Organic nanoscale drug carriers coupled with ligands for targeted drug delivery in cancer

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Nanoscale carriers are of increasing interest for use in anticancer therapy because they can deliver drugs in a targeted way. Ideally, nanocarriers combine passive and active delivery mechanisms, by integrating targeting ligands on the surface of their stealth corona using simple and efficient conjugation chemistry. This feature article summarizes the conventional and newly explored conjugation chemistries that are used to bind ligands in organic nanoparticle systems, providing strategic guidance for the preparation of targeted nanocarriers.

1. Introduction

Targeted delivery of drugs provides therapeutic concentrations of anticancer agents at the desired sites while sparing normal tissues, thereby reducing systemic toxicity and enhancing therapeutic efficacy.[1–3] There is a wide range of strategies available for drug delivery in cancer therapy, among which systemic delivery using nanoscale drug carriers (*e.g.*, liposomes, polymeric nanoparticles or polymer-drug conjugates) has been demonstrated to be efficacious.[4–8] Nanoscale drug carriers promise both prolonged circulation time—due to the nanoscale size and hydrophilic outer shell which inhibit phagocytic and renal clearance—and selective tumor accumulation *via* the enhanced permeability and retention (EPR) effect. Having their composition tuned to allow functionalization, nanocarriers are designed to incorporate targeting ligands by covalent coupling, which combine passive and active targeting in one platform. These "smart" drug delivery systems are capable of targeting specific cell types exclusively through ligand-receptor interactions.

There are many reviews available in the literature focusing on various nanocarrier formulations and strategies for the delivery of therapeutic molecules.[1,4,5,7–14] In the present feature article, the aim is to provide an updated, comprehensive review on organic nanocarriers with targeting ligands coupled on the surface. A brief overview on targeting mechanisms and the structure of nanocarriers will be presented first, followed by a comprehensive summary on the methodologies for functionalizing nanocarriers and coupling targeting ligands. Special emphasis will be placed on the conjugation chemistries that are used in nanoparticle systems for surface modification.

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Meng Shi

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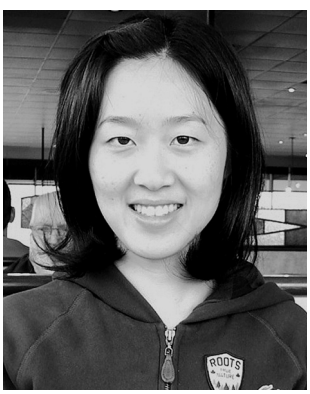


Jiao Lu

Jiao Lu received her B.Sc. degree in Chemistry from Simon Fraser University in B.C. in 2006 while working with Professor Erika Plettner on the synthesis of a moss pheromone analog. In 2006, she joined the laboratory of Professor Molly S. Shoichet in the Department of Chemistry at the University of Toronto, where she is conducting research on the synthesis and modification of polymeric nanoparticles for drug delivery.

2. Targeting mechanisms

There are two main mechanisms through which nanoscale drug carriers achieve tumor targeting, namely passive targeting and active targeting. Passive targeting is based on prolonged circulation time provided by a hydrophilic outer shell that reduces phagocytic and renal clearance, thereby promoting selective accumulation in tumor tissues *via* the EPR effect. Active targeting builds on passive targeting by incorporating ligands in the nanocarriers which bind specifically with receptors on the cancer cells, thereby promoting nanocarrier-cell interaction and cellular internalization (Fig. 1).

2.1 Passive tissue targeting

It has been demonstrated that the vascular endothelium is more permeable in tumor sites than normal tissues as a result of the hyperpermeable vasculature that surrounds cancerous tissues.[15–17] Relatively large macromolecular drugs or nanoscale drug carriers, with sizes ranging from 20 nm to 200 nm, can extravasate and accumulate inside the interstitial space of a tumor tissue due to the loosely connected vasculature of the endothelium therein. In contrast to normal tissues, cancer tissues do not have a well-defined functioning lymphatic network, which limits the penetrated particles from being cleared rapidly and thus promotes their accumulation.[16] The ability of nanoscale particles to accumulate selectively and be retained for a prolonged period in cancer tissues has been termed the EPR effect[17,18] (Fig. 1). Interestingly, the EPR effect does not apply to free drugs with low molecular weights due to their rapid diffusion back into the circulating blood and their clearance from the circulation by renal filtration. Therefore, the EPR effect is a key mechanism for selective solid tumor targeting of nanoscale drug carriers and has been the basis for novel drug carrier design.[4,6]

To achieve selective tissue accumulation *via* the EPR effect, nanoscale drug carriers should circulate for prolonged times in the bloodstream to provide a sufficient level of target accumulation. The ability to bypass the recognition of the reticuloendothelial system (RES) is crucial for achieving prolonged circulation time in blood. The RES or mononuclear phagocyte system (MPS), which is a class of cells, including monocytes and macrophages, is responsible for engulfing and clearing old cells, miscellaneous cellular debris, foreign substances, and pathogens from the bloodstream. Non-biocompatible foreign substances are recognized by the RES *via* complement activation, followed by elimination from circulation. For colloidal nanoparticles, proteins will often adsorb to the surface of the nanoparticles within the first few minutes of its exposure, especially if the material is either charged or hydrophobic. Surface adsorption of opsonins enhances RES clearance of the nanoparticles from circulation, thereby impeding accumulation in the tumor and preventing the drug from reaching its intended site of action. The recognition of nanoparticles by the RES is largely determined by their physical and biochemical properties such as particle size and surface interaction with blood components.[19–22] Nanoparticles with diameters less than 200 nm have been shown to be less susceptible to RES clearance.[23,24] Furthermore, the presence of a biocompatible and hydrophilic corona, such as poly(ethylene glycol) (PEG), will sterically stabilize the nanoparticles by creating entropic and osmotic forces which resist protein adsorption and reduce RES uptake.[20–22,25,26]

These properties are widely recognized, and PEG incorporation has become a requisite in drug carrier design. By incorporating PEG-lipids in the lipid bilayers, the circulating half-life of liposomes in the blood was extended greatly (*e.g.*, $t_{1/2}$ > 5 h in

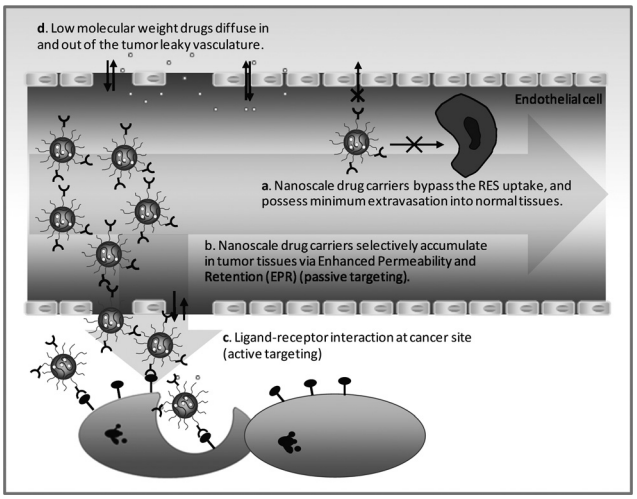


Fig. 1 Nanoscale drug carriers deliver anticancer drugs to tumor sites by passive targeting and active targeting mechanisms. a. Nanoscale drug carriers, which have a hydrophilic polymer corona, achieve prolonged circulation time by bypassing uptake by the reticuloendothelial system (RES) and having minimum extravasation in normal tissues. b. The passive targeting of nanoscale drug carriers to tumor sites is achieved due to the enhanced permeability and retention (EPR) effect. c. By incorporating cell-specific targeting ligands, nanoscale drug carriers target cancer cells specifically by ligand-receptor interactions where the drug carriers can release the incorporated drugs in the tumor tissue and/or be internalized *via* receptor-mediated endocytosis for intracellular drug delivery. d. Nanoscale drug carriers allow selective delivery to the tumor, thereby limiting systemic toxicity whereas drugs diffuse freely across the tumor leaky vasculature, limiting specific targeting.

