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DRUG DELIVERY (A HATEFI, SECTION EDITOR)

Recent Advances in Immunoliposome-Based Cancer Therapy

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Abstract Advances in protein engineering have revolutionized nanomedicine by introducing novel nanocarriers for delivery of cancer therapeutics. Designing assemblies that simultaneously incorporate therapeutics and target the site of disease has been the focus of recent studies. Such devices have been developed to behave dynamically in response to certain cues to trigger drug release. Immunoliposomes have been regarded as an attractive drug targeting vehicle for cancer treatment. In the present review, we focus on recent advances in the design of immunoliposomes incorporating a variety of chemotherapeutics that simultaneously exhibit specific target-cell interactions and stimuli-sensitivity. We provided an overview of different stimuli-responsive immunoliposomes that are capable of controlling release of drug in response to either exogenous (temperature, light, and magnetic field) or endogenous (changes in pH and enzyme concentration) stimuli. We have discussed examples of stimuli-sensitive immunoliposomes with respect to each stimuli and their therapeutic potential for cancer treatment.

Keywords Immunoliposomes · Stimuli-responsive carriers · Antibody · Temperature · Enzymes

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Introduction

Cancer is still one of the major causes of death worldwide, with more than 10 million new cases every year [1]. From a drug delivery perspective, general distribution of cancer therapeutic within the whole body and the subsequent toxicity to normal tissues limit drug bioavailability at the tumor site [2, 3]. It would be therefore desirable to develop site-specific pharmaceutical nanocarriers that can either passively or actively target cancerous cells [4–8]. Among different drugs and gene delivery approaches, liposomes offer several advantages such as biocompatibility, low toxicity, and the capacity to alter the pharmacokinetic profile of therapeutic agents [9]. Specifically, the incorporation of polyethylene glycol (PEG) coating on the surface of liposomes greatly enhances blood circulation half-life and promotes preferential localization at desired target tissue through the EPR [10, 11]. This phenomenon, also termed as passive targeting, exploits the general features of tumor vasculature, including leaky blood vessels and poor lymphatic drainage, characteristics of rapid and defective angiogenesis which provide oxygen and nutrients for fast proliferating tumor cells (Fig. 1) [12]. Thus, carriers that are more likely to be cleared by reticuloendothelial system (RES) exploit this phenomenon to accumulate in tumor via multiple passages through the tumor sites [13••]. Though passive targeting approach forms the rational basis for the design

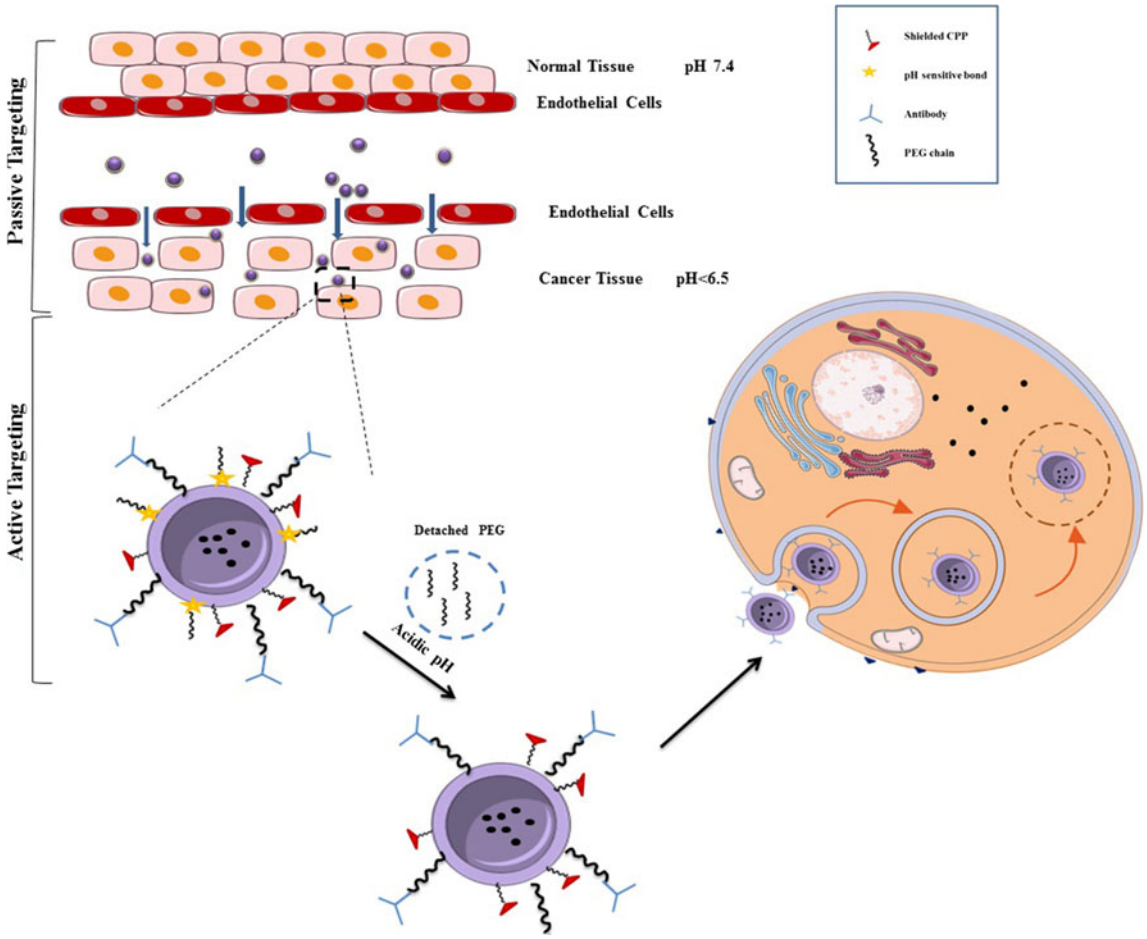

Fig. 1 Targeted drug delivery from pH-sensitive immunoliposomes. For the intracellular drug delivery to the targeted site, the liposomes should possess (1) PEG coating for prolonged circulation, (2) targeting motifs such as monoclonal Abs for cell-specific interaction, and (3) stimuli-sensitive capabilities to respond to the pathological site characteristic to either release the cargo in the cytoplasm by escaping endosome or expose the hidden cell penetrating peptide moieties (CPP) by surface-attached pH-sensitive coatings

