Published in final edited form as:

Adv Drug Deliv Rev. 2013 January ; 65(1): 24–35. doi:10.1016/j.addr.2012.08.009.

Immunoconjugates and long circulating systems: Origins, current state of the art and future directions*

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Abstract

Significant progress has been made recently in the area of immunoconjugated drugs and drug delivery systems (DDS). The immuno-modification of either the drug or DDS has proven to be a very promising approach that has significantly improved the targeted accumulation in pathological sites while decreasing its undesirable side effects in healthy tissues. The arrangement for both prolonged life in the circulation and specific target recognition represents another potent strategy in the development of immuno-targeted systems. The longevity of immuno-targeted DDS such as immunoliposomes and immunomicelles improves their targetability even in the presence of the additional passive accumulation in areas with a compromised vasculature. The added use of the immuno-targeted systems takes advantage of the specific microenvironment of pathological sites including lowered pH, increased temperature, and variation in the enzymatic activity. “Smart” stimulus-responsive systems combine different valuable functionalities including PEG-protection, targeting antibody, cell-penetration, and stimulus-sensitive functions. In this review we examined the evolution, current status and future directions in the area of therapeutical immunoconjugates and long-circulating immuno-targeted DDS.

Keywords

Antibody; Immunoconjugation; Long-circulating systems; PEGylation; Immunoliposomes; Immunomicelles

1. Introduction

One of the main problems for effective drug delivery is to increase the therapeutic efficacy of toxic drugs while minimizing their non-specific side effects. Targeting of drugs towards disease sites can bring a solution to this problem. The idea of the “magic bullet” drug, which was introduced by Paul Ehrlich early in the twentieth century, considered that one part should consist of a moiety recognizing and binding the target, while the second part should provide a therapeutic action at this target. With the discovery of antibodies, a targeting molecule with high specificity for the ligand, the concept of “magic bullet” was successfully implemented with a variety of immuno-target drugs (immunoconjugates) including immunotoxins, immunoisotopes, antibody-chemotherapeutic conjugates and immuno-targeted drug delivery systems (DDS).

*This review is part of the *Advanced Drug Delivery Reviews* theme issue on “25th Anniversary issue — Advanced Drug Delivery: Perspectives and Prospects”.

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To date, as many as 31 therapeutic antibodies and immunocjugates (Table 1) are clinically approved for a variety of applications, with many more under investigation [1]. Despite the recent clinical success of individual antibodies, they are still rarely curative. Conjugation of pharmaceutical agents with an antibody provides an opportunity to improve the therapeutic efficacy of the antibody itself or/and potency of the conjugated drug. Although, immunoconjugates are considered a very promising therapeutic tool, still, the problems associated with low drug/antibody ratios, complexity of the conjugation chemistry, the high cost of production and instability in the circulation remain.

On the other hand, nanosized DDS, such as liposomes and micelles, have certain advantages that might offer a solution to some of these problems. The obvious advantages of immuno-targeted DDS over other immunoconjugated systems include: 1) a high capacity of drug loading; 2) the relatively few mAbs necessary to achieve high levels of drug targeting; 3) protection of the encapsulated drug from degradation in the biological environment, and 4) the ability to provide stimulus-sensitive compositions for controlled and enhanced release of the drug in the targeting areas.

The concept of long-circulating immuno-targeted DDS requires an attempt to combine the capacity for prolonged circulation with specific target recognition by mobilized mAbs (or their fragments) in one preparation. The longevity of immuno-targeted DDS in the blood is routinely achieved by modification of their surface with flexible hydrophilic polymers, such as poly-ethylene glycol (PEG) [2] to protect them from the elimination by cells of the reticulo-endothelial system (RES). The prolonged circulation can help to achieve a better targeting effect for immuno-targeted DDS by allowing more time for opportunities for their interaction with the target antigen [3]. In situations when the target has a diminished blood supply (ischemic or necrotic areas), or the expression of the targeting antigen is very low, the longevity of the immuno-targeted DDS becomes a critical parameter for their therapeutic efficiency. The other important aspect of longevity is the ability of many drugs and DDS to be accumulated passively in the areas with leaky vasculature (including tumors, infarcts, and inflammation sites) due to the Enhanced Permeability and Retention (EPR) effect [4]. In this case, the EPR effect additionally can contribute to the targeted accumulation of immunoconjugates and immuno-targeted DDS [5].

Recently the concept of “smart multifunctional” nanocarriers that gather various functionalities including PEG-protection, targeting ligand, cell-penetrating and stimulus-sensitive functions was introduced as a novel direction in the development of immuno-targeted DDS [6].

This review focuses on recent developments, design, applicability and future directions in the development of therapeutic immunoconjugates and long-circulating immuno-targeted DDS.

2. Immunoconjugates: past, present and future in targeted drug delivery

2.1. Evolution of therapeutic immunoglobulins

The invention of a hybridoma technique by Köhler and Milstein in 1975 [7], which allowed obtaining large quantities of monoclonal antibodies (mAbs) with a single specificity, accelerated the introduction of antibodies and immunoconjugates for therapy. However, the initial use of full-length mouse mAbs was restricted because of their low efficacy and rapid clearance due to the immune response initiated in human patients [8]. These limitations on the use of murine mAbs have largely been overcome by the development of the chimerization technique, in which the murine constant domains are replaced with human equivalent regions [9,10]. Another humanization strategy involves transfer of the

complementarity-determining regions (CDRs) from a murine to a human antibody [11]. A low or negligible level of immunogenicity of humanized mouse mAbs has provided higher therapeutical efficiency [11,12].

