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Review

Structure and design of polymeric surfactant-based drug delivery systems

Vladimir P. Torchilin*

Department of Pharmaceutical Sciences, Bouve College of Health Sciences, Northeastern University, 360 Huntington Avenue, Boston, MA 02129, USA

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Abstract

The review concentrates on the use of polymeric micelles as pharmaceutical carriers. Micellization of biologically active substances is a general phenomenon that increases the bioavailability of lipophilic drugs and nutrients. Currently used low-molecular-weight pharmaceutical surfactants have low toxicity and high solubilization power towards poorly soluble pharmaceuticals. However, micelles made of such surfactants usually have relatively high critical micelle concentration (CMC) and are unstable upon strong dilution (for example, with the blood volume upon intravenous administration). On the other hand, amphiphilic block co-polymers are also known to form spherical micelles in solution. These micelles have very high solubilization capacity and rather low CMC value that makes them very stable in vivo. Amphiphilic block co-polymers suitable for micelle preparation are described and various types of polymeric micelles are considered as well as mechanisms of their formation, factors influencing their stability and disintegration, their loading capacity towards various poorly soluble pharmaceuticals, and their therapeutic potential. The basic mechanisms underlying micelle longevity and steric protection in vivo are considered with a special emphasis on long circulating drug delivery systems. Advantages and disadvantages of micelles when compared with other drug delivery systems are considered. New polymer–lipid amphiphilic compounds such as diacyillipid–polyethylene glycol, are described and discussed. These compounds are very attractive from a practical point of view, since they easily micellize yielding extremely stable micelles with very high loading capacity. Micelle passive accumulation in the areas with leaky vasculature (tumors, infarct zones) is discussed as an important physiology-based mechanism of drug delivery into certain target zones. Targeted polymeric micelles prepared by using thermo- or pH-sensitive components or by attaching specific targeted moieties (such as antibodies) to their outer surface are described as well as their preparation and some in vivo properties. The fast growing field of diagnostic micelles is analyzed. Polymeric micelles are considered loaded with various agents for gamma, magnetic resonance, and computed tomography imaging. Their in vitro and in vivo properties are discussed and the results of the initial animal experiments are presented.

Keywords: Drug carriers; Polymeric micelles; Amphiphilic co-polymers; PEG conjugates

1. Introduction

To minimize drug degradation and loss, to prevent harmful side-effects and to increase drug bioavail-

*Tel.: +1-617-373-3206; fax: +1-617-373-8886.
E-mail address: vtorchil@lynx.neu.edu (V.P. Torchilin).

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ability and the fraction of the drug accumulated in the required zone, various drug delivery and drug targeting systems are currently developed or under development. Among drug carriers one can name soluble polymers, microparticles made of insoluble or biodegradable natural and synthetic polymers, microcapsules, cells, cell ghosts, lipoproteins, liposomes, and micelles. Each of those carrier types offers its own advantages and has its own shortcomings, so the choice of a certain carrier for each given case can be made only taking into account the whole bunch of relevant considerations. Those carriers can be made slowly degradable, stimuli-reactive (for example, pH- or temperature-sensitive), and even targeted (for example, by conjugating them with specific antibodies against certain characteristic components of the area of interest).

Micelles as drug carriers are able to provide a set of unbeatable advantages — they can solubilize poorly soluble drugs and thus increase their bioavailability, they can stay in the body (in the blood) long enough providing gradual accumulation in the required area, their size permits them to accumulate in body regions with leaky vasculature, they can be targeted by attachment of a specific ligand to the outer surface, and they can be prepared in large quantities easily and reproducibly. Being in a micellar form, the drug (poorly soluble drug, first of all) is well protected from possible inactivation under the effect of biological surroundings, it does not provoke undesirable side effects, and its bioavailability is usually increased.

The micelle is structured in such a way that the outer surface of the micelle exposed into the aqueous surrounding consists of components that are hardly reactive towards blood or tissue components. This structural peculiarity allows micelles to stay in the blood (tissues) rather long without being recognized by certain proteins and/or phagocytic cells. This longevity is an extremely important feature of micelles as drug carriers.

