Passive and Active Drug Targeting: Drug Delivery to Tumors as an Example

Vladimir P. Torchilin

Contents

Abstract The paradigm of using nanoparticulate pharmaceutical carriers has been well established over the past decade, both in pharmaceutical research and in the clinical setting. Drug carriers are expected to stay in the blood for long time, accumulate in pathological sites with affected and leaky vasculature (tumors, inflammations, and infarcted areas) via the enhanced permeability and retention (EPR) effect, and facilitate targeted delivery of specific ligand-modified drugs and drug carriers into poorly accessible areas. Among various approaches to specifically target drug-loaded carrier systems to required pathological sites in the body, two seem to be most advanced – passive (EPR effect-mediated) targeting, based on the longevity of the pharmaceutical carrier in the blood and its accumulation in pathological sites with compromised vasculature, and active targeting, based on the attachment of specific ligands to the surface of pharmaceutical carriers to recognize and bind pathological cells. Here, we will consider and discuss these two targeting approaches using tumor targeting as an example.

V.P. Torchilin
Department of Pharmaceutical Sciences and Center for Pharmaceutical Biotechnology and Nanomedicine, Northeastern University, 360 Huntington Avenue, Boston, MA, 02115, USA
e-mail: v.torchilin@neu.edu

M. Schäfer-Korting (ed.), *Drug Delivery*,
Handbook of Experimental Pharmacology 197,
DOI 10.1007/978-3-642-00477-3_1,

Keywords Drug delivery · Drug targeting · Tumors · Liposomes · Polymeric micelles

1 Drug Targeting: General Considerations

By drug targeting, one usually understands an increased accumulation of an active pharmaceutical ingredient (API) in the required area of the body compared to other tissues and organs mediated by a spontaneous or external force or targeting moiety. For the majority of pharmaceuticals currently in use, the specificity and activity of pharmaceuticals towards disease sites or individual diseases is usually based on the API's ability to interfere with local pathological processes or with defective biological pathways, but not on its selective accumulation in the specific intracellular compartment or in the target cell, organ or tissue. Usually, pharmaceutical agents, practically independently on the route of administration, are distributed within the body rather evenly, proportionally to the regional blood flow. Moreover, to reach the site of action, the API has to cross many biological barriers, such as other organs, cells and intracellular compartments, where it can be inactivated or express undesirable effects on organs and tissues that are not involved in the pathological process. As a result, to achieve a required therapeutic concentration of an API in a certain body compartment or certain tissue, one has to administer the drug in large quantities (thus increasing the cost of the therapy), the great part of which, even in the best case scenario, is just wasted in normal tissues, while cytotoxic and/or antigenic/immunogenic agents can become the cause of many negative side-effects.

Drug targeting can bring a solution to all these problems. In a very general sense, one understands drug targeting as the ability of the API to accumulate in the target organ or tissue selectively and quantitatively, independent of the site and method of its administration. Ideally, under such conditions, the local concentration of the agent at the disease site(s) should be high, while its concentration in other nontarget organs and tissues should be below certain minimal levels to prevent any negative side-reactions. The following advantages of drug targeting are evident: drug administration protocols may be simplified; the drug quantity required to achieve a therapeutic effect may be greatly reduced, as well as the cost of therapy; and drug concentration in the required sites can be sharply increased without negative effects on nontarget compartments. The same is true, to a great extent, for the use of many diagnostic agents.

Although the concept of drug targeting, suggested by Paul Ehrlich early in the twentieth century, considered a hypothetical "magic bullet" as an entity consisting of two components – the first one should recognize and bind the target, while the second one should provide a therapeutic action in this target – currently, the whole set of suggested targeting protocols includes many different approaches to targeted drug delivery. These approaches do not necessarily involve the use of specific targeting moieties. In certain cases various physical principles and/or some physiological

features of the target area may be utilized for a successful targeting of pharmaceuticals and pharmaceutical carriers.

The concept of targeted pharmaceuticals includes a coordinated interaction of several components: pharmaceutical agent, targeting moiety, pharmaceutical carrier (soluble polymers, microcapsules, microparticles, cells, cell ghosts, liposomes, and micelles) used to load many drug molecules per single targeting moiety, and a target. The recognition of the target may proceed on various levels: on the level of a whole organ, certain cells specific for this organ, or even individual components of these cells (cell surface antigens). Recognition on the molecular level is certainly the most universal form of target recognition, because in each organ or tissue certain components can be found specific only to that organ or tissue.

Currently, numerous approaches for drug targeting have been described, permitting the specific delivery of therapeutic and diagnostic pharmaceutical agents to a variety of tissues and organs; some of them are discussed in many review-type publications (see for example Francis and Delgado 2000; Gregoriadis 1977; Muzykantov and Torchilin 2003; Torchilin 1995).

Attempts have been made to directly administer drugs into the affected zone, to load APIs into certain carriers sensitive to specific values of pH or temperature in pathological areas, or even use certain external forces, such as magnetic fields or ultrasound, to guide drugs to required targets or to release APIs there. The passive accumulation of many drugs and drug carriers due to their extravasation through leaky vasculature (named the Enhanced Permeability and Retention [EPR] effect) works very well for tumors, infarcts, and inflammation areas. The use of specific delivery vectors (certain moieties possessing high specific affinity towards the target areas) can target drugs and drug carriers almost everywhere.

Direct drug administration into an affected area was used in the intracoronary infusion of thrombolytic enzymes for therapy of coronary thrombosis (Chazov et al. 1976), and in the intra-articular administration of nonhormonal drugs for the treatment of arthritis (Williams et al. 1996). However, the direct administration of a drug into an affected organ or tissue is technically quite difficult in a majority of cases; in addition, many diseases are spread over a variety of cells or tissues. All this limits the applicability of this approach to a very few clinical situations.

Various endogenous and/or exogenous physical factors have been shown to mediate targeted delivery of pharmaceuticals. This approach utilizes, for example, the differences in pH and temperature values between normal tissues and pathological areas (tumors, inflammation, etc.) that are characterized by acidosis (decreased pH) and hyperthermia. With this in mind, it was suggested to load various pharmaceutical agents onto pH- or temperature-responsive drug carriers that can change their properties and release an encapsulated agent when they are brought to the areas with lower (compared to normal) pH or higher temperature. The advantage of this approach is that even though the drug-loaded carrier is evenly distributed within the circulation, it degrades and releases the drug only in the target area. Moreover, the target area can be additionally heated from the outside by applying external heat or ultrasound irradiation. Thus, it was shown that on intravenous administration, the anti-cancer drug methotrexate accumulated in tumors in mice

several times faster when it was incorporated into temperature-sensitive liposomes and external heat was applied locally onto the tumor area (Weinstein et al. 1979). Drug-loaded pH-sensitive liposomes are also frequently used for experimental delivery of API and genetic material into a variety of compromised tissues (see Budker et al. 1996; Torchilin et al. 1993 for just a few of many reviews). In many cases, however, a pathological site does not differ much from normal tissues in terms of temperature or local pH value, which makes the use of targeting based on pH or temperature differences inapplicable.

An intentionally applied external magnetic field can also be used for the targeted delivery of pharmaceuticals. In this case, the API of choice has to be attached to a drug carrier possessing ferromagnetic properties. As a result, one can expect the accumulation of a drug-loaded ferromagnetic carrier in the area to which an external magnetic field is applied. High magnetic field gradient together with high blood flow velocity and accompanying high shear strength in large blood vessels do not allow this approach to work in blood vessels such as the aorta; however, magnetic field-mediated drug accumulation in smaller blood vessels with slower blood flow and located closer to the body surface was clearly demonstrated (Widder et al. 1983). Dextran-coated microparticles of iron oxide have been used to couple the thrombolytic enzyme streptokinase, and the preparation was successfully used for the targeted thrombus lysis of artificially formed thrombi in carotid arteries of experimental dogs when a small strong permanent magnet was implanted into the tissues next to a vessel in the area of thrombus (Torchilin et al. 1988). The local prevention of thrombosis in experimental dogs and rabbits was achieved by the intravenous application of autologous red blood cells loaded with ferromagnetic colloid compound and aspirin, when a strong magnet was positioned externally to the blood vessel where the thrombus was initiated (Orekhova et al. 1990). Quite a few examples exist (McBain et al. 2008; Pauwels and Erba 2007; Sun et al. 2008) of magnetic targeting of various anticancer drugs to tumors, when such drugs have been co-loaded together with magnetically sensitive nanoparticles into various pharmaceutical carriers and concentrated in tumors under the action of an external magnetic field. However, magnetic drug delivery has its limitations connected with the blood flow rate in the target, and is virtually impossible in large vessels or in "deep" tissues.

In this chapter, we will concentrate on drug targeting based on the EPR effect (passive targeting) and on the use of targeting moieties (active targeting) and will use as examples the studies related to drug delivery into tumors, which are the most numerous and advanced.

2 Concepts of Passive and Active Targeting

It is now a well-established phenomenon that under certain circumstances the endothelial lining of the blood vessel wall becomes more permeable than in the normal state. This was clearly demonstrated in many tumors (Hobbs et al. 1998; Jain 1999) and in infarcted areas (Palmer et al. 1984; Torchilin et al. 1992). As a

result, in such areas, large molecules and even relatively large particles, such as micelles and liposomes ranging from 10 to 500 nm in size, can leave the vascular bed and accumulate inside the interstitial space. Assuming these large (polymeric) molecules/particles are loaded with a pharmaceutical agent, they can bring this agent into the area with the increased vascular permeability, where the API can be eventually released from a carrier. Because the cut-off size of the permeabilized vasculature varies from case to case (Hobbs et al. 1998; Yuan et al. 1995), the size of a drug-carrying particle may be used to control the efficacy of such spontaneous "passive" drug delivery or EPR effect (Maeda 2003; Maeda et al. 2000; Fig. 1). This type of targeting requires drug delivery systems to be long-circulating (i.e., to stay in the blood for extended periods of time) in order to provide a sufficient level of accumulation in the target. The most usual way to keep drug carriers in the blood long enough is to "mask" them by modifying (grafting) their surface with certain water-soluble polymers with a well-solvated and flexible main chain, such as polyethylene glycol (PEG) (Klibanov et al. 1990; Torchilin and Trubetskoy 1995). The surface-grafted "protective" polymers effectively prevent (slow down) the opsonization of drug carriers and their clearance by the reticuloendothelial system. The approach is best developed for liposomes (Lasic and Martin 1995; Lasic and Papahadjopoulos 1998), although it has a rather broad applicability (Torchilin 1998). The anticancer drug doxorubicin incorporated into long-circulating PEG-coated liposomes, which is currently used in clinical conditions, demonstrates

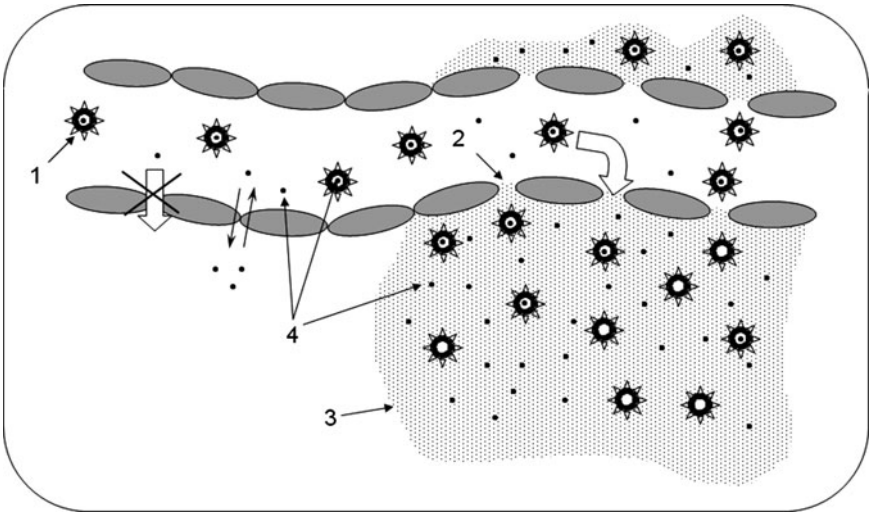

Fig. 1 Schematics of the Enhanced Permeability and Retention (EPR) effect or "passive" targeting. (1) Drug-loaded nanocarrier; it cannot extravasate through normal endothelium and only small molecules of free drug (4) can traverse normal endothelium to a certain extent in both directions; (2) gaps between endothelial cells appear in pathological areas (3) (such as tumors, infarcts, and inflammations), through which nanoparticles can extravasate and accumulate in such areas creating high local drug concentrations

high efficacy in EPR-based tumor therapy and strongly diminishes the side-effects (Gabizon 1995, 2001) characteristic of free doxorubicin. Long-circulating polymeric micelles (Torchilin 2001) may be used as carriers for drug delivery into tumors with a smaller cut-off size (Hobbs et al. 1998; Yuan et al. 1995), as was shown in Lewis lung carcinoma-bearing mice (Weissig et al. 1998).