The development of phage display technology [13] and transgenic mice [14] that express human immunoglobulin genes contributed to a trend towards substantial increase in the clinical study of human mAbs [15]. Despite the success of full-length mAb for therapeutic applications, the current trends are shifting towards the development of antibody fragment technologies [16]. Antibody fragments, including antigen-binding fragments (Fab), single chain variable fragments (scFv), and “third generation” (3G) molecules — present advantages of both small molecules and mAbs: lower costs of production, improved efficacy, flexibility of their composition, low immunogenicity, better tissue and tumor penetration and an ability to bind cryptic epitopes not accessible to full-sized mAbs [17].

The one serious disadvantage of small antibody fragments is decreased circulation half-lives comparing to full-length mAb, primarily because of renal clearance [18]. However, the circulation time of antibody fragments can be prolonged significantly by their PEGylation [19]. FDA-approved certolizumab pegol (Cimzia®), an anti-TNF-alpha humanized Fab conjugated to a 40 kDa polyethylene glycol (PEG) moiety, is an example of a commercially available antibody fragment with a prolonged circulation half-life [20].

Bispecific antibodies (bsAbs) represent another interesting trend in the development of therapeutical mAbs [21]. In contrast to a natural antibody that consists of two identical antigen-binding domains, bispecific antibodies are capable of interacting with two different antigens. Catumaxomab (Removab; Fresenius Biotech/TRION Pharma) represents a successful example of a trifunctional bispecific mAb, which binds to both the epithelial cell adhesion molecule (EPCAM) on tumor cells and T-cell antigen CD3 [22].

It is worth mentioning that the further success of antibody technologies relies on decreasing the cost of production with improved immunogenicity and greater therapeutic efficiency by optimization of the antibody design.

2.2. Design of immunoconjugates

A typical immunoconjugate consists of an antibody conjugated via a chemical linker (or fused with a protein, as in the case of immunotoxins) to a drug molecule or drug carrier.

Undeniably, the most important component of immunoconjugates is the antibody. The desired antibody should enhance accumulation of the conjugated drug in targeted areas with minimal cross-reactivity with healthy tissues. To date, the majority of targets involved in clinical trials are the tumor-associated antigens (TAA). This includes antigens over-expressed in B-cells (e.g., CD20, CD22, CD40, CD79), T-cells (CD25, CD30), carcinoma cells (HER2, EGFR, EpCAM, EphB2, PSMA, Cripto), endothelial (endoglin), stroma cells (fibroblast activated protein) and the tumor vasculature antigens [23,24].

One of the most important parameters that should be taken under consideration when designing immunoconjugates is the antigen-binding affinity of the mAbs. The antigen-binding affinity (K_d) of existing therapeutical antibodies varies from 0.08 to 30 nM [23]. The antigen-binding affinity can be improved from the nanomolar to the picomolar range using phage-display libraries, as shown for human anti-HIV-1 antibody [25] and HER2-specific mAb fragments [26].

Linkers represent another key component of immunoconjugates. A “perfect” linker between an antibody and drug must not only provide stability during circulation in blood but also

should allow for the rapid release of the effector molecule in the targeting zone. The amino groups of lysine or the sulfhydryl groups of the inter-chain cysteine residues are the major antibody sites used for drug conjugation [27]. The conjugation process begins with functionalization of the mAb by attachment of a bifunctional linker [28] or with a preformed drug-linker molecule such as maleimidocaproyl-valine-citrulline-p-aminobenzyloxycarbonyl-monomethylauristatin E [29,30]. One of the most important aspects in conjugation chemistry is to avoid binding of the drug molecule to the antigen site of the antibody. A number of highly regioselective techniques have been developed to introduce molecules of interest onto mAbs without deterioration of the affinity to the antigen. These methods include reductive amination of oxidized oligosaccharide moieties [31], introduction of new more favorably located oligosaccharide sites [32] or serine residues [33] using genetic engineering.

Two different types of linkers are in the use: non-reducible and reducible [34]. Non-reducible linkers, such as thioether [35], are stable in a chemical or enzymatic environment, and release the drug only after complete degradation of the mAb in the lysosome. Hydrazone, peptide, and disulphide bonds are examples of reducible linkers that can be cleaved either intracellularly or extracellularly under lowered pH conditions or by hydrolytic enzymes [24]. Still, the main problem for conjugation technology remains the heterogeneity of antibody–drug complex with respect to both the distribution and loading of drug molecule on the mAb.

The third module of an immunoconjugate is represented by the effector drug. Immunoconjugates can be divided into numerous groups according to their payloads: immunotoxins [36]; a chemotherapeutic agent chemically linked to the antibody; antibody–radionuclides [37] and nanocarriers modified with an antibody, such as immunoliposomes [38] and immunomicelles [39].

2.3. Immunotoxins

The immunotoxins represent a very potent class of the chemotherapeutical agents due to their extreme potency and ability to kill drug-resistant cells [36]. The first generation of immunotoxins, which an intact mAb linked by chemical conjugation methods [28] to the full-length natural toxin, were usually therapeutically inefficient because of their nonspecific toxicity, high immunogenicity and poor tumor infiltration [40]. The next generation of immunotoxins (recombinant immunotoxins) was produced by recombinant DNA techniques [41] and consisted of a chimeric protein with a tumor-specific antibody fragment (Fv), cytokine genetically fused with a toxin molecule [42]. The recombinant immunotoxins are homogeneous and fairly inexpensive.

At the moment, denileukin diftotox (OntakTM) is the only immunotoxin approved by the FDA for patients with persistent or relapsed CD25-positive cutaneous T-cell lymphoma (CTCL) [43]. It targets the IL2 receptor, which is highly expressed in numerous malignancies such as CTCL, adult T-cell leukemia (ATL), Hodgkin's disease (HD), and other B- and T-cell leukemias and lymphomas [44]. Denileukin diftotox is a fused protein (DAB389IL2) that consists of IL-2 moiety merged with diphtheria toxin [42].