Molly Shoichet

Molly Shoichet holds the Canada Research Chair in Tissue Engineering and is a Professor at the University of Toronto. She is an expert in the study of Polymers for Regeneration, holds numerous patents on drug delivery and scaffold design and has founded two spin-off companies. She is a recipient of the Killam Research Fellowship, NSERC's Steacie Fellowship, CIHR's Young Explorer's Award (to the top 20 scientists under 40 in Canada), Canada's Top 40 under 40, and was elected into the Canadian Academy of Sciences. She received her S.B. in Chemistry from MIT (1987) and Ph.D. in Polymer Science and Engineering from the University of Massachusetts, Amherst (1992).

mice) relative to that of conventional liposomes without PEG decoration where there had been more than 80% blood clearance within 2 h in mice.[27–29] Relative to solid polymeric nanoparticles without PEG, polymeric nanoparticles of amphiphilic copolymers, which have a flexible PEG corona, circulate for a prolonged time in the blood.[30–34] For example, poly(lactide-*co*-glycolide)-poly(ethylene glycol) (PLGA-PEG) nanoparticles have a blood half-life $t_{1/2} > 2$ h in mice whereas plain PLGA nanoparticles have 95% of the particles removed from the circulation within 1 h.[30]

Prolonged circulation increases the probability that nanoparticles reach the tumor leaky vasculature, where, mediated by the EPR effect, nanoscale drug carriers accumulate selectively allowing for significantly elevated drug concentrations. This passive targeting[4,19] is a prerequisite for the specific binding of drug carriers to a localized recognition moiety—that is active targeting.

2.2 Active cellular targeting

Actively targeted delivery vehicles are the second generation nanocarriers for intravenous administration. While non-targeted nanoparticle formulations demonstrate selective tumor targeting as a consequence of passive targeting, they do not interact with cancer cells directly and have limited tumor penetration. By linking targeting ligands to the surface of the long-circulating nanoparticles, specific binding with receptor-expressing cancer cells can be designed to enhance the therapeutic activity of anticancer drugs.[1,2,4,7,8] The rationale for the specific binding of ligand-modified nanoparticles with receptors on cancer cells is based on: i) the overexpression of specific antigenic receptors on the surface of cancer cells relative to cells in normal tissues; ii) the specificity and high binding affinity of targeting ligands to receptors; and iii) the intracellular delivery possible by cell-mediated endocytosis *via* the ligand-receptor interaction. For example, Herceptin®, a therapeutic monoclonal antibody (Ab), binds to human epidermal growth factor receptor-2 (HER2) which is overexpressed on 20–30% of breast and ovarian cancer cell surfaces, providing a basis for selective immunotargeting.[35] By using Herceptin®-modified liposomes, the enhanced therapeutic index of doxorubicin was achieved relative to non-targeted liposomes in a metastatic model of breast cancer where the HER2 receptor expression level was 10^5 copies per cell,[36] demonstrating the clinical potential of targeted drug delivery *via* an antibody-mediated targeting mechanism.

Ligand-nanoparticles promise intracellular drug delivery *via* receptor-mediated endocytosis.[5,6,37,38] The internalized nanoparticles end up in small vesicles of endosomes which then undergo a rapid maturation to late endosomes and fuse with each other or lysosomes. Receptor-mediated endocytosis provides a means for nanocarriers entering into the cells. By incorporating stimuli-response mechanisms in drug carrier designs, such as acidic pH or enzymatic cleavage in the endosomes/lysosomes, anticancer drugs can be released intracellularly and targeted to specific organelles.[1,11,13,39] For those DNA-interacting drugs such as doxorubicin and paclitaxel, the therapeutic efficacy can be increased dramatically due to receptor-mediated cellular uptake and intracellular drug release.[36,39–43]

Active drug targeting has the potential to suppress multiple-drug resistance (MDR), where tumors develop resistance to a wide range of chemotherapeutic agents. MDR lowers the therapeutic efficacy and renders many chemotherapeutic drugs of limited utility. It is believed that MDR is associated with pumping anticancer drugs out of the cell through efflux pumps from the ATP-binding cassette (ABC)-transporter family such as p-glycoprotein (P-gp).[44] Nanoscale drug carriers decorated with targeting ligands gain cellular entry by means of receptor-mediated endocytosis, thus circumventing MDR by bypassing P-gp-mediated drug efflux and achieving improved therapeutic efficacy as has been shown with drug-resistant cell lines *in vitro*.[45,46]

3. Nanoscale drug carriers

Advances in material chemistry are now enabling the preparation of functional nanostructures with great potential and versatility for defined drug delivery purposes. These nanocarriers possess size dimensions of 1–1 000 nm, and are able to incorporate drugs and strategies for localized drug release. Based on the materials of which nanocarriers are mainly comprised, nanocarriers can be broadly classified as polymeric nanocarriers (*e.g.*, nanospheres, micelles, polymer-drug therapeutics, polymersomes and dendrimers),[6,8,10,13,14,47] liposomal nanocarriers (*e.g.*, liposomes),[1,5] inorganic nanoparticles (*e.g.*, nanoshells),[48,49] or other nanostructures (*e.g.*, human serum albumin (HSA) nanoparticles[50]).

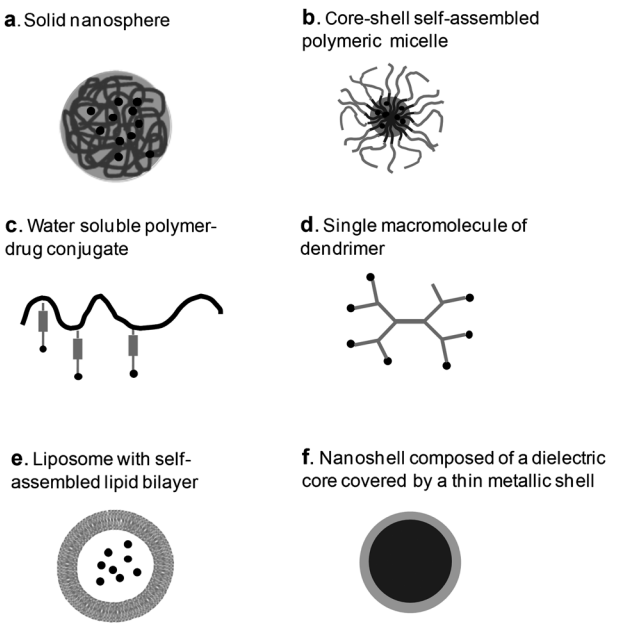


Fig. 2 Nanocarriers incorporating drugs: a. solid nanosphere from polymers or other materials incorporating drugs inside;[50–52] b. core-shell micellar polymeric nanoparticles self-assembled from well-defined synthetic amphiphilic polymers where drugs are physically or chemically incorporated in the inner core;[47] c. water-soluble polymer-drug conjugate with biologically defined linkers between the drugs and polymers;[6] d. dendrimers, branched polymeric macromolecules carrying drugs by physical encapsulation or covalent binding;[14] e. liposomes, a spherically arranged bilayer structure with drug loaded either in the inner aqueous phase or between the lipid bilayers;[5] and f. nanoshell as one example of inorganic nanoparticles.[49]

Polymer-based nanocarriers have been explored in anticancer therapy because the versatility of polymer chemistry and precision engineering of materials at a molecular level allow for diverse formulations of polymeric nanocarriers. As shown in Fig. 2a, solid polymer nanospheres have drug molecules physically encapsulated within and drug release is controlled by polymer degradation.[50–52] For prolonged circulation time in the bloodstream, these solid nanospheres require surface modification with hydrophilic PEG. In contrast, amphiphilic copolymers composed of defined hydrophobic and hydrophilic blocks self-assemble into a core-shell micellar nanostructure upon contact with aqueous environments (Fig. 2b).[53] These polymeric micelles usually incorporate drugs within the inner hydrophobic microenvironment by either physical encapsulation or chemical coupling.[8,9,11] As seen in Fig. 2c, polymer-drug conjugates covalently bind drugs along a water-soluble polymer chain, altering the biodistribution of small drugs and enabling passive targeting *in vivo*.[6,13] In addition, advanced polymer chemistry can be used to create an environment-sensitive linker between the drug and polymer for specific drug release at desired sites.[11] Dendrimers are highly branched polymeric molecules and used to deliver drugs (Fig. 2d).[14] The multivalent nature resulting from the well-defined chemical structure and many terminal groups allow for drugs, targeting ligands and/or labeling molecules to be incorporated into one dendrimer scaffold. In addition to polymer-based nanocarriers, liposomal nanocarriers are also frequently used for anticancer drug delivery (Fig. 2e) and many of these are approved by regulatory agencies for clinical treatment. As highly ordered lipid molecules in lamellar arrangement, liposomes can encapsulate drugs either inside or between the bilayers. To overcome the problem of a short half-life in the bloodstream, long-circulating liposomes decorated with a PEG corona are being widely investigated currently. Inorganic nanoparticles such as nanoshells have found their application in thermal ablative cancer therapy or cancer imaging[48] (Fig. 2f). The solid core of these inorganic nanocarriers has limited capacity for loading drugs; yet surface modification to introduce therapeutic agents is possible.