Important advantages of prolonged circulation of drugs and drug carriers in the blood flow include the possibility of maintaining a required concentration of an API or drug carrier in the blood for a long time after a single administration; the ability to utilize the EPR effect for the accumulation of pharmaceuticals in the areas with leaky vasculature; and the possibility of enhancing targeting of drugs and drug carriers into the areas with a limited blood supply and/or low concentration of a target antigen, where an extended time is required to allow for a sufficient quantity of a drug in the target zone. More information regarding the importance of drug carrier longevity is given below.

It is important to mention, however, that in many pathological situations the integrity of vascular endothelium remains unaffected and there is no opportunity for EPR.

Many of the approaches to drug targeting described so far are not universal. Thus, direct administration of a drug into an affected organ or tissue may be technically difficult, or the disease site may be delocalized. Often, the affected area does not differ much from normal tissues in terms of vascular permeability, temperature or local pH value. Magnetic drug delivery also has limitations connected with the blood flow rate in the target. The most natural and universal way to impart to a nonspecific drug affinity towards its target is the binding of this drug with another molecule (usually referred to as a targeting moiety or vector molecule) capable of specific recognition and binding to a target site (Fig. 2). The following substances can be used as targeting moieties: antibodies and their fragments, lectins, other proteins, lipoproteins, hormones, charged molecules, mono-, oligo- and polysaccharides, and some low-molecular-weight ligands, such as folate. Monoclonal antibodies against characteristic components of target organs or tissues are the most frequently used vector molecules.

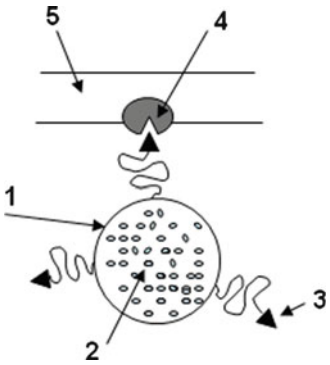

Fig. 2 Schematics of the specific ligand-mediated active targeting. Nanocarrier (1) loaded with the drug (2) is modified with the moieties of a specific ligand (3) capable of recognizing certain binding sites (4) on the cell surface (5). As a result, the carrier remains attached to the cell surface and releases its drug load there or can be internalized bringing the drug inside target cells

Direct coupling of a drug to a targeting moiety seems the simplest way to prepare a targeted drug. Immunotoxins represent the most vivid examples of this approach (Vitetta et al. 1983). A natural toxin can be "cut" into active moiety (the toxic one) and recognizing moiety, and then the latter is separated and the former is conjugated with an antibody. As a result, a toxic unit may be delivered only in those cells that express an appropriate antigen (usually cancer cells), while antigen-free cells will not be recognized by the immunotoxin and damaged. However, in this case every single antibody molecule is able to carry just one active moiety. Since toxic moieties of toxins/immunotoxins are extremely active (just one catalytic moiety of the plant toxin ricin can kill a cell if gets inside and destroys thousands of ribosomes), immunoxins may still find clinical application, primarily for cancer treatment (Goldmacher et al. 2002; Vitetta et al. 1983).

Another example of this kind is the attachment of various thrombolytic enzymes to some antibodies specific towards different components of thrombi. Thus, it was clearly demonstrated in hamsters and baboons that effective thrombolysis may be achieved by using the conjugate between single-chain urokinase-type plasminogen activator and a bispecific monoclonal antibody against this activator and fibrin (Imura et al. 1992). The data on enzyme–antibody conjugates for thrombolysis, as well as on a variety of antibodies used to deliver the thrombolytic therapy directly to the occlusion site, are numerous and well reviewed (Haber 1994; Khaw 2002).

Certain attempts have been also made to use direct drug–antibody conjugates for targeted treatment of malignant diseases, such as human small cell lung cancer (SCLC). Antibody against the proliferative compartment of mammalian squamous carcinomas was conjugated with daunomycin and sharply enhanced drug potency in the murine model (Ding et al. 1990). Murine monoclonal antibody NCC-LU-243 was conjugated with mitomycin C and used for the targeted therapy of nude mice with the transplanted antigen-positive cell line of human SCLC (Kubota et al. 1992).

In general, however, the load of a pharmaceutical agent onto a single targeting moiety should be much higher than a simple 1:1 ratio to make the whole approach beneficial and practically applicable. Alternatively, some soluble or insoluble carrier can be loaded with multiple active moieties and then conjugated additionally with the targeting unit according to the scheme suggested by Ringsdorf in the mid-1970s (Ringsdorf 1975). Different reactive and biocompatible soluble polymers can be used as soluble carriers, whereas the family of insoluble carriers includes microcapsules, nanoparticles, liposomes, micelles and cell ghosts. Various reservoir-type systems, such as liposomes or microcapsules, demonstrate the following important advantages over other drug carriers: (a) maximum volume at a given surface (i.e., maximum load of the drug); (b) few targeting moieties can carry multiple drug moieties loaded into the reservoir; (c) the possibility to control size and permeability.

To date, it has already been shown that body compartments and pathologies that can be successfully targeted via different mechanisms include components of cardiovascular system (blood pool, vascular walls, lungs, heart), reticuloendothelial system (liver and spleen); lymphatic system (lymph nodes and lymphatic vessels), tumors, infarcts, inflammations, infections, and transplants.

The parameters determining the efficacy of drug targeting include: the size of the target, blood flow through the target, number of binding sites for the targeted drug/drug carrier within the target, number and affinity of targeting moieties on an API molecule (drug carrier particle), and multipoint interaction of a drug/drug carrier with the target.

3 Pharmaceutical Carriers: Liposomes and Micelles as Examples

Numerous drug delivery and drug targeting systems, such as synthetic polymers, microcapsules, cells, cell ghosts, lipoproteins, liposomes, and micelles (Cohen and Bernstein 1996; Müller 1991), are currently developed or under development. Their use aims to minimize drug degradation upon administration, prevent undesirable side-effects, and increase drug bioavailability and the fraction of the drug accumulated in the pathological area. To better achieve these goals, all listed drug carriers can be made slowly degradable, stimuli-reactive (for example, pH- or temperature-sensitive), and targeted (for example, by conjugating them with ligands specific towards certain characteristic components/receptors of the area of interest). In addition, drug carriers are expected to stay in the blood for prolonged time intervals (Lasic and Martin 1995; Torchilin and Trubetskoy 1995) in order to maintain the required therapeutic level of pharmaceuticals in the blood over an extended period, to allow for their slow accumulation in pathological sites with affected and leaky vasculature (tumors, inflammations, and infarcted areas) via the enhanced permeability and retention (EPR) effect (Maeda et al. 2000; Palmer et al. 1984), and facilitate targeted delivery of specific ligand-modified drugs and drug carriers into poorly accessible areas (Torchilin 1998).

Pharmaceutical drug carriers, especially those for parenteral administration, are expected to be easy and reasonably cheap to prepare, biodegradable, have small particle size, possess high loading capacity, demonstrate prolonged circulation, and, ideally, accumulate specifically or nonspecifically in required pathological sites in the body (Gref et al. 1994).

The paradigm of using nanoparticulate pharmaceutical carriers to enhance the in vivo efficiency of many drugs, anti-cancer drugs first of all, has been well established over the past decade both in pharmaceutical research and in the clinical setting and does not need any additional proofs. Various pharmaceutical nanocarriers, such as nanospheres, nanocapsules, liposomes, micelles, cell ghosts, lipoproteins and some others are widely used for experimental (and even clinical) delivery of therapeutic and diagnostic agents (Alonso 2004; Gregoriadis 1988; Müller 1991; Rolland 1993). Surface modification of these carriers is often used to control their properties in a desirable fashion and make them simultaneously perform several different functions. The most important results of such modification(s) include increased longevity and stability in the circulation, changed biodistribution, targeting effect, sensitivity to

stimuli (pH or temperature), and contrast properties. Frequent surface modifiers (used separately or simultaneously) include: soluble synthetic polymers (to achieve carrier longevity); specific ligands, such as antibodies, peptides, folate, transferrin, sugar moieties (to achieve targeting effect); pH- or temperature-sensitive copolymers (to impart stimuli sensitivity); and chelating compounds, such as EDTA, DTPA or deferoxamine (to add a diagnostic/contrast moiety onto a drug carrier). Evidently, different modifiers can be combined on the surface of the same nanoparticular drug carrier providing it with a combination of useful properties (for example, longevity and targetability, targetability and stimuli sensitivity, or targetability and contrast properties).

Liposomes, artificial phospholipid vesicles, can be obtained by various methods from lipid dispersions in water. Preparation of liposomes, their physico-chemical properties and possible biomedical application have already been extensively discussed in several monographs (Gregoriadis 2007; Lasic and Martin 1995; Lasic and Papahadjopoulos 1998; Torchilin and Weissig 2003; Woodle and Storm 1998). To date, many different methods have been suggested for preparing liposomes of different sizes, structure and size distribution. To increase liposome stability towards the action of the physiological environment, cholesterol is incorporated into the liposomal membrane (sometimes up to 50% mol). The size of liposomes depends on their composition and preparation method and can vary from around 50 nm to greater than 1 μm in diameter. MLVs (multilamellar vesicles) range from 500 to 5,000 nm and consist of several concentric bilayers. LUVs (large unilamellar vesicles) range from 200 to 800 nm. SUVs (small unilamellar vesicles) are around 100 nm (or even smaller) in size and are formed by a single bilayer (see Fig. 3). The encapsulation efficacy for different substances is also variable depending on the liposome composition, size, charge, and preparation method. The use of the reverse phase evaporation method (Szoka and Papahadjopoulos 1980) permits the inclusion of 50% or more of the substance to be encapsulated from the water phase into the liposomes. Besides, a variety of methods have been developed to obtain lyophilized liposomal preparations possessing good storage stability (Madden et al. 1985). The in vitro release rate of different compounds from liposomes, including proteins of moderate molecular weight, such as lysozyme or insulin, is usually under 1% per hour (assuming that the incubation temperature sufficiently differs from the phase transition temperature of a given phospholipid).

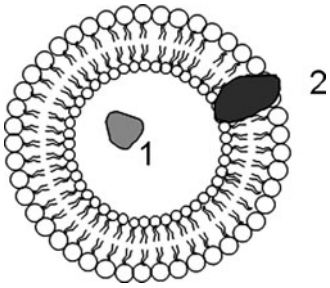

Fig. 3 Liposomes can vary in size between 50 and 1000 nm. Structures and drug loading: soluble hydrophilic drugs are entrapped into the aqueous interior of the liposome (1), while poorly soluble hydrophobic drugs are localized in the liposomal membrane (2)

In vivo, this parameter can vary within wide limits (minutes to hours) and depends on the liposome membrane composition and cholesterol content, and location of the liposome in the body.

Liposomes are biocompatible, cause no or very little antigenic, pyrogenic, allergic and toxic reactions; they easily undergo biodegradation; they protect the host from any undesirable effects of the encapsulated API, at the same time protecting an entrapped API from the inactivating action of the physiological medium; and, last but not least, liposomes are capable of delivering their content inside many cells. Different methods of liposomal content delivery into the cytoplasm have been elaborated (Connor and Huang 1986). According to one of these methods, the liposome is made of pH-sensitive components and, after being endocytosed in the intact form, it fuses with the endovacuolar membrane under the action of lowered pH inside the endosome, releasing its content into the cytoplasm. In addition, liposomes have been shown to fuse with the microscopic pores on the cell surface (which appear, for example, as a result of ischemia) (Khaw et al. 2001, 1995) and deliver their contents including DNA into the cell cytoplasm. Liposomes modified on the surface with TAT-peptide (Torchilin and Levchenko 2003) (or other cell-penetrating peptides, such as Antp, penetratin, or synthetic polyarginines; see the review in Torchilin 2008) are also capable of delivering their cargo inside cells (Torchilin et al. 2003a).