Among other potent immunotoxins, which are currently in different phases of development, it's worth mentioning the anti-CD22 dsFv PE38 (BL22) and anti-CD25 PE38 (LMB-2), that have showed potent therapeutical effect against a variety of hematologic malignancies [36].

2.4. Antibody–radionuclide conjugates

Because lymphoma cells are especially sensitive to radiation and readily accessible to antibody conjugates, radioimmunoconjugates became a standard treatment option for

patients with hematological cancers such as non-Hodgkin's lymphoma (NHL) [45]. So far, two radiolabeled anti-CD20 murine antibodies, ^{131}I -Tositumomab (Bexxar®; GlaxoSmithKline) and ^{90}Y -ibritumomab tiuxetan (Zevalin®; Spectrum Pharmaceuticals) have been approved FDA for treatment of patients with NHL [46,47].

While the use of radioimmunoconjugates for treatment of lymphomas has been successful, the response in solid tumors was insufficient, presumably due to their reduced radiosensitivity and the inability of the radioimmunoconjugates to deliver the dose of radiation required to eradicate solid tumors [48,49]. Higher efficiency of radioimmunoconjugate-based treatment for solid tumors can be achieved by improvement of the antibody uptake, enhancement of the tumor's sensitization to radiation and by new co-treatment strategies [45].

2.5. Antibody–drug conjugates (ADCs)

Cytotoxic drugs conjugated with an antibody via a chemical linker represent another important class of immunoconjugates. The earliest generations of ADCs were combined with anticancer drugs already clinically approved, including doxorubicin, daunorubicin, methotrexate, mitomycin C, 5-fluorouracil and vinca alkaloids [50,51].

BR96–doxorubicin is an example of the first generation of ADCs [52]. In this immunoconjugate, about eight molecules of doxorubicin were linked via a pH sensitive hydrazone bond to the anti-Lewis Y chimeric human/mouse mAb BR96. Preclinical studies on xenografted mouse and rat models demonstrated significant antitumor effects, including cures [53,54]. However, only limited antitumor activity of this immunoconjugate has been observed in clinical trials [55].

The poor therapeutic efficacy of the first generations of ADCs, including BR96–doxorubicin, was a product of insufficient potency of the toxic drug and limited internalization ability of the immunocjugates: the amount of the effector molecule that could be delivered intracellularly by the antibody was not enough to cause cell death [56].

The next generation of ADCs was directed to the use of highly potent cytotoxic agents such as maytansinoids, auristatins, and calicheamicin [57]. Despite the potent cytotoxicity of these compounds towards cancer cells in vitro, which was 100–1000 higher than the cytotoxicity of the traditional chemotherapeutic drugs used in ADCs, the results of clinical trials were disappointing due to the poor therapeutic window [58,59]. However, the picomolar IC_{50} of these drugs makes them attractive candidates for targeted delivery with mAbs.

The most well-known auristatin-based ADCs is brentuximab vedotin (SGN-35), which is composed of the chimeric anti-CD30 mAb linked through a dipeptide linker with the synthetic tubulin-disrupting agent monomethyl auristatin E (MMAE). In August 2011, brentuximab vedotin was approved by the FDA for the treatment of Hodgkin lymphoma after the failure of autologous stem cell transplant [60].

A set of the maytansinoid–disulfide linker derivatives (DM1, DM3, DM4) conjugated to different tumor-specific antibodies is currently in various stages of clinical development [56]. The most prominent maytansine-based ADC is trastuzumab–DM1 [61], a maytansine derivative (DM1) linked to trastuzumab (Herceptin), a clinically approved anti-HER2 mAb. After successful results in phase I–II studies [62,63], trastuzumab–DM1, either alone or in combination with other anticancer drugs, have led to several ongoing phase II and III trials [64].

Inotuzumab ozogamicin is an example of calicheamicin-based immunoconjugate composed of an anti-CD22 IgG4 isotype (G544) and the derivative of calicheamicin [65]. The combined treatment of inotuzumab ozogamicin plus rituximab has demonstrated encouraging clinical responses in patients with B-cell NHL in phase I–II studies [66], and is currently under phase III (NCT01232556) trials in relapsed/refractory aggressive NHL patients who are not candidates for intensive high-dose chemotherapy.

Summarize, considerable progress has been made in the development of immunoconjugates over the past few decades. Numerous immunoconjugates, including different immunotoxins, radioimmunoconjugates and ADCs, have been approved for clinical applications. Still, the problems associated with stability of immunoconjugates in the circulation, low drug loading, short half-life and controlled release of the effector molecule in the pathogenic areas need to be fixed. Among the other immunoconjugates, immuno-targeted DDS, such as immunoliposomes or immunomicelles, can be considered a new therapeutical opportunity in the field. The new perspective with immuno-targeted and long-circulating DDS will be discussed further.

3. Long-circulating immuno-targeted drug delivery systems (DDS)

The one of the problems with immunocojugates is that only a limited number of drug molecules (up to eight) can be attached to the antibody without compromising its targeting properties [67]. This was the main reason for the clinical failure of immunocojugates that utilized traditional cytotoxic drugs including doxorubicin, daunorubicin and mitomycin C. As mentioned above, this limitation can be overcome by conjugation of highly toxic compounds with a picomolar range for their cytotoxicity, such as toxins, maytansinoids or auristatins. Alternatively, well established lipid-based DDS, such as liposomes and micelles, present another approach that may resolve this problem. The properties, current state and future perspective of immuno-targeted long-circulating DDS will be discussed using immunoliposomes and immunoliposomes as an example.

