzumab monotherapy; thus, combination therapy with other anti-cancer drugs with distinct mechanisms of action, such as paclitaxel (PTX), dox, or lapatinib, improves the therapeutic outcome. Furthermore, as an overexpressed cyto-membrane protein, HER2 is considered an attractive marker for targeted drug delivery to tumor cells [90,93–95].

Anthracyclines, particularly dox (Adriamycin®), have been employed for the treatment of breast cancer, both in the adjuvant and metastatic settings. However, the low therapeutic index of dox is a major drawback. Due to dox cardiotoxicity, it is not clinically combined with trastuzumab. However, PEGylated liposomal dox is effective for breast cancer treatment and reduces the toxicity associated with conventional dox, including myelosuppression, alopecia, nausea, vomiting, and, most importantly, cardiac toxicity. Also, Doxil®, the first liposome approved by the FDA, is a commercially available dox liposome for cancer treatment, including breast tumors [96]. Combining trastuzumab with liposomal dox has been proving clinical benefit [97,98]. Within this context, anti-HER2 immunoliposomes have been developed for dox delivery to breast cancer. Fab' portions against the HER2

receptor were conjugated to dox-loaded liposomes and evaluated in HER2-overexpressing human breast cancer xenografts. Immunoliposomes behaved better: penetrated and accumulated in the tumors and increased antitumor cytotoxicity with less systemic toxicity than free dox [99]. Trastuzumab fragments, have been successfully conjugated to dox-loaded liposomes, resulting in better uptake in HER2-expressing cells, long circulation with favorable pharmacokinetics, and potent *in vivo* anticancer activity, which was better in the immunoliposome group compared to the other groups [100].

PTX, a taxane drug that inhibits microtubule stabilization during mitosis, has been widely employed in combination with trastuzumab in the clinic, with significant improvements in the time of disease progression, duration of response, and time to treatment failure, resulting in increased patient survival compared to monotherapy [93]. Yang and collaborators developed PTX-loaded immunoliposomes using trastuzumab as the targeting moiety. PEGylated immunoliposomes had higher cellular uptake in HER2-positive cancer cell lines than non-functionalized liposomes. Also, the circulation time of the immunoliposome formulation was prolonged compared to the commercial PTX formulation (Taxol®) [34]. Subsequently, the authors observed that immunoliposomes exhibited superior efficacy compared to Taxol® or liposomes in a HER2-positive BT-474 xenograft model. Conversely, in the HER2-negative MDA-MB-231 xenograft model, immunoliposomes and liposomes had the same tumor distribution and antitumor activity.

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase member of the cellular phosphatidylinositol 3-kinase (PI3K) pathway, which plays a critical role in cell survival. Therefore, inhibitors of mTOR, such as rapamycin (RAP), also known as sirolimus, have been shown to reduce the growth of several tumors. Additionally, mTOR inhibition may overcome the resistance of some HER2-positive tumors to trastuzumab [101]. Recently, RAP was loaded with high efficiency into immunoliposomes functionalized with trastuzumab. The formulations were able to cause cytotoxicity to HER2-positive SKBR3 cells [35]. In another study, RAP was combined with PTX in trastuzumab-targeted immunoliposomes to utilize RAP and PTX synergistic effects. The immunoliposomes showed higher uptake in SKBR3 cells and more effectively controlled tumor growth *in vivo* compared to liposomes [32].

Moreover, other drugs have been loaded into HER2-targeted immunoliposomes. For instance, melittin, an antimicrobial peptide with anticancer activity, was encapsulated into trastuzumab-functionalized liposomes. Immunoliposomes decreased cancer cell viability in a dose–response manner and in correlation with their level of HER2 expression, showing specific binding to SKBR3 cells [29]. PE38KDEL, an immunotoxin, was loaded into immunoliposomes targeted to the HER2 receptor through Fab' portions. Cell studies indicated receptor-specific binding and internalization, resulting in enhanced cytotoxicity [42]. Small interference RNA (siRNA)-based gene therapy was able to silence a breast cancer target gene, with consequent SKBR3 cell invasion inhibition, when delivered by anti-HER2 immunoliposomes [43].

