[Frontiers in Bioscience 16, 1388-1412, January 1, 2011]

Recent developments in lipid-based pharmaceutical nanocarriers

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1. ABSTRACT

Within the broad spectrum of nanoparticulate carriers, polymeric and lipid-core micelles, liposomes, solid nanoparticles and many others have demonstrated great biological properties which make them excellent pharmaceutical delivery systems. In particular, micelles and liposomes have been shown to have good longevity in the blood that allows their accumulation in pathological areas with a compromised vasculature; can possess specific targeting to disease sites when various targeting ligands are attached to the surface of the nanocarriers or to surface-attached cell-penetrating molecules (like TAT peptide) to enhance intracellular penetration; possess stimulus-sensitivity allowing for drug release from the carriers under certain pathological conditions; and show contrast properties with carrier loading of various contrast materials that allow for direct carrier visualization *in vivo*. The engineering of "multifunctional pharmaceutical nanocarriers" based on the combination of several useful properties in the same system can significantly enhance the efficacy of many therapeutic and diagnostic protocols. This review considers the current status and next future directions in the emerging area of nanomedicine with particular attention to two lipid-based nanoparticulate systems: liposomes and micelles.

2. INTRODUCTION

Pharmaceutical nanotechnologies represent a new horizon for medicine. They can be defined as the nanoscale technological innovations referred as "nanomedicine". This rapidily growing field provides the opportunity to design and develop several devices that can target, treat and diagnose several diseases including tumors. Among drug delivery nanosystems developed over the last four decades, are hydrogels, micelles, liposomes, solid lipid nanoparticles, dendrimers, nanotubes, and polymersomes (1-8). There are three main reasons for the development of various drug delivery systems (DDS): to protect a drug against the inactivating action of the biological microenvironment, to protect non-pathological tissues against the non-specific toxic action of a drug, and to favorably change and control drug pharmacokinetics. In this review, we will discuss the latest advances in nanomedicine by focusing on the recent scientific accomplishments reached through the application of two of the lipid-based carriers, micelles and liposomes. Both can significantly modify and improve pharmacological properties of the carrier-loaded drugs and enhance their therapeutic activity. In fact, nanosystems like micelles (mainly, for water-insoluble drugs) and liposomes (mainly, for water-soluble drugs) have particular advantages over other DDS, including ease of control of composition, size, and *in vivo* stability; relatively simple preparation and scale up; good drug loading efficiency; and the ability to be made specifically targeted with small quantities of a targeting component. Just a few targeting moieties attached to their surface can carry multiple kinds of drugs loaded into the particle reservoir. In addition, liposomes and micelles with prolonged circulation in the blood are capable of "passive" targeting to pathological site via the enhanced permeability and retention (EPR) effect, a penetration into the tissue through its compromised ("leaky") vasculature which is characteristic of several pathological states, such as a tumor, an infarcts, and an inflammation (9, 10). In this chapter, we will introduce the properties and discuss the application of the lipid-based nanocarriers, liposomes and micelles.

3. PROPERTIES OF LIPOSOMES

Liposomes are artificial phospholipid vesicles that can be designed for effective encapsulation of an active drug. Whether the drug is encapsulated in the core or in the bilayer of the liposome depends on the characteristics of the drug and the encapsulation process (11) (see Figure 1). Many different methods have been suggested to prepare liposomes of different sizes, structure and size distribution (12-16). The most frequently used methods are ultrasonication, reverse phase evaporation and detergent removal from mixed lipid-detergent micelles by dialysis or gel-filtration. To increase liposome stability in the presence of the physiological environment, cholesterol is incorporated into the liposomal membrane (up to 50% mol). The size of liposomes depends on their composition and preparation method and can vary from around 80 nm to greater than 1 μm in diameter in various lamellar configurations. 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 (17) permits the inclusion of more than 50 percent of the substance to be encapsulated from the water phase into the liposomes.

The *in vitro* release rate of different compounds from liposomes, including proteins of moderate molecular weight (such as a lysozyme or insulin) is usually under 1 % per hour under the condition that the incubation temperature differs sufficiently from the phase transition temperature of the given liposomal phospholipid(s). Maximal permeability of liposomes is usually observed at temperatures close to the phase transition temperature of the liposomal phospholipid. The *in vivo* release rate from liposomes varies within wide limits (minutes to hours) and depends on its membrane composition, cholesterol content and location in the body.

From the clinical point of view, biodistribution of liposomes is a very important parameter. Liposome characteristics can influence both the tissue distribution and the rate of clearance of the drug by making the drug acquire the pharmacokinetic properties of the carrier (18, 19). Pharmacokinetic variables of the liposomes depend on the physiochemical characteristics of the liposomes, such as size, surface charge, membrane lipid packing, steric stabilization, dose and route of administration. As with other microparticulate delivery systems, conventional liposomes suffer from rapid elimination from the systemic circulation by the cells of the reticulo-endothelial system (RES) (20). Many studies have shown that within the first 15-30 min after intravenous administration of liposomes, between 50 and 80% of the dose is adsorbed by the cells of the RES, primarily by the Kupffer cells of the liver (21-23).

In order to improve the biological properties of the loaded drugs and enhance their therapeutic activity, several liposome formulations have been suggested and studied.

In the next paragraphs we will illustrate the most significant examples of liposomal formulations primarly by focusing on those in which the use of polymers incorporated in their formulation improves their intrinsic properties to make them ideal candidates for clinical applications.

4. LONG CIRCULATING LIPOSOMES

One of the drawbacks of the use of the so-called "plain" liposomes is their rapid elimination from the blood through capture by the cells of the RES. Two approaches have been suggested to increase liposomal drug accumulation in the areas of interest: a) targeted liposomes with surface-attached ligands capable of recognizing and binding to cells of interest, to potentially induce the liposomal internalization and b) PEGylated liposomes, i.e. liposomes surface-grafted with chains of polyethylene glycol (PEG). This biologically inert, non-toxic, highly soluble polymer with a very flexible main chain forms a sterically protective layer over the liposome surface that