3.1. EPR effect and need for long circulation

It is now well known that many pathological tissues (tumors, infarcts) have increased blood vessel permeability due to their compromised integrity or rapid and defective angiogenesis [68,69]. Such “leaky” vessels allow increased accumulation of large molecules (>40 kDa) and nano-carriers in such pathological tissues. After accumulation, they are retained in the tumor interstitium due to the absence of functioning lymphatics [70]. Thus, macromolecules entering tumors are retained with higher efficiency than in normal tissues. This is called as the Enhanced Permeability and Retention (EPR) effect and is the basis for passive targeting [4,71]. Most of the studies report a threshold size of ~400 nm for extravasation in tumors [72]. More effective extravasation has been shown with particles <200 nm [38,68].

A second criterion important for passive targeting by the EPR effect is prolonged residence time in the blood (i.e. long circulation). Such long-circulating macromolecules or nanocarriers are able to extravasate more efficiently due to the repeated passages through the leaky tumor vasculature and will show enhanced accumulation compared to normal tissues.

3.2. Drug–polymer conjugates

Many anticancer agents, including small molecules, proteins and peptides, are biologically active and potent therapeutic molecules. However, upon administration they suffer rapid elimination from the circulation by renal filtration (for molecules with molecular weight below the threshold for kidney excretion), by uptake by the reticuloendothelial system (RES) and through distribution into various non-targeted organs. This has sometimes led to

administration of large doses to reach the desired therapeutic effect but with exacerbation of toxic side effects.

Most of the aforementioned limits of the small molecular weight anticancer drugs can be overcome by their conjugation with water-soluble polymers. In general, polymer–drug conjugates can increase the aqueous solubility, reduce toxicity and prolong the blood circulation time compared to free drugs [73]. The various polymers have been used successfully for conjugation of anticancer drugs (e.g. paclitaxel, doxorubicin, camptothecin oxaliplatin, etc.) include polyethyleneglycol (PEG) [74], poly(glutamic acid) [75,76], dextran [77,78], N-(2-hydroxypropyl) methacrylamide copolymer (HPMA) [79–81] and cyclodextrin [82,83]. The molecular complexity of cancer seems to demand drug combinations that improve long-term patient prognosis, and now many studies are evaluating strategies for such polymer-combination therapeutics [84,85].

Proteins and peptidic anticancer therapeutics, in addition to the aforementioned drawbacks, commonly suffer from fast proteolytic degradation and immunogenicity. The conjugation with hydrophilic polymers is employed to overcome these drawbacks [86]. Although several polymers have been used for protein delivery and stabilization, PEG has been the most widely used [73]. PEG is a highly water soluble, flexible, non-toxic, non-degradable and non-immunogenic polymer. The PEG is also non-biodegradable, does not form any toxic metabolites, and PEG molecules with <40 kDa molecular weight are readily excreted by the kidneys. The PEG, when attached to protein or peptides, interferes with binding to active sites of proteases, opsonins and antigen-processing cells by creating steric hindrances. In addition, PEG conjugation increases the hydrodynamic volume and molecular weight of proteins resulting in decreased clearance by renal filtration [87]. The evolution of PEGylation chemistry now allows for site-specific modification of the attached proteins and peptides [88]. This can lead to better preservation of the activity of the native protein and peptide. Several anticancer formulations based on PEG conjugation have shown promising results for delivery of L-asparaginase, interferon, G-CSF, etc. [86].

3.3. Longevity of DDS

For most therapeutics, nano-sized DDS may represent a better alternative to conjugation with polymers. Drug delivery using nanocarriers has several advantages. As was already mentioned, these include a higher drug/carrier ratio compared to polymer conjugates, higher degree of protection against enzymatic degradation, reduced toxicity to normal healthy tissues, improved safety and anti-tumor efficacy and the ability to deliver multiple drug moieties [89]. Over the last two decades, nanotechnology has undergone an explosive growth in areas of nanoparticle drug delivery systems for various disease specialties, especially cancer. These nanocarriers include liposomes, polymeric micelles, polymersomes, niosomes, nanoparticles and nanoshells [90,91]. Oncology has particularly benefited from this growth since nanocarrier therapeutic systems and liposomes (10–200 nm) were the first commercially available injectable nano-therapeutics [92].

Among the nanocarriers, liposomes are the most extensively studied. Liposomes are aqueous phospholipid vesicles formed by spherical phospholipid bilayers with an aqueous inner space [93]. The first conventional liposomal formulation of anticancer drugs did not improve the therapeutic activity of the drug against implanted tumors [94]. Upon intravenous administration these liposomes were quickly captured (within 15–30 min) by the RES, primarily by liver where they released up to 50% of their contents [95,96]. Later, the liposomes were designed to stably circulate in the blood for a prolonged period of time. As with polymeric conjugates, longevity in the blood is important for nanocarrier DDS for several reasons: one of them by maintenance of a sufficient amount of drug in blood for an

extended period of time and the other, to achieve sufficient accumulation in desired target areas such as tumors by the EPR effect [97,98].

The first approach was to extend the liposome longevity by mimicking the liposomal surface to the erythrocyte membrane. Gangliosides and sialic acid derivatives, such as monosialoganglioside (GM1), were used for liposomal surface modification [99]. This led to a prolonged plasma circulation time for the liposomes [100]. The other approach was to 'mask' the surface of liposomes with hydrophilic polymers, such as polyethylene glycol (PEG) [100–103], similar to the conjugation of PEG to proteins [104]. Coating with PEG sterically hindered binding interactions with opsonins and reduces the capture by the RES [105–107]. PEGylation resulted in increased surface hydrophilicity, shielding of surface charges, increased repulsive interaction between PEGylated nanocarriers and blood components and took advantage of impermeable nature of the polymeric layer formed on the surface of the nanocarrier for other solutes [105,108–110]. Although this approach was studied for liposomes [110], it can be easily adapted for other nanocarriers [111].