Finally, hyperthermia is a promising strategy for adjuvant cancer treatment, particularly using magnetite nanoparticles. Hyperthermia is able to kill cancer cells directly, and also cause activation of anticancer immunity. Within this context, magnetite nanoparticles were developed for specific tumor accumulation through targeting of the HER2 receptor, and results showed a HER2-mediated antiproliferative effect on SKBR3 cells under an alternating magnetic field [49]. In another paper, anti-HER2 immunoliposomes containing magnetite nanoparticles caused tumor regression in the hyperthermic group, which was sustained for 10 weeks after hyperthermia [102].

4.3. Cancer targeting: EGFR

EGFR belongs to the ErbB family of receptor tyrosine kinases and is involved in the pathogenesis and progression of different types of cancers, including lung, head and neck, brain, colon, and breast carcinomas [103]. The intracellular domains of ErbB receptors contain a highly-conserved tyrosine kinase domain, while the extracellular domains are less conserved; the latter are bound and activated by growth factors, generating complex signal transduction pathways that involve the Ras/ERK signaling cascade [104]. As a strategy for target inhibition, anti-EGFR monoclonal antibodies bind to the extracellular domain of the receptor, blocking ligand-induced EGFR tyrosine kinase activation. Cetuximab (Erbitux®), a human–murine chimeric IgG, binds to EGFR with higher affinity than natural ligands, such as TGF-α and EGF, causing receptor inactivation and cell apoptosis. Clinically, cetuximab has been used in combination with other chemotherapeutics, such as platinum drugs or 5-FU, for the treatment of squamous cell carcinoma of the head and neck, and colorectal cancers [105]. Another therapeutic option is the fully human IgG antibody panitumumab, which is also employed for the treatment of metastatic colorectal cancer, and associated with overcoming cetuximab-induced resistance [106].

A variety of drugs have been loaded into EGFR-targeted immunoliposomes. For cancer treatment, some small molecule drugs have been reported, including dox, sodium borocaptate, gemcitabine, 5-FU, oxaliplatin, and boron compound [18,36,37,40,50]. Immunoliposomes functionalized with cetuximab were tested *in vitro* in EGFR-overexpressing cell lines. Receptor-specific intracellular drug delivery by targeted liposomes correlated with receptor density, reaching up to 3-fold higher levels than with non-targeted liposomes [50]. Cetuximab acted synergistically with 5-FU on EGFR-positive skin squamous cell carcinoma A431 cells [37]. Also, cetuximab mediated potent uptake of boron in EGFR-positive F98 glioma cells [36]. Using an anti-EGFR Fab', immunoliposomes enabled enhanced cytotoxicity and uptake in EGFR-overexpressing hepatocellular carcinoma cells [18]. In murine xenograft models of cancer *in vivo*, specific targeting to glioma tumors has been demonstrated in a system that combined imaging with drug delivery [40]. Interestingly, targeting with an anti-EGFR Fab' or cetuximab was compared in a colorectal xenograft model, and the Fab'-targeted liposomes outperformed the cetuximab-targeted liposomes in both tumor accumulation and tumor growth control [50].

Stimuli-responsive nanoparticles are an interesting approach for triggered release upon intrinsic or extrinsic stimuli [107]. Thermosensitive liposomes, with the ability to trigger drug release upon temperature increase, are being researched as an alternative to improve therapeutic efficacy. Thermodox®, for dox delivery, is a formulation that has reached clinical trials [3]. In the field of targeted delivery, Fab' portions of cetuximab were covalently attached to thermosensitive liposomes for dox delivery. The liposomes facilitated EGFR-mediated cellular association and temperature-dependent release, with consequent enhanced tumor cell cytotoxicity, demonstrating the potential of the nanocarrier for further evaluation [108].

RNA interference is an endogenous regulatory pathway that causes sequence-specific gene silencing, which can be used as a potent, targeted therapeutic tool applicable to a variety of diseases, including cancer [109]. Within this context, EGFR-targeted immunoliposomes have been used for siRNA delivery. Gao et al. developed an siRNA-loaded immunoliposome functionalized with anti-EGFR Fab' for hepatocellular carcinoma therapy, and observed enhanced cellular uptake and gene silencing activity compared to the untargeted liposome both *in vitro* and *in vivo*. Furthermore, higher tumor accumulation was observed for the immunoliposomes [44]. In a subsequent work, the authors further improved their therapeutic strategy by co-encapsulating ribonu-

cleotide reductase M2 siRNA and dox in an anti-EGFR Fab'-targeted immunoliposome. Results showed potent cytotoxicity, apoptosis, and senescence-inducing activity, with consequent reduced orthotopic tumor weight [18]. Finally, an anti-EGFR Fab' mediated siRNA-loaded liposome delivery to the EGFR-overexpressing hepatocellular carcinoma cells SMMC-7721 [110].