Long-circulating pharmaceuticals and pharmaceutical carriers represent currently a fast growing area of biomedical research, see for example Refs. [1–5]. There are several reasons for the search for long-circulating drugs and drug carriers. At least three of them seem to be the most important. First, one often needs to keep certain pharmaceuticals in the blood long enough. Thus, long-circulating microparticulates may be used to maintain a required level of a pharmaceutical agent in the blood for extended time intervals for better drug availability. Long-circulating diagnostic agents are of primary importance for blood pool imaging. Blood substitutes represent another important area for the use of long-circulating pharmaceuticals, when artificial oxygen carriers should be present in the circulation long enough to provide sufficient time for the restoration of the normal physiological mechanisms for blood cell production [6].

Second, long-circulating drug-containing microparticulates or large macromolecular aggregates can slowly accumulate (so-called enhanced permeability and retention effect, EPR, known also as a ‘passive’ targeting or accumulation via an impaired filtration mechanism) in pathological sites with affected and leaky vasculature (such as tumors, inflammations, and infarcted areas) and improve or enhance drug delivery in those areas [7–9].

Third, prolonged circulation can help to achieve a better targeting effect for those targeted (specific ligand-modified) drugs and drug carriers since it increases the total quantity of targeted drug/carrier passing through the target, and the number of interactions between targeted drugs and their targets [5].

The most important biological consequence of modification of microparticular carriers with protecting polymers is sharp increase in their circulation time and decrease in their RES (liver) accumulation [2,10–12]. Interactions of non-extravasating or very slowly extravasating drugs/carriers are limited to blood components and cells exposed into the blood. Usually, such substances are removed from the circulation via opsonization-mediated phagocytosis, and surface coating with polyethylene glycol [PEG] or PEG-like polymers slows opsonization down. From the clinical point of view, it is extremely important that various long-circulating microparticulate drug carriers of a relatively small size (100–200 nm) were shown to really effectively accumulate in many tumors via the enhanced permeability and retention (EPR) effect or ‘impaired filtration’ mechanism [8,9]. The most important pathways for the clearance of long-circulating drugs and drug carriers — extravasation, renal clearance, and uptake by cells

from the blood — are independent processes. However, namely extravasation determines interstitial and lymphatic transport, interstitial and lymphatic uptake of long-circulating substances, and, generally speaking, their bioavailability. With this in mind, it was suggested [13] to divide all long-circulating drugs/carriers into extravasating and non-extravasating ones. The main difference between these two groups is their size, with a border zone being at ca. 5–10 nm. The non-extravasating group includes cells, cell ghosts, particles, and large liposomes, while the extravasating group includes micelles, small liposomes, proteins and their derivatives (including antibodies and PEG-modified enzymes) and various polymers which are large enough (more than 40 kDa) to avoid fast renal clearance. In certain pathological areas with increased endothelial permeability the pattern can change completely. However, such conditional division allows to build idealized models describing the biological behavior of long-circulating substances [13].

An important analysis of pharmacokinetics of long-circulating (PEG-coated) particulate drug carriers was performed by Allen [14,15] using liposomes as an example. It clearly follows from this analysis that association of drugs with carriers such as liposomes has pronounced effects of pharmacokinetic profiles of both the drug and the carrier. The most important consequences of such association for the drug are: delayed absorption, restricted biodistribution, decreased volume of biodistribution, delayed clearance, and retarded metabolism [16]. All these effects are determined by hindered interstitial penetration of a drug and lesser drug accessibility for the biological milieu because of entrapment into liposome. Needless to say that the same regularities are applicable to various particulate drug carriers [17–19] including micelles.

An interesting problem is how to combine the unique properties of long-circulating (PEG-coated) nanocarriers (liposomes and others) and targeted antibody-modified carriers in one preparation. The concern about using PEG for immunocarriers protection is that the grafted polymer can create steric hindrances for normal antibody–target interaction. However, in certain cases this problem can be avoided as follows from consideration of different cases for the co-immobilization of antibody and water-soluble flexible polymer on the same carrier [20,21]. Alternatively, antibodies were immobilized on the surface of a drug carrier via the long spacer group or even directly on termini of some protecting polymer (such as PEG) molecules [22–25]. Naturally, protocols developed can be used with the whole variety of sterically protected drug carriers, micelles being among them.