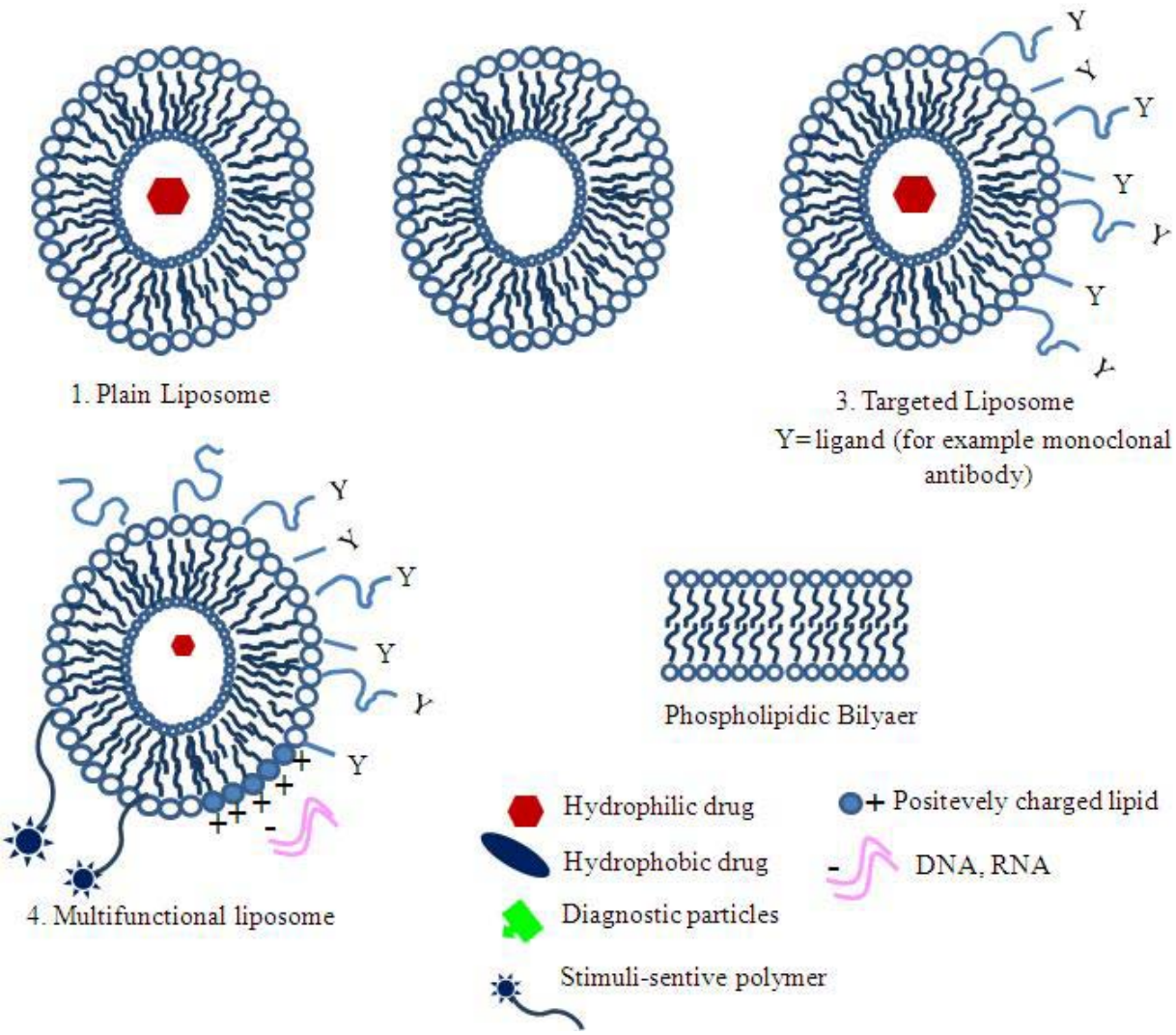

Figure 1. Liposome structures. 1. Plain liposome with a water-soluble drug in in the aqueous environment and lipophilic drug inthe phospholipidic bilayer. 2. Long circulating liposome sterically protected by a polymeric chain (usually PEG chain). 3. Targeted liposome modified by a ligand (such as antibodies, transferrin, folate, peptides, proteins, etc.) attached to the liposomal surface or to the protective polymer. 4. Multifunctional liposome modified simultaneously or separately by different active molecules, such as protective polymer, protective polymer and targeting ligand , diagnostic contrast moieties, incorporation of positevely charged lipid (allowing the DNA and RNA complexation), stimuli-sensitive polymers changing liposome permeability at certain conditions.

slows down liposome recognition by opsonins with subsequent clearance (24, 25). Furthermore, the PEG chains on the liposome surface prevent vesicle aggregation and improve stability of formulations (26). In general, PEGylated liposomes demonstrate dose-independent, non-saturable, log-linear kinetics, and increased bioavailability (21). The incorporation of PEG-lipids allows the liposomes to remain in the blood circulation for extended periods of time (i.e., $t_{1/2} > 20$ hours) and circulate through an organism relatively evenly with most of the dose remaining in the blood with only 10% to 15% of the dose captured by the liver (21, 27, 28). Recent papers describe other long-circulating liposomes prepared with different polymers such as *poly(N-(2-hydroxypropyl)methacrylamide))(29)*, *poly-N-vinylpyrrolidones* (30), *L-amino acid-based biodegradable polymer-lipid conjugates (31)*, and *polyvinyl alcohol* (32). On the other hand, some additional research have revived the early strategy of liposome surface modification with *gangliosides* (GM1 and GM3), analogous to erythrocyte membrane, which has demonstrated prolonged circulation in mice and rats (33, 34).

An alternative way to achieve higher drug accumulation in the desired areas of interest is to target the drug-loaded liposomes directly to an affected tissue. Ideally, the two strategies, prolonged circulation and ligand-mediated targeting, should be combined. In fact, the

further development of the concept of long-circulating liposomes involves the combination of the properties of long-circulating liposomes and targeted liposomes (in particular, immunoliposomes) in one preparation (9, 10, 26). To improve selective targeting of PEG-coated liposomes, the targeting ligand has been attached to the particles indirectly, via a PEG spacer arm, so that the ligand is extended outside of the dense PEG layer to exclude steric hindrances to its binding to the targeted sites and receptors. The PEG moiety must be modified with a phospholipid on one terminus with the targeting ligand on the other. Currently, with various advanced technologies, the targeting moiety is usually attached externally to the protecting polymer layer, by coupling it with the distal water-exposed terminus of an activated liposome-grafted polymer molecule (9, 35). In general, the attachment by the conjugation methodology is based on three main reactions, which are sufficiently efficient and selective. These reactions are between: a) activated carboxyl groups and amino groups to yield an amide bond; b) pyridyldithiols and thiols to yield disulfide bonds; c) maleimide derivatives and thiols to yield thioether bonds. Many of the lipid derivatives used in these techniques are commercially available (36). Another single-step technique for attachment of specific ligands, including monoclonal antibodies, to PEGylated liposomes involves the use of the p-nitrophenyloxycarbonyl-terminated PEG-phosphatidylethanolamine (pNP-PEG-PE) (see paragraph "Immunomicelles") to allow for the easy formation of the carbamate bond between the *p*NP group and ligand amino-group at neutral or slightly basic pH values (37-39).

5. TARGETED LIPOSOMES

5.1. Immunoliposomes

Various monoclonal antibodies and their fragments have been used to promote delivery of liposomes to many targets, including tumors. Antibody attachment can improve therapeutic efficacy of liposomal drugs, as shown with the internalizable epitope CD19 (40) on B-lymphoma cells, anti-HER2 (41) against HER2-overexpressing tumors and nucleosome-specific antibodies (such as 2C5) capable of recognition of various the tumor cells via tumor cell's surface-bound nucleosomes (42-44). Other examples include GD2-targeted immunoliposomes (with the novel antitumoral drug, fenretinide) which showed strong anti-neuroblastoma activity both *in vitro* and *in vivo* in mice (45) and liposomes targeted with CC52 antibody against rat colon adenocarcinoma CC531 which provided specific accumulation of liposomes in a rat model of metastatic CC531 (46).

Combining an immunoliposome with an endosome-disruptive peptide improves cytosolic delivery of the liposomal drug, increases cytotoxicity, and has opened a new approach to constructing targeted liposomal systems. This was demonstrated with a diphteria toxin A chain incorporated together with pH-dependent fusogenic peptide INF-7 into liposomes which was specific towards ovarian carcinoma (47). Optimization of properties of immunoliposomes still continues.

5.2. Transferrin- and Folate- mediated targeting

In addition to antibodies, a variety of other ligands, including low-molecular-weight ones can target liposomes to certain cells and tissues. Thus, since transferrin (Tf) receptors (TfR) are overexpressed on the surface of many tumor cells, antibodies against TfR as well as Tf itself are among popular ligands for liposome targeting to tumors and tumor cell interiors (48). Recent studies have involved the coupling of Tf to PEGylated liposomes to combine longevity and targetability for drug delivery into solid tumors (49). Tf-coupled doxorubicin-loaded liposomes have increased binding and toxicity against the C6 glioma cell line (50). 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 (51). Tf (52) as well as anti-TfR antibodies (53, 54) were also used to facilitate gene delivery into cells with cationic liposomes.

Another good example of receptor mediated targeting is the use of folate-bearing liposomes. Since folate receptor (FR) is overexpressed in many tumor cells the use of liposomes represents a very popular approach. After early studies, where folate demonstrated the possibility of delivery of macromolecules (55) and then liposomes (56) into living cells utilizing FR-mediated endocytosis to bypass the multidrug resistance mechanism of cancer cells, the interest in folate-targeted drug delivery by liposomes grew rapidly (57, 58) . Studies that utilized this approach involved liposomal daunorubicin (59), doxorubicin (60) and 5-fluorouracyl (61), all of which demonstrated an increased cytotoxicity (both *in vivo* and *in vitro*) when delivered into various tumor cells via folate receptor interaction with folate residues attached to drug-loaded liposomes. For gene therapy, folate-targeted liposomes were utilized for both gene targeting to tumor cells (62) and for targeting tumors with antisense oligonucleotides (63).