Liposome surface modification with PEG can be performed by covalent attachment onto the surface of preformed liposomes. Both the amount of the PEG grafted and the molecular weight of the polymer are important factors for longevity in blood [112]. Usually, the liposomes with higher molecular weight PEG (PEG 1900 and PEG 5000) sustained higher blood levels than liposomes containing PEG-lipid with a shorter chain PEG (e.g. PEG 750 and PEG 120). A sharp increase in circulation time (up to six times) was noted with Doxil® (PEGylated liposomal doxorubicin) compared to free doxorubicin and also led to greater therapeutic efficacy [98,113]. Various other anticancer drugs such as arabinofuranosylcytosine, adriamycin, cisplatin and vincristin [114–118] have been used in long-circulating liposomes.

Another advantage of PEGylation is that the clearance of the liposomes becomes less dependent on size, membrane fluidity, and surface charge density [119]. Usually, liposomes of similar composition show increase in RES uptake with an increase in size [120]. The addition of PEG-DSPE into the liposome composition resulted in clearance rates that were relatively insensitive to size in the range of 80 to 250 nm [103,121]. Liposomes containing unsaturated lipids, such as egg PC, are cleared more rapidly than those containing high-phase transition phospholipids (DSPC/cholesterol). However, after modification with PEG-DSPE, liposomes composed of low-phase transition lipids with some charge were found in plasma after 24 h, similar to those with neutral high-phase transition lipids [103,110]. It should be noted that PEGylated liposomes are not completely biologically inert and can still induce activation of complement systems [122].

Several alternative sterically protecting polymers such as poly [N-(2-hydroxypropyl)methacrylamide] [123], poly-N-vinylpyrrolidones [124], L-amino-acid-based biodegradable polymer-lipid conjugates [125] and polyvinyl alcohol [126] have also been studied for preparation of long-circulating liposomes.

3.4. Immunoliposomes

The first attempts to create the immuno-conjugated liposomes were performed in the late 70s–early 80s [127–131]. Initially, immunoliposomes were obtained by hydrophobic interaction of antibodies specifically modified with hydrophobic residues on the surface of plain non-PEGylated liposome [131]. Still, despite improvements in targeting efficacy, the earlier generations of immunoliposomes suffered from fast elimination from the blood and capture by the cells of the RES, primarily in the liver [132].

The earlier attempts to improve of the circulation longevity of immunoliposomes were performed by simple co-immobilization of an antibody and PEG on the surface of the same liposomal composition [133]. Although PEG molecules can interfere sterically with an antibody's ability to recognize the antigen, a significant increase of their circulation time was achieved [134,135]. Multiple conjugating procedures have been developed to attach antibodies to the surface of PEG layer in PEGylated liposomes, so that the ligand is extended outside of the dense PEG layer to exclude steric hindrances to the binding of antibodies to the target [136,137].

The majority of long-circulating immunoliposomes are targeted for delivery of anticancer drugs [138]. Thus, the recent clinical success of doxorubicin-loaded long-circulating PEGylated liposomes (Doxil®/Caelyx®) [139] in the treatment of metastatic breast cancer, progressive ovarian cancer, multiple myeloma and AIDS-related Kaposi's sarcoma stimulated numerous experimental attempts for the improvement of their targeting properties by surface immobilization of different antibodies or their fragments against specific tumor antigens.

The modification of PEGylated doxorubicin-liposomes with mAbs or their Fab fragments against HER2, a member of the epidermal growth factor receptor (EGFR/ErbB) family frequently over-expressed on various cancer cells, successfully improved the tumor delivery and therapeutical efficiency of liposomal doxorubicin in different HER2-overexpressing mouse xenograft models [140–144].

Doxorubicin-loaded long-circulating liposomes modified with the nucleosome (NS)-specific mAbs (2C5), which recognize a broad variety of tumors via the tumor cell surface-bound NSs, demonstrated an increased antitumor effect both in vitro and in vivo [145–150].

CD19, an internalizing receptor overexpressed in most types of B-lymphoid malignancies, is another promising targeting antigen. Introduction of anti-CD19 mAbs or its Fab' fragments to PEG-liposomes loaded with doxorubicin clearly enhanced targeting and therapeutic efficacy in mice bearing a human CD19+ B-lymphoma [151,152].

Other potent immuno-modifications that improved the targeting properties of PEGylated liposomal doxorubicin, include mAbs or their fragments against CD22 [153], membrane type-1 matrix metalloproteinase MT1-MMP [154], VEGFR2 [155], EGFR [156], epithelial cell adhesion molecule (EpCAM) [157], disialoganglioside (GD2) [158,159] and hemoglobin scavenger receptor CD163 [160]. Doxorubicin-loaded PEG-liposomes modified with chimeric TNT-3 mAbs towards degenerating cells located in the tumor's necrotic areas demonstrated enhanced therapeutic efficacy in nude mice bearing H460 tumors [161].

Interestingly, the tumor accumulation of antibody-targeted liposomes was, in some cases, comparable to the accumulation of long-circulating liposomes not modified with an antibody [162]. However, therapeutic activity was higher for long-circulating immunoliposomes [141–143]. PEGylated immunoliposomes modified with anti-HER2 mAb fragments are internalized much better by cancer cells compared to non-modified liposomes, which allows higher drug dose delivery inside cancer cells for, i.e., more efficient cancer cell killing [163].

In other cases, liposome internalization seems not to be important. The therapeutical effects are dependent on the type of the encapsulated drug and the rate of drug release from the immunoliposomes in the targeted areas [152,164].

The cytotoxic efficiency of immunoliposomes is also dependent on the surface density of the membrane antigen against which liposomes were targeted. It was estimated that about 4×10^4

antigen sites per single cell are required to exert the immunoliposomal targeting effect [61,165].