of drug carriers and modern therapies, it faces challenges related to variable vascular permeability, high tumor interstitial fluid pressure, inhomogeneous targeting of tumor cells within a tumor, and the likely occurrence of multiple drug resistance (MDR) [14]. Thus, improved specificity can be imparted by designing more sophisticated systems to selectively deliver drugs to the targeted tissue.

Liposomes can be surface functionalized with targeting ligands through a variety of conjugation chemistries available in review articles [15–17]. The grafting of targeting ligands is generally implemented to facilitate target cell recognition and cellular uptake of nanocarriers rather than improving overall tumor accumulation [18]. This suggested that after extravasation of targeted liposomes into the tumor interstitial space, cellular internalization would be enhanced through ligand-receptor interactions (Fig. 1) [19]. The choice of target receptor or antigen on cancer cell as well as the type of targeting ligand is among the most important parameters of efficient ligand-mediated liposomal drug delivery [20]. A number of targeting ligands have been investigated for the development of targeted liposomes including antibodies, nucleic acids, and small molecules (peptides, carbohydrates, vitamins, etc.); see review in [21••]. Among several homing devices, antibody coupled liposomes, also termed as immunoliposomes, have attracted considerable attention for the targeted therapies [22, 23].

Immunoliposomes

Immunoglobulins of IgG class and their fragments (Fragment antigen binding (Fab) or single-chain variable fragments (scFV)) are among the most extensively studied ligands for targeted therapy and diagnosis of tumors by virtue of drug-loaded liposomes. Following their first description in the 1980s [24], several promising studies on tumor targeting related to immunoliposomes have been conducted; see Ref [19] for review. Several methods using various advanced chemistries and techniques have also been reviewed for antibody coupling to the surface of long-circulating PEGylated

liposomes [17, 25]. Further, since PEG molecules can sterically interfere with the targeting ability of an antibody, numerous methods have been developed for antibody conjugation to the surface of PEG to exclude steric hindrance of ligand binding to the target [26, 27].

Interestingly, it was shown that the accumulation of antibody-modified liposomes was in some cases similar to that of nontargeted liposomes and based on EPR phenomenon [28]; thus, the higher anti-cancer activity of immunoliposomes might be associated with internalization of modified liposomes and efficient drug delivery inside cancer cells [29, 30]. The therapeutic effect also depends on antibody density on the surface of liposomes, since over-modifications of the surface have shown to compromise the longevity of liposomes in vivo [31]. Other essential parameters that determine the degree of immunoliposome targeting are the type of encapsulated drugs, the rate of drug release, and specific overexpression of antigens on tumor cells to ensure the optimized binding of liposomes to the target cancer cells [32].

Therapeutic Availability of Immunoliposomes

The fate of immunoliposomes following binding to the target influences the therapeutic outcome. After binding to tumor cell, the delivery of encapsulated compound can take place via four different mechanisms: (1) adsorption onto the cell surface, (2) fusion with the cell membrane, (3) exchange of lipid components with the cell membrane, and (4) endocytic pathways [19]. In the case of receptor-mediated endocytosis, drug-containing nanocarriers have to be able to penetrate inside cells, bypassing lysosomal degradation for efficient targeting of the intracellular compartment [33, 34]. Likewise, if the carrier is taken up by the cell via receptor-mediated endocytosis, the PEG coat on the surface may prohibit endosomal escape and the subsequent delivery to cytoplasm. Further, cell surface binding via the high-affinity interactions of immunoliposomes with receptors limits the distribution and deeper penetration of carrier within tumor through binding-site barrier mechanism [35]. Extracellular release on the other hand seems preferable due to the bystander effect which allows drugs to reach the cells that do not express the targeted antigens or that are not readily accessible to immunoliposomes [20]. Generally, the ability of antibody-targeted liposomes to increase the bioavailable drug concentrations depends on the rate of release of the entrapped drug from liposomes at the target site. Such a delivery system should simultaneously show longevity in blood stream to allow for target accumulation and possessing the ability to switch on and off certain functions when necessary. Recent reviews on switchable nano-systems for medical application have mainly focused on stimuli-responsive nanocarriers for drug delivery [36, 37, 38•, 39••]; a further aim of the present review is to briefly include an overview of the literature regarding stimuli-responsive targeted immunoliposomes which offers an additional platform for the successful drug and gene delivery to the tumors (Fig. 2).

Smart Stimuli-Responsive Immunoliposomes

In order to efficiently deliver the encapsulated compounds to the target cells, the stability of drugs in circulation must be maintained. Immunoliposomes have to exert actions after binding to their target receptors on the cell surface; however, this is not a guarantee for the intracellular delivery of the liposome contents. Generally, there are two main pathways for drug entry into targeted cells: first, selective uptake of liposomes by endocytosis or fusion with the cell membrane, leading to the intracellular release of drug and secondly, release of the entrapped contents in close proximity to target cells. Methods used to fabricate immunoliposomes for triggering the release of encapsulated drug provide a novel strategy in the assembly of smart multifunctional immunoliposomes, see [40, 41••] for reviews. The ideal smart immunoliposome carriers should specifically accumulate in the target tissue and possess the capability to "switch on" certain functions upon the action of biological stimuli. This stimuli can be either intrinsic to the diseased area such as a change in the enzyme levels [42], pH [43] and temperature (noted for inflamed and neoplastic area), or external stimuli like magnetic fields [44], ultrasound [45], light, and heat (see Table 1). These novel approaches that exploit the altered tumor microenvironment may avoid the complications associated with receptor-specific targeting and give new insights for achieving better therapeutic outcome.