5.3. New ligands

The search for new ligands for liposome targeting has concentrated on the specific receptors overexpressed on target cells (particularly cancer cells) and certain specific components of pathologic cells. Thus, liposome targeting to tumors also has been achieved with vitamin and growth factor receptors (64). Vasoactive intestinal peptide (VIP) was used to target PEG-liposomes with radionuclides to VIP-receptors of tumor, which resulted in an enhanced breast cancer inhibition in rats (65). PEG-liposomes were targeted by RGD peptides to integrins of the tumor vasculature and, after being loaded with doxorubicin, demonstrated increased efficiency against C26 colon carcinoma in a murine model (66). RGD-peptide was also used for targeting liposomes to integrins on activated platelets and, thus, could be used for specific cardiovascular targeting (67) as well as for selective drug delivery to monocytes/neutrophils in the brain (68). Similarly, an angiogenic homing peptide was used for targeted delivery of drug-loaded liposomes to vascular endothelium in experimental treatment of tumors in mice (69). Epidermal growth factor receptor (EGFR)-targeted

immunoliposomes were specifically delivered to a variety of tumor cells overexpressing EGFR (70).

Other recent examples include mitomycin C liposomes (71), oligomannose-coated liposomes (72), cisplatin-loaded liposomes (against tumors) (73), and galactosylated liposomes (for gene delivery) (74). Tumor-selective targeting of PEGylated liposomes was also achieved by grafting these liposomes with basic fibroblast growth factor-binding peptide (75). Interestingly, liposomes modified by the ascorbate moiety (palmitoyl-modified ascorbate was incorporated into the liposomes) were shown to target cancer cells via glucose transporters (76).

5.4. pH-sensitive liposomes

To take advantage of the altered pH environment of the particular cell organelles, the intracellular drug delivery by liposomal DDS can be facilitated if the liposomes are made of pH-sensitive components. After being endocytosed in the intact form, pH-sensitive liposomes have been shown to fuse with the endovacuolar membrane at lowered endosomal pH and permeabilize it, releasing their drug content into the cytoplasm (77, 78). This optimized approach combines pH-sensitivity of liposomes with their increased longevity and ligand-mediated targeting. Long-circulating PEGylated pH-sensitive liposomes, although demonstrating a decreased pH-sensitivity, still effectively deliver their contents into cytoplasm (79). Antisense oligonucleotides are delivered into cells by anionic pH-sensitive PE-containing liposomes, which are stable in the blood. However, they still undergo a phase transition at the acidic endosomal pH and facilitate the release of the incorporated oligonucleotides into the cell cytoplasm (80). New pH-sensitive liposomal additives have recently been described including oleyl alcohol (81) and pH-sensitive morpholine lipids (mono-stearoyl derivatives of morpholine) (82). The combination of liposome pH-sensitivity and specific ligand targeting for cytosolic drug delivery that utilizes decreased endosomal pH values has been described for both folate and Tf-targeted liposomes (83-86).

5.5. Liposomes modified by cell-penetrating peptides

As will be further discussed for micelles, a new approach to targeted drug delivery is based on the use of viral proteins that demonstrate a unique ability to penetrate cells by a “protein transduction” phenomenon.

Complexes of TAT-peptide-liposomes with a plasmid (plasmid pEGFP-N1 encoding for the Green Fluorescence Protein, GFP) were used successfully for *in vitro* transfection of various tumor and normal cells as well as for *in vivo* transfection of tumor cells in mice bearing Lewis lung carcinoma (87). TAT-peptide liposomes have also been successfully used for transfection of intracranial tumor cells in mice after intracarotid injection (88). As of today, there are quite a few examples of successful intracellular delivery of liposomes by surface attachment of CPPs (88-90). An interesting example of intracellular targeting of liposomes was described recently, where liposomes containing mitochonriotropic amphiphilic cations with delocalized positive charge in their membrane were shown to specifically target mitochondria of intact cells (91, 92).

Many of the listed functions/properties of liposomes, such as circulatory longevity, targetability, stimuli-sensitivity, and the ability to deliver drugs intracellularly could reasonably be combined in a single preparation to yield a so-called multifunctional liposomal nanocarrier (93).

6. CLINICAL APPLICATIONS

Liposomes as pharmaceutical nanocarriers have found various clinical applications in addition to those already mentioned.

6.1. Delivery of therapeutics

Several liposomal formulations are now available in the market or are undergoing clinical trials. This includes AmBisome® (Gilead Sciences, Foster City, CA, USA) in which the encapsulated drug is the antifungal amphotericin B (94); Myocet® (Elan Pharmaceuticals Inc., Princeton, NJ, USA) encapsulating the anticancer agent doxorubicin (95); and Daunoxome® (Gilead Sciences), where the entrapped drug is daunorubicin (96). Concerning long-circulating liposomes, PEGylated liposomal doxorubicin DOXIL®/ Caelyx® was the first and it is still the only long-circulating liposome formulation approved in both the USA and Europe for the treatment of Kaposi’s sarcoma (97) and recurrent ovarian cancer (98, 99). Currently, (DOXIL®/ Caelyx®) is undergoing trials for treatment of other malignancies such as multiple myelomas(100), breast cancer (101, 102), and recurrent high-grade glioma (103). A very similar stealth liposome formulation, encapsulating cisplatin, SPI-077™ (Alza Corporation, Mountain View, CA, USA), has demonstrated the same evident stealth behavior with an apparent $t_{½}$ of approximately 60–100 hours. Phase I/II clinical trials of the drug to treat head and neck cancer and lung cancer (104), have shown a promising toxicity profile, yet therapeutic efficacy was low (105), due mainly to delayed drug release. Similarly, S-CKD602 (Alza Corp., Mountain View, CA, USA), a PEGylated liposomal formulation of CKD-602 - a semisynthetic analog of camptothecin – has been submitted for a Phase I trial (106, 107). Lipoplatin™ (Regulon Inc., Mountain View, CA, USA), another pegylated liposomal cisplatin formulation (108) showed no nephrotoxicity up to a dose of 125 mg/ml given every 14 days and without the serious side effects of the free cisplatin. Clinical evaluation of a PEGylated liposomal formulation of mitoxantrone (Novantrone®, Wyeth Lederle, Madison, NJ, USA), has shown promising therapeutic results in acute myeloid leukemia, and prostate cancer (109).

6.2. Photo-dynamic therapy

Photo-dynamic therapy (PDT) is rapidly developing as a modality for the treatment of superficial/skin tumors, where administered photosensitizing agents are used for photochemical eradication of malignant cells. In PDT, liposomes are used both as drug carriers and enhancers. Targeting, as well as

the controlled release of the photosensitizing agent in tumors, may still further increase the outcome of the liposome-mediated PDT (110).

6.3. Liposomes as diagnostic agents

The fundamentals concerning diagnostic imaging medicine will be more extensively described in the micelle section since same basic requirements apply to the case of liposomes.

There is a variety of different methods to label/load the liposomes with a contrast/reporter group to make liposomes useful as delivery vehicles for imaging agents (111-113). Thus, the label could be: a) added to liposomes in the process of liposome preparation (label is incorporated into the aqueous interior of the liposome or into the liposomal membrane); b) adsorbed onto the surface of preformed liposomes; c) incorporated into the lipid bilayer of preformed liposomes; d) loaded into preformed liposomes using membrane-incorporated transporters or ion channels. In any case, clinically acceptable diagnostic liposomes have to meet certain requirements: a) the labeling procedure should be simple and efficient; b) the reporter group should be affordable, stable and safe/easy to handle; c) liposomes should be stable *in vivo* without release of free label; d) liposomes should be stable on storage. The relative efficacy of entrapment of contrast materials into different liposomes as well as the advantages and disadvantages of various liposome types were analyzed by Tilcock (114). Liposomal contrast agents have been used for experimental diagnostic imaging of liver, spleen, brain, cardio-vascular system, tumors, inflammations and infections (115). Recently, tumor-targeted antibody-modified liposomes were described which could be heavily loaded with contrast agent (with metals, such as ^{III}In for gamma-imaging) via liposome-incorporated polychelating polymers and thus serve as effective agents for experimental tumor imaging (111, 116, 117).