Another essential factor that determines the degree of immunoliposomal targeting is the extent of heterogeneous expression of antigens in the targeting area. It was suggested that a co-mobilization of antibodies against different antigens on a single immunoliposome will provide better and more uniform targeting of all cells within the tumor [164,166]. Alternatively, the antigen-negative cells can be killed by so-called “bystander” effect, i.e., an action of the drug released from the immunoliposomes attached to a cancer cell expressing a particular antigen on neighboring cancer cells devoid of a similar receptor [164,166].

Among studies with targeted delivery of anticancer drugs, PEG-liposomes modified with different antibodies have been successfully applied for targeted delivery of siRNA [167] and DNA [168,169]. Another interesting application of immunoliposomes includes targeted delivery of “bioenergetic” substrates, such as ATP, to the ischemic myocardium [170]. Thus, ATP-loaded PEGylated liposomes with attached 2G4, an anti-myosin mAb, have shown very promising results in an isolated rat heart model [171].

3.5. Immunomicelles

Micelles, self-assembling colloidal particles with a size ranging from 5 to 100 nm, represent another attractive pharmaceutical carrier for immuno-targeted delivery of water-insoluble drugs [39]. Lipid-core micelles formed by self-assembled amphiphilic polymers (such as PEG-PE) demonstrate high stability both in vitro and in vivo, good biocompatibility, and longevity in the circulation [172].

An antibody or its fragments can be attached to the activated water-exposed free terminus of a hydrophilic block of the micelle-forming polymer (PEG) ensuring that targeted micelles are created without any steric hindrances for the antibody [173,174]. A simple method for a one-step attachment of an antibody via its amino-group to the distal terminus of a PEG block activated with a p-nitrophenyl (pNP) group has been developed [137,175]. 2C5-conjugated immunomicelles prepared by this method contain about 10 mAbs per a single micelle, have a uniform size (about 20 nm), and the antibodies conjugated to the micelles retain their specific binding ability to the target antigen [176]. Paclitaxel-loaded 2C5-immunomicelles demonstrated increased intra-tumoral accumulation and a greater ability to inhibit tumor growth than plain micelles [177]. The 2C5-immunomicelles loaded with a photosensitizer, meso-tetraphenylporphine (TPP), have also shown promising anticancer effects both in vitro and in vivo [178–180].

Doxorubicin-loaded immunomicelles made from poly(D,L-lactic-co-glycolic acid)-PEG and modified with the bivalent fragment HAb18 F(ab')₂ against hepatocellular carcinoma (HCC) significantly suppressed in vivo tumor growth [181].

Anti-HER2 Fab fragment-conjugated immunomicelles prepared from thermosensitive poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide)₁₁₈ and loaded with doxorubicin showed high serum stability, improved intratumoral accumulation and tumor growth inhibition in mice bearing N87 tumors with overexpressed HER2 antigen [182].

High loading, small size, the possibility of surface modification and long circulation time of micelle-based systems assure a promising role in medical diagnostic imaging as carriers for contrast agents [183,184]. PEG-PE-micelles modified with mAb-2C5 and loaded with superparamagnetic iron oxide nanoparticles (SPION) have demonstrated their potential to serve as an MRI contrast agent due to better MRI cell-associated T2 relaxation times [185].

Tumor-targeted quantum dot (QD)-loaded PEG-PE 2C5-immunomicelles that have been developed recently for whole body near infrared imaging (NIR), had a doubled increase in the QD signal intensity and improved detection of metastases in mice [186].

An interesting example of the multi-functional immunomicelles with a capability of both imaging and anti-cancer therapy has been recently published [187]. NIR QD-loaded micelles containing poly(ethylene glycol)-10,12-pentacosadiynoic acid (PEG-PCDA) and PCDA-Herceptin® conjugates provided simultaneous imaging and strong anti-cancer efficacy in vivo.

Polymeric immunomicelles also present a promising platform for targeted delivery of small interfering RNA (siRNA). Still, few in vitro studies that use antibody-targeted micelles for delivery siRNA have been performed to this date [188,189]

4. New “smart” stimulus-sensitive immuno-targeted DDS

In addition to passive targeting (via the EPR effect) and active targeting (via mAbs, small molecules or peptides) of long-circulating PEGylated DDS, the attachment of certain stimulus-sensitive moieties to these nanocarriers provides a novel strategy for the assembly of “smart” multifunctional nanocarriers (Fig. 1), which function in response to intrinsic or externally applied stimuli in a coordinated manner to maximize the antitumor efficacy and minimize the drug side effects [190,191].

In many cases, if drug loaded, PEG-stabilized nanoparticles accumulate in the tumor, they may not readily release the drug into the tumor cells. Also, if the uptake of the nanocarrier is to be via endocytic pathway, the PEG chains on its surface may prevent its drug contents from escaping the endosomes and their delivery into the cell cytoplasm [6,191]. These multifunctionalities can be used to detach the long-circulating polymer (PEG) chains and release the nanocarrier contents at pathological areas, such as infarcts, zones of inflammation and tumors, in the presence of certain intrinsic stimuli such as decreased pH, hyperthermia, altered enzyme levels [192] or redox conditions characteristic of these zones. Intratumoral pH is around 6.5, lower than the normal circulating blood (pH 7.4) due to hypoxia and cell death in tumors [193,194] and is further lowered within the cell compartments, especially the endosomes, to about 5.5 or below [195]. In tumor cells, the redox potential is altered largely by intracellular glutathione levels that are 100-fold greater than the normal extracellular glutathione levels [196]. Apart from these intrinsic local conditions, several stimuli can also be applied externally to these areas, such as temperature [197,198], magnetic fields [198,199] and ultrasound [200], for the activation of stimulus-sensitive multifunctional carriers.