It is essential that drug carriers retain the encapsulated/conjugated drug in circulation to minimize systemic cytotoxicity and release the drug upon interaction with cancer cells to yield optimal therapeutic effect at targeted sites. Physically entrapped drugs, the release of which is usually diffusion-controlled, have a tendency to leak out of carriers rapidly.[54,55] There are a variety of strategies used to retain drugs in their carriers including new polymeric materials that form complexes with drugs[56] and liposomes coated on the outside to retard their breakdown.[57] Alternatively, drug incorporation through covalent binding (*e.g.*, in polymer-drug conjugates) where the chemical linkage is stable under physiological environments, is desirable in terms of retaining drugs during blood circulation. To efficiently release entrapped/conjugated drugs at the cancer tissue or within cancerous cells, the stability of the carrier has to change to allow release of free drugs at their therapeutic target. Functional components of environment-sensitive polymers or drug-polymer linkers have been employed to incorporate stimuli-responsive strategies where a transformation is induced by an external stimulus such as intracellular change in pH, temperature or the presence of specific enzymes.[11,13,52,58,59]

Drug incorporation in nanoscale carriers increases drug delivery, protects drugs from premature degradation, and controls drug tissue distribution. By incorporating targeting ligands on the surface of long-circulating PEG-modified nanocarriers, the drug carriers gain the ability to target the diseased cell or tissues by both the passive mechanism (*i.e.*, the EPR effect) and active mechanism (*i.e.*, ligand-receptor interactions).

4. Conjugation of targeting ligands to nanocarriers

The preparation of targeted nanocarriers involves either chemical conjugation or physical adsorption/interaction of targeting ligands with the outer surface of the nanocarriers. Chemically binding targeting ligands to nanocarriers is more desirable because it provides more precise control in terms of the density and orientation of the attached ligands and forms a stable linkage under *in vivo* conditions. As shown in Fig. 3, the chemical modification can be carried out either before or after nanocarrier formation and drug incorporation. Targeting ligands are usually coupled to the terminal groups of the "stealth" PEG corona (or the corona of other hydrophilic polymers) which are easily accessible by targeting ligands for conjugation. Importantly, targeting ligands exposed on the surface of nanocarriers facilitate their interaction with cell-surface receptors relative to those nanocarriers having targeting ligands hidden within the PEG corona; however, some types of targeting ligands (*e.g.*, antibodies or antibody fragments) may negatively impact circulation time due to increased opsonisation.[60]

Fig. 3a presents the preparation of PEG-grafted solid nanoparticles where difunctional PEGs decorate solid nanospheres (*e.g.*, polymeric nanospheres, nanoshells) by either chemical reaction or physical interaction.[52,53,61,62] For example, NHS-PEG-maleimide was covalently grafted on nanoparticles through an NHS-ester-amine reaction and presented the maleimide functional groups on the surface of the PEG corona. Similarly, the terminal carboxyl groups on PLGA nanospheres were activated sequentially by NHS and EDC which then reacted with bifunctional NH_2-PEG-COOH (obtained by the deprotection of the amine of Fmoc-PEG-COOH), to create carboxylic acid-functionalized long-circulating nanospheres.[52] Fig. 3b describes the preparation of targeting ligand-coupled polymeric micelles. Polymeric micellar systems have hydrophilic segments oriented toward the aqueous environment where the functional ends on the PEG corona bind the targeting ligands.[11,41,60,63–70] Polymeric building blocks have been designed with various functionalized amphiphilic copolymers such as: PLGA-PEG-COOH,[69] poly(ε-caprolactone)-poly(ethylene glycol)-maleimide (PCL-PEG-Mal),[41] *p*-nitrophenylcarbonyl-poly(ethylene glycol)-phosphatidylethanolamine (pNP-PEG-PE),[66] and poly(2-methyl-2-carboxytrimethylene carbonate-*co*-*D,L*-lactide)-*graft*-poly(ethylene glycol)-furan (poly(TMCC-*co*-LA)-*g*-PEG-furan).[64] Relatively small targeting ligands, such as folic acid, can be conjugated with amphiphilic copolymers before drug incorporation or self-assembly,[63,65,67] whereas large targeting ligands, such as targeting antibodies, are coupled to pre-formed micelles[11,40,64,66,68–70] because the addition of large end groups may alter the bulk properties of the polymer and negatively impact the subsequent self-assembly process. As seen in Fig. 3c, polymer-drug conjugates have both drugs and targeting ligands conjugated on the same

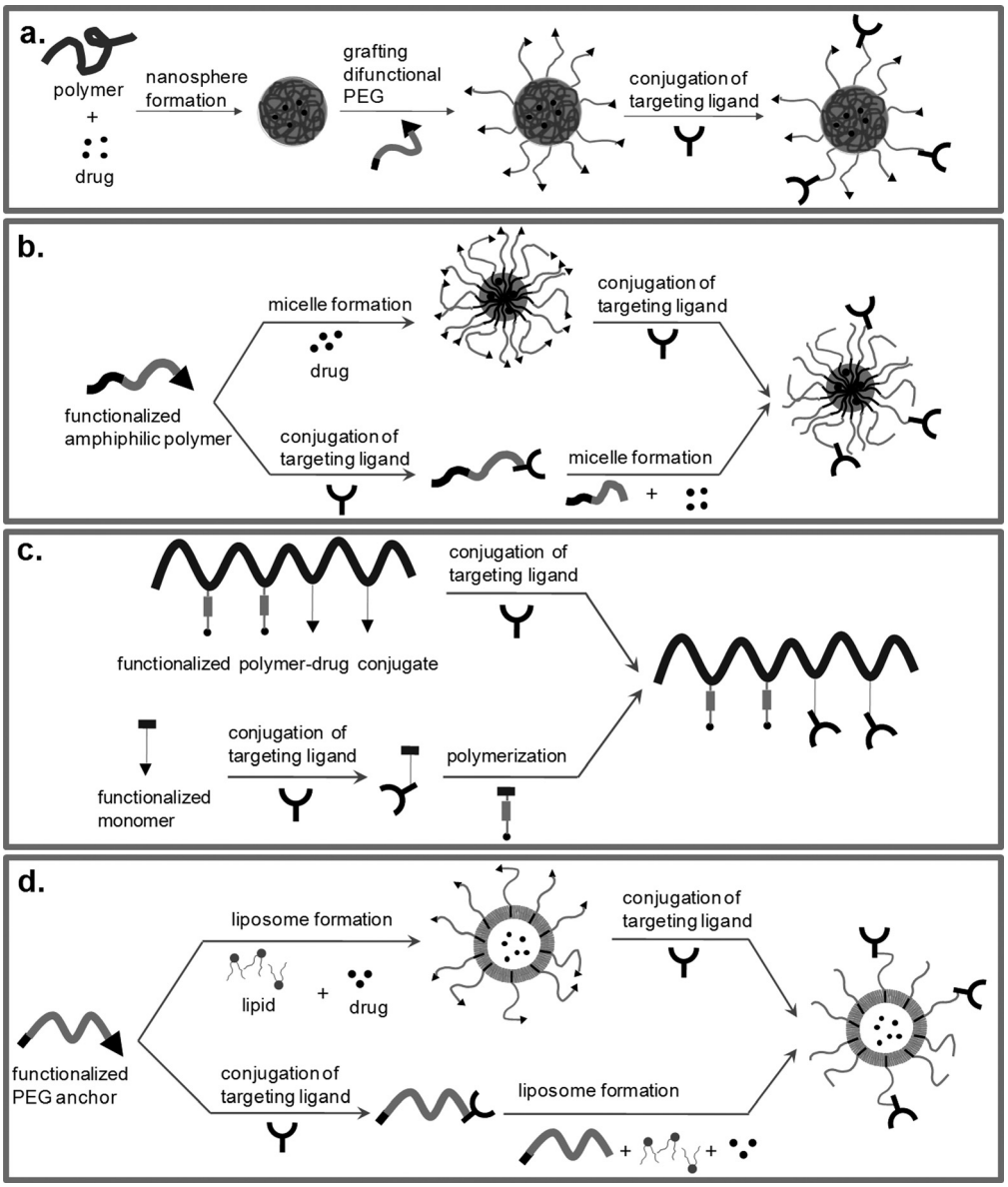


Fig. 3 The preparation of targeting ligand-coupled nanocarriers. a. Polymeric nanospheres are grafted with bifunctional PEG brushes on the surface and the functional groups on the PEG corona are used to couple targeting ligands.[50–52] b. Amphiphilic polymers bearing functional groups at the termini of hydrophilic segments either self-assemble into micellar nanoparticles where the functional groups available on the surface of hydrophilic corona couple targeting ligands,[11,41,64,66,68–70] or couple targeting ligands and then form micellar nanoparticles.[63,65,67] c. Targeting ligand-coupled polymer-drug conjugates are prepared by either coupling ligands with functional groups on the polymers,[40,71–75,83,106] or the polymerization of the monomers containing targeting ligands.[76] d. Functional PEG-anchors are either incorporated into the liposomal membrane during liposome formation, and then targeting ligands on the surface of PEG-liposomes coupled[45,64,77–99] or covalently modified by targeting ligands at the PEG terminus, and then incorporated into the liposomal membrane during liposome formation.[100,101]