7. PROPERTIES OF MICELLAR CARRIERS

Micelles represent colloidal dispersions with a particle size normally ranging from 5 to 100 nm. They belong to a group of association or amphiphilic colloids which form spontaneously under certain conditions of concentration and temperature from amphiphilic or surface-active agents (surfactants), molecules which consist of two distinct regions with opposite affinities towards a given solvent (118). At low concentrations in an aqueous medium, such amphiphilic molecules exist as unimers. However, as their concentration is increased, aggregation takes place within a rather narrow concentration interval. The concentration of a monomeric amphiphile at which micelles appear is called the "critical micelle concentration" (CMC), while the temperature, below which amphiphilic molecules exist as unimers, and above as aggregates, is called the "critical micellization temperature" (CMT). The formation of micelles is driven by the decrease of free energy in the system due to the removal of hydrophobic fragments from the aqueous environment and the replacement of hydrogen bonds network in water. Hydrophobic fragments of amphiphilic molecules form the core of a micelle, while hydrophilic fragments form the micelle's shell (119-123).

Micelles as drug carriers provide a set of clear advantages (124-126). Thanks to their small size, micelles demonstrate an effective "passive targeting" (127, 128) via the previously mentioned EPR effect. It has been repeatedly shown that micelle-incorporated anticancer drugs, such as adriamycin (129), accumulate better in tumors than in non-target tissues, thus minimizing the undesired drug toxicity towards normal tissue. In addition, micelles may be made targeted by chemical attachment of target-specific molecules to their surface. In this case, the local release of free drug from the micelles in the target organ can lead to the drug's increased efficacy. Furthermore, in a micellar form, the drug is better protected from possible inactivation by the effect of biological surroundings, and undesirable side-effects on non-target organs and tissues are reduced. At the usual size of a pharmaceutical micelle between 10 and 80 nm, its CMC value is expected to be in a low millimolar region or even lower, and the loading efficacy towards a hydrophobic drug is ideally between 5 and 25 % wt. The solubilization of drugs using micelle-forming surfactants results in an increased water solubility of sparingly soluble drug with its improved bioavailability, reduction of toxicity and other adverse effects, enhanced permeability across physiological barriers, and substantial and favorable changes in drug biodistribution. Micellar compositions of various drugs have been suggested for parenteral (130-132), oral (133, 134), nasal (135, 136), and ocular (136, 137) application.

8. POLYMERIC MICELLES

Micelles prepared from amphiphilic co-polymers for solubilization of poorly soluble drugs has attracted much attention recently (124, 126, 138, 139). Polymeric micelles are formed by block-copolymers represented by hydrophilic and hydrophobic monomer units with the length of a hydrophilic block exceeding to some extent that of the hydrophobic one (126). When the length of the hydrophilic segment is too high, copolymers exist in water as single polymer chains, while molecules with very long hydrophobic segment forms structure with non-micellar morphology, such as rods and lamellae (140). The major driving force leading to self-association of amphiphilic polymers is again the decrease of the free energy of the system, as in case of surfactants. Similar to micelles formed by conventional detergents, polymeric micelles comprise the core of the hydrophobic blocks stabilized by the corona of hydrophilic chains. Polymeric micelles are often more stable compared to micelles prepared from conventional detergents (have lower CMC value), with some amphiphilic co-polymers having CMC values as low as 10^{-6} M (141, 142), which is about two orders of magnitude lower than that for surfactants such as Tween 80. The core compartment of a pharmaceutical polymeric micelle should possess a high loading capacity, a controlled release profile for the incorporated drug and good compatibility between the core-forming chain and incorporated drug, while the micelle corona should provide an effective steric protection for the micelle and determine the micelle's hydrophilicity,

charge and the length and surface density of hydrophilic blocks. The corona also serves as the site of reactive groups suitable for further micelle derivatization, such as an attachment of targeting moieties (143, 144). These properties control important biological properties of a micellar carrier, including its pharmacokinetics, biodistribution, biocompatibility, longevity, surface adsorption of biomacromolecules, adhesion to biosurfaces and targetability (143, 145, 146). The use of polymeric micelles can allow for the achievement of an improved circulation time, a favorable biodistribution and lower toxicity of a drug (124, 126, 129).

Usually, amphiphilic micelle-forming unimers include poly(ethylene glycol) (PEG) blocks with a molecular weight from 1 to 15 kDa as hydrophilic corona-forming blocks (147). This polymer is inexpensive, has a low toxicity, offers efficient steric protection for various biologically active macromolecules (28, 148-151) and particulate delivery systems (13, 25, 152, 153), and has been approved for internal applications by regulatory agencies (151, 154). Still, some other hydrophilic polymers, *poly(N-vinyl-2-pyrrolidone)* (PVP), *poly(vinyl alcohol)* and *poly(vinylalcohol-co-vinyloleate)* co-polymer may be used as hydrophilic blocks (155) as alternatives to PEG. New materials for pharmaceutical micelles include new copolymers of PEG (156) and completely new macromolecules, such as scorpion-like polymers (157, 158) and some other star-like and core-shell constructs (159). Hydrophobic blocks of polymeric micelles are represented with propylene oxide, L-lysine, aspartic acid, β-benzoyl-L-aspartate, γ-benzyl-L-glutamate, caprolactone, D,L-lactic acid and spermine (see the review in references (160-169)).

9. LIPID-CORE MICELLES AS DRUG CARRIERS

In the polymeric micelle panorama, phospholipid residues used as hydrophobic core-forming groups (170) have had greatly increased importance in the last decade. This is due to the additional advantages for particle stability when compared with conventional amphiphilic polymer micelles provided by the existence of two fatty acid acyls, which can contribute considerably to an increase in the hydrophobic interactions between the polymeric chains in the micelle's core. Conjugates of lipids with water-soluble polymers are commercially available. The diacyllipid-PEG molecule represents a characteristic amphiphilic polymer with a bulky hydrophilic (PEG) portion and a very short but very hydrophobic diacyllipid part. Micelle preparation from the lipid-polymer conjugates is a simple process, since polymers form micelles spontaneously in an aqueous media (see Figure 2). All versions of polyethylenglycol-phospholipid (PEG-PE) conjugates form micelles with a size of 7 to 35 nm. Micelles formed from conjugates with polymer (PEG) blocks of higher molecular weight have a slightly larger size. This suggests that micelle size may be tailored for a particular application by varying the length of the PEG. Such micelles have a spherical shape and uniform size distribution (171). From a practical point of view, it is important that micelles prepared from these polymers remain intact at concentrations much lower than required for drug delivery purposes. Another important property is that PEG_{2000}-PE and PEG_{5000}-PE micelles retain the size characteristic for micelles even after 48 h incubation in the blood plasma (172), i.e. the integrity of PEG-PE micelles should not be immediately affected by components of biological fluids upon parenteral administration.

Amphiphilic PVP-lipid conjugates with various polymer lengths have also been prepared by the free-radical polymerization of vinylpyrrolidone and further modified by the attachment of long-chain fatty acid acyls, such as palmityl (P) or stearyl (S) residues, to one of the polymer termini (155, 173). Amphiphilic PVPs with a MW of the PVP block between 1,500 and 8,000 Da form micelles in an aqueous environment (173). CMC values and the size of micelles formed depend on the length of the PVP block and vary between 10^{-4} and 10^{-6} M at 5 and 20 nm, respectively. Similar to PEG-PE-based micelles, micelle made of amphiphilic PVP could also be used for the solubilization of poorly water soluble drugs to yield highly stable biocompatible formulations. The application of micelles prepared from a similar lipidated polymer, polyvinyl alcohol substituted with oleic acid, for transcutaneous delivery of retinyl palmitate has also been proposed (174).