2. Micelles and micellization. Relevance to drug delivery

2.1. Micelles

Micelles represent so-called colloidal dispersions that belong to a large family of dispersed systems consisting of particulate matter or dispersed phase, distributed within a continuous phase or dispersion medium. In terms of size, colloidal dispersions occupy a position between molecular dispersions with particle size under 1 nm and coarse dispersions with particle size greater than 0.5 μm. More specifically, micelles normally have particle size within 5 to 50–100 nm range. Among colloidal dispersions normally divided into three principal groups — lyophilic, lyophobic and association colloids — micelles belong to a group of association or amphiphilic colloids. Such colloids, under certain conditions (concentration and temperature), 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 [26]. At low concentrations in a liquid medium, these amphiphilic molecules exist separately; however, as their concentration is increased, aggregation takes place within a rather narrow concentration interval (see the principal scheme of micelle formation from an amphiphilic molecule in an aqueous medium in Fig. 1). Those aggregates include several dozens of amphiphilic molecules, usually have a shape close to spherical, and are known as micelles. The concentration of a monomeric amphiphile at which micelles appear is called the critical micelle concentration (CMC), while the number of individual molecules forming a micelle is called the aggregation number of the micelle. In aqueous solutions, the CMC value for a

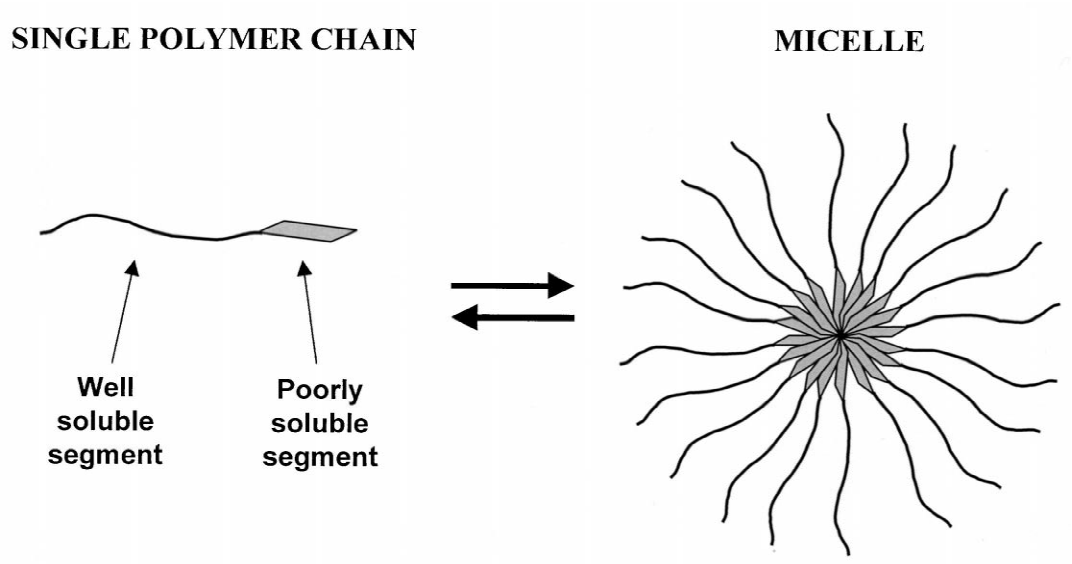


Fig. 1. The general scheme of micelle formation from amphiphilic molecules.

given amphiphile can be lowered in the presence of electrolytes.

There exist several methods to determine the CMC value for a given amphiphilic compound. Strictly speaking, almost any physical parameter can be used to register the change in the system properties at or near CMC. However, because of sensitivity issues only certain methods are applied to actually determine the CMC value. Among those methods are HPLC, particle size measurement by using small angle light scattering, and fluorescent spectroscopy. The latter method is the most sensitive and precise one [27,28]. It is based on the fact that some fluorescent probes, such as pyrene, have a tendency to associate with micelles rather than with water phase and their fluorescence changes depend on surroundings [29]. Below the CMC, the marker (pyrene) is solubilized in a polar solvent (water) to a very small extent; however, in the presence of micelles a hydrophobic, non-polar micelle core solubilizes pyrene. Following the fluorescence intensity of a corresponding marker at different concentrations of an amphiphilic polymer, one can see the increase in fluorescence intensity when micelles begin to appear in the system and the marker becomes associated with the micelle core. Fig. 2 presents a typical curve describing fluorescence changes of a marker (pyrene) in the presence of