polymer chain where conjugation can be completed after polymer synthesis.[39,40,71–75] Alternatively, monomers coupled with the antigen binding fragment of antibodies (Fab′) have been copolymerized with drug-conjugated monomers to form, for example, a targeted N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-drug conjugate, using synthetic conditions that were not harsh for the pre-coupled targeting ligands.[76] With this methodology, the amount of conjugated ligands was precisely controlled by the monomer feed ratio. Long-circulating liposomes with a hydrophilic protective layer (*e.g.*, PEG) over the liposome surface are usually prepared by inserting PEG-anchors (*e.g.*, distearoyl phosphoethanolamine-polyethylene glycol (DSPE-PEG)) into lipid bilayers (Fig. 3d). It is essential that the PEG-anchors bear functional groups at the PEG termini so that they can couple targeting ligands on liposome surfaces.[45,64,77–101] Similarly, the conjugation of targeting ligands with PEG chains can be completed either before or after the formation of PEG-liposomes.

Since targeting ligands (classified as antibodies and their Fab′ fragments, nucleic acids, peptides, vitamins and carbohydrates) are usually biomolecules that are much more chemically sensitive than typical small organic molecules, the methods available for

Table 1 Conjugate chemistry for coupling targeting ligands with nanocarriers[a]

Chemistry	Functional groups on nanocarriers	Functional groups on targeting ligands	Reaction conditions (additional agents, pH, reaction time, temperature)	Bioactivity tested	Refs
Electrophilic addition of thiol to alkene	Maleimide	Thiol	pH > 7.0, 4–24 h, RT	*In vitro, in vivo*	41,61,62,82–95,106–109
	Carboxylic acid	Thiol	EDAC, cystamine, DTT or TCEP, 1 h, RT,	*In vitro*	110–112
	PDP	Maleimide	DTT, pH 7.4, 2–24 h, RT	*In vitro*	60,81,109,113
	VS	Thiol	pH 7.4, 16–24 h, 4 °C or RT	*In vitro*	75,95
Nucleophilic acyl substitution reaction	Carboxylic acid	Amine	EDC, NHS, pH 7.5–8.5, 2–24 h, 4 °C or RT	*In vitro, in vivo*	52,65,69,79,80,87, 98,115,116
	Amine	Amine	DSP, NHS, pH 7.4, 2 h, RT	*In vitro*	117
	pNP	Amine	pH 8–9.5, 2–3 h, RT	*In vitro, in vivo*	66,73,97,101
Hydrazide coupling	Hydrazide	Aldehyde	24 h, 5–6 °C	*In vitro, in vivo*	74,82,94,96,104
Disulfide exchange	PDP	Thiol	pH 8.0, 2–24 h, 4 °C or RT	*In vitro*	90–93,102
Biotin-streptavidin	Biotin	Streptavidin	Water, 30 min, RT	*In vitro, in vivo*	50,68,104,122
Diels–Alder	Furan	Maleimide	pH 5.5, 2–6 h, 37 °C	*In vitro*	64
Click chemistry	Azide	Alkyne	Copper(I), RT, 2–3 d	*In vitro*	154–156

[a] Abbreviations: RT, room temperature; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, N-hydroxysuccinimide; DTT, dithiothreitol; TCEP, tris(2-carboxyethyl)phosphine; DSP, dithiobis(succinimidylpropionate); PDP, pyridyldithiopropionate; pNP, *p*-nitrophenylcarbonyl; VS, vinylsulfone.

their immobilization to micelles are restricted by limited solubility in organic solvents, pH and temperature sensitivity, and side reactions which may all decrease their bioactivity.[5,70,103–105] To maintain the bioactivity of coupled targeting ligands/incorporated drugs, the conjugation chemistry should be simple, efficient, site-selective, occur under mild aqueous conditions (with pH values between 6 and 8 and at temperatures <40 °C), and produce a stable and non-toxic chemical bond.

The following subsections summarize the conventional and newly developed coupling methodologies that have been used in the field (Table 1).

4.1 Maleimide and thiol reaction

Due to its high efficiency in aqueous environments, the reaction between thiol and maleimide functional groups is one of the most useful and efficient reactions in bioconjugate chemistry.[104] Drug carriers can bear either maleimide (Fig. 4a)[41,61,62,82–95,106–109] or activated thiol groups (Fig. 4b and c)[60,104,110–113] on the surface for functionalization. Since maleimide groups are relatively stable under synthesis conditions, maleimide-containing molecules have been used during the synthesis of liposomes,[77,78,82–84,89,100,108] nanoparticles,[62] polymeric micelles,[41] or water-soluble polymer-drug conjugates.[106] For example, N-(4-(*p*-maleimidophenyl)butyryl)-phosphatidylethanolamine (MPB-PE)[82,85,108] and N-[(3-maleimide-1-oxopropyl)aminopropyl polyethyleneglycol-carbamyl] distearoylphosphatidyl-ethanolamine (DSPE-PEG-Mal)[77,78,83,88,100] have been widely employed as maleimide-functionalized anchors in the formulation of maleimide-functionalized liposomes. The amphiphilic copolymer, poly(ε-caprolactone)-*block*-poly-(ethylene glycol)-maleimide (PCL-*b*-PEG-Mal), self-assembled into micellar nanoparticles with maleimide groups exposed on the outer surface.[41] A water-soluble polymer of *N*-(2-hydroxypropyl)methacrylamide (HPMA) bearing pendant maleimide groups was synthesized.[106] After the formation of maleimide-containing drug carriers, targeting antibodies containing thiol groups were coupled to the drug carriers by a simple addition reaction which occurs within a short period (hours) under mild conditions (at room temperature in aqueous solution). In the case of Fab′-thiol fragments conjugated to maleimide-activated liposomes, the coupling efficiency, which is normally expressed as the percentage of conjugated antibody relative to the original antibody feed for conjugation, was up to 100% when the Fab′ was conjugated to the surface of the PEG corona through a maleimide-terminated PEG-DSPE anchor (Fig. 5).[77] The conjugation resulted in 60–120 Fab′/liposome with long-term stability and cell-binding ability.[77] It is known that the thioether bond formed is quite stable without cleavage during 24 h in the presence of reducing agent dithiothreitol (DTT) or 50% human serum.[114]

As thiol groups are very reactive towards various electrophiles and rapidly deteriorate when in solution, it is difficult to introduce sufficient amounts of thiol residues into biomolecules such as antibodies. The most frequently used modification techniques involve antibody activation N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP)[83–85,108] and N-succinimidyl-S-acetylthioacetate (SATA)[82] followed by a deprotection procedure using reducing reagents such as dithiothreitol (DTT). Alternatively, the endogenous disulfide groups in the hinge region of the immunoglobulin structure can be reduced to generate half-antibody fragments containing active thiol groups.[89,100,107]

Although maleimide retains alkylating activity for hours in acidic environments (pH 4.5–6.5), it may undergo gradual degradation under the conditions of liposome preparation or drug loading.[60] An alternative way to use thiol-maleimide chemistry is to introduce thiol groups on drug carriers by activating amines[60,81,109,113] or carboxylic acid groups,[110–112] and then linking maleimide-containing targeting ligands (Fig. 4b and c).