Intrinsic Stimuli

The upcoming sections discuss immuno-conjugated liposomes with the ability to tune the release of therapeutic payload upon exposure to intrinsic stimuli such as altered pH and differentially expressed enzymes.

pH-Responsive Immunoliposomes

Improving Endosomal Escape to Enhance Cytoplasmic Delivery of Drugs and Genes Certain ligands improve intracellular levels of the entrapped cargo by induction of receptor-mediated endocytosis, thus exposing the endocytosed material to acidic lysosomal compartment and the subsequent enzymatic degradation which limits its therapeutic potential. In particular, this is critical in the case of drugs susceptible to degradation including nucleic acid-related materials such as pDNA, siRNA, and shRNA, and also peptidic drug [18]. For these molecules, delivery vectors capable of releasing the

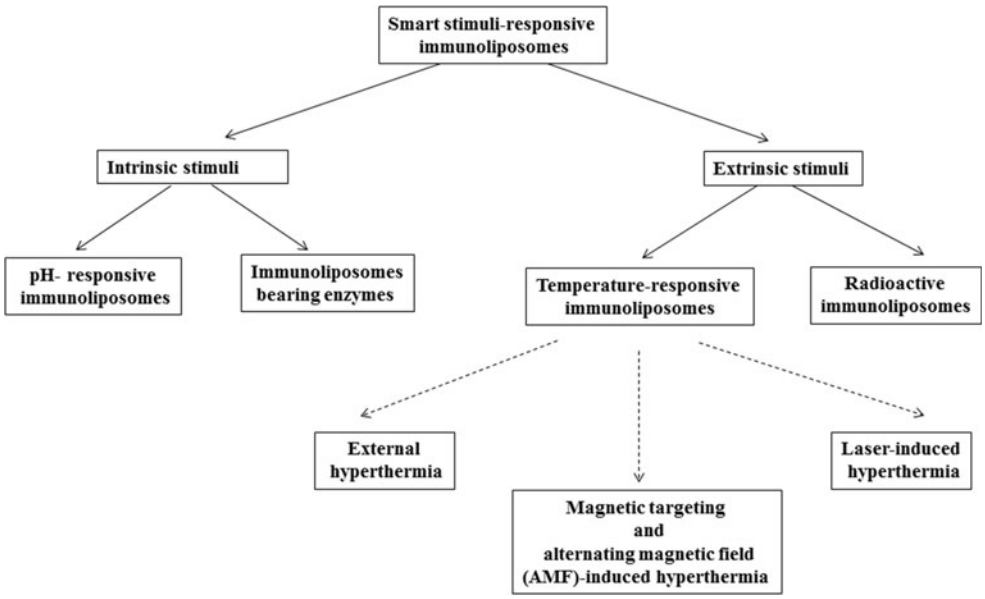

Fig. 2 Schematic representation of stimuli-sensitive targeted immunoliposomes

entrapped content into the cytoplasm of the target cells are advantageous; see review in [77]. There are generally two approaches through which cytosolic delivery can be achieved. The first mechanism involves fusion of immunoliposome with plasma membrane after their binding to the target cells. The endosomal escape of bioactive component into cytosol may also be enhanced by incorporating pH-dependent fusogenic properties into the liposomal vehicle. In this case, pH-dependent fusion of ligand-targeted liposomes with the endosomal membrane following internalization via receptor-mediated endocytosis inhibits intralysosomal degradation [78]. The extracellular pH of tumors and other pathological sites is lower than that of blood and normal tissues (~6.5 versus 7.4), due to the difference in their metabolic environment [79]. This feature has long been exploited by pH-responsive systems bearing pH-cleavable bonds; while stable in blood and normal tissues, they disintegrate and release the drug load in areas with lowered pH [80, 81]. Different classes of pH-sensitive liposomes have been proposed by tailoring liposomes with various pH-labile components including phosphatidylethanolamine (PE) variants [82, 83], pH-sensitive lipids [84, 85], synthetic fusogenic peptides [20, 86, 87], polymers [88, 89], and polyethylene glycol derivatives [90]. So far, multiple attempts have been made to deliver drugs and genes into cytoplasm of tumor cells by virtue of pH-sensitive liposomes, see reviews in [91, 92]. For example, the synthesis of a hydrazine-functionalized PEG-PE-BASED amphiphilic polymer has been reported which could conjugate a variety of ligands via a reversible pH-labile bond, allowing for the targeting of liposomes to the desired site [46]. The majority of the initial works on pH-sensitive systems focused on the combination of DOPE with amphiphilic stabilizers in liposomes with a strong tendency toward acquiring the inverted hexagonal phase upon acidic pH in the endosome [93]. Recent studies have mainly focused on combination of pH-sensitivity, longevity, and targeting ability of liposomes; see [94••] for reviews. Folate-targeted liposomes composed of cationic and anionic lipid combinations exhibited superior serum stability compared to DOPE-based pH-sensitive liposomes. Besides pH sensitivity, folate-mediated endocytosis provides liposomes with an ideal mechanism for cell-specific delivery of araC molecules [47]. The combination of NIPAM copolymer or vesicles containing DOPE and CHEMS with PEGylated lipid provided liposomes with pH-responsive property, as well as long circulation half-lives. Coupling these pH-sensitive liposomes with anti CD-33 and EGFR mAbs has shown benefits in terms of tumor accumulation and endosomal escape for efficient cytoplasmic delivery [48, 49]. Additionally, using lipid pairs with nonmatching acyl chain length in anti-HER2 lipid vesicles loaded with doxorubicin induced pH-dependent permeability of the targeted bilayer due to defective packing of the lipids at the interphases of lipid domains [50].