The micelles made of such lipid-containing conjugates can be loaded with various poorly soluble drugs (tamoxifen, paclitaxel, camptothecin, porphyrins, etc.) demonstrate good stability, longevity and the ability to accumulate in a damaged vasculature via the EPR effect such as myocardial infarcts and tumors (170, 172, 175). Mixed micelles made of PEG-PE and other micelle-forming components are also very interesting. They provide even better solubilization of certain poorly soluble drugs due to the increase in the capacity of the hydrophobic core for the drug (171, 176, 177). A drug incorporated in lipid-core polymeric micelles is firmly associated with micelles: when PEG-PE micelles loaded with various drugs were dialyzed against aqueous buffer at sink conditions, all tested preparations retained more than 90% of an encapsulated drug for the first 7 h of incubation (178).

10. SOLUBILIZATION PROCESS AND DRUG LOADING

The process of solubilization of water-insoluble drugs by micelle-forming amphiphilic block-copolymers has been investigated in detail (179). The mathematical simulation of the solubilization process (180) indicated, that the solubilization proceeds via the initial displacement of solvent (water) molecules from the micelle core, and later a solubilized drug begins to accumulate in the very center of the micelle core "pushing" hydrophobic blocks away from this area. Extensive solubilization may result in some increase of the micelle size due to the expansion of its core with drug. Among other factors influencing the efficacy of drug loading into the micelle, the size of both core-forming and corona-forming blocks are important (181). In the former case, the larger the hydrophobic block the bigger the core size and its ability to entrap hydrophobic drugs. In the latter case, the increase in the length of the hydrophilic block results in the increase of the CMC value, i.e. at a given concentration of the amphiphilic

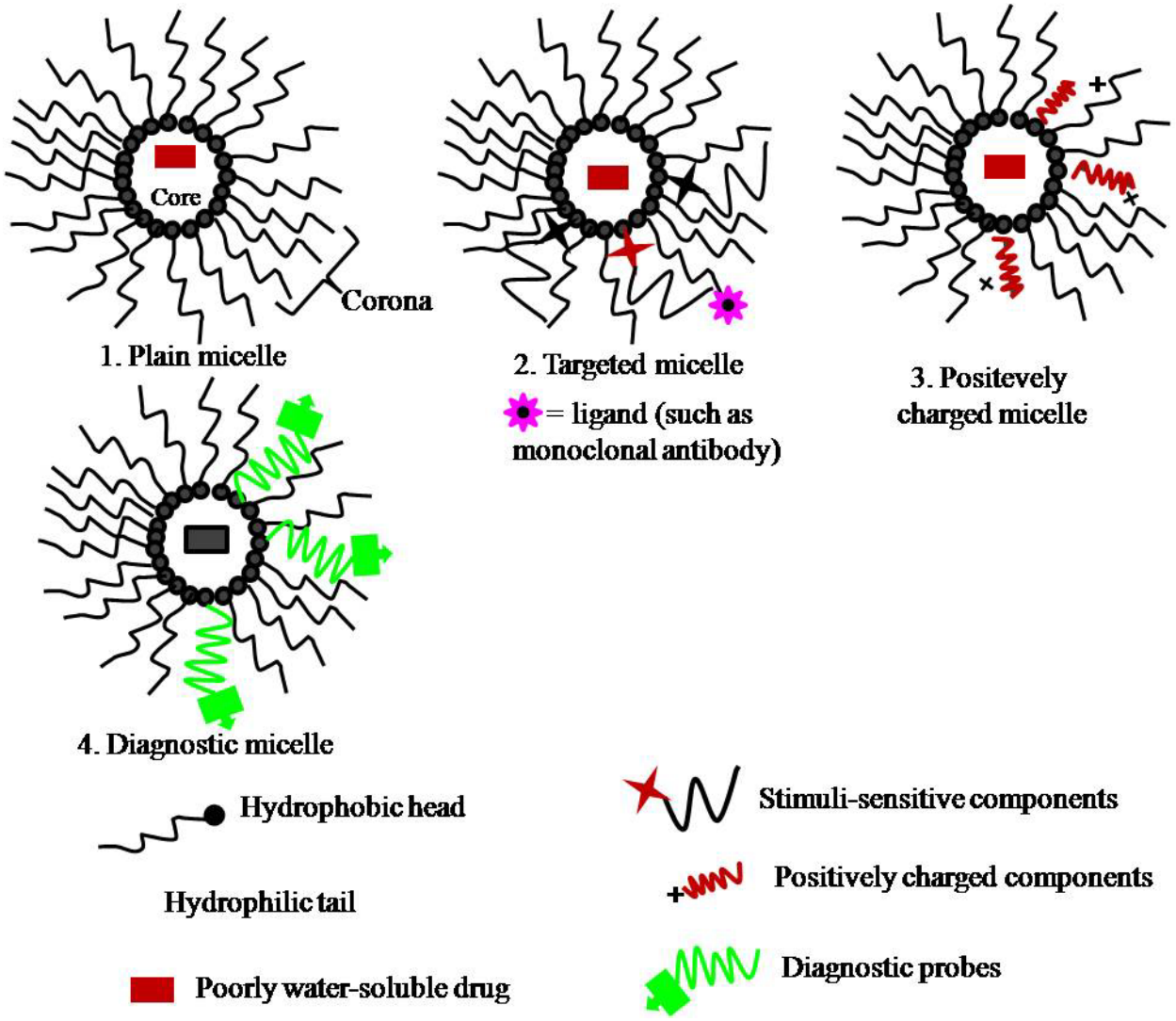

Figure 2. Lipid-core micelle structure. 1. Plain micelle with a poorly-soluble drug in the hydrophobic core. 2. Targeted micelle surface –modified with a ligand (monoclonal antibody, transferrin,etc.) and/or with a stimuli-sensitive component. 3. Positively charged micelle components to improve the intracellular drug delivery. 4. Diagnostic micelle loaded with amphiphilic modified chelating probes (such as gadolinium, manganese, etc.).

polymer in solution the smaller fraction of this polymer will be present in the micellar form and the quantity of the micelle-associated drug will drop. Drugs, such as diazepam and indomethacin (182, 183), adriamicin (163, 184, 185), anthracycline antibiotics (186), polynucleotides (187, 188), and doxorubicin (189) were effectively solubilized by various polymeric micelles, including micelles made of Pluronic® (block co-polymers of PEG and polypropelene glycol) (141). Doxorubicin incorporated into Pluronic® micelles demonstrated superior properties compared with free drug in the experimental treatment of murine tumors (leukemia P388, myeloma, Lewis lung carcinoma) and human tumors (breast carcinoma MCF-7) in mice (189). Micellar drugs also show a lower non-specific toxicity (190) than free drugs. To prepare drug-loaded micelles by direct entrapment of a drug into the micelle core a whole set of micelle-forming co-polymers of PEG with poly(L-amino acids) was used (142). PEG-b-poly(caprolactone) co-polymer micelles were successfully used as delivery vehicles for dihydrotestosterone (191). PEG-PE micelles can efficiently incorporate a variety of low soluble and amphiphilic substances including paclitaxel (192-194), tamoxifen, camptothecin (195-197) porphyrine, vitamin K3, and others (194, 198, 199).

Numerous studies have dealt with micellar forms of platinum-based anti-cancer drugs (200-202) and cyclosporin A (138, 203). Mixed polymeric micelles made of positively charged polyethyleneimine and Pluronic were used as carriers for antisense oligonucleotides (204). A typical protocol for the preparation of drug-loaded polymeric micelles from amphiphilic co-polymers involves the following steps. Solutions of an amphiphilic polymer and a drug of interest in a miscible volatile organic solvents

are mixed, and organic solvents are evaporated to form a polymer/drug film. The film obtained is then hydrated in an aqueous buffer, and the micelles are formed by intensive shaking. If the amount of a drug exceeds the solubilization capacity of micelles, the excess drug precipitates in a crystalline form and is removed by filtration. The loading efficiency for different compounds varies from 1.5 to 50% by weight. This value apparently correlates with the hydrophobicity of a drug. In some cases, to improve drug solubilization, additional mixed micelle-forming compounds may be added to polymeric micelles. Thus, to increase the encapsulation efficiency of paclitaxel, egg phosphatidylcholine (PC) was added to the PEG-PE-based micelle composition, which approximately doubled the paclitaxel encapsulation efficiency (from 15 to 33 mg of the drug per g of the micelle-forming material (176, 198, 205).