Liposomes have been considered promising drug carriers for over two decades (Ringsdorf 1975). However, upon intravenous administration, plain liposomes are very quickly (usually within 15–30 min) opsonized and sequestered by cells of the reticuloendothelial system (RES), primarily by the liver (Ringsdorf 1975). From this point of view, the use of targeted liposomes, i.e., liposomes with a specific affinity for the affected organ or tissue, may both increase the efficacy of liposomal drug, and decrease the loss of liposomes and their contents in RES.

To obtain targeted liposomes, different methods have been developed to bind corresponding vectors (antibodies) to the liposome surface. These methods are relatively simple and allow binding of sufficient numbers of antibody molecules to a liposome surface without affecting the liposome integrity and antibody affinity and specificity. At present, over 100 antibody molecules can be bound to a single 200 nm liposome, allowing for firm multi-point liposome binding with a target. The routine methods for antibody coupling to liposomes include covalent binding to a reactive group on the liposome membrane, and hydrophobic interaction of proteins specifically modified with hydrophobic residues with the membrane (Francis and Delgado 2000; Ringsdorf 1975).

A potentially important problem with liposomes (or any other microparticulate drug carrier) is their inability to reach extravascular targets.

Despite some promising results with immunoliposomes as pharmaceutical carriers, the whole approach is limited because of the short lifetime of liposomes and immunoliposomes in the circulation. The majority of antibody-modified liposomes still end up in the liver as a consequence of an insufficient time for the interaction between the target and targeted liposome. This is certainly the case when the target has a diminished blood supply (ischemic or necrotic areas). Even high liposome

affinity towards the target cannot provide substantial liposome accumulation because of the small quantity of liposomes passing through the target with the blood during the time period when liposomes are still present in the circulation. The same lack of targeting can happen if the concentration of the target antigen is very low, and even sufficient blood flow (and liposome passage) through the target does not result in good accumulation due to the small number of "productive collisions" between antigens and immunoliposomes. In both cases, better accumulation can be achieved if liposomes can remain in the circulation long enough. This is why long-circulated (usually PEGylated) liposomes have attracted so much attention over the last decade.

Micelles represent colloidal dispersions with particle size from 5 to 50–100 nm. At a certain concentration and temperature, such colloids are spontaneously formed by amphiphilic or surface-active agents (surfactants), molecules of which consist of two clearly distinct regions with opposite affinities towards a given solvent (Mittal and Lindman 1991). At low concentrations, these amphiphilic molecules exist separately as unimers; however, as their concentration is increased, aggregation begins to take place at a certain concentration called the critical micelle concentration (CMC). The aggregates known as micelles include several dozens of amphiphilic molecules and usually have a shape close to spherical. Hydrophobic fragments of amphiphilic molecules form the core of a micelle, which can solubilize poorly soluble pharmaceuticals (Lasic 1992). This solubilization phenomenon was extensively investigated and reviewed in many publications (see, for example, Attwood and Florence 1983). In aqueous systems, nonpolar molecules will be solubilized within the micelle core, polar molecules will be adsorbed on the micelle surface, and substances with intermediate polarity will be distributed along surfactant molecules in certain intermediate positions (Fig. 4).

Polymeric micelles are usually prepared of amphiphilic block-copolymers of hydrophilic PEG and various hydrophobic blocks. Numerous studies (see, for example, Gao and Eisenberg 1993; Hunter 1991; Kabanov et al. 1992) have been published on polymeric micelle formation and properties. Many good recent reviews exist dealing with various aspects of polymeric micelle preparation, physicochemical and biological properties, and possible applications as pharmaceutical carriers (Adams et al. 2003; Jones and Leroux 1999; Kabanov et al. 2002a, b; Kakizawa and Kataoka 2002; Kwon 1998, 2003; Lukyanov and Torchilin 2004; Otsuka et al. 2003; Torchilin 2001).

In the majority of cases, amphiphilic unimers include PEG blocks with a molecular weight from 1 to 15 kDa as corona-forming blocks, and the length of a hydrophobic core-forming block is close to or somewhat lower than that of a hydrophilic block (Cammas et al. 1997). Though some other hydrophilic polymers may be used as hydrophilic blocks (Torchilin et al. 1995), PEG still remains the corona block of choice. At the same time, a variety of polymers may be used to build hydrophobic core-forming blocks: propylene oxide (Miller et al. 1997), L-lysine (Katayose and Kataoka 1998), aspartic acid (Harada and Kataoka 1998), β-benzoyl-L-aspartate (La et al. 1996), γ-benzyl-L-glutamate (Jeong et al. 1998), caprolactone (Allen et al. 1998), and D, L-lactic acid (Hagan et al. 1996). In certain

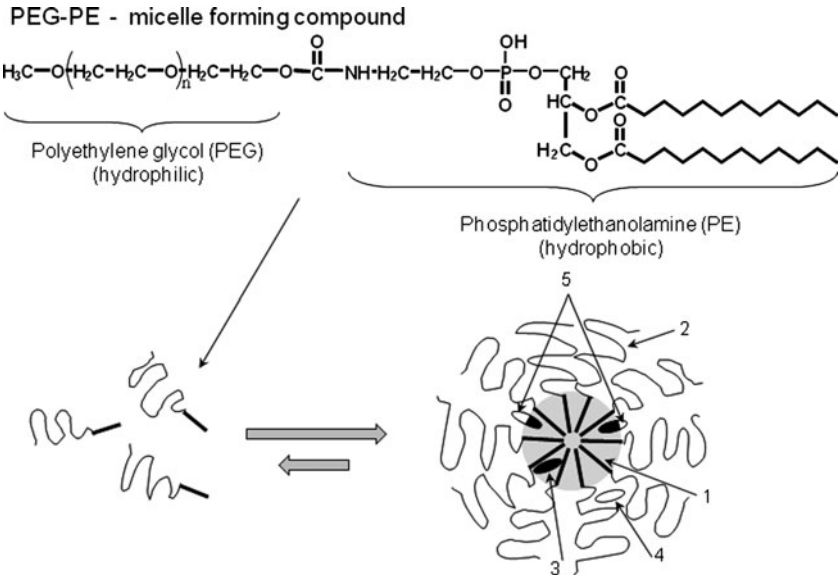

Fig. 4 A micelle as it self-assembles in the aqueous medium from amphiphilic unimers (such as polyethylene glycol–phosphatidylethanolamine conjugate, PEG–PE; see on the top) with the hydrophobic core (1) and hydrophilic corona (2). In water, nonpolar molecules will be solubilized within the micelle core (3), polar molecules will be adsorbed on the micelle surface (4), and substances with intermediate polarity will be distributed along surfactant molecules in certain intermediate positions (5)

cases, the starting copolymers can be prepared from two hydrophilic blocks and then one of those blocks is modified by the attachment of a hydrophobic pharmaceutical agent (such as paclitaxel, cisplatin, antracyclin antibiotics, hydrophobic diagnostic units, etc.) yielding amphiphilic micelle-forming copolymers (Katayose and Kataoka 1998; Kwon and Kataoka 1995; Trubetskoy et al. 1997).

In some cases, phospholipid residues – short but extremely hydrophobic due to the presence of two long-chain fatty acyl groups – can also be successfully used as hydrophobic core-forming groups (Trubetskoy and Torchilin 1995). The use of lipid moieties as hydrophobic blocks capping hydrophilic polymer (such as PEG) chains can provide additional advantages for particle stability when compared with conventional amphiphilic polymer micelles, due to the existence of two fatty acid acyls which might contribute considerably to an increase in the hydrophobic interactions between the polymeric chains in the micelle's core. Similar to other PEG-containing amphiphilic block-copolymers, diacyllipid–PEG conjugates (such as PEG–phosphatidyl ethanolamine, PEG–PE) were found to form very stable micelles in an aqueous environment (Klibanov et al. 1990; Lasic et al. 1991b). Their CMC values can be as low as 10^{-6} M (Kabanov et al. 2002a; Torchilin 2001), which is at least 100-fold lower than those of conventional detergents (Rowe et al. 2003), so that micelles prepared from these polymers will maintain their integrity even upon strong dilution (for example, in the blood during a therapeutic application).

The high stability of polymeric micelles also allows for good retention of encapsulated drugs in the solubilized form upon parenteral administration.

Three targeting mechanisms can be seen for micelles, as for any other pharmaceutical long-circulating drug carrier. The first one is based on spontaneous penetration of micelles into the interstitium through the leaky vasculature (EPR effect) and is considered "passive targeting" (Gabizon 1995; Maeda et al. 2000; Palmer et al. 1984). Thus, it was repeatedly shown that micelle-incorporated anticancer drugs (such as adriamycin, see, for example, Kwon and Kataoka 1999) accumulate much better in tumors than in nontarget tissues (such as the heart muscle), which minimizes undesired drug toxicity. In certain cases, it is the small size of micelles which makes them superior to other nanoparticulates, including liposomes. The transport efficacy and accumulation of microparticulates, such as liposomes and/or micelles, in the tumor interstitium is to a great extent determined by their ability to penetrate the leaky tumor vascular endothelium (Jain 1999; Yuan et al. 1995); see the schematic representation of this phenomenon in Fig. 1. Diffusion and accumulation parameters were recently shown to be strongly dependent on the cut-off size of tumor blood vessel wall, and the cut-off size varies for different tumors. As a result, the use of PEG–PE micelles for the delivery of a model protein drug to a murine solid tumor with a low permeability, Lewis lung carcinoma, provided the best results compared to other particulate carriers (Weissig et al. 1998).

The second targeting mechanism is based on the fact that many pathological processes in various tissues and organs are accompanied by local temperature increase and/or acidosis (Helmlinger et al. 1997). Micelles made of temperature- or pH-sensitive components, such as poly(*N*-isopropylacrylamide) and its copolymers with poly(D,L-lactide) and other blocks, can disintegrate in such areas releasing the micelle-incorporated drug (Jones and Leroux 1999).

Finally, specific ligands can be attached to the water-exposed termini of hydrophilic blocks, such as antibodies and/or certain sugar moieties (Rammohan et al. 2001). In this case, in order to make the micelles targeted without creating any steric hindrances for the antibody, the antibody of choice or its fragment can be chemically attached to an activated water-exposed free terminus of a hydrophilic block of micelle-forming polymer. For this purpose, relatively simple chemistry can be applied similar to that developed earlier for liposomes (Torchilin et al. 2001a) and involving the use of amphiphilic PEG–PE with a protein-reactive *p*-nitrophenylcarbonyl (pNP) group on the distal tip of the hydrophilic PEG block.

4 Chemistry Used to Provide Pharmaceutical Nanocarriers with Various Functions

Preparing various functional nanocarriers with controlled properties requires the conjugation of proteins, peptides, polymers, cell-penetrating moieties, reporter groups and other functional ligands to the carrier surface (although in certain

cases functional components may be loaded inside the nanocarrier or distributed within the nanocarrier structure: thus, for example, fine ferromagnetic particles can be loaded inside liposomes or polymeric nanoparticles to make them magnetic). This attachment can proceed noncovalently, via the hydrophobic adsorption of certain intrinsic or specially inserted hydrophobic groups in the ligands to be attached onto or into the surface of the nanocarrier. Thus, amphiphilic polymers or hydrophobically modified proteins can adsorb on the hydrophobic surface of polystyrene nanoparticles (Yuan et al. 1995) or incorporate into the phospholipid membrane of liposomes (Torchilin 1998) or hydrophobic core of micelles (Torchilin 2001). More frequently, the attachment is performed chemically, via the interaction of reactive groups generated on the carrier surface and certain groups in the molecule to be attached. In the case of liposomes, the most popular drug delivery system and convenient example of the techniques used, the conjugation methodology, is based on three main reactions, which are quite efficient and selective: reaction between activated carboxyl groups and amino groups yielding an amide bond; reaction between pyridyldithiols and thiols yielding disulfide bonds; and reaction between maleimide derivatives and thiols yielding thioether bonds (Torchilin and Klibanov 1993). Some other approaches also exist, for example yielding the carbamate bond via the reaction of the *p*-nitrophenylcarbonyl groups introduced onto the surface of nanocarriers with amino group of various ligands (Torchilin et al. 2001b). The detailed review of numerous coupling procedures and protocols used for attaching a whole variety of surface modifiers to drug carriers can be found in Klibanov et al. (2003) and Torchilin et al. (2003c).