Targeted pH-sensitive liposomes have also been designed to induce the cytoplasmic release of oligonucleotides [95]. Guo et al. have recently prepared C-X-C chemokine receptor type 4 (CXCR4)-targeted pH-sensitive immunoliposomes encapsulating Lcn2 siRNA, to target metastatic breast cancer [51]. They have shown that the simultaneous targeting of CXCR4 and silencing Lcn2 via lowered pH was more effective in impeding breast cancer cell migration. Turner et al. have also developed PEGylated anionic liposomes as carrier of plasmid DNA to transfect CD3 T lymphocytes. The polyplex has shown significant gene transfer activity dependent on the charge ratio of the components [96].

Table 1 Illustrative examples of stimuli-sensitive liposomes for drug and gene delivery

Platform	Stimuli	Payload	Target	Status	Ref.
Liposome	pH	Carboxyfluorescein Con-A, avidin	Anti-2C5, 2G4 mAbs	In vitro	Biswas [46]
Liposome	pH	Cytosine-b-D-arabinofuranoside luciferase reporter gene	Folate	In vitro	Shi [47]
Liposome	pH	Cytosine arabinoside	Anti-CD33 mAb	In vitro	Simard [48]
Liposome	pH	Gemcitabine	Anti-EGFR mAb	In vitro, in vivo	Kim [49]
Liposome	pH	Doxorubicin	Anti-HER2 mAb	In vitro	Karve [50]
Liposome	pH	Lipocalin-2 (Lcn2) siRNA	Anti-CXCR4 mAb	In vitro	Guo [51]
Lipopolyplex	pH	Plasmid DNA	Anti-CD3 mAb	In vitro	Turner [96]
Liposome	pH	GFP	TAT	In vitro, in vivo	Kale [103]
Liposome	pH	Doxorubicin	Anti-2C5 mAb, TAT	In vitro	Koren [52•]
Liposome	pH	Doxorubicin	Anti-2C5 mAb, TAT	In vitro, in vivo	Apte [53]
Liposome Micelle	pH	Doxorubicin	Anti-2G4 mAb, TAT, biotin	In vitro	Sawant [104]
Liposome	pH	Doxorubicin	Anti-CD19 mAb	In vitro, in vivo	Ishida [54]
Liposome	Enzyme	Fluorescent-labeled	Anti-2C5 mAb, TAT	In vitro, in vivo	Zhu [55]
Liposome	Enzyme pH	shVEGF and DOX	Cell penetrating peptide	In vitro, in vivo	Huang [56]
Liposome	Enzyme	Epirubicin-glucuronide	Anti-OV-TL3 F (ab′) of mAb	In vitro	Vingerhoeds [57]
Liposome	Enzyme	Daunorubicin-glucuronide	Anti-OV-TL3 F (ab′) of mAb	In vitro	Vingerhoeds [58]
Liposome	Enzyme	Epirubicin-glucuronide	Anti-OV-TL3 F (ab′) of mAb Anti-323.A3 F (ab′) of mAb	In vitro	Storm [59]
Liposome	Enzyme	Calcein	anti 323.A3 F (ab′) of mAb	In vitro	Fonseca [60]
Liposome	Temperature	Uridine	Anti-H2K^{k}mAb	In vitro	Sullivan [61]
Liposome	Temperature	Doxorubicin	Anti-HER2/neu F (ab′) of mAb	In vitro	Gaber [62]
Liposome	Temperature	Calcein	Anti-HER2/neu affibody	In vitro	Puri [115]
Liposome	Temperature	Doxorubicin	Anti-HER2/neu affibody	In vitro	Smith [63]
Liposome	Temperature	Doxorubicin	CREKA peptide	In vitro, in vivo	Wang [118]
Liposome	Magnetic	Fe_3O_4	Anti-HER2 and CD20 mAb	In vitro	Ito [64]
Liposome	Magnetic	Fe_3O_4	Anti-HER2 mAb	In vivo	Kikumori [65]
Liposome	Magnetic	Doxorubicin and Fe_3O_4	Folate	In vitro	Pradhan [66]
Polymeric liposome	Magnetic	Epidoxorubicin and Fe_3O_4	RGD peptide	In vitro	Su [67]
Liposome	Laser	Magneto-plasmonic nanoshells	Anti-HER2 mAb	In vitro	Khosroshahi [68]
Liposome	UV	Doxorubicin	Anti-CD20 mAb	In vitro	Li [69]
Liposome	Radioactive	(Et4N) 2(10) B10H10	Anti-MGb 2 mAb	In vitro	Xu [70]
Liposome	Radioactive	Docetaxel	Anti-carcinoembryonic Ab	In vitro	Wang [71]
Liposome	Radioactive	Cisplatin	Anti-EGFR mAb	In vitro, in vivo	Jung [72]
Liposome	Radioactive	^{111}In-labelled	Anti-2C5 mAb	In vivo	Elbayoumi, [73]
Liposome	Radioactive	Technetium-99m	Anti-GAH F (ab′) of mAb	In vivo	Kitamura [74]
Liposome	Radioactive	Technetium-99m	Anti-EGFR and VEGF mAb	In vivo	Li [75]
Liposome	Radioactive	Combretastatin	Cyclo RGD	In vivo	Pattillo [76]

Despite all the advances in preclinical and clinical studies, there are still many challenges in cancer therapy particularly siRNA therapeutics. The main hurdle is the delivery mechanism of the naked siRNA. Other than susceptibility to enzymatic degradation and unfavorable distribution, the surface charge and size of siRNA limit its passage through cell membrane. Numerous nanoparticulate systems have been heavily researched to enhance siRNA stability, cellular entry, and its biodistribution [97]. Lipid-based system carriers including cationic, fusogenic, neutral, and anionic lipids are versatile siRNA delivery vehicles that provide multifunctional features such as targeting, membrane fusion, and triggered release. However, due to the complexity of the biological environment, siRNA delivery vehicles are associated with toxicity-related nonspecific immune stimulation, inflammatory responses, and consequently poor in vivo performance [98].