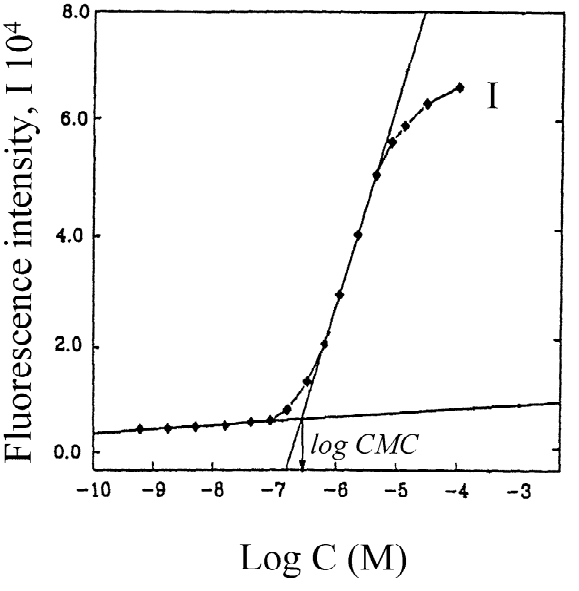


Fig. 2. Determination of CMC value using a sparingly soluble fluorescent dye with different solubility in water and in micellar phase. The typical curve is presented of the dye fluorescence in the presence of variable concentration of a given amphiphilic micelle-forming polymer. The increase in dye fluorescence evidences its solubilization via the incorporation into the micellar phase, i.e. the appearance of micelles in the system. CMC value is calculated as shown.

varying concentrations of a micelle-forming amphiphile and demonstrates how CMC value is calculated.

Below the CMC amphiphilic molecules have a strong tendency to be adsorbed at the air–water interface [30]. With the increase of amphiphile concentration in the system, a point is reached when both the interface and the bulk of the solvent (water) become saturated with monomeric amphiphiles. At this point (CMC point), any further increase in amphiphile concentration leads to the formation of micelles within the bulk phase and subsequent decrease in the free energy of the system. Below the CMC, the increase in amphiphile concentration leads to the decrease of the surface tension, while above CMC the surface tension remains constant at increasing concentrations of an amphiphile, evidencing the saturation of the interface with an amphiphile and micelle formation in the bulk phase. Mixed micelles can be composed of several amphiphiles, and in the ideal case, the CMC of the mixture can be calculated from CMC values of individual components and their molar fraction (M) in the mixture:

$$1/\mathrm{CMC} = M_1/\mathrm{CMC}_1 + M_2/\mathrm{CMC}_2$$

Another important parameter describing the micellization process is a Critical Micellization Temperature or CMT. Below this temperature amphiphilic polymers exist as unimers, while above it both unimers and aggregates (micelles) are already present in the system. The same methods that are used to find a CMC value may be successfully applied to determine CMT. The analysis of CMC and CMT values, as well as their dependencies on temperature and concentration, correspondingly, provide a simple method to determine thermodynamic parameters of micelle formation, such as free energy and enthalpy of micellization.

2.2. Solubilization by micelles

An important property of micelles that has a particular significance in pharmacy is their ability to increase the solubility of sparingly soluble substances. This solubilization phenomenon was extensively investigated and reviewed in many publications (see, for example, Refs. [31,32]). Micelles made of nonionic surfactants (the most frequently used pharmaceutical micelles) are known to have an anisotropic water distribution within their structure — water concentration decreases from the surface towards the core of the micelle. Due to this anisotropy, such micelles demonstrate a polarity gradient from the highly hydrated surface to the hydrophobic core. As a result, the spatial position of a certain solubilized substance (drug) within a micelle will depend on its polarity. 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 (see Fig. 3). The capacity of surfactants for drugs depends on many various factors, such as chemical structure of a drug and surfactant, polarity of a drug, the location of a drug within the micelles, temperature, pH, etc. Thus, an increase in the length of a hydrophobic region of a surfactant facilitates the solubilization of a hydrophobic drug inside the micelle core. Simultaneously,