It was shown, for example, that carboxylic groups of immunoglobulins can be activated by water-soluble carbodiimide; activated protein can then be bound to free amino-group-containing surfaces, such as PE-containing liposomes (Dunnick et al. 1975). For further ligand attachment, corresponding reactive groups on the surface of nanocarriers can be premodified with the aid of heterobifunctional cross-linking reagents, such as the popular *N*-succinimidyl-3(2-pyridyldithio)-propionate (SPDP) used to synthesize a PE derivative further used for coupling to SH-containing proteins (Leserman et al. 1980). Another possibility is to rely on the reaction of the thiol groups on a ligand (protein) with the maleimide-carrying surfaces (phospholipid molecules, in the case of liposomes). This approach (Martin and Papahadjopoulos 1982) is now one of the most widely used in research and practical applications. Different commercially available maleimide reagents can be used for the preparation of maleimide-carrying phospholipids in a simple single-step procedure. Various high and low molecular weight compounds have been attached to liposomes by using pyridyldithiopropionyl-PE or maleimide reagents (Klibanov et al. 2003; Torchilin et al. 2003c). The application of free thiol groups located on immunoglobulin Fab fragments is also attractive. It is believed that these SH groups are positioned far from the antigen-binding sites, enabling the nanocarrier-bound antibody fragments to retain their specific interaction with antigens.

Some ligands carry carbohydrate residues, which can be easily oxidized to yield aldehyde groups that can react with surface aminogroups with the formation of Schiff bases (Heath et al. 1980). Nanocarriers (such as liposomes) containing

surface-exposed carboxylic groups were used for the attachment of different ligands (Kung and Redemann 1986). In the case of liposomes, they can be prepared by various techniques and activated with water-soluble carbodiimide directly prior to ligand addition. The same chemical reactions can be used to attach nonmodified proteins and peptides to various nanocarriers, including preformed liposomes, containing membrane-incorporated reactive lipid derivatives, such as *N*-glutaryl-PE or glutaryl-cardiolipin (Bogdanov et al. 1988; Weissig and Gregoriadis 1992; Weissig et al. 1990). The use of a four-tailed hydrophobic cardiolipin derivative instead of a two-tailed PE derivative allows for a decrease in the number of amino groups involved in the conjugation reaction at the same degree of hydrophobicity. This results in better preservation of the activity of the hydrophobized and liposome-attached protein (Niedermann et al. 1991; Weissig et al. 1986). Some current methods for attaching various (mainly, targeting) ligands to nanocarriers are reviewed in Nobs et al. (2004).

Some special methods are designed to attach various sterically protective polymers to the surface of nanocarriers (see below). Thus, for example, to make PEG capable of incorporation into the liposomal membrane, the reactive derivative of hydrophilic PEG is single terminus-modified with a hydrophobic moiety (usually, the residue of PE or long-chain fatty acid is attached to PEG-hydroxysuccinimide ester) (Klibanov et al. 1991, 1990). In the majority of protocols, PEG–PE is used, which must be added to the lipid mixture prior to liposome formation. Alternatively, it was suggested to synthesize single-end-reactive derivatives of PEG able to be coupled with certain reactive groups (such as maleimide) on the surface of already prepared liposomes, referred to as the postcoating method (Maruyama et al. 1995). Currently, numerous studies on the preparation and properties of polymer-modified liposomes are well reviewed in several important books (Gregoriadis 1993; Lasic and Barenholz 1996; Lasic and Martin 1995). Spontaneous incorporation of PEG-lipid conjugates into the liposome membrane from PEG-lipid micelles was also shown to be very effective and did not disturb the vesicles (Sou et al. 2000).

5 Longevity of Nanocarriers in the Blood and its Importance for Drug Delivery

Longevity in the blood is one of the key properties of nanoparticulate drug delivery systems for both passive and active targeting, and long-circulating pharmaceuticals and pharmaceutical carriers currently represent an important and still growing area of biomedical research (see, for example, Cohen and Bernstein 1996; Lasic and Martin 1995; Moghimi and Szebeni 2003; Torchilin 1996b, 1998; Trubetskoy and Torchilin 1995). There are quite a few important reasons for making long-circulating drugs and drug carriers. One of them is to maintain a required level of a pharmaceutical agent (both therapeutic and diagnostic) in the blood for an extended

time interval. Long-circulating diagnostic agents are of primary importance for blood pool imaging, which helps in evaluating the current state of blood flow and discovering its irregularities caused by pathological lesions. Blood substitutes represent another important area for the use of long-circulating pharmaceuticals, when artificial oxygen carriers should be present in the circulation for long enough (Winslow et al. 1996). Then, as was discussed above, long-circulating drug-containing microparticulates or large macromolecular aggregates can slowly accumulate ("passive" targeting: Maeda 2001; Maeda et al. 2000) in pathological sites with affected and leaky vasculature (primarily tumors) and improve or enhance drug delivery in those areas (Gabizon 1995; Maeda 2001; Maeda et al. 2000). In addition, the prolonged circulation can help to achieve a better targeting effect for targeted (specific ligand-modified) drugs and drug carriers, allowing more time for their interaction with the target (Torchilin 1996b) due to larger number of passages of targeted pharmaceuticals through the target.

Chemical modification of drugs and drug carriers with certain synthetic polymers is the most frequent way to add in vivo longevity to other functions of drugs and drug carriers. Hydrophilic polymers have been shown to protect individual molecules and solid particulates from interaction with different solutes. This phenomenon relates to the stability of various aqueous dispersions (Molyneux 1984), and within the pharmaceutical field it helps to protect APIs and drug carriers from undesirable interactions with components of the biological milieu. The term "steric stabilization" has been introduced to describe the phenomenon of polymer-mediated protection (Naper 1983). The most popular and successful method to obtain long-circulating biologically stable nanoparticles is coating with certain hydrophilic and flexible polymers, primarily with poly(ethylene glycol) (PEG), as was first suggested for liposomes (Allen et al. 1991; Klibanov et al. 1990; Maruyama et al. 1991; Senior et al. 1991). On the biological level, coating nanoparticles with PEG sterically hinders interactions of blood components with their surface and reduces the binding of plasma proteins with PEG particles, as was demonstrated for liposomes (Allen 1994; Chonn et al. 1991, 1992; Lasic et al. 1991a; Senior et al. 1991; Woodle 1993). This prevents drug carrier interaction with opsonins and slows down their fast capture by RES (Senior 1987; Fig. 5). The mechanisms of preventing opsonization by PEG include shielding of the surface

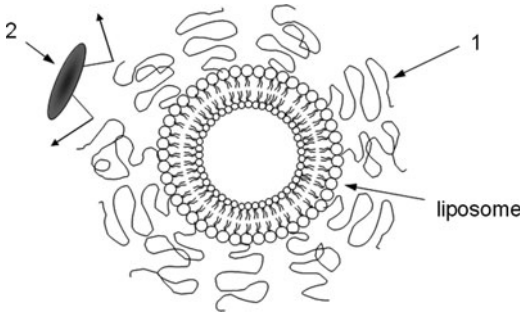

Fig. 5 The mechanism of steric protection of pharmaceutical nanocarriers by surface-grafted polymers. As an example, PEG chains (1) on the liposome surface prevent opsonin (2) from being adsorbed on the liposome and allow for its prolonged circulation

charges, increased surface hydrophilicity (Gabizon and Papahadjopoulos 1992), enhanced repulsive interaction between polymer-coated nanocarriers and blood component (Needham et al. 1992), and formation of a polymeric layer over the particle surface which is impermeable for other solutes even at relatively low polymer concentrations (Gabizon and Papahadjopoulos 1992; Torchilin et al. 1994).

Although quite a few polymers have been tried as steric protectors for nanoparticular drug carriers (Torchilin and Trubetskoy 1995), which will be discussed further, the majority of research on long-circulating drugs and drug carriers was performed with the use of PEG as a sterically protecting polymer because of the very attractive combination of properties of PEG: its excellent solubility in aqueous solutions and its ability to bind many water molecules, high flexibility of its polymer chain, very low toxicity, immunogenicity, and antigenicity, lack of accumulation in the RES cells, and minimum influence on specific biological properties of modified pharmaceuticals (Pang 1993; Powell 1980; Yamaoka et al. 1994; Zalipsky 1995). It is also important that PEG is not biodegradable and subsequently does not form any toxic metabolites. On the other hand, PEG molecules with molecular weight below 40 kDa are readily excreted from the body via the kidneys. From the practical point of view, PEG is easily commercially available in a variety of molecular weights. PEGs which are normally used for the modification of drug and drug carriers have a molecular weight from 1,000 to 20,000 Da. Single-terminus reactive (semitelehelic) PEG derivatives are often used for modification of pharmacologically important substances without the formation of cross-linked aggregates and heterogenic products. Currently, there exist many chemical approaches to synthesize activated derivatives of PEG and to couple these derivatives with a variety of drugs and drug carriers. Extensive reviews of these methods and their applicability for solving various problems in the drug delivery area were done by several authors (Torchilin 2002; Veronese 2001; Zalipsky 1995). Despite well-developed chemistry of PEG coupling, the search for alternative sterically protecting polymers is quite active. These polymers should be biocompatible, soluble, hydrophilic, and have a highly flexible main chain (see some data in Chonn et al. 1992; Lasic et al. 1991a; Maruyama et al. 1994; Takeuchi et al. 1999; Torchilin 1996b; Torchilin et al. 2001b, 1995; Torchilin and Trubetskoy 1995; Trubetskoy and Torchilin 1995; Woodle et al. 1994).

The most important biological consequence of nanocarrier modification with protecting polymers is a sharp increase in their circulation time and decrease in their RES (liver) accumulation (Klibanov et al. 1990; Lasic and Martin 1995; Torchilin et al. 1994). From the clinical point of view, it is extremely important that various long-circulating liposomes of a relatively small size (100–200 nm) were shown to effectively accumulate in many tumors via the "impaired filtration" mechanism (Gabizon and Papahadjopoulos 1988; Gabizon 1995; Maeda 2001; Maeda et al. 2000). As a result, PEG-coated and other long-circulating liposomes were prepared containing a variety of anticancer agents, such as doxorubicin, arabinofuranosylcytosine, adriamycin, and vincristin (Allen et al. 1992; Boman et al. 1994; Gabizon et al. 1994; Huang et al. 1994). The biggest success was achieved with PEG-liposome-incorporated doxorubicin, which has already demonstrated very good

clinical results (Ewer et al. 2004; Gabizon 1995; Rose 2005). An analysis of the pharmacokinetics of long-circulating nanocarriers (using PEG-liposomes) was performed by Allen (Allen et al. 1995b). In general, the association of drugs with nanocarriers has pronounced effects on pharmacokinetics: delayed drug absorption, restricted drug biodistribution, decreased volume of drug biodistribution, delayed drug clearance, and retarded drug metabolism (Hwang 1987). All these effects are determined by hindered interstitial penetration of a drug and lesser drug accessibility for the biological milieu because of entrapment into the drug carrier. The presence of protective polymer on the carrier surface changes all these parameters still further (Klibanov et al. 1990; Senior et al. 1991). Thus, while "plain" liposomes have nonlinear, saturable kinetics, long-circulating liposomes demonstrate dose-independent, nonsaturable, and log-linear kinetics (Allen and Hansen 1991; Huang et al. 1992; Mayhew et al. 1992). All pharmacokinetic effects depend on the route of liposome administration and their size and composition, and are always less expressed for sterically protected PEG-carriers (Allen et al. 1989; Liu et al. 1991, 1992; Maruyama et al. 1992).