So, in order to carry these molecules into clinical trials, many challenges including rapid degradation, poor cellular uptake, and untoward side effects need to be clearly addressed. Despite this, the new class of therapeutics has shown improved efficacy in animal models and are currently entering clinical trials for the treatment of solid tumors [99].

Detachable PEG Coating to Improve Target Cell Interaction Though longevity of immune-targeted liposomes in plasma is achieved by modification of the surface with PEG polymers, evidence demonstrates that steric stabilization is not desirable for all steps of drug delivery and may hinder drug release and target cell interaction. So, attempts have been made to enhance efficacy of PEG-liposomes by the loss of PEG after localization at the target site, thus facilitating liposome-cell interaction for efficient cargo delivery to the cells [100, 101]. Torchilins' lab developed TAT-modified pH-sensitive liposomes which showed improved internalization in tumor cells when administrated intratumorally in mice [102, 103]. They hypothesized that after accumulation via EPR, the PEGylated liposomes lose their PEG coating inside the acidified tumor due to the hydrolysis of hydrazone bond and penetrate inside the cells via the exposed TAT moieties. Multifunctional pH-sensitive immunoDoxil® conjugated with anti-nucleosome mAb 2C5 on the surface and further showed enhanced cytotoxicity after exposure to a lower pH environment [52•]. This system has successfully enhanced therapeutic efficacy in mice tumor xenografts through overcoming MDR in drug-resistant cells [53]. After deposition in the targeted organ, the pH-labile hydrazone bond between PEG and PE is cleaved in the acidified milieu, stimulating cell penetration via exposed TAT moieties [104]. The pH-sensitive liposomes with chemically cleavable surface-grafted PEG (mPEG-S-S-DSPE) have also been reported to actively deliver doxorubicin to the CD19 epitope of B-lymphoma cells, where cleavage of disulfide linkage by lysosomal enzymes allowed the rapid release of doxorubicin and consequently enhanced cytotoxicity [54]. To sum up, upon target-cell interaction of immunoliposomes, pH-sensitivity could enhance the release of the entrapped contents into the cytoplasm. However, first, we have to consider that the pH difference is not uniform throughout the tumor tissue and can also fluctuate depending on the cancer type [39••], and second, the applicability of this mechanism largely depends on the endocytosis of the liposomes by the target cells and those systems that are not actively endocytosed may require alternative strategies.

Immunoliposome Bearing Enzymes

The significant internal physiological changes, such as an altered pattern of extracellular protein expression, have been used to develop stimuli-responsive nanocarriers. Enzymes for examples are crucial for the biochemical processes, and their expression varies greatly by tissue types. Extracellular matrix (ECM) remodeling proteinases such as the matrix metalloproteinases (MMPs) are known as the chief mediators of changes in the tumor microenvironment associated with the progression of tumors. The overexpression of MMP-2 has been shown in several clinical and preclinical investigations. Numerous MMP-2 substrates which designed with cleavable peptides have shown to be degradable in the presence of MMP-2 [105, 106]. Zhue et al. reported the design of mAb 2C5-targeted liposomes bearing MMP-2 cleavable octapeptide as a labile bond between long-chain PEG and lipid. When tested for in vitro cellular uptake, results indicated that the highly expressed extracellular MMP-2 degrades octapeptide linker in the tumor cells. This resulted in the exposure of TAT moieties on the surface of nanocarriers allowing for the enhanced cellular internalization and a greater cellular uptake [55]. In a more recent study, Huang et al. developed nanoparticles to co-deliver plasmid expressing interfering RNA targeting VEGF, and doxorubicin to effectively shut down blood vessels and induce cell apoptosis within the tumor. They decorate smart nanoparticles with a cell-penetrating peptide that was dual-triggered by the lowered tumor extracellular pH and MMP-2 to achieve efficient tumor targetability [56].

Another strategy concerns using enzymes with the potential to locally convert relatively nontoxic prodrugs into active cytotoxic agents. In this approach, known as antibody-directed enzyme prodrug therapy (ADEPT), administration of enzyme-coupled antibody prior to prodrug could selectively activate prodrug at the tumor site; see [107] for review. In an alternative approach, enzymes can be conjugated to the surface of immunoliposomes targeted to specific cancer cells, referred to as immune enzymosomes. The obvious advantages of immune-enzymosomes over enzyme-antibody conjugates include (1) efficient conversion of prodrug to active agent due to the high density of enzymes at tumor cell surface and (2) strong affinity of immunoliposomes for target cells through their multivalent binding features [59]. Storm's group first coupled the enzyme β-glucuronidase (GUS) to the surface of immunoliposomes bearing Fab fragments of the mAb OV-TL3. They indicated that the target binding potential of Fab, directed against human ovarian carcinoma cells, was preserved after coupling to immune-enzymosomes. Only in the cases of pretreatment of the target cells with immune-enzymosomes, the cytotoxicity enhancement of epirubicin-glucuronide (epi-glu) prodrug was observed [57], which was further improved by increasing the enzyme density on the surface of immune-enzymosomes [58, 59]. However, above certain levels, the impairment of target cell binding of immune-enzymosomes occurred which was due to the steric hindrance effect mediated by high enzyme density on the surface of immunoliposomes [60]. Overall, designing nanoparticles which respond to enzymes is a fairly new strategy; however, with the rapid progress in biomarkers development and

enzyme-sensitive substrate, in the years to come, we can expect a higher degree of sophistication in the design of theses smart nanocarriers.