Drugs other than conventional chemotherapeutics can be explored through EGFR targeting. For instance, celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, was loaded into immunoliposomes targeted with cetuximab. The rationale for this approach is that there is crosstalk between COX-2 and EGFR. The outcome of this approach was higher uptake in EGFR-overexpressing cells, resulting in higher cytotoxicity using the targeted liposomes [111].

4.4. Cancer targeting: other receptors

Antibody-based therapy for cancer has been used over the past 20 years and is now one of the most successful and important strategies to treat patients with hematological malignancies and solid tumors [112]. The outcome of this therapy, targeted drug delivery to cancer cells, is an interesting and promising field due to the many targeted receptors overexpressed on cancer cells, in addition to HER2, EGFR, and the vascular receptors, compared to normal cells. The level of expression of these receptors must be sufficient to allow delivery of anticancer drugs in sufficient amounts, and an ideal target receptor will generally be expressed on the apical surface of a cancer cell and not within its cytoplasm or nucleus [113]. In Table 1, we summarized a series of studies reporting targeted liposomes using antibodies for a variety of cancer receptors. Most studies reported enhanced specific cell binding and internalization with immunoliposomes compared to untargeted liposomes.

Among others, potential targets for cancer treatment and diagnosis are the folate and transferrin receptors, which are overexpressed in a variety of cancer types. Folate receptor is necessary for folate uptake and DNA synthesis and is commonly overexpressed in ovarian cancer [114]. Farletuzumab is an anti-folate receptor monoclonal antibody, which has been clinically evaluated in combination with carboplatin and pegylated liposomal doxorubicin, and a safe profile has been demonstrated [115]. Therefore, a logical approach for ovarian targeted delivery of anticancer drugs would the synthesis of liposomes functionalized with farletuzumab. Transferrin receptor is involved with iron cellular uptake and is overexpressed in many cancer types, usually correlated with higher level of malignancy [116]. For example, an anti-transferrin receptor monoclonal antibody, OX26, was conjugated onto plasmid DNA-loaded liposomes as an approach to enhance the transport to blood brain barrier (BBB), where transferrin receptor is overexpressed. A further functionalization was done with chlorotoxin for specific binding to glioma cells and for facilitated binding to matrix metalloproteinases (MMP-2). Results evidenced higher *in vitro* transfection and, *in vivo*, immunoliposomes reduced tumor volume and increased survival rate [117].

An interesting cancer target is represented by MMPs, a family of zinc-dependent endopeptidases, which are a driving factor for cancer progression and patient prognosis. MMPs are present in nearly all human cancers and are involved in remodeling the extracellular matrix in tumor microenvironments [118]. For this reason, MMP2-responsive liposomes have been constructed for tumor targeting. Liposomes were conjugated with TAT, a cell penetrating peptide, to enhance the intracellular uptake, and the formulation was further functionalized with a tumor cell-specific anti-nucleosome monoclonal antibody (mAb 2C5). The liposomal composition included an MMP2-sensitive bond between PEG and lipids, which undergoes cleavage in the tumor by the highly expressed extracellular MMP2, causing the removal of PEG chains for improved uptake. When PEG chain is cleaved, TAT peptide is then exposed, resulting in TAT peptide-mediated endocytosis, causing increased cell uptake. This strategy allows the prevention of the nonspecific intracellular uptake on the way to the tumor by steric shielding of TAT peptide with the long-chain of PEG [119]. In another work, Fab' portions of antibody against MT1-MMP were conjugated to dox-encapsulating liposomes, resulting in significant tumor growth suppression [56].