An additional function can be added to long-circulating PEGylated pharmaceutical carriers, which allows for the detachment of PEG chains under the action of certain local stimuli characteristic of pathological areas, such as the decreased pH value or increased temperature usually noted in inflamed and neoplastic areas. The problem is that the stability of PEGylated nanocarriers may not always be favorable for drug delivery. In particular, if drug-containing nanocarriers accumulate inside the tumor, they may be unable to easily release the API to kill the tumor cells. Likewise, if the carrier has to be taken up by a cell via an endocytic pathway, the presence of the PEG coat on its surface may preclude the contents from escaping the endosome and being delivered in the cytoplasm. In order to solve these problems, for example, in the case of long-circulating liposomes, the chemistry was developed to detach PEG from the lipid anchor in the desired conditions. Labile linkage that would degrade only in the acidic conditions characteristic of the endocytic vacuole or the acidotic tumor mass are well-known from the area of controlled drug release. Such linkages can be based, e.g., on diortho ester acid-labile chemistry (Guo and Szoka 2001), or vinyl ester chemistry (Boomer and Thompson 1999). The latter reference describes the preparation of an acidic medium-cleavable PEG-lipid. Cysteine-cleavable lipopolymers were also described (Zalipsky et al. 1999). When the PEG brush is cleaved (e.g., from the liposome surface), membrane destabilization should occur, and the liposome contents would be delivered to its target (e.g., by escaping from the primary endosome into the cell cytoplasm). Polymeric components with pH-sensitive (pH-cleavable) bonds are widely used to produce stimuli-responsive drug delivery systems that are stable in the circulation or in normal tissues. However, they acquire the ability to degrade and release the entrapped agents in body areas or cell compartments with lowered pH, such as tumors, infarcts, inflammation zones or cell cytoplasm or endosomes (Roux et al. 2002a, 2004; Simoes et al. 2004). Since in "acidic" sites the pH drops from the normal physiological value of 7.4 to pH 6 and below, chemical bonds used so far to prepare the acidic pH-sensitive carriers have included vinyl esters, double esters,

and hydrazones that are quite stable at pH around 7.5 but hydrolyze relatively fast at pH values of 6 and below (Guo and Szoka 2001; Kratz et al. 1999; Zhang et al. 2004). To date, a variety of liposomes (Leroux et al. 2001; Roux et al. 2002b) and micelles (Lee et al. 2003a, b; Sudimack et al. 2002) have been described that include components with the above-mentioned bonds, as well as a variety of drug conjugates capable of releasing such drugs as adriamycin (Jones et al. 2003), paclitaxel (Suzawa et al. 2002), doxorubicin (Potineni et al. 2003; Yoo et al. 2002), and DNA (Cheung et al. 2001; Venugopalan et al. 2002) in acidic cell compartments (endosomes) and pathological body areas under acidosis. New detachable PEG conjugates are also described in Zalipsky et al. (1999), where the detachment process is based on the mild thiolysis of the dithiobenzylurethane linkage between PEG and the amino-containing substrate (such as phosphatidyl ethanol amine). Serum stable, long-circulating PEGylated pH-sensitive liposomes were also prepared using, on the same liposome, the combination of PEG and pH-sensitive terminally alkylated copolymer of *N*-isopropylacrylamide and methacrylic acid (Roux et al. 2004). The attachment of the pH-sensitive polymer to the surface of liposomes might facilitate liposome destabilization and drug release in compartments with decreased pH values. Numerous in vitro and in vivo experiments have shown great potential for improved efficiency of drug delivery and targeting.

6 Passive Accumulation of Liposomes and Micelles in Tumors

Since it was repeatedly shown that, like macromolecules, long-circulating liposomes are capable of accumulating in various pathological areas with affected vasculature via the EPR effect (Maeda et al. 2001; Yuan et al. 1994), long-circulating polymer (PEG)-coated liposomes have been repeatedly used for drug delivery into tumors via passive accumulation. An important feature of protective polymers is their flexibility, which allows a relatively small number of surface-grafted polymer molecules to create an impermeable layer over the liposome surface (Torchilin et al. 1994; Torchilin and Trubetskoy 1995). Although PEG remains the gold standard in liposome steric protection for passively targeted preparations, attempts continue to identify other polymers that could be used to prepare long-circulating liposomes. Earlier studies with various water-soluble flexible polymers have been summarized in Torchilin and Trubetskoy (1995) and Woodle (1998). More recent papers describe long-circulating liposomes prepared using poly[*N*-(2-hydroxypropyl)methacrylamide)] (Whiteman et al. 2001), poly-*N*-vinylpyrrolidones (Torchilin et al. 2001b), L-amino acid-based biodegradable polymer-lipid conjugates (Metselaar et al. 2003), and polyvinyl alcohol (Takeuchi et al. 2001).

As was already mentioned, long-circulating liposomes demonstrate dose-independent, nonsaturable, log-linear kinetics, and increased bioavailability (Allen and Hansen 1991). The relative role of the liposome charge and protective polymer molecular size was investigated, showing that opsonins with different

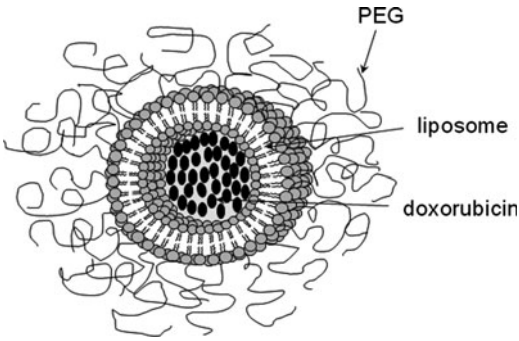

Fig. 6 Schematic structure of Doxil® – doxorubicin in PEG-coated liposomes

molecular sizes may be involved in the clearance of liposomes containing different charged lipids (Levchenko et al. 2002). PEG was also attached to the liposome surface in a removable fashion to facilitate liposome capture by the cell after PEG-liposomes accumulate in target site via the EPR effect (Maeda et al. 2001), and the PEG coating is detached under the action of local pathological conditions (decreased pH in tumors). New detachable PEG conjugates are described in Zalipsky et al. (1999), where the detachment process is based on the mild thiolysis of the dithiobenzylurethane linkage between PEG and an amino-containing substrate (such as PE). Low pH-degradable PEG-lipid conjugates based on the hydrazone linkage between PEG and lipid have also been described (Kale and Torchilin 2007; Sawant et al. 2006).

Doxorubicin in PEG-coated liposomes (Doxil® and Caelyx®; see the schematic structure in Fig. 6) is successfully used for the treatment of solid tumors in patients with breast carcinoma metastases, with subsequent survival improvement (O'Shaughnessy 2003; Perez et al. 2002; Symon et al. 1999). The same set of indications was targeted by the combination therapy involving liposomal doxorubicin and paclitaxel (Schwonzen et al. 2000) or Doxil/Caelyx and carboplatin (Goncalves et al. 2003). Caelyx is currently also in Phase II studies for patients with squamous cell cancer of the head and neck (Harrington et al. 2001) and ovarian cancer (Johnston and Gore 2001). Clinical data showed the impressive effect of doxorubicin in PEG-liposomes against unresectable hepatocellular carcinoma (Schmidinger et al. 2001), cutaneous T-cell lymphoma (Wollina et al. 2003), and sarcoma (Skubitz 2003). The recent review on the successful use of Caelyx in the treatment of ovarian cancer can be found in Perez-Lopez et al. (2007). It should, however, be noted here that recent evidence showed that PEG-liposomes, previously considered biologically inert, could still induce certain side-reactions via activation of the complement system (Moein Moghimi et al. 2006; Moghimi and Szebeni 2003).

Different methods of liposomal content delivery into the cytoplasm have been elaborated by adding the pH-sensitivity function to liposomal preparations (Torchilin 1991). When the liposome is made of pH-sensitive components then, after being endocytosed, it fuses with the endovacuolar membrane under the action of lowered pH inside the endosome and destabilizes it, releasing its content into the cytoplasm (Torchilin et al. 1993). Thus, endosomes become the gates from

the outside into the cell cytoplasm (Sheff 2004). This approach has been reviewed many times in various publications (in 2004, endosomal escape by pH-sensitive drug delivery systems was specifically discussed in a special issue of *Advanced Drug Delivery Reviews* #56, J.C. Leroux, ed.). It is usually assumed that inside the endosome, the low pH and some other factors destabilize the liposomal membrane, which, in turn, interacts with the endosomal membrane provoking its secondary destabilization and drug release into the cytoplasm. The presence of fusogenic lipids in the liposome composition, such as unsaturated DOPE (dioleoyl-*sn*-glycero-3-phosphatidylethanolamine), is usually required to render pH sensitivity to liposomes (Shalaev and Steponkus 1999). Multifunctional long-circulating PEGylated DOPE-containing pH-sensitive liposomes, although having a decreased pH sensitivity, still effectively deliver their contents into cytoplasm after being passively accumulated in the tumor (Varga et al. 2000). Antisense oligonucleotides (ODN) were delivered into cells by anionic pH-sensitive PE-containing liposomes, which are stable in the blood but undergo phase transition at acidic endosomal pH and facilitate oligonucleotide release into cell cytoplasm (Fattal et al. 2004). New pH-sensitive liposomal additives were recently described including oleyl alcohol (Sudimack et al. 2002) and pH-sensitive morpholine lipids (mono-stearoyl derivatives of morpholine) (Asokan and Cho 2003).

In the case of micellar preparations of anticancer drugs, passive micelle targeting to pathological organs or tissues can further increase the pharmaceutical efficiency of a micelle-encapsulated drug. Direct correlations between the longevity of a particulate drug carrier in the circulation and its ability to reach its target site have been observed on multiple occasions (Gabizon 1995; Maeda et al. 2001). The results of the blood clearance study of various PEG–PE micelles clearly demonstrated their longevity: the micelle formulations studied had circulation half-lives in mice, rats, and rabbits from 1.2 to 2.0 h depending on the molecular size of the PEG block (Lukyanov et al. 2002). The increase in the size of a PEG block increases the micelle circulation time in the blood probably by providing a better steric protection against opsonin penetration to the hydrophobic micelle core. However, circulation times for PEG–PE micelles are somewhat shorter than those for PEG-coated long-circulating liposomes (Klibanov et al. 1990), which could be explained in part by the more rapid extravasation of the micelles from the vasculature associated with their considerably smaller size compared to liposomes (Weissig et al. 1998). Slow dissociation of micelles under physiological conditions due to continuous clearance of unimers, with a micelle–unimer equilibrium being shifted towards the unimer formation (Trubetskoy et al. 1997), can also play its role.

As with long-circulating liposomes (Gabizon 1992, 2001; Papahadjopoulos et al. 1991), PEG–PE-based micelles formed by PEG_{750}–PE, PEG_{2000}–PE, and PEG_{5000}–PE accumulate efficiently in tumors via the EPR effect. It is worth mentioning that micelles prepared with several different PEG–PE conjugates demonstrated much higher accumulation in tumors compared to nontarget tissue (muscle), even in the case of an experimental Lewis lung carcinoma (LLC) in mice known to have a relatively small vasculature cut-off size (Hobbs et al. 1998; Weissig et al. 1998).

In other words, because of their smaller size, micelles may have additional advantages as a tumor drug-delivery system which utilizes the EPR effect compared to particulate carriers with larger sizes of individual particles. Thus, the micelle-incorporated model protein (soybean trypsin inhibitor or STI, MW 21.5 kDa) accumulates to a higher extent in subcutaneously established murine Lewis lung carcinoma than the same protein in larger liposomes (Weissig et al. 1998).

The accumulation pattern of PEG–PE micelles prepared from all versions of PEG–PE conjugates is characterized by peak tumor accumulation times of about 3–5 h. The largest total tumor uptake of the injected dose 5 h post-injection (as AUC) was found for micelles formed by the unimers with a relatively large PEG block (PEG_{5000}–PE). This may be explained by the fact that these micelles have the longest circulation time and a lesser extravasation into the normal tissue than micelles prepared from the smaller PEG–PE conjugates. Micelles prepared from PEG–PE conjugates with shorter versions of PEG, however, might be more efficient carriers of poorly soluble drugs because they have a greater hydrophobic-to-hydrophilic phase ratio and can be loaded with drug more efficiently on a weight-to-weight basis. Similar results have been obtained with another murine tumor model, EL4 T-cell lymphoma (Lukyanov et al. 2002). Some other recent data also clearly indicate spontaneous targeting of PEG–PE-based micelles into other experimental tumors (Torchilin et al. 2003b) in mice, as well as into the damaged heart areas in rabbits with experimental myocardial infarction (Lukyanov et al. 2004b).

Among drugs delivered by passively targeted micelles, one can name paclitaxel, which was shown to accumulate in tumors much better than its commercial formulation Taxol®, when loaded into micelles made of PEG-*b*-poly(4-phenyl-1-butanoate)-l-aspartamide conjugates (Hamaguchi et al. 2005). With this preparation, an almost 100-fold increase in the AUC, a 15-fold decrease in the volume of distribution and a significant decrease of drug clearance was achieved, which resulted in 25-fold improved drug accumulation in C-26 tumor in mice and corresponding increase in antitumor activity. Some other micellar preparations for passive targeting of paclitaxel have also been tested with variable degrees of success (Hamaguchi et al. 2005; Kim et al. 2004).

PEG-*b*-poly(amino acid)-based micelles loaded with cisplatin (CDDP) were designed for passive drug targeting into tumors and are undergoing clinical trials (Uchino et al. 2005). Among other micellar preparations for passive drug targeting in clinical trials, one can also mention doxorubicin in micelles made of PEG-*block*-poly(*l*-aspartate)–doxorubicin conjugate (these micelles contain both free and hydrophobic block-conjugated drug) (Matsumura et al. 2004) as well as doxorubicin in micelles made of Pluronic® (Danson et al. 2004).