11. MICELLES AS THERAPEUTIC AGENTS

11.1. Targeted micelles

Micelles targeted to pathological organs or tissues can further increase pharmaceutical efficiency of a micelle-encapsulated drug. Several approaches have been used to enhance the accumulation of various drug-loaded pharmaceutical nanocarriers, including pharmaceutical micelles, in a pathological area.

11.2. Passive targeting

It proceeds via the previously mentioned enhanced permeability and retention (EPR) effect based on the spontaneous penetration of long-circulating macromolecules, particulate drug carriers, and molecular aggregates into the interstitium through the compromised leaky vasculature, which is characteristic for solid tumors, infarcts, infections and inflammation (127, 128). Clearly, the prolonged circulation of drug-loaded micelles facilitates the EPR-mediated target accumulation since it gives a better chance to reach and/or interact with its target. The results of blood clearance study of various micelles clearly demonstrated their longevity: micellar formulations, such as PEG-PE-based micelles, had circulation half-lives in mice and rats of around 2 hrs with certain variations depending on the molecular size of the PEG block (172). The increase in the size of a PEG block probably increases the micelle circulation time in the blood by providing better steric protection against opsonin penetration to the hydrophobic micelle core. Still, circulation times for long-circulating micelles are somewhat shorter compared to those for long-circulating PEG-coated liposomes (25). Diffusion and accumulation parameters were shown to be strongly dependent on the cutoff size of the tumor blood vessel wall, and the cutoff size varied for different tumors (206-208). An increased accumulation of PEG-PE based micelles in such areas with a leaky vasculature such as tumors and infarcts was clearly demonstrated (172).

Micelles formed by PEG_{750}–PE, PEG_{2000}–PE, and PEG_{5000}-PE (like long-circulating liposomes (19, 35, 209)) accumulate efficiently in tumors via the EPR effect (47). It is worth mentioning that micelles prepared from several different PEG-PE conjugates studied demonstrated much higher accumulation in tumors compared to non-target tissue (muscle) even in the case of an experimental Lewis lung carcinoma (LLC) in mice known to have a relatively small vasculature cutoff size (206, 210). 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 a larger size 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 (210).

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-to-5 hours. The largest total tumor uptake of the injected dose at 5 h post-injection (as AUC) was found for micelles formed by the unimers with 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 compared to 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 (172). Some other recent data also clearly indicate spontaneous targeting of PEG-PE-based micelles to other experimental tumors (171) in mice as well as into the damaged heart areas in rabbits with an experimental myocardial infarction(175) (299).

11.3. Stimuli-responsive micelles

A different delivery approach is based on the fact that many pathological processes in various tissues and organs are accompanied by a local temperature increase (by 2-to-5 °C) and/or a pH decrease of 1-to-2.5 units (acidosis) (211, 212). Thus, the efficiency of the micellar carriers in local drug delivery can be improved by making micelles capable of disintegration and local drug released 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 thermo- or pH-sensitive components (112, 213-215), such as *poly(N-isopropylacrylamide)* and its co-polymers with *poly(D,L-lactide)* and other blocks, and acquire the ability to disintegrate in target areas releasing the micelle-incorporated drug (149, 216).

pH-responsive polymeric micelles loaded with phtalocyanine seem to be promising systems for the photodynamic cancer therapy (217), while doxorubicin-loaded polymeric micelles containing acid-cleavable linkages have provided an enhanced intracellular drug delivery into tumor cells and thus higher efficiency (218). Similarly, pH-sensitive unimolecular polymeric micelles – star-shaped polymers – have been made of hydrophobic ethyl methacrylate and t-butyl methacrylate and hydrophilic poly(ethylene glycol)methacrylate (219). With micelle size

of 10 to 40 nm, their ionization and possibly drug release should depend on pH. Such micelles can also be considered for oral delivery. Micelles based on poly(2-ethyl-2-oxazoline)-b-poly(L-lactide) diblock copolymer have also been described that are loaded with doxorubicin and capable of releasing the drug at pH values typical for late endosomes (pH at 5.5) and secondary lysosomes (pH around or below 5.0) (220, 221). Phosphorylcholine-based diblock copolymer micelles also demonstrated distinct pH-sensitivity.

Thermo-responsive polymeric micelles showed an increased drug release upon temperature changes (222). Micelles combining thermosensitivity and biodegradability have also been suggested (132) . The penetration of drug-loaded polymeric micelles into cells (tumor cells) as well as drug release from the micelles can also be enhanced by an externally applied ultrasound (223, 224).

11.4. Ligand mediated targeting

The drug delivery potential of polymeric micelles may be still further enhanced by attaching targeting ligands to the micelle surface (225), i.e. to the water-exposed termini of hydrophilic blocks (226). Among those ligands we can mention antibodies (137, 171, 227, 228), sugar moieties (229, 230), transferrin (228, 231, 232) and folate residues (233, 234). The last two ligands are especially useful in targeting to cancer cells. It was shown that galactose- and lactose-modified micelles made of PEG-polylactide co-polymer specifically interact with lectins thus modeling targeting delivery of the micelles to hepatic sites (227, 230).

Transferrin-modified micelles based on PEG and polyethyleneimine with a size between 70 and 100 are expected to target tumors with over-expressed transferrin receptors(228). Mixed micelle-like complexes of PEGylated DNA and PEI modified with transferrin(232) have been designed for the enhanced DNA delivery into cells over-expressing transferrin receptors. A similar approach was successfully tested with increasingly more popular folate-modified micelles (134, 233, 235). Poly(L-histidine)/PEG and poly(L-lactic acid)/PEG block co-polymer 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 with combined targetability and pH-sensitivity (236, 237). Mixed micelles made of folate-PEO-*b*-poly(D,L-lactic-*co*-glycolic acid) and PEO-*b*-PLGA-DOX conjugates demonstrated superior cell uptake of folate-modified micelles compared to folate-free micelles by human squamous carcinoma cells expressing the folate receptor and better activity against these cells both *in vitro* and *in vivo* (238). Folate-targeted for PEO-*b*-poly(ε-caprolactone) micelles loaded with paclitaxel demonstrated significantly higher cytotoxicity against human breast adenocarcinoma MCF-7 and human uterine cervix adenocarcinoma HeLa 229 cells compared to unmodified micelles(134). Lactose-modified PEO-*b*-poly(2-(dimethylamino) ethyl methacrylate) that form an electrostatic micellar complex with plasmid DNA demonstrated a significantly higher transfection efficiency in $HepG_2$ cells (239). Tumor-specific peptide sequences, such as RGD (RGD peptides are small synthetic peptides containing an RGD-sequence, Arg-Gly-Asp) have also been used to target drug-loaded micelles to tumors. Thus, tumor endothelial cells have been successfully targeted with doxorubicin-loaded PEO-*b*-PCL micelles modified with the cyclic pentapeptide C (Arg-Gly-Asp-d-Phe-Lys) specifically recognizing $\alpha_v\beta_3$ integrins overexpressed in the tumor vasculature (240).

11.5. Immunomicelles

PEG-PE-based immunomicelles modified with monoclonal antibodies were prepared by using PEG-PE conjugates with the free PEG terminus activated with a *p*-nitrophenyloxycarbonyl (*p*NP) group (37). Diacyllipid fragments of such bifunctional PEG derivative firmly incorporate into the micelle core, while the water-exposed *p*NP group, stable at pH values below 6, interacts with amino groups of various ligands (antibodies and their fragments or peptides) at pH values above 7.5 to yield a stable urethan (carbamate) bond. To prepare immunotargeted micelles, the corresponding antibody can be simply incubated with drug-loaded *p*NP-PEG-PE-containing micelles at pH around 8.0. Using fluorescent labels or by SDS-PAGE (171, 205), it was calculated that several antibody molecules could be attached to a single 20 nm micelle.