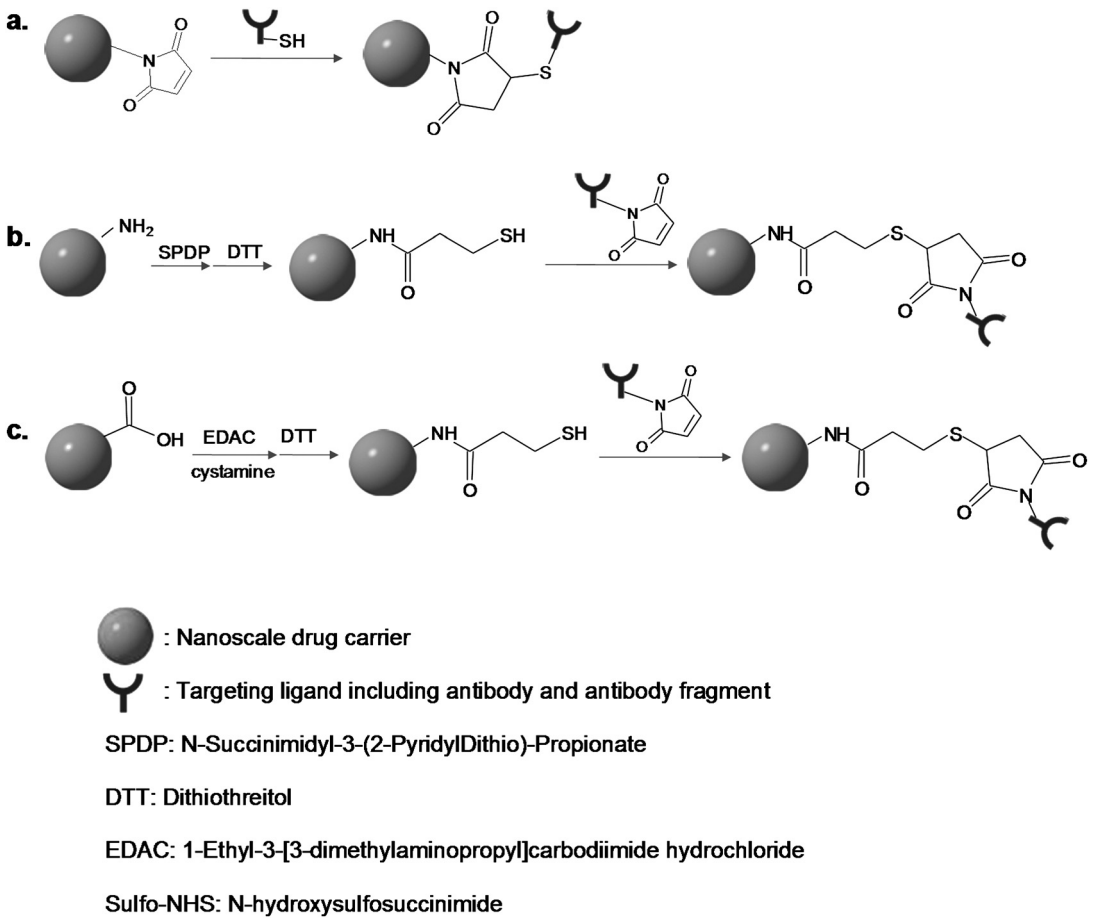


Fig. 4 Thiol-maleimide reactions to attach targeting ligands to nanoscale drug carriers: a. maleimide-containing drug carriers couple the targeting ligands that contain activated thiol groups;[41,61,62,82–95,106–109] b. amine groups on the drug carriers are activated by SPDP to introduce thiol groups which react with maleimide groups on the targeting ligands;[60,81,109,113] and c. carboxylic acid groups on the drug carriers are activated by EDAC to introduce thiol groups which react with the maleimide groups on the targeting ligands.[110–112]

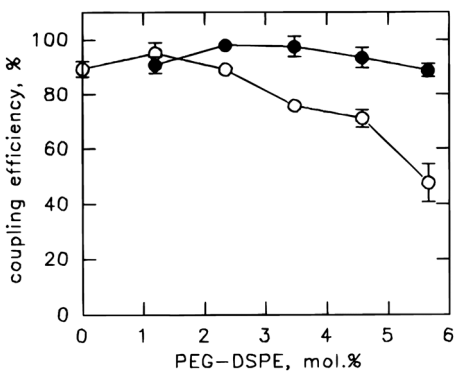


Fig. 5 The coupling efficiency of Fab′-thiol fragments to maleimide-activated liposomes was affected by the location of the maleimide group. (○) The coupling efficiency decreased with increased PEG-DSPE when the maleimide group was close to the liposome bilayer as the PEG chains sterically interfered with this reaction; however, (●) the coupling efficiency was high and independent of PEG-DSPE above 1 mol% when the maleimide group was at the PEG terminus, demonstrating that Fab′ can gain easy access to its coupling sites.[77]

By acylation of the terminal amino group of PEG-DSPE anchors with SPDP, PDP-PEG-DSPE was prepared and then incorporated easily into liposomes during their formation.[60] Following the reduction of the pyridyldithiopropionate (PDP) group with DTT, maleimide-modified antibodies were efficiently coupled to the surface of liposomes. As shown in Fig. 6, a higher molar ratio of PDP-PEG-DSPE/Ab in the reaction solution resulted in greater coupling efficiency of antibody to liposome, reaching a maximum of more than 80% for a 10 fold molar excess of PDP-PEG-DSPE vs. Ab.[60]

Thiol-maleimide chemistry meets many of the criteria which would be desirable for biomolecule immobilization on nanocarriers including high coupling efficiency, controlled antibody density, compatible chemistry with drug loading, and creating bioactive targeting ligand-decorated nanocarriers with the ability to bind with specific cell types.[60,81] Notwithstanding the ease of this reaction, a series of protection and deprotection reactions are often required and unreacted thiol groups can lead to undesirable side reactions.

4.2 NHS-ester and primary amine reaction

Carboxylic acid groups on drug carriers have been frequently activated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC or EDC) in the presence of N-hydroxysulfosuccinimide (sulfo-NHS) to catch amine-containing ligands (Fig. 7).[52,65,69,79,80,87,98,115,116] For example, COOH-PEG-anchors

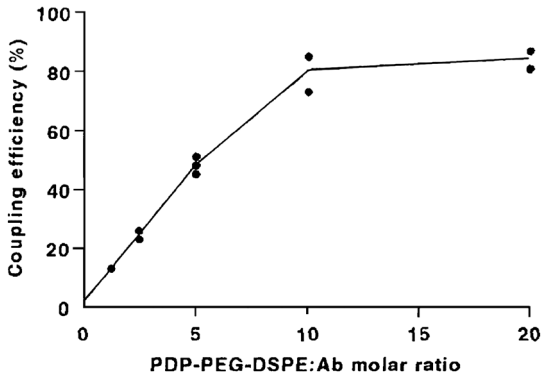
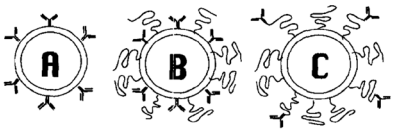


Fig. 6 Coupling efficiency of maleimide-modified antibodies conjugated to PDP-functionalized liposomes where the PDP groups were reduced to thiol groups by the addition of DTT before antibody conjugation: after overnight reaction at room temperature, a high coupling efficiency of greater than 80% was achieved when the feed molar ratio of PDP-PEG-DSPE to Ab was over 10-fold. The coupling efficiency is expressed as the % of Ab initially available to bind the liposomes.[60]



	34A-Immunoliposomes[a]		
	Type A	Type B	Type C
PEG content (mol% of total lipid)	0	6	6
Mean diameter (nm)	121	111	122
Initial antibody: lipid ratio (w/w)	1:6	1:6	1:5
Conjugation efficiency (%)	35.6	31.8	24.8
Number of antibody molecules per liposome	35	30	30

[a]34A, rat monoclonal IgG2a antibody

Fig. 8 Antibodies conjugated with EDAC activated carboxylic acid-functionalized liposomes: after 8 h of reaction at 4 °C, three types of immunoliposomes were achieved including PEG-free liposomes with antibodies covalently linked to short anchors (Type A), PEG–liposomes with antibodies covalently linked to short anchors (Type B), and PEG-liposomes with antibodies covalently linked to the distal terminal of DSPE-PEG-COOH (Type C). The coupling efficiencies of antibodies conjugated with different types of liposomes are 25–35%, with approximately 30–35 antibodies attached to each liposome.[98]

were incorporated into liposomes during liposome formation and activated to form liposome-NHS which bound to amine-containing targeting ligands.[80,98,116] The methodology conjugated antibodies close to the bilayer or on the outer surface of liposomes at a modest coupling efficiency (25–35%) as shown in Fig. 8.[98] Similarly, poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles with carboxylic acid-terminated functional groups[115] or micellar nanoparticles with carboxy-terminated poly(ethylene glycol) (PEG-COOH) hydrophilic segments[65,69] were both activated to attach amine-functional targeting ligands. Alternatively, dithiobis(succinimidylpropionate) (DSP), a water-insoluble homobifunctional crosslinker, was used to activate the primary amine groups on nanocarriers to generate NHS-esters which readily react with primary amine functionalized targeting ligands[117] (Fig. 7b). These target-modified liposomes[79,98] and polymeric nanoparticles[52,65] bound to receptor-expressing cells *in vitro*.