Another targeting mechanism is based on the fact that many pathological processes in various tissues and organs are accompanied by local temperature increase and/or acidosis (Vutla et al. 1996; Yerushalmi et al. 1994). Hence, the efficiency of the micellar carriers can be further improved by making micelles capable of disintegration under the increased temperature or decreased pH values in pathological sites, i.e., by combining the EPR effect with stimuli responsiveness.

For this purpose, micelles are made of temperature- or pH-sensitive components, such as poly(*N*-isopropylacrylamide) and its copolymers with poly(D,L-lactide) and other blocks, and acquire the ability to disintegrate in target areas, releasing the micelle-incorporated drug (Cammas et al. 1997; Chung et al. 1998; Kohori et al. 1998; Kwon and Okano 1999; Meyer et al. 1998). pH-responsive polymeric micelles loaded with phthalocyanine seem to be promising carriers for photodynamic cancer therapy (Le Garrec et al. 2002), while doxorubicin-loaded polymeric micelles containing acid-cleavable linkages provided an enhanced intracellular drug delivery into tumor cells and thus higher efficiency (Yoo et al. 2002). Thermo-responsive polymeric micelles were shown to demonstrate an increased drug release upon temperature changes (Chung et al. 1999).

Passively targeted micelles (polymeric micelles) can also demonstrate pH sensitivity and ability to escape from endosomes. Thus, micelles prepared from PEG-poly(aspartate hydrazone adriamycin) easily release an active drug at lowered pH values typical for endosomes and facilitate its cytoplasmic delivery and toxicity against cancer cells (Bae et al. 2005). Alternatively, micelles for intracellular delivery of antisense ODN were prepared from ODN–PEG conjugates complexed with a cationic fusogenic peptide, KALA, and provided much higher intracellular delivery of the ODN than could be achieved with free ODN (Jeong et al. 2003). One could also enhance an intracellular delivery of drug-loaded micelles by adding to their composition the lipid components used in membrane-destabilizing Lipofectin®. Thus, PEG–lipid micelles, for example, carry a net negative charge (Lukyanov et al. 2004b), which might hinder their internalization by cells. On the other hand, it is known that the net positive charge usually enhances the uptake of various nanoparticles by cells, and after endocytosis the drug/DNA-loaded particles could escape from the endosomes and enter a cell's cytoplasm through disruptive interaction of the cationic lipid with endosomal membranes (Hafez et al. 2001). The compensation of this negative charge by the addition of positively charged lipids to PEG–PE micelles could improve the uptake by cancer cells of drug-loaded mixed PEG–PE/positively charged lipid micelles. It is also possible that after the enhanced endocytosis, such micelles could escape from the endosomes and enter the cytoplasm of cancer cells. With this in mind, an attempt was made to increase the intracellular delivery and, thus, the anticancer activity of micellar paclitaxel by preparing paclitaxel-containing micelles from a mixture of PEG–PE and Lipofectin® lipids (LL) (Wang et al. 2005). Multifunctional polymeric micelles capable of pH-dependent dissociation and drug release when loaded with doxorubicin and supplemented with biotin as cancer cell-interacting ligand were also described in Lee et al. (2005).

The problems with drug delivery using micelles for passive targeting are usually associated with too fast drug release from the micelles and with the difficulties of intracellular drug delivery (Aliabadi and Lavasanifar 2006). In order to minimize drug release from the micelles, the drug can be chemically conjugated with the hydrophobic blocks of micelle-forming components or drug-loaded micelles can be additionally chemically cross-linked (Kang et al. 2005; Lavasanifar et al. 2002; Shuai et al. 2004; Yuan et al. 2005).

7 Active Tumor Targeting with Drug-Loaded Liposomes

Current development of liposomal carriers often involves the attempt to combine the properties of long-circulating liposomes and targeted liposomes in one preparation (Abra et al. 2002; Blume et al. 1993; Torchilin et al. 1992). To achieve better selectivity of PEG-coated liposomes, it is advantageous to attach the targeting ligand via a PEG spacer arm, so that the ligand is extended outside of the dense PEG brush, excluding steric hindrances for the ligand binding to the target. Various advanced technologies are used for this purpose, and the targeting moiety is usually attached above the protecting polymer layer, by coupling it with the distal water-exposed terminus of an activated liposome-grafted polymer molecule (Blume et al. 1993; Torchilin et al. 2001a), see Fig. 7.

Since PEG–lipid conjugates used for the steric protection of liposomes and other pharmaceutical nanocarriers, and for the preparation of polymeric micelles, are derived from methoxy-PEG (mPEG) and carry nonreactive methoxy terminal groups, several attempts have been made to functionalize PEG tips in PEG–lipid conjugates. For this purpose several types of end-group functionalized lipopolymers were introduced of general formula X-PEG–PE (Zalipsky 1995; Zalipsky et al. 1998), where X represents a reactive functional group-containing moiety, and PEG–PE represents the conjugate of PE and PEG.

An interesting approach to couple various ligands, such as antibodies, to liposomes, including PEGylated liposomes, involves a so-called "postinsertion" technique (Ishida et al. 1999). This technique is based on the preliminary activation of ligands with any reactive PEG–PE derivative and subsequent co-incubation of unstable micelles formed by the modified ligand–PEG–PE conjugates with preformed drug-loaded plain or PEGylated liposomes. Eventually, modified ligands spontaneously incorporate from their micelles into the more thermodynamically favorable surroundings of the liposome membrane. This method was used, in particular, to prepare immuno-Doxil by modifying it with *p*-nitrophenylcarbonyl

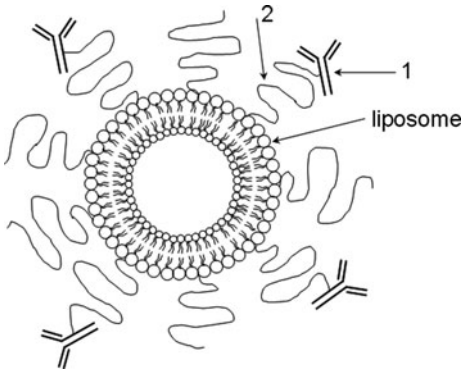

Fig. 7 The attachment of the targeting moiety (mostly monoclonal antibody) to PEGylated pharmaceutical nanocarrier (with liposome as an example). Although the targeting ligand could be co-immobilized on the surface together with PEG (1), the targeting moiety is usually attached above the protecting polymer layer, by coupling it with the distal water-exposed terminus of the activated liposome-grafted polymer molecule (2)

(pNP)–PEG–PE-modified anticancer 2C5 monoclonal antibody (Elbayoumi and Torchilin 2007; Lukyanov et al. 2004a).

Since antibodies are the most diverse and broadly used specific ligands for experimental targeted chemotherapy of various tumors with drug-loaded liposomes, there exist multiple original papers and reviews on antibody-targeted drug-loaded liposomes in cancer (see for reviews Kontermann 2006; Park et al. 2004; Sapra and Allen 2003; Sofou and Sgouros 2008; Torchilin 1996a, 2000; Vingerhoeds et al. 1994).

Antibody-modified liposomes of the "first generation" have been used to estimate certain parameters of their interaction with target cells in vitro (Klibanov et al. 1985) and also perform liposome targeting to certain model and real targets both in vitro and in vivo, such as extracellular matrix antigens or infarcted areas in the myocardium (Chazov et al. 1981; Torchilin et al. 1985). Importantly, it was noted that the modification of antibody-bearing liposomes with PEG (to make them long-circulating) usually results in decreased binding efficacy because of steric shielding of surface-attached antibodies by the liposome-grafted PEG (Klibanov et al. 1991; Torchilin et al. 1992). This eventually led to the development of multiple methods to attach antibodies onto the surface of the PEG layer in PEGylated liposomes.

In general, antibody attachment can decrease the circulating time of liposomes because of increased uptake of the modified liposomes via Fc receptors of circulating or liver macrophages, or opsonization of the liposome-tagged antibody molecules (Allen et al. 1995a; Kamps and Scherphof 1998). Whole antibodies can also trigger complement-mediated cytotoxicity and antibody-dependent cellular cytotoxicity (Sapra and Allen 2003). These effects could be minimized by using antibody Fab fragments instead of whole antibodies (Flavell et al. 1997). Although Fab fragments can also accelerate liposome clearance (Maruyama et al. 1997), in general Fab-liposomes circulate significantly longer than full antibody-modified liposomes (Maruyama et al. 1997). In the case of antibody-modified PEGylated liposomes, even a certain decrease in the circulation time still allows for their sufficiently long circulation, permitting good target accumulation. Clearly, attention should be paid not to over-modify PEG-liposomes with the antibody to the level at which their longevity is seriously compromised.

Interestingly, in some cases tumor accumulation of antibody-modified long-circulating liposomes is comparable with the accumulation of long-circulating liposomes without antibody attached (Moreira et al. 2001; Park et al. 2002, 1997, 2001). However, therapeutic activity is higher for antibody-targeted liposomes. As explained in Kirpotin et al. (2006) using PEGylated liposomes modified or nonmodified with anti-HER2 antibody, although intratumoral accumulation is similar for both preparations, antibody-modified preparations are much better internalized by tumor cells, which allows for higher drug doses delivered inside cancer cells, i.e., for more efficient cancer cell killing.

In some other cases, however, the liposome internalization seems not to be important. Thus, it was shown in Sapra and Allen (2004) that PEGylated liposomes loaded with vincristine or doxorubicin and modified (or nonmodified) with antibodies against internalizing CD19 antigen or noninternalizing CD20 antigen

demonstrate therapeutic effects which depended more on the type of drug used than on its ability to be internalized. As expected, the cytotoxicity of targeted liposomes depended also on the rate of drug release from the liposomes (Allen et al. 2005).

An interesting phenomenon was described in Hosokawa et al. (2003), the authors of which have demonstrated that, while nontargeted doxorubicin-containing liposomes were toxic to various cancer cells to the extent reflecting cell sensitivity to the drug, the cytotoxicity of antibody-targeted liposomes was proportional to the surface density of the surface antigen against which liposomes were targeted. The critical antigen surface concentration was about 4×10^4 sites per single cell, and after this value a further increase in antigen density was not important any more. Similar observations have also been made in Lopes de Menezes et al. (1998) and Park et al. (2002). Since cancer cells are often rather heterogenous in respect to antigens they express, it was suggested in Sapra and Allen (2003) to use a combination of antibodies against different antigens on a single liposome to provide better and more uniform targeting of all cells within the tumor. Alternatively, the "bystander" effect can also be relied upon (Sapra and Allen 2003), i.e., the action of the drug released from the liposomes attached to a certain cancer cell on the neighboring cancer cells devoid of a similar receptor.

An antibody which has gained popularity in cancer targeting is the monoclonal antibody against HER2, the antigen frequently over-expressed on various cancer cells. Monoclonal anti-HER2 antibodies including the humanized ones as well as currently clinically used Herceptin antibodies have been used to render drug-loaded liposomes (long-circulating liposomes) specific for HER2-positive cancer cells (Kirpotin et al. 1997, 2006; Park et al. 1995, 1997, 2001; Yang et al. 2007). This antibody was successfully used to deliver doxorubicin, both in plain and long-circulating liposomes, to breast tumors xenografts in mice, which resulted in significantly enhanced therapeutic activity of the drug. PEGylated liposomes decorated with anti-HER2 antibody were shown to undergo effective endocytosis by HER2-positive cancer cells, allowing for better drug (doxorubicin) accumulation inside tumor cells with better therapeutic outcome. Compared to doxorubicin in plain PEGylated liposomes (Doxil®), which normally accumulates in the tumor interstitial space, in the case of antibody-targeted Doxil, more drug molecules were discovered inside cancer cells, i.e., targeting with the antibody increases drug internalization by target cells.

Another promising antibody to target tumors with drug-loaded liposomes is the monoclonal antibody against CD19 antigen, which is also frequently over-expressed on various cancer cells. Anti-CD19 antibody-modified liposomes loaded with doxorubicin demonstrated clearly enhanced targeting and therapeutic efficacy both in vitro and in vivo in mice with human CD19+ B-lymphoma cells (Lopes de Menezes et al. 1998). Similar results have also been obtained with doxorubicin-loaded liposomes modified with antibodies against internalizable C19 antigen and against noninternalizable CD20 antigen (Sapra and Allen 2004). Anti-CD19 antibodies have also been used to target doxorubicin-loaded liposomes with variable drug release rates to experimental tumors (Allen et al. 2005). Recently, a successful attempt was made to target doxorubicin-loaded long-circulating liposomes to

CD19-expressing cancer cells with single chain Fv fragments of CD19 antibodies (Cheng and Allen 2008; Cheng et al. 2007).