In case of pH-responsive systems, the pH-cleavable bonds stabilize the nanocarrier in normal tissues and blood, but they disintegrate and release the drug load in areas with lowered pH, including neoplastic, ischemic and inflamed tissues, or endosomes and cell cytoplasm. Commonly used pH-labile linkages, which when they break down, detach the protective polymer covering, expose any ligands present and aid drug release, including poly(ortho-esters) [201], vinyl ethers [202,203], hydrazones [204,205], cis-aconityls [206,207], acetals [208,209], polyketals [210], and thiopropionates [211]. These linkages are stable at pH 7.4 but quickly degrade at pH 5–6, and can allow site-specific pH-dependent drug release.

The synthesis of a hydrazine-functionalized poly (ethylene glycol)-phosphatidylethanolamine (PEG-PE)-based amphiphilic polymer capable of conjugating several ligands such as anti-nucleosome 2C5 mAb or anti-myosin 2G4 mAb and proteins such as concanavalin A (Con-A) and avidin via a reversible pH-labile bond has been

reported [212]. The PE portion of the polymer helps to integrate the polymer–ligand complex into the liposomes with targeting functionalities attached to the distal end of the PEG block, which allow targeting of the liposomes to the desire site. The pH-sensitive PEG-conjugate was then detached from the liposomal surface because of lowered pH resulting in improved drug delivery into cancer cell [212].

The long-circulating, targeting and stimulus-responsive functions of nanocarriers can also be combined with certain cell penetrating proteins and peptides (CPPs), such as trans-activating transcriptional activator (TATp) for improved intracellular drug delivery [213,214]. These CPPs efficiently translocate across the plasma membrane directly into cell cytoplasm, avoiding the endocytic pathway and so prevent lysosomal drug degradation and lead to increased cellular uptake [175,215]. Such multifunctional DDS (Fig. 2) must be assembled in such a way that during the initial step, the organ- or tissue-specific delivery is attained by passive targeting via the EPR effect, or by active targeting with attached antibody, while long-chain PEG sterically-protecting an internalizable ligand such as CPP. After the deposition in the targeted organ or tissue, the long-chain PEG or PEG-antibody conjugates that attached to the nanocarrier surface via stimulus-responsive linkages is expected to be detach in the presence of the corresponding stimulus (pH, temperature or enzyme levels) and deshield the otherwise hidden cell-penetrating moiety, which stimulates cargo delivery to the cells [6]. Such double-targeted pH-sensitive liposomes have been developed using a pH-cleavable hydrazone bond between PEG and PE (PEG-Hz-PE) [216]. These liposomes were targeted a specific cell or organ due to the attachment of the specific mAbs to their surface through p-nitrophenylcarbonyl-PEG-PE (pNP-PEG-PE) moieties. The liposomes were modified further with TATp attached to their surface via TATp-short PEG-PE derivative. At normal pH, the TATp function was hidden by the long PEG chains. However at lowered pH (5–6), the PEG coating was removed due to acidic hydrolysis of PEG-Hz-PE leading to increased internalization of the liposome via the TATp [216]. A multifunctional immunoDoxil® with enhanced cytotoxicity contained mAb 2C5 attached to the liposomal surface through PEG_{3,4k}-PE, aTATp function attached with the help of TATp-PEG_{1k}-PE, and a pH-sensitive bond PEG_{2k}-Hz-PE (long PEG blocks) were developed [217]. TATp-modified/pH-sensitive liposomes loaded with pGFP loaded have shown improved internalization in the tumor cells when administered intratumorally in tumor-bearing mice [218,219].

More recently, a novel MMP2-responsive multifunctional immunoliposomal nanocarrier which utilizes the up-regulated levels of extracellular matrix metalloprotease 2 (MMP2) in tumor conditions as a stimulus for effective tumor targeting and cellular penetration was reported [220]. The system was comprised of a TATp function shielded sterically by long-chain PEG, mAb 2C5 for active tumor targeting attached to the liposomal surface and a MMP2 cleavable octapeptide (Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln) as a labile bond between long chain PEG and lipid. It was shown that the octapeptide linker was degraded by the extracellular MMP2 in the tumor cells, which exposes the TAT moiety and results in greater cellular uptake [220].

Other stimulus-responsive systems also have important applications. An example of a thermo-sensitive nanocarrier were liposomes prepared with a poly-(N-isopropylacrylamide) (NIPAM) [221]. Such polymers precipitate at temperatures above a lower critical solution temperature (LCST) and solubilize below an LCST because they exhibit an LCST above the normal tissue LCST. Thus, the liposome membrane breaks down during the thermal-induced precipitation and leads to the release of its cargo [222,223]. A review on thermo-sensitive polymer-modified liposomes can be found in [222].

Multifunctional nanocarriers can also have diagnostic or imaging functions that may be used to study their real-time biodistribution and deposition at target sites by incorporation of certain contrast agents. The contrast moieties can be integrated both in the liposomal membrane and hydrophilic core. Monoclonal antibody mAb 2C5-modified ^{111}In -labeled PEGylated doxorubicin loaded multifunctional liposomes have shown enhanced tumor accumulation and improved visualization of tumors by γ -scintigraphy or MRI when injected into tumor-bearing mice [145,224].

5. Conclusion

The progress in antibody technologies (hybridoma technology, chimerization, humanization and phage-display libraries) has resulted in the appearance of new potent immuno-conjugated drugs and immuno-targeted DDS. Although, immunoconjugation has been proven to be an effective approach that improves the therapeutical potency of many toxic drugs, the problems associated with low drug loading efficacy, heterogeneity of their composition, short half-life, high complexity and cost of production remain to be solved.