As no prior modification is required, the NHS-ester and primary amine reaction demonstrates a straightforward route to prepare protein or antibody-coupled nanocarriers. However, there are often numerous amine functional groups available on the proteins or antibodies, making it difficult to control their conformation and the number of sites modified. This can result in lost bioactivity. For example, the antigen binding capacities of antibodies modified randomly by amine groups were significantly lower compared to those antibodies modified specifically at the hinge region.[118] Moreover, the multiple binding sites can result in crosslinked proteins and nanoparticles, which in turn increase the nanoparticle size and/or precipitate the polymer and protein complex, further complicating the methodology. The thiol-maleimide coupling chemistry does not suffer from these many side reactions due to the relatively fewer cysteine thiols than lysine amine groups available on proteins and antibodies coupled.

4.3 Other conventional conjugation methods

Besides the most popular thiol-maleimide and NHS-ester and primary amine reactions, there are a few conventional methods developed for nanocarrier modification including *p*-nitrophenylcarbonyl and amine reaction, disulfide exchange,

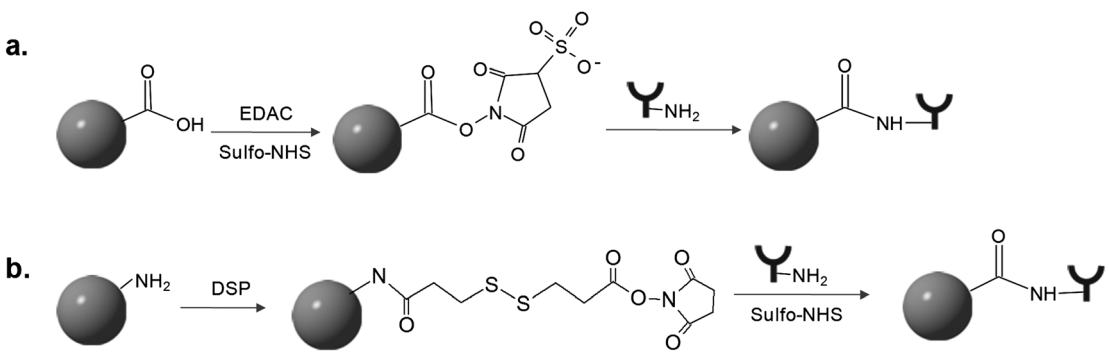


Fig. 7 Nanocarriers react with primary amine-containing targeting ligands by the formation of NHS-esters: a. carboxylic acid-functionalized nanocarriers are first activated by EDAC in the presence of sulfo-NHS to create NHS ester-nanocarriers which react with the primary amines on the targeting ligand, yielding a stable amide bond;[52,65,69,79,80,87,98,115,116] b. the primary amines on nanocarriers are activated to create NHS-esters on the surface which react with primary amines on targeting ligands.[117]

hydrazide coupling, vinylsulfone and thiol reaction, and non-covalent avidin-biotin interaction. The proved feasibility of these methods in ligand conjugation allows for multiple choices for ligand modification and great opportunities for material design.

Recently, a new amphiphilic PEG derivative, *p*-nitrophenylcarbonyl-PEG-PE (pNP-PEG-PE), was designed and incorporated into liposomes[97,101] or polymeric self-assembled micelles.[66] The water-exposed pNP groups react with the amine groups on antibodies or proteins readily and quantitatively at pH values greater than 8, producing a stable and non-toxic carbamate (urethane) bond (Fig. 9a). It has been demonstrated that approximately 100 protein molecules were bound to a single 200 nm liposome even though the content of pNP-PEG in the total lipid was as low as 1 mol%.[97] Similar chemistry was used as well in the preparation of immuno-polymer-drug conjugates where pNP-modified HPMA was copolymerized with HPMA to yield water soluble functionalized polymers.[73] Although the reaction proceeded readily and the biological activity of targeting ligands was preserved, the pNP derivatives are pH sensitive and undergo hydrolysis at pH values above neutral. At the preferred reaction conditions of pH $\geq$ 8, where the reaction between pNP and amine is most efficient, pNP is most susceptible to hydrolysis. The spontaneous hydrolysis of pNP groups within 1 to 2 h[97] ultimately decreases the coupling efficiency of antibodies.

Liposomes have had the following incorporated into their design to facilitate the binding of thiol-containing Abs: *N*-[3-(2-pyridyldithio)propionyl]phosphatidylethanolamine (PDP-PE),[91–93] *N*-[3-(2-pyridylthio)propionyl]-stearylamine (PDP-SA)[90] and *N*-(3′-(pyridyldithio)propionoylamino)-PEG-DSPE (PDP-PEG-DSPE)[102] (Fig. 9b). The PDP groups contain disulfide groups that are able to participate in disulfide exchange reactions with thiols present on targeting ligands in the presence of reducing agents. The reaction proceeds rapidly at pH 8.0[93] with a modest coupling efficiency greater than 20%;[90] yet thiols undergo oxidation in this alkaline medium. In addition, the disulfide bonds formed between antibody and nanocarriers are less stable compared to thioether bonds (Fig. 4): 62% of disulfide-coupled Fab′ were lost in the presence of human serum for 24 h.[93] Compared to the reaction of Fab′-thiol with maleimide-liposome, the reaction between Fab′-thiol and PDP-liposome is less efficient, achieving only half the amount of Fab′ conjugated to liposomes.[93]

The reaction between hydrazide (HZ) end groups and oxidized antibodies has been employed to synthesize immunoliposomes[74,82,94,96,104] (Fig. 9c) and HZ-PEG anchors have been designed to incorporate into liposomes for this purpose. The site-specific oxidation of carbohydrate chains, within the Fc region, results in the introduction of aldehyde groups on antibodies.[119] By avoiding the antigen binding regions while allowing for the use of intact antibody molecules, the site-specific modification method is believed to result in the highest retention of antigen binding capability.[94,120] Although the chemistry offers the advantage of site-specific modification of antibodies, the coupling has been shown to be inefficient relative to other conventional approaches: with a yield of 17% of the antibody attached relative to 60–70% *via* maleimide and thiol chemistry under the same conditions.[104] The antibody coupling with liposomes by this approach has been shown to induce aggregation of the immunoliposomes, likely manifested by the considerable size increase due to antibody conjugation.[94] In addition, the