Since neuroblastoma cells usually over-express disialoganglioside GD2, antibodies against GD2 and their Fab′ fragments have been suggested to target drug-loaded liposomes to corresponding tumors (Brignole et al. 2003; Pastorino et al. 2006, 2003). Fab′ fragments of anti-GD2 antibodies covalently coupled to long-circulating liposomes loaded with doxorubicin allowed for increased binding and higher cytotoxicity against target cells both in vitro and in vivo, including in models of human tumors in nude mice and in metastatic models. GD2-targeted immunoliposomes with the novel antitumoral drug, fenretinide, inducing apoptosis in neuroblastoma and melanoma cell lines, have also demonstrated strong anti-neuroblastoma activity both in vitro and in vivo in mice (Raffaghello et al. 2003). The combination of doxorubicin-loaded PEGylated liposomes targeted with anti-GD2 and with NGR-peptides specifically binding with the tumor vasculature, produced an improved therapeutic effect by acting on both tumor cells and tumor blood vessels (Pastorino et al. 2006).

An interesting novel target for anti-tumor drug delivery by means of targeted liposomes is the membrane type-1 matrix metalloproteinase (MT1-MMP), playing an important role in tumor neoangiogenesis and over-expressed both on tumor cells and on neoangiogenic endothelium. The modification of doxorubicin-loaded long-circulating liposomes with anti-MT1-MMP antibody resulted in an increased uptake of the targeted liposomes by MT1-MMP-over-expressing HT1080 fibrosarcoma cells in vitro and in more effective inhibition of tumor growth in vivo compared to antibody-free doxorubicin-loaded PEGylated liposomes (Hatakeyama et al. 2007). It was demonstrated that anti-MM1-MMP antibody enhances the endocytic internalization of drug-loaded liposomes, thus increasing their cytotoxicity (Atobe et al. 2007). Strong action of such preparation on tumor endothelial cells was noted.

Epidermal growth factor receptor (EGFR) and its variant EGFRvIII can serve as valuable targets for intracellular drug delivery into tumor cells over-expressing these receptors. Fab′ fragments of the monoclonal antibody C225, which binds both EGFR and EGFRvIII, and scFv fragment of the monoclonal antibody, which binds only to EGFR, were coupled to drug-loaded liposomes and allowed for substantially enhanced binding of such targeted liposomes with cancer cells over-expressing corresponding receptors, such as glioma cells U87 and carcinoma cells A0431 and MDA-MB-468. The better binding resulted in enhanced internalization and increased cytotoxicity (Mamot et al. 2003). In vivo therapy with such targeted drug-loaded liposomes (doxorubicin, epirubicin and vinorelbine were used as drugs) always resulted in better tumor growth inhibition than therapy with nontargeted liposomal drugs (Mamot et al. 2005). Fab′ fragment derived from the humanized anti-EGFR monoclonal antibody EMD72000 was shown to provide efficient intracellular delivery of the liposomal drugs into colorectal tumor cells (Mamot et al. 2006). The authors of this study have also shown that the attachment of the targeting moiety to PEGylated liposomes requires the length of the spacer arm sufficient to overcome possible steric shielding of antibody fragments by sterically-protecting PEG chains. An interesting method to construct anti-EGFR-targeted

liposomes was suggested in Pan and Lee (2007), where the anti-EFGR antibody (cetuximab or C225) was covalently linked to the folate-binding protein via a thioester bond and then coupled to the preformed folate-containing liposomes. Cetuximab-liposomes loaded with boron derivatives for boron neutron capture therapy were also prepared using the cholesterol-based anchor and micelle transfer technology (Pan et al. 2007).

Various proteins of the extracellular matrix expressed on the surface of cancer cells have also been used as targets for the antibody-mediated delivery of liposomal drugs. Thus, β_1-integrins expressed on the surface of human nonsmall cell lung carcinomas were targeted by doxorubicin-loaded liposomes modified with Fab′ fragments of anti-β_1-integrin monoclonal antibodies (Sugano et al. 2000). Treatment of SCID mice with lung tumor xenografts with such liposomes resulted in significant suppression of tumor growth compared to all controls and also inhibited metastases. The idea of targeting various antigens (preferably, the internalizable ones) on the endothelial cells by antibody–liposome conjugates was tested long ago (Trubetskaya et al. 1988). However, the approach attracted real attention only in the last few years. Thus, liposomes modified with anti-E-selectin antibodies were successfully internalized by activated endothelial cells in vitro through E-selectin-mediated endocytosis (Asgeirsdottir et al. 2008). Another possible target for antibody-mediated cancer therapy with drug-loaded liposomes is the epithelial cell adhesion molecule (EpCAM), which is expressed in many tumors but not in normal cells (Hussain et al. 2007). EpCAM-targeted immunoliposomes were generated by covalent attachment of the humanized scFv fragment of the 4D5MOCB monoclonal antibody to the surface of PEGylated doxorubicin-loaded liposomes and demonstrated significantly improved binding, internalization and cytotoxicity with EpCAM-positive cancer cells. Similarly, liposomes coupled with antibodies against vascular cell adhesion molecule-1 (VCAM-1) can be effectively targeted to activated endothelial cells over-expressing VCAM-1 (Voinea et al. 2005). Liposomes loaded with cytotoxic drugs were also targeted to ED-B fibronectin using scFv fragments of the corresponding antibody (Marty and Schwendener 2005). Proliferating endothelial cells have been targeted with doxorubicin-loaded liposomes modified with scFv fragments of the antibody against endoglin over-expressed on such cells (Volkel et al. 2004).

Lipid-based drug carriers have also been conjugated with antibodies (or their fragments) against transferrin receptor (TfR), frequently over-expressed on the surface of various cancer cells. For example, such carriers were modified with the OX26 monoclonal antibody against TfR via liposome-incorporated maleimide-modified PEG_{2000}–PE molecules and demonstrated strong binding with cells over-expressing TfR (Beduneau et al. 2007). The same antibody was attached to daunomycin-loaded liposomes noncovalently via the avidin–biotin couple, and the modified liposomes demonstrated good accumulation in multidrug-resistant RBE4 brain capillary endothelial cells both in vitro and in vivo (Schnyder et al. 2005).

Liposomes loaded with a lipophilic prodrug 5-fluorodeoxyuridine and modified with the monoclonal antibody CC531 against rat colon carcinoma demonstrated good binding with target cells (Koning et al. 1999) and effective intracellular drug

delivery compared to all controls (Koning et al. 2002). Antibody CC52 against rat colon adenocarcinoma CC531 attached to PEGylated liposomes provided specific accumulation of liposomes in a rat model of metastatic CC531 tumors (Kamps et al. 2000).

Nonpathogenic antinuclear autoantibodies (ANAs), frequently detected in cancer patients and in healthy elderly individuals, represent a subclass of natural anticancer antibodies. Earlier, we have shown that certain monoclonal ANAs (such as mAbs 2C5 and 1G3) recognize the surface of numerous tumor, but not normal, cells (Iakoubov et al. 1995a, b; Iakoubov and Torchilin 1997). Nucleosome-restricted specificity was shown for some of these monoclonal ANAs, and tumor cell surface-bound nucleosomes (NSs) have been shown to be their universal molecular target on the surface of a variety of tumor cells (Iakoubov and Torchilin 1997, 1998). Because these antibodies can effectively recognize a broad variety of tumors, they may serve as specific ligands to deliver other drugs and drug carriers into tumors. These antibodies were used to prepare drug-loaded tumor-targeted long-circulating immunoliposomes (with doxorubicin), which demonstrated highly specific binding with various cancer cells (murine Lewis lung carcinoma, 4T1, C26, and human BT-20, MCF-7, PC3 cells) in vitro (Elbayoumi and Torchilin 2007; Lukyanov et al. 2004a), significantly increased tumor accumulation in model tumors in mice including intracranial human brain U-87 MG tumor xenografts in nude mice, decreased side-effects, and superior antitumor activity in vivo (Elbayoumi and Torchilin 2006, 2008; Gupta and Torchilin 2007).

Doxorubicin-loaded PEGylated liposomes were also modified with Fab′ fragments of an anti-CD74 antibody via a PEG-based heterobifunctional coupling reagent and demonstrated significantly accelerated and enhanced accumulation in Raji human B-lymphoma cells in vitro (Lundberg et al. 2007). Anti-CD166 scFv attached to drug-loaded liposomes facilitated doxorubicin internalization by several prostate cancer cell lines (Du-145, PC3, LNCaP) (Roth et al. 2007). scFv fragments of antibodies against leukemia stem cells and oncogenic molecules participating in acute myeloid leukemia pathogenesis were used to target acute leukemia stem cells (Wang et al. 2008). Doxorubicin-loaded liposomes were successfully targeted to the kidney by using Fab′ fragments of the monoclonal OX7 antibody directed against Thy1.1 antigen in rats (Tuffin et al. 2005). Since fibroblast activation protein (FAP) represents a cell surface antigen expressed by the tumor stromal fibroblasts in different cancers, scFv from the antibody cross-reacting with human and mouse FAP was used to target PEGylated liposomes to tumor stromal cells (Baum et al. 2007). Tumor necrotic zones were effectively targeted by doxorubicin-loaded liposomes modified with chimeric TNT-3 monoclonal antibody specific towards degenerating cells located in necrotic regions of tumors and demonstrated enhanced therapeutic efficacy in nude mice bearing H460 tumors (Pan et al. 2008). The combination of immunoliposome and endosome-disruptive peptide improves cytosolic delivery of liposomal drugs, increases cytotoxicity, and opens new approaches to constructing targeted liposomal systems, as shown with diphtheria toxin A chain incorporated together with pH-dependent fusogenic peptide diINF-7 into liposomes specific towards ovarian carcinoma (Mastrobattista et al. 2002).

Early clinical trials of antibody-targeted drug-loaded liposomes have already demonstrated some promising results. Thus, doxorubicin-loaded PEGylated liposomes (with a size of approx. 140 nm) modified with $F(ab')_2$ fragments of the GAH monoclonal antibody specific for stomach cancer were tested in a Phase I clinical study and demonstrated pharmacokinetics similar to that of Doxil® (Matsumura et al. 2004).

Since transferrin (Tf) receptors (TfR) are over-expressed on the surface of certain tumor cells, antibodies against TfR as well as Tf itself are among popular ligands for liposome targeting to tumors and inside tumor cells (Hatakeyama et al. 2004) (although TfR expression in normal cells, particularly in the liver, can compete with tumor targeting of Tf-liposomes). Recent studies involve the coupling of Tf to PEG on PEGylated liposomes in order to combine longevity and targetability for drug delivery into solid tumors (Ishida et al. 2001). A similar approach was applied to deliver into tumors agents for photodynamic therapy, including hypericin (Derycke and De Witte 2002; Gijsens et al. 2002), and for intracellular delivery of cisplatin into gastric tumors (Iinuma et al. 2002). Tf-coupled doxorubicin-loaded liposomes demonstrate increased binding and toxicity against C6 glioma (Eavarone et al. 2000). Interestingly, the increase in the expression of the TfR was also discovered in post-ischemic cerebral endothelium, and was used to deliver Tf-modified PEG-liposomes to post-ischemic brain in rats (Omori et al. 2003). Tf (Joshee et al. 2002) as well as anti-TfR antibodies (Tan et al. 2003; Xu et al. 2002) were also used to facilitate gene delivery into cells by cationic liposomes. Tf-mediated liposome delivery was also successfully used for brain targeting. Immunoliposomes with OX26 monoclonal antibody to rat TfR were found to concentrate on brain microvascular endothelium (Huwyler et al. 1996).

Targeting tumors with folate-modified liposomes represents a very popular approach, since folate receptor (FR) is frequently over-expressed in many tumor cells. After early studies demonstrated the possibility of delivery of macromolecules (Leamon and Low 1991) and then liposomes (Lee and Low 1994) into living cells utilizing FR endocytosis, which could bypass multidrug resistance, the interest in folate-targeted drug delivery by liposomes grew quickly (see important reviews in Gabizon et al. 2004; Lu and Low 2002a). Liposomal daunorubicin (Ni et al. 2002) as well as doxorubicin (Pan et al. 2003) and 5-fluorouracil (Gupta et al. 2007) were delivered into various tumor cells both in vitro and in vivo via FR and demonstrated increased cytotoxicity. Recently, the application of folate-modified doxorubicin-loaded liposomes for the treatment of acute myelogenous leukemia was combined with the induction of FR using all-*trans*-retinoic acid (Pan et al. 2002). Folate-targeted liposomes have been suggested as delivery vehicles for boron neutron capture therapy (Stephenson et al. 2003) and used also for targeting tumors with haptens for tumor immunotherapy (Lu and Low 2002b). Within the frame of gene therapy, folate-targeted liposomes were utilized for both gene targeting to tumor cells (Reddy et al. 2002) as well as for targeting tumors with antisense ODN (Leamon et al. 2003).