4.5. Infectious disease targeting

Immunoliposomes may be employed for antiviral therapy, with advantages over traditional antiviral therapies. Viruses display a varied repertoire of proteins for that can be targeted with antibodies. Furthermore, antibodies against viral proteins are more specific than those against cancer cell receptors because cancerous and normal cells share most receptor proteins and only differ in expression levels. Importantly, immunoliposomes may be able to target not

Table 1
Monoclonal antibody and antibody fragments-mediated targeted delivery to cancer.

Antibody	Cancer type	Drug	Main findings	Reference
Anti- T-24	Bladder carcinoma	Pheophorbide a	Enhanced uptake and cytotoxicity	[46]
Mab CC52	Colon cancer	5-Fluorodeoxyuridine	Cell uptake by endocytic process	[16,53]
Mab 2C5	Lung carcinoma and mammary adenocarcinoma	Doxorubicin	Recognition, binding and cytotoxicity to cancer cells	[120]
Anti-B-cell lymphoma Mab LL2	Lymphoma	Doxorubicin	Increased cellular association and internalization and better cytotoxicity than untargeted liposome	[60]
Anti-CD32 and anti-CD2 antibodies	Leukemia	Oligonucleotides	Improved cellular uptake	[121]
Anti-CD19	Myeloma	Doxorubicin	Receptor- mediated internalization and selective cytotoxicity	[64]
Mab AF-20	Hepatocarcinoma	Fluorescent dye	Improved specific cell interaction	[122]
Anti-ovarian carcinoma Fab'	Ovarian carcinoma	Not employed	Binding to cancer cells	[123]
Anti-CD133 Mab	Glioblastoma	Gemcitabine and bevacizumab	Increased cytotoxicity and antitumor efficacy in xenograft model	[59]
anti-FAP (fibroblast activator protein)' scFv antibody	Metastatic fibrosarcoma	Fluorescent dye	Image-guided detection of the spontaneous metastases and accumulation in mice models	[124]
Anti-CD19, anti-CD20 and anti-CD37 Mab	Leukemia	Fluorescent dye	Dual-ligand immunoliposome enable a better strategy of personalized treatment of B-cell malignancies	[125]
Anti-transferrin scFv antibody fragment	Several cancer cell lines and prostate cancer (*in vivo*)	Plasmid DNA	Enhanced *in vitro* and *in vivo* transfection. *In vivo* gene delivery and expression	[126]

only the virus but also infected cells presenting viral proteins on their surfaces [127].

Some papers have reported immunoliposomes targeted to viruses. For HIV treatment, HLA could serve as a target because CD4+ T cells express substantial levels of the HLA-DR determinant of the major histocompatibility complex class II molecules. Anti-HLA immunoliposomes loaded with the antiviral drug indinavir were successfully delivered to lymphoid tissues and were as effective as the free drug *in vitro*. Also, liposomes bearing Fab' portions were 2.3-fold less immunogenic than liposomes bearing the entire IgG [58]. In another approach, phosphatidylserine immunoliposomes targeted with antibodies that bind HIV-1 virus-like particles were initially protected from macrophage uptake, in order to provide enough time to circulate through the body and achieve maximum virus binding [128]. Moreover, anti-CD4 conjugated immunoliposomes containing 2 antiretroviral drugs (nevirapine and saquinavir) inhibited viral proliferation at a lower concentration than free drugs [41].

Other viruses have been targeted with immunoliposomes. For instance, for targeting to influenza A (H5N1) viral infection, immunoliposomes were functionalized with a humanized scFv antibody against the hemagglutinin (HA) of H5N1, for influenza virus nucleoprotein siRNA delivery. The authors demonstrated specific binding to HA-expressing Sf9 cells, enhanced siRNA transfection efficiency, and a pronounced silencing effect [129]. Falco et al. encapsulated melittin, a pore-forming lytic amphiphilic peptide with antiviral activity, in immunoliposomes targeted against viral hemorrhagic septicemia rhabdovirus (VHSV). The authors claimed that immunoliposomes were able to inhibit VHSV infectivity by 95.2% *via* direct inactivation of the virus [127].