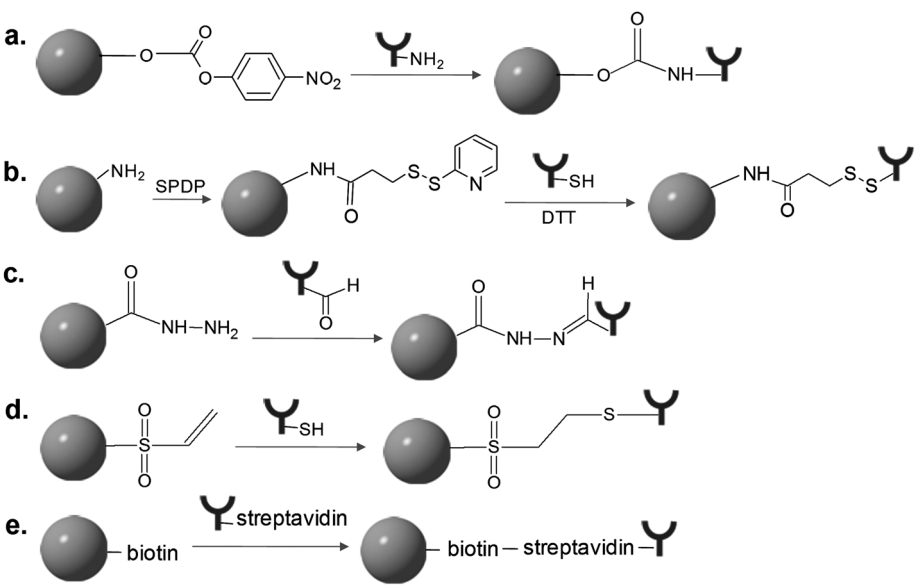


Fig. 9 a: pNP-functionalized nanocarriers readily couple targeting ligands through primary amine groups, forming a stable and non-toxic carbamate bond. The methodology is simple and applicable to a large variety of primary amine-containing targeting ligands,[66,73,97,101] yet limited by the hydrolysis of pNP groups at basic pH. b: Reaction of heterobifunctional cross-linker SPDP with primary amine-functionalized nanocarriers introduces PDP groups which undergo disulfide exchange with thiolated targeting ligands, yielding a disulfide bond.[90–93,102] c: The hydrazide end groups on the periphery of a nanocarrier react with the carbohydrate moieties of targeting ligands oxidized to aldehyde to form a hydrazone linkage.[74,82,94,96,104] d: VS functionalized nanocarriers bind with free thiol groups of targeting ligands.[75,95] e: The preparation of targeting ligand-decorated nanocarriers through non-covalent linking of biotin and streptavidin.[50,68,104,122]

hydrazone linkage formed by this reaction is somewhat labile, but can be stabilized by reacting with sodium cyanoborohydride to reduce the double bond.

Reactive double bonds, such as those in vinylsulfone (VS) groups, are capable of undergoing addition reactions with thiol groups in aqueous environments under mild conditions (Fig. 9d). The design has been incorporated into VS-exposed PEG-liposomes to attach thiol-containing antibodies.[95] Block copolymer poly(L-glutamic acid)-*co*-polyethylene glycol (PG-PEG) bearing VS groups at the PEG terminus has been developed for the preparation of immunoconjugates.[75] The addition reaction is similar to the thiol-maleimide addition; however, the rate of thiol addition to reactive vinylsulfone is slower than that with maleimide groups. For example, when the C225 antibody was conjugated through the VS group at the PEG terminus of PG-PEG using a molar ratio of C225 to copolymer of 1:8, 8.7% of the original antibodies were bound to the polymer[75] whereas a similar reaction with thiol-maleimide resulted in approximately 80% yield.[60]

Owing to the very high affinity of avidin and biotin, which has a $K_D = 10^{-15}$ M and a dissociation $t_{1/2} = 89$ days,[121] this non-covalent conjugation was used in the preparation of immunoliposomes or immuno-nanoparticles[50,68,104,122] (Fig. 9e). Streptavidin-targeting ligands bind quickly with biotin-polymeric nanoparticle under mild conditions. For example, a 30 min incubation resulted in a sufficient amount of bound antibodies for a polymeric nanoparticle to cross the blood-brain barrier *via* antibody-mediated transport,[68] demonstrating the creation of a bioactive and functionalized drug carrier. Since biotin is larger than other functional groups, biotin-nanoparticles exhibit a significant increase in size relative to the original nanoparticles, likely due to nanoparticle aggregation in the presence of bulky groups on the surface.[68] Since streptavidin has four biotin binding sites, cross-linking among nanocarriers and ligands is a concern.

4.4 Diels–Alder (DA) chemistry

DA chemistry involves the cycloaddition of an electron-rich diene and electron-poor dienophile to form a substituted cyclohexene system. DA chemistry has great synthetic importance with its broad scope and simplicity of operation.[123,124] Water was not a popular solvent for DA chemistry until Rideout and Breslow (1980) demonstrated that, in aqueous environments, the rate as well as the stereo-selectivity of DA chemistry is dramatically increased.[125] Since then, various applications of the aqueous acceleration of DA chemistry have been exploited in the fields of synthetic and physical organic chemistry.[126–131] The rate acceleration in aqueous solutions has been ascribed to two main factors: i) greater hydrophobic interactions destabilize the initial state relative to the activated complex, leading to a significant rate enhancement; ii) hydrogen bonding of water to the activating groups stabilizes the polarized activated complex, thereby increasing the rate of reaction in aqueous solutions.[132–134]

The aqueous DA reaction is diverse in scope and efficient in reactivity, resulting in very high yields, producing no byproducts, and occurring under simple and mild reaction conditions. Thus, the DA reaction provides a competitive alternative to conventional techniques for biomolecule conjugation. The compatibility of DA chemistry with biomolecules has been exploited elegantly in the bioconjugation and/or immobilization of protein, peptides and oligonucleotides.[135–145]

As a newly developed conjugation method in nanocarrier systems, DA chemistry has been recently employed for Ab conjugation to polymeric nanoparticle surfaces.[64] An amphiphilic copolymer, poly(2-methyl-2-carboxytrimethylene

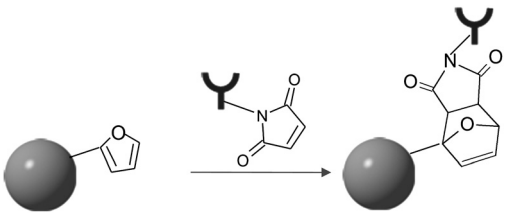


Fig. 10 One-step reaction of DA chemistry is employed in a polymeric nanoparticle system where the furan (dienophile) functional groups located on the surface readily react with maleimide (diene) groups of targeting antibodies.[64]

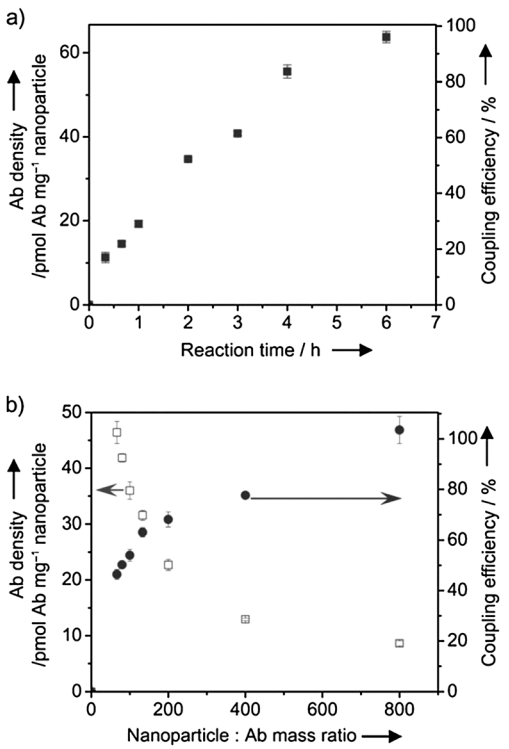


Fig. 11 The high coupling efficiency of maleimide-modified antibody conjugated with furan-functionalized polymeric nanoparticles by DA chemistry. a. When the nanoparticle/antibody feed mass ratio was kept constant (for example at 100:1), both the antibody density on the immuno-nanoparticles and the coupling efficiency (expressed as the % of the initial feed Ab bound to the micellar nanoparticles) increased as a function of reaction time. Almost 100% of the initial antibody added was coupled within six hours of incubation, resulting in an antibody density of 64 pmol/mg nanoparticle (approximated at 5 antibodies/nanoparticle). b. When the reaction time was fixed at 2 h and the initial nanoparticle/antibody mass ratio was increased, the coupling efficiency increased as the nanoparticle/antibody mass ratio increased. By using DA chemistry, either prolonged reaction times or high nanoparticle/antibody feed ratios can be used to achieve maximum coupling efficiency.[64]

carbonate-*co*-*D,L*-lactide)-*graft*-poly(ethylene glycol)-furan (poly(TMCC-*co*-LA)-*g*-PEG-furan), was designed to have furan (diene) functional groups at the PEG terminus. After self-assembly, these furan functional groups are located on the surface of the PEG corona of polymeric nanoparticles, and accessible for reaction with maleimide (dienophile)-functionalized antibodies in aqueous solution (Fig. 10). Maleimide-functional groups were introduced to Abs by the established site-specific oxidation of the carbohydrate chains within the Fc region followed by reaction with a bifunctional amine-maleimide thereby preserving the intact Fab fragment for antigen-binding and minimizing the loss of the bioactivity.[64,120] Immuno-nanoparticles were prepared by incubating maleimide-modified antibodies with furan-nanoparticles in MES buffer at pH 5.5, 37 °C for several hours. Requiring neither coupling agents nor catalyst, the one-step DA reaction is highly selective, achieving high coupling efficiency of antibody of up to 100% (Fig. 11). The immuno-nanoparticles created by DA chemistry exhibited specific binding with receptor-overexpressing cancer cells. The mild aqueous conditions for DA cycloaddition of the Ab to the nanoparticles avoid both long reaction times and the use of coupling or potentially denaturating reagents, which are attributed to the success in creating bioactive immuno-nanoparticles. This is the first time that DA chemistry has been employed in a polymeric nanoparticle system for biomolecule immobilization, opening many exciting opportunities in the broad field of surface modification of nanocarriers.