The search for new ligands for liposome targeting concentrates on specific receptors over-expressed on target cells (particularly cancer cells) and certain

specific components of pathological cells. Thus, liposome targeting to tumors has been achieved by using vitamin and growth factor receptors (Drummond et al. 2000). Vasoactive intestinal peptide (VIP) was used to target PEG-liposomes with radionuclides to VIP-receptors of the tumor, which resulted in an enhanced breast cancer inhibition in rats (Dagar et al. 2003). PEG-liposomes were also targeted by RGD-peptides to integrins of the tumor vasculature and, being loaded with doxorubicin, demonstrated increased efficiency against C26 colon carcinoma in a murine model (Schiffelers et al. 2003). RGD-peptide was also used for targeting liposomes to integrins on activated platelets and, thus, could be used for specific cardiovascular targeting (Lestini et al. 2002) as well as for selective drug delivery to monocytes/neutrophils in the brain (Qin et al. 2007). A similar angiogenic homing peptide was used for targeted delivery to vascular endothelium of drug-loaded liposomes in experimental treatment of tumors in mice (Asai et al. 2002). Epidermal growth factor receptor (EGFR)-targeted immunoliposomes were specifically delivered to a variety of tumor cells over-expressing EGFR (Mamot et al. 2003). Mitomycin C in long-circulating hyaluronan-targeted liposomes increases its activity against tumors over-expressing hyaluronan receptors (Peer and Margalit 2004). The ability of galactosylated liposomes to concentrate in parenchymal cells was applied for gene delivery to these cells; see Hashida et al. (2001) for review. Cisplatin-loaded liposomes specifically binding chondroitin sulfate, over-expressed in many tumor cells, were used for successful suppression of tumor growth and metastases in vivo (Lee et al. 2002). Tumor-selective targeting of PEGylated liposomes was also achieved by grafting these liposomes with basic fibroblast growth factor-binding peptide (Terada et al. 2007).

8 Active Tumor Targeting with Drug-Loaded Micelles

As with other delivery systems, the drug delivery potential of polymeric micelles may also be still further enhanced by attaching targeting ligands to the micelle surface (Fig. 8). Among those ligands one can name various sugar moieties

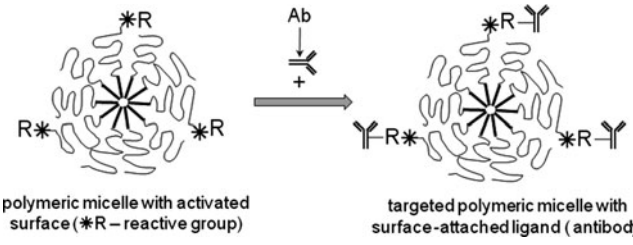

Fig. 8 As with other delivery systems, the drug delivery potential of polymeric micelles may be still further enhanced by attaching targeting ligands to the micelle surface

(Nagasaki et al. 2001), transferrin (Vinogradov et al. 1999), and folate residues (Ota et al. 2002), since many target cells, especially cancer cells, over-express appropriate receptors (such as transferrin and folate receptors) on their surface. Thus, it was shown that galactose- and lactose-modified micelles made of PEG–polylactide copolymer specifically interact with lectins, thus modeling targeting delivery of the micelles to hepatic sites (Jule et al. 2003; Nagasaki et al. 2001). Transferrin-modified micelles based on PEG and polyethyleneimine with a size between 70 and 100 nm are expected to target tumors with over-expressed transferrin receptors (Vinogradov et al. 1999). Mixed micelle-like complexes of PEGylated DNA and polyethyleneimine modified with transferrin (Dash et al. 2000; Ogris et al. 1999) were designed for enhanced DNA delivery into cells over-expressing the same transferrin receptors. A similar targeting approach was successfully tested with folate-modified micelles (Leamon and Low 2001). Poly(L-histidine)/PEG and poly (L-lactic acid)/PEG block copolymer micelles carrying folate residue on their surface were shown to be efficient for the delivery of adriamycin to tumor cells in vitro demonstrating potential for solid tumor treatment and combined targetability and pH sensitivity (Lee et al. 2003a).

Among all specific ligands, antibodies provide the broadest opportunities in terms of diversity of targets and specificity of interaction. Several attempts to covalently attach an antibody to surfactant or polymeric micelles (i.e., to prepare immunomicelles) have been described (Kabanov et al. 1989; Torchilin 2001; Torchilin et al. 2003b; Vinogradov et al. 1999). Thus, micelles modified with fatty acid-conjugated Fab fragments of antibodies to antigens of brain glia cells (acid gliofibrillar antigen and α_2-glycoprotein) loaded with neuroleptic trifluoperazine increasingly accumulated in the rat brain upon intracarotide administration (Chekhonin et al. 1991; Kabanov et al. 1989).

By adapting the coupling technique developed for attaching specific ligands to liposomes (Leamon and Low 2001), PEG–PE-based immunomicelles modified with monoclonal antibodies have been prepared. The approach uses PEG–PE with the free PEG terminus activated with a *p*-nitrophenylcarbonyl (pNP) group. Diacyllipid fragments of such a bifunctional PEG derivative firmly incorporate into the micelle core, while the water-exposed pNP group, stable at pH values below 6, efficiently interacts with amino-groups of various ligands (such as antibodies and their fragments) at pH values above 7.5 yielding a stable urethane (carbamate) bond. All nonreacted pNP groups spontaneously hydrolyze at the same pH values. To prepare immunotargeted micelles, the antibody to be attached was simply incubated with drug-loaded micelles at pH around 8.0 (Lee et al. 2003a; Torchilin et al. 2003b). Both the original and antibody-modified micelles have a spherical shape, and a uniform size of about 20 nm. The micelle-attached protein was quantified using fluorescent labels or by SDS-PAGE (Gao et al. 2003; Torchilin et al. 2003b). It was calculated that 10 to 20 antibody molecules could be attached to a single micelle. Antibodies attached to the micelle corona preserve their specific binding ability. Blood clearance data in mice showed similar pharmacokinetic profiles for 2C5-modified and plain PEG–PE micelles, confirming the long circulation of prepared immunomicelles.

To specifically enhance the tumor accumulation of PEG–PE-based micelles, the latter have been modified with tumor-specific anti-nucleosome monoclonal antibodies, such as mAb 2C5 (Lee et al. 2003a; Torchilin et al. 2003b). Rhodamine-labeled 2C5-immunomicelles effectively bind to the surface of several unrelated tumor cell lines: human BT20 (breast adenocarcinoma) and murine LLC (Lewis lung carcinoma) and EL4 (T-lymphoma) cells. Paclitaxel-loaded 2C5-immunomicelles also demonstrated the same specific properties as "empty" immunomicelles and effectively bound various tumor cells. In studies in vivo, ^{111}In-labeled 2C5-immunomicelles demonstrated significantly higher accumulation in LLC-tumor-bearing female C57BL/6J mice than plain micelles, were able to bring more micelle-incorporated drug into the tumor, and demonstrated significantly higher ability to inhibit tumor growth (Lee et al. 2003a; Torchilin et al. 2003b).

A few other specific ligands (glycoproteins, lipoproteins, carbohydrates, peptides) have also been used to achieve active targeting by polymeric micelles (Jule et al. 2003; Vinogradov et al. 1999; Wakebayashi et al. 2004). Polymeric micelles modified with sugar moieties (glucose, galactose, mannose, lactose) have been particularly successful (Jule et al. 2003; Nagasaki et al. 2001). Folate-targeted mixed block-copolymer micelles have been prepared consisting of folate-PEG-poly (DL-lactic-glycolic acid) and folate-free copolymers bearing a single doxorubicin moiety per polymeric chain (Yoo and Park 2004). Such folate-targeted doxorubicin-loaded micelles demonstrated better uptake by folate receptor over-expressing human squamous carcinoma cells of oral cavity and higher cytotoxicity against these cells both in vitro and in vivo compared to folate-free micelles. There also exist quite a few other examples of drug-loaded targeted polymeric micelles for cancer therapy (see, for example, Park et al. 2005; Vinogradov et al. 1998, 1999; Xiong et al. 2007).

In case of targeted micelles, a local release of a free drug from micelles in the target organ should lead to the increased efficacy of the drug, while the stability of the micelles en route to the target organ or tissue should contribute drug solubility and toxicity reduction due to less interaction with nontarget organs.

9 Conclusion

Summing up this section, one must note that there are several clear aims when using antibody-mediated tumor targeting of drug-loaded nanocarriers compared to more traditional dosage forms: (1) such delivery systems should accumulate in target tumors fast and effectively; (2) the quantity of the drug delivered into the tumor by such systems should be higher than in the case of other delivery systems; (3) ideally, drugs in nanocarriers should not only accumulate in the interstitial space inside tumors but also be internalized by the target cells creating high intracellular drug concentration and allowing multidrug resistance to be bypassed.

To achieve these goals, certain considerations should be taken into account when developing targeted preparations for chemotherapy. First, a target should be

identified which is present (over-expressed) on the surface of tumor cells in sufficient quantity providing good opportunity for the targeted liposomes to firmly bind with cancer cells (Hosokawa et al. 2003). Second, the specific ligand (antibody or its fragment) should be attached to the surface of the drug-loaded nanocarrier in a way which does not affect its specific binding properties (optimal choice should be made from the variety of coupling methods available, keeping in mind that the method suitable for one antibody will not necessarily be suitable for another one), and in sufficient quantity to provide multipoint binding with the target; and in the case of PEGylated long-circulating carriers the quantity of the attached antibodies should not be excessive so as not to compromise the longevity too much (Lukyanov et al. 2004a; Moreira et al. 2002). Third, it is highly desirable that the targeting antibody is internalizable and facilitates the internalization of the carrier and carrier-incorporated anti-cancer drug (Kirpotin et al. 2006; Mamot et al. 2005). Fourth, drug release from the carrier inside the tumor or inside the tumor cell should deliver the therapeutic concentration of the drug in the target and maintain it for a reasonable period of time (a few hours) (Allen et al. 2005; Sapra and Allen 2004).

References

Abra RM, Bankert RB, Chen F, Egilmez NK, Huang K, Saville R, Slater JL, Sugano M, Yokota SJ (2002) The next generation of liposome delivery systems: recent experience with tumor-targeted, sterically-stabilized immunoliposomes and active-loading gradients. J Liposome Res 12:1–3

Adams ML, Lavasanifar A, Kwon GS (2003) Amphiphilic block copolymers for drug delivery. J Pharm Sci 92:1343–1355

Aliabadi HM, Lavasanifar A (2006) Polymeric micelles for drug delivery. Expert Opin Drug Deliv 3:139–162

Allen C, Yu Y, Maysinger D, Eisenberg A (1998) Polycaprolactone-*b*-poly(ethylene oxide) block copolymer micelles as a novel drug delivery vehicle for neurotrophic agents FK506 and L-685,818. Bioconjug Chem 9:564–572

Allen TM (1994) The use of glycolipids and hydrophilic polymers in avoiding rapid uptake of liposomes by the mononuclear phagocyte system. Adv Drug Deliv Rev 13:285–309

Allen TM, Brandeis E, Hansen CB, Kao GY, Zalipsky S (1995a) A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. Biochim Biophys Acta 1237:99–108

Allen TM, Hansen C (1991) Pharmacokinetics of stealth versus conventional liposomes: effect of dose. Biochim Biophys Acta 1068:133–141

Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A (1991) Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. Biochim Biophys Acta 1066:29–36

Allen TM, Hansen C, Rutledge J (1989) Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. Biochim Biophys Acta 981:27–35

Allen TM, Hansen CB, de Menezes DEL (1995b) Pharmacokinetics of long-circulating liposomes. Adv Drug Deliv Rev 16:267–284

Allen TM, Mehra T, Hansen C, Chin YC (1992) Stealth liposomes: an improved sustained release system for 1-beta-D-arabinofuranosylcytosine. Cancer Res 52:2431–2439