Active targeting with pathogen-binding ligands conjugated to the surfaces of nanoparticles may be an effective strategy to target bacteria [130]. Within this context, immunoliposomes have been developed as an approach for targeting the oral bacterium *Streptococcus oralis*; however, the results were not very promising, considering that the positively charged liposomes adsorbed to the bacteria with greater affinities than the immunoliposomes [131]. Later, the authors developed another immunoliposome loaded with the bactericides chlorhexidine and triclosan for *S. oralis* immobilized in polystyrene. The immunoliposomes enhanced growth inhibition of *S. oralis*, compared to free bactericide [132]. For detection purposes, Staphylococcus enterotoxin B fluorescent immunoliposomes were developed for immunochromatographic testing [54].

For protozoa targeting, some approaches have been described, including the development of chloroquine- and primaquine-loaded liposomes functionalized with antibodies against *Plasmodium*. Exciting *in vivo* results in mice showed that immunoliposomes cleared the pathogen below detectable levels [30]. More recently, the same research group used polyclonal antibodies against the NTS-DBL1α N-terminal domain of a *Plasmodium falciparum* protein for targeting lumefantrine-containing liposomes for malaria treatment, with marked inhibition of parasite growth [133]. Additionally, targeting against *P. falciparum*-infected red blood cells was achieved with immunoliposomes carrying chloroquine and fosmidomycin for improved efficacy. Importantly, increasing the number of antibodies on the liposome surface correspondingly enhanced antiparasitary performance [134]. Finally, immunoliposomes have been reported as vaccines targeted to *Leishmania* antigens, which induced stronger cell-mediated immunity in mice compared to untargeted liposomes [69].

4.6. Autoimmune and degenerative disease targeting

Although targeting autoimmune and degenerative diseases is a promising field, it has not been thoroughly explored with immunoliposomes. An important application for them is the treatment of rheumatoid arthritis, an autoimmune disease, which is the leading cause of disability. In this disease, degradation of the cartilage matrix is followed by the loss of proteoglycans and other proteins from the surface, which exposes type II collagen (CII) fibrils and makes them accessible to CII antibodies. The most common treatments for rheumatoid arthritis involve nonsteroidal anti-inflammatory drugs, corticosteroids, and antirheumatic drugs and biological agents, including antibodies [135]. For the purpose of arthritis treatment and diagnosis, near infrared immunoliposomes conjugated with CII antibody were developed. Researchers demonstrated selective binding and quantified cartilage degradation *in vivo* in guinea pigs [136].

Lupus nephritis is a serious consequence of systemic lupus erythematous, an autoimmune disease that also affects other organs, including the skin, pericardium, lungs, and nervous system. Lupus nephritis may lead to potentially fatal renal and cardiovascular damage [137]. Within this context, anti-alpha integrin immunoliposomes (anti-glomerular mesangial cells) were loaded with a fluorescent dye and specifically delivered *in vivo*, predominantly to glomeruli, with little nonspecific uptake by CD11b+ cells [138].

Targeting the blood–brain barrier is an interesting approach for the treatment of Alzheimer's disease, a neurodegenerative brain pathology that causes a decline in cognitive abilities. Loureiro and coworkers developed dual ligand immunoliposomes for drug delivery to the brain and demonstrated cellular uptake in brain capillary cells, as well as the ability to cross the blood barrier *in vivo* [73]. The accumulation of extracellular Aβ is a determinant for the development of Alzheimer's disease. Thus, a logical approach for its treatment would be the use of anti-Aβ, which has been attempted, through the development of anti-Aβ immunoliposomes able to capture Aβ. The formulation was able to reduce circulating and brain levels of Aβ in aged animals. Interestingly, the therapeutic efficacy of the immunoliposome treatment was superior to free monoclonal antibody administration [45]. Moreover, immunoliposomes have already been employed to detect Aβ using time-of-flight secondary ion mass spectrometry and were demonstrated to specifically bind Aβ, providing information on lipid–protein interactions [139]. It is noteworthy that anti-Aβ immunoliposomes have been shown to deposit in post-mortem Aβ brain samples, confirming the potential of the immunoliposomal strategy for Alzheimer's disease therapy and diagnosis [140].