Extrinsic Stimuli

Temperature-Responsive Immunoliposomes

External Hyperthermia Despite the great advantage of tumor-specific drug delivery and high uptake of immunoliposomal chemotherapeutics, further improvement could be achieved by applying an external trigger to smartly control drug bioavailability from liposomes [41••]. Mild hyperthermia (HT) has been shown to play a key role in modifying tumor microenvironment by increasing blood flow velocity, oxygenation, and vascular permeability [108]. Pioneering work by Dewhirst's group reported on increased vascular hyperpermeability after HT which lasted for several hours, resulting in increased liposome extravasation [109, 110]. In addition, HT may be used to trigger content release from liposomes incorporating thermosensitive moieties, like lysolipids or olygoglycerol-PG which induces a gel-to-liquid phase transition at the desired temperature leading to release of the entrapped contents [111]. Generally, the triggered release from thermosensitive liposomes is based on the lipid-destabilizing mechanism, phase transition effect, and the time and temperature schedule of the external HT [112]. Ideally, targeted immunoliposomes would result in an increased intracellular localization provided that ligands initiate receptor internalization [113]. Thus, combining the targeting functionality of immunoliposomes and their internalizing features with heat-triggerable characteristics ensures efficient release of the liposome-entrapped drug at the target site. Although literature on targeted immunoliposomes is limited, several groups have investigated this novel approach for targeted thermoresponsive drug delivery, as reviewed in [114••]. In 1986, Huang et al. described the first heat-sensitive immunoliposome that enhanced cellular uptake of uridine. They demonstrated that the enhancement of drug uptake was due to the release of drugs in close proximity to the cell surface by virtue of bound immunoliposomes [61]. Gaber et al. created sterically stabilized thermosensitive liposomes conjugated to the Fab fragments of HER2/neu, for the delivery of doxorubicin to the tumor using postinsertion technique. Uptake of HER2-immunoliposomes by HER2-overexpressing SK-BR-3 cells was shown to be eight times higher than those of nontargeted liposomes for thermoresponsive and nonthermoresponsive liposomes. Though the toxicity of targeted thermosensitive liposomes was quite similar to that of free drug, heating cells to 42 °C after liposome incubation did not enhance the cytotoxicity of targeted immunoliposomes. On the whole, it was shown that the fast release of the entrapped drug by virtue of an external trigger does not necessarily contribute to the efficiency of targeted liposomes [62]. Puris' group investigated affibody affinity ligands as an alternative to antibodies for targeting drug delivery. They conjugated HER2-specific affibody to the surface of thermosensitive liposomes and determined thermosensitivity by temperature-induced leakage of calcein from liposomes. The DPPC-based thermoresponsive liposomes showed an optimal leakage (90–100 %) at 41 °C and appropriate retention of the entrapped calcein in the presence of serum [115]. The same research group modified HER2 affibody by introducing a glycine-serine spacer to achieve more access to HER2 expressed on the target cells. Results indicated that the accumulation of HER2+ liposomes was at least twofold to threefold greater than control liposomes. However, cytotoxicity studies demonstrated that brief exposure of liposome-cell complexes at 45 °C before the onset of incubation improved cell killing for both targeted and control liposomes [63].

It has been suggested that HT in combination with chemotherapy is playing an important role in the treatment of MDR [116, 117]. In a more recent study, a CREKA-modified lysothermosensitive liposome containing doxorubicin was designed to overcome multidrug resistance in tumor cells. It was hypothesized that the tumor homing peptide CREKA would target the clotted plasma proteins in tumor vessels and releases a burst of the encapsulated doxorubicin in the heated tumor site. The in vivo anti-tumor results indicated that the highest drug concentration and antitumor activity during HT treatment was observed with targeted doxorubicin-loaded thermosensitive liposome [118].

Magnetic Targeting and Alternating Magnetic Field (AMF)-Induced HT An alternative approach in applying local HT treatment is magnetic targeting and alternating magnetic field (AMF)-induced HT. Iron oxide nanoparticles, known as superparamagnetic iron oxide nanoparticles (SPIONs), have been extensively utilized for simultaneous imaging and stimuli-responsive drug delivery [119••]. Additionally, magnetic nanoparticles can generate HT following the application of an alternating magnetic field which could damage tumor cells (Fig. 3), while normal cells remain unaffected. Ito et al. reported that Fe_3O_4-loaded anti-HER2 immunoliposomes under 42.5 °C heat generated by AMF induced strong cytotoxic effects on SK-BR-3 breast cancer cells in vitro [64]. Another targeted magnetoliposomes to HER2+ breast cancer cells were developed by Kikumori et al. They investigated the feasibility of this modality in tumor-bearing mouse models and demonstrated a substantial tumor regression in the AMF-HT-treated animals [65]. Pradhan et al. also combined biological (folate) and physical (magnetic) drug targeting to use these features in magnetic HT-triggered drug release. To this end, they developed folate-targeted doxorubicin magnetoliposomes and showed that under a permanent