4.5 Alkyne-azide click chemistry

The concept of "click chemistry" was first introduced by Sharpless *et al.* in 2001. This type of reaction has been defined as being broad in scope, having high yields, simple in product isolation, stereospecific, and compatible with both organic and aqueous reaction conditions.[146] If two small units can be joined together with heteroatom links and to fulfill the above requirements, then it can be classified as "click chemistry". Among all the carbon-heteroatom bond formation reactions, such as cycloadditions of unsaturated species, nucleophilic substitution chemistry, carbonyl chemistry of the "non-aldol" type and additions to carbon-carbon multiple bonds, copper-catalyzed Huisgen 1,3-dipolar cycloaddition is the most popular conjugation method and is now often referred to as "click chemistry".

As shown in Fig. 12, an azide functional group readily reacts with an alkyne group in the presence of a copper(I) catalyst, forming a stable 1,2,3-triazole bond. Click chemistry offers the advantage of a simple, one-step reaction without side reactions and protecting/deprotecting procedures. It is highly specific and efficient under moderate reaction conditions. Recently, click chemistry gained interest as a means of biomolecule conjugation.[147–160] Its opportunity as a new conjugation methodology in conjugated nanocarriers has been recognized as well.[148,154–157] For example, polymers have been functionalized to bio-orthogonally couple a wide range of biomolecules such as peptides, folic acid, sugars, proteins, nucleic acids or even bacteria, viruses and cells.[158–160] The mild and aqueous reaction conditions permit functional biomolecules to be introduced either before or after the formation of an amphiphilic polymeric micelle.[156] An azide terminated amphiphilic block copolymer of *N*-isopropylacrylamide (NIPAM) and *N*,*N*-dimethylacrylamide (DMA) was conjugated with propargyl folate before its self-assembly resulting in folic acid residues being efficiently conjugated to end-functionalized polymers *via* click chemistry.[154] Alternatively, click-functionalized amphiphilic copolymers self-assemble to form surface-functionalized "clickable" micelles in aqueous solutions, where the high selectivity and orthogonality of click chemistry allows for quantitative incorporation of fluorescent dye molecules on the micelle surface,[155] creating a platform of azide- or alkyne-functionalized nanostructures. In another example, click chemistry was used to couple an alkyne-protein to an azide-functionalized gold nanoparticle at RT for 3 days, resulting in a bioactive protein-nanoparticle conjugate.[157] Similarly, alkyne-peptides were coupled to azide polymeric nanoparticles resulting in peptide-modified nanoparticles that selectively bound with cells expressing the integrin receptor.[156]

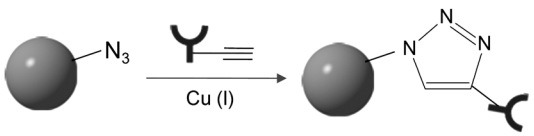


Fig. 12 Copper-catalyzed Huisgen 1,3-dipolar cycloaddition is employed in bioconjugated nanocarriers where functionalized nanocarriers present azide groups on the surface and catch alkyne-modified targeting ligands.[155–157]

The Cu(I) catalyzed alkyne-azide click reaction holds numerous advantages for biomolecule conjugation; however, when the coupling is conducted with sensitive biological molecules, such as proteins or nucleic acids, the toxicity of the copper ion may result in undesirable modification or loss of bioactivity. For example, the presence of the copper catalyst induced the cleavage of a single copper-sensitive peptide bond at the hinge region of IgG.[161] This cleavage was demonstrated to depend on the reaction time, temperature, pH and copper ion concentration. Cu(II) has high affinity to thiol groups of cysteine residues and can oxidize them to disulfide bonds, which may reduce the biological function of biomolecules involved.[162] Moreover, in the presence of dioxygen, Cu(I) rapidly damages DNA *via* the generation of reactive oxygen species.[163] To circumvent such problems, metal free click chemistry has been developed. The use of catalyst-free [3 + 2] Huisgen cycloadditions of cyclooctyne and azide by a strain-promoted mechanism has been reported.[150,160] It has been demonstrated that this cycloaddition reaction can be used for selective modification of biomolecules and living cells without apparent physiological harm. As the synthesis of cyclooctyne is complicated, coupling ligands such as tris(benzyltriazolylmethyl)amine (TBTA) or sulfonated bathophenanthroline (BCDS) have been introduced as an alternative, which can accelerate the cycloaddition reaction while overcoming the redox chemistry associated with Cu(I) and oxygen.[147,149,151–153,158,164]

4. Conclusions

Targeted delivery of anticancer drugs by nanoscale drug carriers promises enhanced drug efficacy and reduced systemic toxicity. By coupling targeting ligands on the surface, passive nanocarriers have the ability to recognize and bind to specific cell

types that express the corresponding receptors, combining passive and active targeting mechanisms in one platform. Targeting ligands can be conjugated to nanocarriers either before or after nanocarrier formation and/or drug incorporation, where a highly efficient and specific chemistry occurring under mild conditions is desirable in order to preserve the biological function of incorporated targeting ligands/drugs and the structure of the nanocarriers. Functionalized polymeric nanocarrier components allow a wide range of conjugation chemistries to be employed, each of which has advantages and limitations. Of the many coupling chemistries available, the thiol-maleimide is likely the most popular due to the efficiency of this chemistry, notwithstanding the sometimes synthetic complexity of this chemistry. Other coupling chemistries described have lower yields, require coupling agents (and therefore further purification), or result in unstable bonds (*i.e.* susceptible to hydrolysis). While these conventional methodologies have resulted in biologically-functionalized targeted nanocarriers, they often require organic reagents and have side-reactions and low coupling efficiency. Alternative coupling chemistries, such as Diels–Alder and alkyne-azide click chemistry, provide competitive alternatives to the conventional methodologies for nanocarrier functionalization. The investigations to exploit simple and highly efficient conjugation chemistries which are applicable to a broad class of biomolecules continue.

The targeted nanocarrier formulation represents a new generation for the delivery of combination immunotherapy/chemotherapy where the improved therapeutic outcome of anticancer therapy has been established in both animal models and clinical trials. Despite the considerable opportunity in formulating these nanocarriers, many challenges remain in translating targeted nanocarriers from bench to bedside including biodistribution and targeting. Therefore the type of materials and ligands, construction of nanocarriers, and density and orientation of coupled ligands must be optimized and determined specifically *in vivo* to lead to successful targeting strategies. The enhanced targeting and reduced blood circulation has to be balanced to achieve maximum therapeutic outcome since the presence of targeting ligands on nanoparticle surfaces sometimes results in increased opsonisation, resulting in reduced circulation time.[60] When availability of cell surface receptors is limited, such as in solid tumors, drug carriers could be designed to selectively bind to other receptors, such as those in tumor vasculature or surrounding matrix.[165,166] While delivering the maximum drug payload to cancer sites is the goal, a highly efficient drug release at the target sites requires more defined material chemistry, or novel strategies to be developed. For example, doxorubin targeting by some doxorubin-polymer conjugates is limited by the low cleavage efficiency of environmentally sensitive linkages, whereas a polymeric nanoparticle with doxorubin conjugated on the surface of the drug carriers (instead of inside) delivers drugs to the cell nucleus, without the cleavage of linkages, which is likely mediated by the surface exposed doxorubin.[167]

Looking to the future, the advances in material chemistry, combined with an increased understating of molecular medicine, will result in the design of more functionalized and sophisticated nanostructured delivery vehicles, resulting in the formulation of therapeutically effective platforms for *in vivo* application.

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