Antibodies attached to the micelle corona (171) preserve their specific binding ability. These immunomicelles specifically recognize their target substrates as was confirmed by ELISA. For tumor targeting, PEG-PE-based micelles were modified with monoclonal 2C5 antibody possessing the nucleosome-restricted specificity (mAb 2C5) and capable of recognition of a broad variety of tumor cells via the tumor cell's surface-bound nucleosomes (241). Such specific targeting of cancer cells by drug-loaded mAb 2C5-immunomicelles resulted in dramatically improved *in vitro* cancer cell killing by such micelles: with human breast cancer MCF-7 cells, paclitaxel-loaded 2C5-immunomicelles clearly showed a superior killing efficiency compared to paclitaxel-loaded plain micelles or free drug (205). *In vivo* experiments with Lewis lung carcinoma-bearing mice have revealed an improved tumor uptake of paclitaxel-loaded radiolabeled 2C5-immunomicelles compared to non-targeted micelles (171). In addition, unlike plain micelles, 2C5-immunomicelles, promote delivery of their load not only to tumors with a mature vasculature, but also to tumors at earlier stages of their development and to metastases.

Additionally, 2C5-targeted PEG-PE micelles that release photosensitizing agents showed an enhanced anticancer activity *in vitro* and *in vivo* and are considered promising for the photochemical eradication of malignant cells (242, 243).

12. MICELLES AS DIAGNOSTIC AGENTS

Medical diagnostic imaging is an emerging area for the use of micelles as carriers for pharmaceuticals.

Medical imagining modalities consist of magnetic resonance (MR), computed tomography (CT), gamma-scintigraphy and ultra-sonography. Imaging is highly important for visualization, localization and detection in organs and tissues for several pathologies at their onset. Regardless of the specific modality used, medical diagnostic imaging requires a signal of sufficient intensity relative to the area of interest in order to differentiate the targeted area from the surroundings. To achieve this goal, appropriate contrast agents for specific imaging modalities are needed so that once accumulated in the targeted area of interest these sites will be clearly visualized and distinguished by applying suitable imagining modalities (244). Depending on the imaging modality and the signal intensity (intended as sensitivity and resolution) the contrast agents will have a different chemical nature and are required in different amounts. In fact, to be delivered into the area of interest and in the appropriate tissue concentration, this amount varies broadly in a range quite low in the case of gamma-imagining and quite high for MR and CT. Thus, to achieve the optimal local concentration of a diagnostic labeled agent, the development of particulate carriers with high efficient and selective delivery is a natural progression. However, micellar transport of contrast agents represents a relatively new field (245, 246). Approaches suggesting the use of micellar contrast agents for both pure diagnostic/imaging purposes and for the visual control over the drug delivery are underway.

Chelated paramagnetic metals, such as gadolinium (Gd), manganese (Mn) or dysprosium (Dy), are of major interest for the design of magnetic resonace (MR) positive (T1) contrast agents. Mixed micelles obtained from monoolein and taurocholate with Mn-mesoproporphyrin, were shown to be a potential oral hepatobiliary imaging agent for T1-weighted MR imaging (MRI) (247). Since chelated metal ions possess a hydrophilic character, to be incorporated into micelles, such structures must acquire an amphiphilic nature. Several amphiphilic chelating probes have been developed earlier for liposomes, where a hydrophilic chelating residue is covalently linked to a hydrophobic (lipid) chain, such as a diethylene triamine pentaacetic acid (DTPA) conjugate with phosphatidyl ethanolamine (DTPA-PE) (248), DTPA-stearylamine, DTPA-SA (249, 250), and amphiphilic acylated paramagnetic complexes of Mn and Gd (250). The lipid part of such an amphiphilic chelate molecule can be anchored in the micelle's hydrophobic core while a more hydrophilic chelating group is localized inside the hydrophilic shell of the micelle. The amphiphilic chelating probes (paramagnetic Gd-DTPA-PE and radioactive 111In-DTPA-SA) were incorporated into PEG(5 kDa)-PE micelles and used *in vivo* for MR and gamma-scintigraphy imaging (251). The main feature that makes PEG-lipid micelles attractive for diagnostic imaging applications is their small size which allows for better penetration into the target tissue to be visualized. In addition, in the case of MRI contrast agents, it is especially important that chelated metal atoms are directly exposed to the aqueous environment to enhance the relaxivity of the paramagnetic ions which lead to the enhancement of the micelle contrast properties.

Computed tomography (CT) represents an imaging modality with high spatial and temporal resolution. Diagnostic CT imaging requires an iodine concentration of millimoles per ml of tissue (252) , so that large doses of low-molecular-weight CT contrast agent (iodine-containing organic molecules) are normally administered to patients. The selective enhancement of blood with such levels of administration is brief due to rapid agent extravazation and clearance. Micelles to be used as a contrast agent for the blood pool CT imagining were prepared from copolymers of PEG with heavily iodinated, and thus insolubilized, polylysine (162, 253). The micellar iodine-containing CT contrast agent was injected intravenously into rats and rabbits, and a 3-to-4-fold enhancement of the X-ray signal in the blood pool was visually observed in both animal species for a period of at least 2 hours following the injection (162, 253).

By combining in a single micelle both, contrast moiety and therapeutic agent, one can directly connect an enhanced accumulation of drug-loaded micelles in the target (tumor) and increased therapeutic efficiency of such micelles, as was done with radiolabeled tumor-targeted PEG-PE micelles loaded with a porphine derivative and used for photo-dynamic tumor therapy (243).

13. OTHER APPLICATIONS OF POLYMERIC MICELLES

13.1. Lipid-core micelles for intracellular delivery

An additional strategy may further improve the efficiency of drug-loaded micelles by enhancing their intracellular delivery to compensate for drug degradation in lysosomes that results from the endocytosis-mediated capture of therapeutic micelles. An attempt to achieve this has been realized by controlling the micelle charge. As known, a net positive charge enhances the uptake of various nanoparticles by cells. Cationic lipid formulations such as Lipofectin® (an equimolar mixture of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride – DOTMA, and dioleoyl phosphatidylethanolamine – DOPE), significantly improved the endocytosis-mediated intracellular delivery of various drugs and DNA entrapped in liposomes and other lipid constructs made of these materials (254-257). Some PEG-based micelles, such as PEG-PE micelles, have been found to carry a net negative charge (175) which might hinder their internalization by cells. The compensation for this negative charge by the addition of positively charged lipids to PEG-PE-based micelles should improve their uptake by cancer cells. It is also possible that after the enhanced endocytosis, drug-loaded mixed micelles made of PEG-PE and positively charged lipids could escape from the endosomes and enter the cytoplasm of cancer cells. With this in mind, an attempt was made to increase an intracellular delivery and, thus, the anticancer activity of micellar paclitaxel by preparing paclitaxel-containing micelles from the mixture of PEG-PE and positively charged Lipofectin® lipids (LL) (194). The cell interaction with breast adenocarcinoma cells BT-20 and intracellular fate of paclitaxel-containing PEG-PE/LL micelles and similar micelles prepared without the addition of the LL were investigated by fluorescence microscopy. It

was clearly demonstrated that fluorescently-labeled PEG-PE and PEG-PE/LL micelles were both endocytosed by cancer cells. However, in the case of PEG-PE/LL micelles, endosomes degraded and released drug-loaded micelles into the cell cytoplasm as a result of the de-stabilizing effect of the LL component on the endosomal membranes (30). The *in vitro* anticancer effects of drug-loaded micelles were significantly improved for intracellularly delivered paclitaxel-containing PEG-PE/LL compared to that of free paclitaxel or paclitaxel delivered using LL-free PEG-PE micelles. In human ovarian carcinoma A2780, the IC_{50} values of free paclitaxel, paclitaxel in PEG-PE micelles, and paclitaxel in PEG-PE/LL micelles were 22.5, 5.8 and 1.2 μM, respectively.