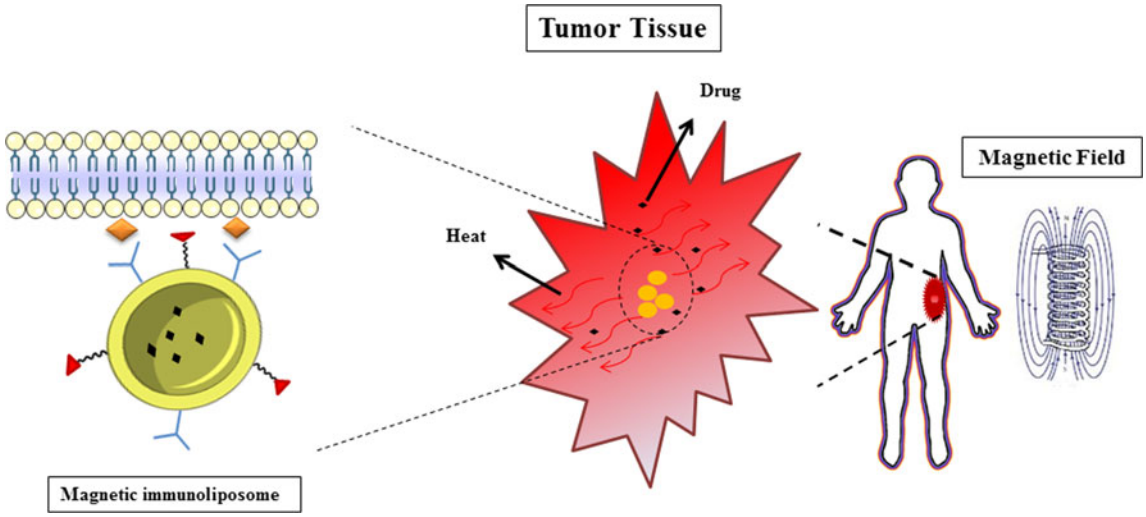

Fig. 3 Magnetic field-induced hyperthermia and drug release. The multifunctional immunoliposome can be targeted physically by means of magnetic field and also biologically by targeting moieties attached to their surface. Targeting immunoliposome would enhance receptor-mediated uptake of nanoparticles into tumor cells followed by trigger release of the entrapped content through application of an external magnetic field

magnetic field, this formulation substantially increased doxorubicin cellular uptake compared to nonmagnetic liposomes in folate-receptor expressing tumor cell lines. Additionally, parallel increase in cell killing was also observed with these formulations [66]. More recently, PEG-RGD magnetic polymeric liposomes were developed as a multifunctional platform for targeted epidoxorubicin delivery to tumor cells. Results indicated that external magnetic field promotes accumulation of magnetic carrier on the surface of tumor cells where cellular uptake can be enhanced by RGD targeting to $\alpha v\beta 3$ on MCF-7 tumor cells [67].

Laser-Induced Hyperthermia Light offers an interesting approach for use as an external stimulus due to noninvasiveness and suitable remote control over release of drugs from nanovehicles. Several photo-sensitive groups and a wide range of mechanisms are involved in light-triggered release of cargos from nanopreparations [39••]. Laser irradiations of gold nanostructures have been shown to induce local heating due to temperature rise, which can be directly transferred to the vicinity of nanostructure. Khosroshahi et al. have designed such magneto-plasmonic nanoshells (MPNS)-loaded liposomes for targeted laser-induced apoptosis in HER2-overexpressing human breast carcinoma. Results demonstrated that the laser-induced release of carboxyfluorescein was due to the direct heating of liposomes and the subsequent effect on the permeability of lipid bilayers. Further, laser irradiation of Herceptin-mediated internalized MPNS-loaded liposomes resulted in cell apoptosis which was suggested to be due to photothermal bubble generation that thermally damaged cellular membrane [68]. In a more recent study, Li et al. used UV irradiation to improve the serum stability of CD20-targeting immunoliposome of doxorubicin [69]. They developed liposomes that can form chains of covalently linked lipids in the liposomal bilayers upon UV irradiation with slower and efficient drug release compared to nonirradiated liposomes.

Radioactive Immunoliposomes

Locoregional recurrence following surgery is a major problem in cancer treatment. To improve the overall patient survival and reduce the local recurrence rate after surgery, adjuvant chemotherapy and radiotherapy (RT) is given preoperatively or postoperatively. Since the discovery of liposomes 40 years ago, various methods of labeling liposomes with both diagnostic and therapeutic radionuclides have been developed [120]. Further development of monoclonal antibody makes it possible to enhance the selectivity of liposomes by a targeted delivery approach. In 1991, Xu et al. showed that SGC-7901 cells pretreated with boron-10 containing immunoliposomes survived only 27 %; this survival rate was significantly lower than nonirradiated or nonpretreated cells with irradiation [70]. Radiation also induces apoptosis in neoplastic cells, and the extent of apoptosis is closely related to radiosensitivity. Some chemotherapeutics possess the radiosensitizing properties for malignant tumors; however, in most instances, poor selectivity for targeted tumors lead to untoward side effects [121]. It has been reported that immunoliposomal docetaxel has strong radiosensitizing effect in LOVO colon carcinoma [71]. When combined with irradiation, antibody-conjugated liposomes substantially decreased the percentage of cells in G2/G1 and S phases compared to liposomal docetaxel. In a more recent study, Jung et al. evaluated the radiosensitizing effect of EGFR-conjugated cisplatin-incorporated liposomes. They showed that treatment with a

combination of immunoliposomes and radiation resulted in increased growth delay in xenograft tumor mouse model with reduced side effects [72].

Immunoliposomes have also been used as carriers of diagnostic agents for different imaging modalities including gamma scintigraphy, MRI, and computed tomography [122]. Torchilin laboratory developed ^{111}In-labelled liposomes for in vivo biodistribution and tumor accumulation of 2C5 antibody-modified liposomes [73]. Gamma scintigraphic imaging of tumor-bearing mice exhibited faster and more efficient tumor accumulation of 2C5-modified liposomes compared to control in tumor models. In another study, immunoliposomes were labeled with Tc-99m, either specifically in fragment portion or entrapped within liposomes. Biodistribution data in xenografted model of human gastric cancer indicated that Tc-99m entrapped in liposomes demonstrated higher stability in vivo and was strongly taken up by the tumor compared to Tc-99m-labled liposomes [74]. Intracavitarily, injection of Tc-99m labeled panitumumab- and bevacizumab-liposomes in the rat breast cancer xenograft model following tumor removal showed high intracavitary retention of liposomes [75]. The results generally indicate the increased potential of targeted liposomes in enhancing radiotherapy to decrease cancer recurrence with minimal systemic toxicity.