Considerable progress has been made in the development of long-circulating immunotargeted DDS that combine particular advantages in one formulation including easy control of composition, relatively simplicity of preparation, high drug loading efficiency, targetability and longevity. The usefulness of long circulation (PEGylation) has been clearly demonstrated for the improvement of the therapeutic value of many small molecules, proteins, peptides and nanocarrier-based DDS. To further improve the therapeutic outcome, many efforts have been directed to the combination of specific targeting with long circulation-based EPR effects.

Among other long-circulating DDS, immunoliposomes and immunomicelles have demonstrated an encouraging therapeutical activity in vivo. “Smart” stimulus-sensitive immunoliposomes represent the future for development of the immunoconjugated DDS. These “smart” immunoliposomes should be considered a new therapeutic opportunity in the future production of highly efficient and specialized delivery systems for drugs, siRNA, and diagnostic agents.

Acknowledgments

This work was supported by the NIH Grant 5U54CA151881.

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Fig. 1.

Evolution of liposomes: from plain to stimulus-sensitive immuno-targeted DDS. (A) Early traditional 'plain' liposomes. (B) Long-circulating liposome grafted with a protective polymer (i) such as PEG, which shields the liposome surface from the interaction with opsonizing proteins. (C) Long-circulating immunoliposome simultaneously bearing both PEG and mAbs (ii) attached to the distal end of the PEG. (D) Stimulus-sensitive immuno-targeted liposomes with mAbs attached to long-chain PEG via stimuli-sensitive bonds (iii) and hidden CPPs (iv) conjugated to the short-chain PEG.

Fig. 2.

Schematic presentation of “smart” stimulus-sensitive long-circulating immunoliposome. The “shielding” long-chain PEG with or without targeting mAbs is attached to the liposomal surface via low pH-degradable bonds. After the accumulation in the tumor due to passive accumulation and/or active targeting, pH-dependent de-shielding of the temporarily hidden cell-penetrating function allows for carrier penetration of tumor cells.

Table 1

FDA-approved mAbs and immunconjugates.

Antibody	Brand name/manufacturer	Approval date	Type of mAbs	Antigen	Indication
Muronomab-CD3 ^a	OKT3/Johnson & Johnson	1986	Murine, IgG2a	CD3	Autoimmune
Abciximab	ReoPro/Johnson & Johnson	1997	Chimeric, IgG1, Fab	PIIb/IIIa	Homeostasis
Rituximab	Rituxan/Genentech	1997	Chimeric, IgG1	CD20	Cancer
Daclizumab ^a	Zenapax/Roche	1997	Humanized, IgG1	CD25	Autoimmune
Basiliximab	Simulect/Novartis	1998	Chimeric, IgG1	CD25	Autoimmune
Palivizuma	Synagis/MedImmune	1998	Humanized, IgG1	RSV	Infections
Infliximab	Remicade/Johnson & Johnson	1998	Chimeric, IgG1	TNF α	Autoimmune
Trastuzumab	Herceptin/Genentech, Roche	1998	Humanized, IgG1	HER2	Cancer
Gemtuzumab ^a ozogamicin	Mylotarg/Wyeth, Pfizer	2000	Humanized, IgG4, immunotoxin	CD33	Cancer
Alemtuzumab	Campath/Genzyme	2001	Humanized, IgG1	CD52	Cancer
Ibritumomab tiuxetan	Zevalin/Biogen Idec	2002	Murine, IgG1, radiolabeled (yttrium-90)	CD20	Cancer
Adalimumab	Humira/Abbott	2002	Human, IgG1		Autoimmune
Onalizumab	Xolair/Genentech, Roche	2003	Humanized, IgG1	IgE	Autoimmune
Tositumomab-I-131	Bexxar/Corixa, GSK	2003	Murine, IgG2a, radiolabeled (iodine-131)	CD20	Cancer
Efalizumab ^a	Raptiva/Genentech, Roche	2003	Humanized, IgG1	CD11a	Autoimmune
Cetuximab	Erbix/Imclone, Lilly	2004	Chimeric, IgG1	EGFR	Cancer
Bevacizumab	Avastin/Genentech, Roche	2004	Humanized, IgG1	VEGF	Cancer
Tocilizumab	Actemra/Roche	2010	Humanized, IgG1	IL-6R	Autoimmune
Panitumumab	Vectibix/Amgen	2006	Human, IgG2	EGFR	Cancer
Ranibizumab	Lucentis/Genentech, Roche	2006	Humanized IgG1 Fab	VEGF	Macular degeneration
Eculizumab	Soliris/Alexion	2007	Humanized IgG2/4	C5	Hemoglobinuria
Certolizumab pegol	Cimzia/USB	2008	Humanized, pegylated Fab	TNF α	Autoimmune
Natalizumab	Tysabri/Biogen Idec	2008	Humanized, IgG4	VLA-4	Autoimmune
Golimumab	Simponi/Johnson & Johnson	2009	Human IgG1	TNF α	Autoimmune
Canakinumab	Ilaris/Novartis	2009	Human IgG1	IL-1 β	Inflammatory
Ustekinumab	Stelara/Johnson & Johnson	2009	Human IgG1	IL-12/23	Autoimmune
Ofatumumab	Arzerra/Genmab	2009	Human IgG1	CD20	Cancer
Denosumab	Prolia	2010	Human IgG1	RANKL	Osteoporosis

Antibody	Brand name/manufacturer	Approval date	Type of mAbs	Antigen	Indication
Iplimumab	Yervoy/BMS	2011	Human IgG1	CTLA-4	Cancer
Belimumab	Benlysta/Human Genome Sciences	2011	Human IgG1	BLyS	Cancer
Brentuximab Vedotin	Adcetris/Seattle Genetics	2011	Chimeric IgG1 MMAE-conjugate	CD30	Cancer

^aWithdrawn by the manufacturer.