Lately, attempts have been made to prepare pharmaceutical nanocarriers, including micelles, which can simultaneously perform targeting of and within tumor cells. To achieve this, micelles have been modified by both cell-penetrating peptides (CPP) and cancer-specific antibodies in such a way that the micelle-attached CPP was sterically shielded in the circulatory system by surrounding longer PEG and antibody-PEG moieties. However after accumulation in the tumor, longer PEG chains conjugated with the carrier via pH sensitive bonds detached under the action of the lowered intratumoral pH, CPP fragments became exposed and facilitated the carrier penetration into cells (258, 259).

It has been demonstrated that the trans-activating transcriptional activator (TAT) protein from HIV-1 enters various cells when added to the surrounding media (260). The recent data assume more than one mechanism for cell penetrating peptides and proteins (CPP) and CPP-mediated intracellular delivery of various molecules and particles. CPP-mediated intracellular delivery of large molecules and nanoparticles was proven to proceed via the energy-dependent macropinocytosis with subsequent enhanced escape from the endosome into the cell cytoplasm (261), while individual CPPs or CPP-conjugated small molecules penetrate cells via electrostatic interactions and hydrogen bonding and do not seem to be energy-dependent (262). Since traversal through cellular membranes represents a major barrier for efficient delivery of macromolecules into cells, CPPs, whatever their mechanism of action is, may serve to transport various drugs and even drug-loaded pharmaceutical carriers into mammalian cells both *in vitro* and *in vivo.*

Thus, an interesting approach to increase the intracellular micellar delivery has been developed based on the attachment of TAT peptide (TATp) moieties to the surface of the nanocarrier with PEG-PE derivatives (258, 263, 264). Recently, it has been demonstrated that TATp-targeted PEG-PE micelles loaded with paclitaxel enhance the interaction with cancer cells compared to non-modified micelles resulting in a significant increase the cytotoxicity to different cancer cells *in vitro* and *in vivo* (265) .

13.2. Micellar complexes as siRNA delivery systems

Lately, siRNA (small interfering RNA) technology has attracted much interest given its several advantages in the panorama of the gene therapy. A siRNA is a short double stranded RNA sequence from 21 to 23 nucleotides which in mammalian cells exhibit a great efficacy in gene silencing via an RNA intereference (RNAi) mechanism (266) . Since their discovery, siRNA molecules and their variously modified derivatives have been implemented as potential candidates for therapeutic applications for different genetic diseases (such as cancer). siRNAs have been chemically conjugated to a variety of bioactive molecules. Previous observations showed that the integrity of the 5′- terminus of the antisense strand, rather than that of the 3′-terminus, is important for the initiation of an RNAi mechanism. Therefore, the 3′- and 5′-terminus of the sense strand and the 3′-terminus of the antisense strand are considered as primary sites for conjugation with minimal influence on RNAi activity (267). Lipids, polymers, peptides, and inorganic nanostructured materials have been conjugated to the siRNA to enhance its pharmacokinetic behavior, cellular uptake, target specificity, and safety. Particularly important are siRNA DDS based on the electrostatic interaction between negative charges of siRNA and positive charges of polymers forming the nanosystems. This interaction results in the formation of electrostatic complexes in which the oligonucleotides are protected. The most popular polymer used to make polyplexes with siRNAs is polyethylenimine (PEI) (268, 269). For example, a polymeric gene carrier was developed to deliver vascular endothelial growth factor (VEGF) siRNA for prostate cancer cells in a target-specific manner. Prostate cancer-binding peptide (PCP) was conjugated with polyethylenimine (PEI) via a poly(ethylene glycol) (PEG) linker (PEI-PEG-PCP). The PEI-PEG-PCP conjugate could effectively condense siRNA to form stable polyelectrolyte complexes. VEGF siRNA/PEI-PEG-PCP polyplexes exhibited significantly higher VEGF inhibition efficiency than PCP-unmodified polycationic carriers (PEI-PEG or PEI) in human prostate carcinoma cells (PC-3 cells) even under serum conditions (270).

13.3. Micelles in immunology

A very interesting and promising area for the use of polymeric micelles concerns immunology. Nonionic block copolymers, first of all, Pluronics® or copolymers of PEG (PEO) and PPG (PPO), are finding application as immunological adjuvants for the modulation of the immune response in the preparation of new and effective vaccines (271). Usually, linear tri-block co-polymers with the linear structure PEG-PPG-PEG are used for this purpose. The adjuvant activity of these polymers is strongly influenced by the length of the PPG block, its increase resulting in the increase of the adjuvant activity. It is important to mention that Pluronics® themselves can provoke macrophage activation. Though the exact mechanism of this activation is still under investigation, there are data suggesting that Pluronics®actually activate the alternative complement pathway (272), and that certain proteins belonging to the complement system, in turn, cause macrophage activation.

Pluronics® have demonstrated their adjuvant properties both in emulsion and micellar forms. In an emulsion form, they not only activate the alternative complement pathway, but also enhance the binding of

protein antigens at the water/oil interfaces increasing antibody responses (273, 274). Pluronics® with higher molecular weights (PPG blocks with MW of about 10 kDa with attached from both sides with shorter PEG blocks) form micelles able to incorporate various antigens. High adjuvant activity of such micelles was demonstrated with an influenza virus vaccine (271). It was also shown that the optimization of vaccine properties can be achieved by controlling the size of PPG and PEG blocks. Thus, with ovalbumin as a model antigen, it was shown that the most potent vaccine was obtained with a 11 kDa core PPG block co-polymer containing between 5 and 10% attached PEG blocks. Naturally, the size of antigen-bearing polymeric micelles depends also on the size of micelle-incorporated protein antigen (275, 276). The mechanism of protein antigen interaction with polymeric micelles is seen as hydrogen bonding between protein antigen molecule and terminal hydroxyl groups of PEG blocks or with multiple hydrogen bond acceptor sites along the hydrophobic PPG block (271).

As noted in (271), studies on cellular immune response provoked by ovalbumin in Pluronic® micelles demonstrated that a more hydrophilic carrier augments mainly Th2 types of responses, while more hydrophobic copolymers augment both Th1 and Th2 responses. These data together with available information on the low toxicity of Pluronic-based compositions for vaccination (277) permit one to speculate that polymeric micelles may have a real clinical future as adjuvants and vaccine components.

14. CONCLUSIONS

The development of 'pharmaceutical' liposomes is a growing research area, with an increasing variety of potential applications, and encouraging results from early clinical applications and clinical trials of different liposomal drugs. The new generation liposomes have frequently demonstrated a combination of different attractive properties such as simultaneous circulatory, longevity and targetability, longevity and stimuli-sensitivity, targetability and contrast properties etc. Thus, liposomes can be successfully utilized in many drug delivery approaches and their use to solve various biomedical problems is likely to become more common in the future.

Furthermore, polymeric micelles possess an excellent ability to solubilize poorly water-soluble drugs and increase their bioavailability. This has been repeatedly demonstrated for a broad variety of drugs, many of them poorly soluble anti-cancer drugs, with micelles of a variety of different compositions. In addition, due to their small size, micelles demonstrate a very efficient spontaneous accumulation in pathological tissues with increased vascular permeability (tumors, infarcts) *in vivo* via the enhanced permeability and retention effect and can bring increased quantities of drugs to a tissue, thereby reducing the whole body toxicity associated with a high drug dose. Micelle-specific targeting to areas of interest can also be achieved by attaching specific targeting ligands (such as target-specific antibodies, transferrin or folate) to the micelle surface. By varying micelle composition and the size of the hydrophilic or hydrophobic blocks of the micelle-forming material, one can readily control the properties of micelles, including size, loading capacity and longevity in the blood. Another interesting option is provided by stimulus-responsive micelles, where increased degradation and subsequent drug release proceeds at the abnormal pH values or temperatures characteristic of many pathological zones.

Combinations of such micellar properties should lead to increased practical applications of micellar drugs in the near future.

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Key Words: Micelles, Liposomes, Phospholipids, Polymers, Nanomedicine, Anticancer drugs, siRNA, DNA, Targeted delivery of therapeutics, Review

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