Another characteristic feature of radiation is upregulation of several adhesion molecules. Thus, carriers bearing on their surface, ligands specific to the radiation-induced upregulated adhesion molecules, would preferentially localize in the irradiated tumor regions. Based on this hypothesis, Kianis' group investigated targeting of antivascular drug, combretastatin, to the irradiated tumor via ligand-bearing liposomes. They showed that treatment with a single dose of irradiation plus immunoliposomes significantly delayed tumor growth compared to other treatment groups [76].

Challenges in Developing Stimuli-Responsive Nanoparticles

Many complexities confound the successful development of multifunctional and stimuli-sensitive drug delivery systems and their transition to clinic. The ideal intelligent nanosystem with the ability to incorporate a sufficient load of drug or nucleic acid-related materials should first deposit at the desired site of action and then deliver its load into targeted cells [123]. So, for both nonspecific and specific targeting, the principle of many drug delivery systems is based on the EPR phenomenon. Clinical outcome from nano-sized delivery systems, however, indicated that EPR-dependent drug delivery could be affected by numerous tumor biological factors. The experimental animal models may not be representative of clinical tumors in several key aspects. Besides, the difficulty of extravasation into the tumors with high interstitial fluid pressure and the reduced intratumoral mobility of drug carrier due to nonspecific interaction with ECM components in a chaotic tumor environment will certainly diminish the reliability of EPR effect. On the basis of tumor heterogeneity and the extremely complex tumor microenvironment, the EPR should only be considered when there is sufficient evidence of the tumor type susceptibility [124]. When developing targeted carriers, one has to keep in mind that though ligand targeting exhibited improved efficacy over passive targeting via enhanced cellular uptake, it raised new challenges including hindered diffusion and penetration through the tumor tissue, immune recognition, and longevity in circulation. Further, surface characteristics, shape, and size of nanoparticle play an important role in the ultimate pharmacokinetic and distribution of these targeted vehicles [18]. Though many ligand-targeted particulate nanomedicines have progressed into clinical trials, yet, none of them have been approved at this moment. In this case, increased understanding of the in vivo fate and interaction of the ligand-targeted vehicles with serum proteins and cells in human is important in advancing the clinical translation of such targeted particles [125]. Going one step further, combining stimuli-sensitive properties with targeted delivery could be beneficial in improving the therapeutic outcome. However, this approach is rather complex and this complexity adds new pitfalls in predicting the behavior of the switchable nano-system. These delivery systems have to carry various functional moieties on the surface and simultaneously possessing the capability to switch on certain functions under the action of local stimuli [39••]. Additional capability means additional synthetic and purification steps as well as elevated complexities and costs [126]. The feasibility of pH-switchable system, for example, has been frequently reported, but the translation from bench to bedside is not straightforward. The applicability of pH-sensitive nanoparticles profits from active targeting where ligand-induced receptor-mediated endocytosis is the principle mechanism [104]. Further, a different pH may be present in in vivo compared to the one tested under experimental in vitro condition. It should be considered that there may be upregulated proteins or high concentrations of enzymes in normal cells in the case of enzyme-responsive nano-vehicles. When the redox potential gradient is the local trigger, the low concentration of reducing agents in blood needs to be taken into account which may result in premature loss of the desired effect. In particular, endogenous stimuli are hard to control and they may vary from one patient to another. Exogenous trigger, on the other hand, offers more freedom in design; however, the biocompatibility of the external stimuli, level of tissue damage, the depth of penetration, and the time and the availability of external source location may restrict the application of the external stimuli [38•, 39••]. On top of this is the difficulty in terms of manufacturing process and reproducibility of smart targeted delivery systems on a commercial

scale which will come at the cost of additional financial hurdles [126]. To date, stimuli-sensitive nano-systems that have reached the clinical stage are based on exogenous stimuli, which may explain the variability associated with endogenous triggers. Thermodox, which is the thermoresponsive liposome of doxorubicin, has been used in clinical trials for the treatment of breast cancer (phase II) and hepatocellular carcinoma (phase III). However, it has recently been suspended due to a lack of sufficient improvement in the life span of patients [37].

Considering the above mentioned problems, clinical translation of the current knowledge on multifunctional delivery systems requires avoiding the complex multistep manufacturing processes. Additionally, scalability of such products should not be problematic and be based on accepted protocols [119••]. Indeed, the development of multifunctional and stimuli-responsive nanoparticles represents a new area in drug delivery systems, and if the requirements are met, the smart carriers could become an important part of the personalized therapy in upcoming years.

Conclusion

Over the past decade, a dramatic increase in the complexity of sophisticated nanocarriers has been observed. Particulate nanomedicines have evolved to overcome the major hurdles of drug targeting including the heterogeneity of the biological target and the limited bioavailability of the drugs. In this context, advances in polymer chemistry enable modification of nanocarriers for on-demand, stimuli-responsive release of the entrapped payload in the targeted tissue. As a result, nanopreparations incorporating multiple functions gained a lot of momentum; however, creating systems to simultaneously achieve targeted delivery through the attached ligands and respond to stimuli to trigger drug release is significantly more challenging than mono-therapies. Though proof of concept has been reported for several targeted stimuli-responsive nanoparticles, only a few have been assessed in in vivo preclinical models and none of them have yet reached clinical phase studies. On the whole, for these systems, complex, multifaceted architectural design, difficulties in scaling-up, insufficient biocompatibility, and the microenvironmental heterogeneity likely hamper the translation from bench to bedside and, however, do not make it impossible. The representative examples highlight just a few of the outstanding potential of nanomedicines for the development of combination therapies. In the next generation of delivery vehicles, we would expect a higher level of sophistications in the design of these systems.

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Compliance with Ethical Standards

Conflict of Interest The authors confirm that this article content has no conflict of interest.

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- •• Of major importance

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