Finally, another approach for delivery to the brain is the use of liposomes modified with transferrin antibodies; given that brain capillary endothelial cells express transferrin receptors, this strategy is potentially applicable to the treatment of brain degenerative diseases [141]. More recently, β targeting with the anti-transferrin OX26 monoclonal antibody was combined with lactoferrin functionalization for the delivery of the selective NK3 receptor agonist senktide, which is typically unable to cross the blood–brain barrier, resulting in higher brain levels of senktide [142]. Furthermore, liposomes loaded with epigallocatechin-3-gallate, a natural antioxidant, have been functionalized with the anti-transferrin receptor antibody OX26 and anti-α-synuclein, as a strategy to cross the blood–brain barrier. The purpose was to treat Parkinson's disease, a motor and cognitive neurodegenerative disorder characterized by impaired dopamine production and the presence of neuronal cytoplasmic inclusions known as Lewy bodies, rich in aggregated α-synuclein. Cellular uptake of the targeted immunoliposomes in the cultured brain endothelial cell line hCMEC/D3 was twice as efficient as that of untargeted liposomes [143].

5. Immunoliposomes: stimulation of endogenous immune response

Cytotoxic T lymphocytes CD8+ play a major role in protection against intracellular infection. In this process, presentation of antigens requires antigen presenting cells (APCs) and association with major histocompatibility complex (MHC) class I. There are several strategies to induce dendritic cells (DCs) to present antigen peptides. Vaccine delivery systems, such as liposomes, promote the uptake of loaded antigens into APCs, enhancing the generation of immune response. Moreover, immunoliposomes have demonstrated increased ability for immune system stimulation, representing a promising strategy to target DCs, leading to efficient stimulation of CD8+ as well as CD4+ T cells [144,145]. Therefore, targeting DCs is considered a very promising approach for the development of vaccines [146]. Noteworthy, the direct delivery of antigens to DCs *via* antibodies has been recently reviewed [147].

For instance, immunoliposomes have been loaded with Soluble *Leishmania* Antigens (SLA) for delivery to a murine model of leishmaniasis. Results showed that liposomes might be effective adjuvant systems to induce protection against *L. major* challenge in BALB/c mice, but stronger cell mediated immune responses were induced when immunoliposomes were utilized, owing to the interaction between IgG and Fc-gamma receptors in DCs [69]. Another strategy for DC targeting involves the C-type lectin receptors, including the DEC-205 target. To this aim, anti-human DEC-205 immunoliposomes showed increased uptake by DCs and were available for antigen processing [148].

Another very interesting approach for immune system stimulation involves the use of synthetic single strand oligodeoxynucleotides (ODNs) containing unmethylated cytosine-guanine (CpG) motifs which mimic the conserved microbial products and bind Toll-like receptor 9 (TLR9), potentiating both humoral and cellular responses [149]. This strategy can be used for effective induction of anti-tumor immunity. Nanoparticles for enhancing the immunostimulatory effect of CpG-ODN were recently reviewed [150]. For example, it has been shown that p5 HER-2/neu derived peptides encapsulated in 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) cationic liposomes co-administered with CpG-ODN greatly enhanced the cytotoxic T lymphocytes response, causing tumor progression inhibition. The antitumor vaccine property was attributed to induction of both CD8+ and CD4+ responses [151]. Additionally, systemic targeting of CpG-ODN to the tumor microenvironment has been achieved through chemical conjugation with an anti-HER-2/neu monoclonal antibody. The conjugate retained its ability to bind HER-2/neu+ tumors, activate DCs and induce antitumor responses [152]. Finally, anti-DCs immunoliposomes would likely be promising CpG-OND vaccines, however this approach needs to be investigated.

6. Immunoliposomes: clinical development

After the introduction of liposomal dox, Doxil®, on the market over 20 years ago, a series of liposomes are now available for the treatment of a variety of diseases, including breast and ovarian cancer, Kaposiís sarcoma, acute lymphoblastic leukemia, meningitis, and fungal infections, such as aspergillosis, as well as for anesthesia, Furthermore, some clinical trials of liposomes are ongoing or recently concluded [96]. A thorough review on nanomedicines for cancer treatment was recently published [153]. It included the promising formulation Thermodox®, a dox-loaded thermosensitive liposome, whose phase 3 trial was recently completed for hepatocellular carcinoma [154].

Furthermore, the variety of reports of successful preclinical use of immunoliposomes, addressed in this paper, led to some clinical trials. Currently, a phase 2 clinical trial for an anti-EGFR immunoliposome loaded with dox is recruiting patients with EGFR-positive, triple-negative breast cancer [155]. In addition, a phase 1 trial for an anti-HER2 liposome functionalized with scFv antibody fragments for the delivery of dox has concluded, which revealed that, as a monotherapy or in combination with trastuzumab, the immunoliposome could be effective for managing previously treated, HER2-positive breast cancer. A phase 2 trial (HERMIONE) aimed to evaluate the efficacy and safety of the formulation plus trastuzumab in patients with refractory HER2-positive, advanced/metastatic breast cancer [156]. However, the clinical trial was stopped due to the absence of clinical benefit evidence. Additionally, a phase 1 trial is recruiting volunteers to evaluate a liposome for targeted delivery of plasmid DNA for gene therapy to solid tumors *via* the transferrin receptor, using scFv antibody fragments [157]. Finally, a melanoma vaccine was evaluated in a phase 1 clinical trial. The formulation Lipovaxin-MM consists of a liposome loaded with melanoma antigens and IFNγ, targeted with a single domain antibody fragment (V_H) against a dendritic cell-specific intracellular adhesion molecule [158].

Despite the success of liposomes for drug delivery and the recent exciting development of monoclonal antibodies for functionalization, no immunoliposome is yet commercially available. However, antibody–drug conjugates (ADCs), which consist of a cytotoxic agent chemically linked to an antibody, therefore combining the target selectivity of antibodies with the potency of cytotoxic agents, are available. Brentuximab vedotin was approved and is commercially available for the treatment of Hodgkin's lymphoma and T-DM1, a trastuzumab emtansine conjugate, was approved for breast cancer treatment. Besides the approval of these ADCs, encouraging clinical responses with safety profile have been observed for other ADCs, which have been previously reviewed [159]. On the other hand, in comparison to ADCs, immunoliposomes have progressed slower to the clinic, which could be attributed to several factors, including the high development cost associated with their development and the lack of successful incorporation of a variety of anticancer drugs [19].

Challenges in bringing the bench to bedside in immunoliposome development involve successful industrial production, which requires a multi-step preparation procedure involving antibody production, coupling, liposome formulation and drug loading, posing challenges for both manufacturing and analytics [160]. For this purpose, Wicki et al., 2015 have developed a large-scale, GMP-compliant production process of anti-EGFR targeted immunoliposomes. Several criteria were considered, such as stability, sterility, pH, drug concentration, endotoxin concentration, leakage, particle size and uptake. The authors claimed that their process was robust, reliable, reproducible and thus suitable for the production of anti-EGFR-targeted nanocarriers in a quantity and quality necessary for clinical trials, which revealed the pharmacokinetics profile and stability of the nanocarrier, *in vivo* [161].

7. Conclusion and future perspective

The clinical success of liposomes combined with the development of monoclonal antibody-based therapies led to the design of immunoliposomes to target diseases while reducing side effects and increasing efficacy. The rationale behind this approach is that targeting ligands may offer the advantage of improved cellular uptake once the nanocarrier arrives at the target, although ligand-targeted nanomedicines are subjected to the same physiological localization as ligand-lacking nanomedicines and, thus, have comparable biodistribution. As described herein, the development of chemical conjugation strategies, particularly through reactions of the sulfhydryl groups of thiolated antibodies and maleimide-

containing liposomes, has enabled a rapid advancement in the immunoliposome field. Furthermore, progress in monoclonal antibody engineering allowed the development of less immunogenic fragments, such as the scFv portion, commonly used for liposome functionalization. Overall, the major focus of immunoliposomes has been on the treatment of many types of cancer, although these formulations have shown potential for the treatment of inflammatory, infectious (*e.g.* HIV and malaria), autoimmune (*e.g.* arthritis), and degenerative diseases (*e.g.* Alzheimer's disease). Regarding cancer treatment, several targets could be explored, but a considerable number of studies have directed their efforts toward HER2 for breast cancer treatment and EGFR for different types of solid tumors. Overall, studies have reported enhanced cellular uptake, resulting in increased cytotoxicity. Moreover, many research groups have conducted *in vivo* studies, with exciting results obtained using cancer xenograft models. Consequently, these promising results have led to clinical trials for the evaluation of safety and efficacy, yet no immunoliposome has reached the market. However, immunoliposomes are expected to become a reality in medicine as soon as more studies reach clinical trials, and after some issues of industrial scale-up are resolved.

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