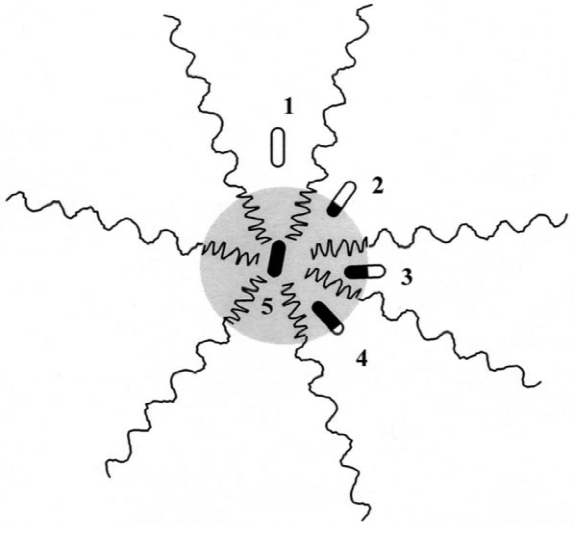


Fig. 3. Possible patterns of drug association with a micelle depending on the drug hydrophobicity (black color on a ‘drug molecule’ shows the hydrophobic area, white, the hydrophilic area). Completely water-soluble hydrophilic drug can only be adsorbed within the micelle corona compartment (case 1); while completely insoluble hydrophobic molecule can only be incorporated in the micelle core compartment (case 5). Drug molecules with intermediate hydrophobic/hydrophilic ratio will have intermediate positions within the micelle particle (cases 2 to 4).

the increase in the size of a micelle core decreases the Laplace pressure resulting from the interface curvature, and also facilitates the incorporation of hydrophobic solubilizate into the micelle core.

From the thermodynamic point of view, the solubilization can be considered as a normal partitioning of the drug between two phases — micellar and aqueous, and the standard free energy of solubilization (ΔG_s^o) can be expressed via the corresponding partition coefficient:

$$\Delta G_s^o = -RT \ln K$$

In general, surfactants play an important role in contemporary pharmaceutical biotechnology, since they are widely used to control such properties of various drug dosage forms as wetting, stability, bioavailability, etc. [30]. It is important to notice that lyophobic colloids used recently as frequent pharmaceutical formulations require a certain energy to be applied for their formation, are quite unstable from the thermodynamic point of view and frequently form large aggregates [33]. At the same time, lyophilic colloids, including micelles, under certain conditions form spontaneously (so-called self-assembling systems), and are thermodynamically more stable towards both dissociation and aggregation. Naturally, they are currently attracting increasing attention. According to [34], 'ideal' self-assembling drug delivery systems should spontaneously form from drug molecules, carrier components and targeting moieties; their size should be of around 10 nm in order to provide them with the ability to penetrate various tissues and even cells; they should be stable in vivo for a sufficiently long time and should not provoke any biological reactions; they should release a free drug upon contact with target tissues or cells; and, at last, the components of the carrier should be easily removed from the body when the therapeutic function is completed.

2.3. Biological significance of micellization

Micellization of biologically-active substances is a rather general phenomenon, since it is believed that the increase in the bioavailability of a lipophilic drug upon oral administration is caused by drug solubilization in the gut by naturally occurring biliary lipid/fatty acid-containing mixed micelles produced by the organism as a result of the digestion of dietary fat. On the other hand, surfactant micelles are widely used as adjuvant and drug carrier systems in many areas of pharmaceutical technology and controlled drug delivery research. As is evident from the numerous publications; see, for example [35,36] and references therein; almost every possible drug administration route has benefited from the use of micellar forms of drugs in terms of either increased bioavailability or reduction of adverse effects. In broad terms, the solubilization of drugs using micelle-forming surfactants (which results in formation of mixed micelles) might be advantageous for drug delivery purposes because of either increased water solubility of sparingly soluble drug or enhanced permeability across the physiological barrier or substantial changes in the drug biodistribution. Use of special amphiphilic molecules as surfactants can introduce the property of micelle extended blood half-life upon intravenous administration. In addition, micelles may be made targeted by chemical attachment of targeting moiety to their surface.

It is believed that the increase in the bioavailability of a lipophilic drug upon oral administration is caused by drug solubilization in the gut by naturally occurring biliary lipid/fatty acid-containing mixed micelles produced by the organism as a result of the digestion of dietary fat. The micellar form of the drug is transferred across the intestinal mucosal membrane into the enterocyte where it enters the lipoprotein biosynthetic pathway and eventually is released into the intestinal lymphatics being incorporated into chylomicron particles [37]. In this example, mixed micelles play a key role in the transport of drug across the mucosal membrane. Even more interestingly, mixed micelles have been found to enhance bioavailability for not only lipophilic drugs (which presumably possess some affinity to the micelle's hydrophobic core) but also for polar and even for macromolecular ones including peptides and proteins. Fatty acid/synthetic surfactant mixed micelles were identified as effective promoters of gastrointestinal absorption and lymphotropic drug delivery of poorly absorbable polar compounds [38]. The proposed molecular mechanism of this effect is likely the disturbance of intestinal epithelial cell

plasma membrane induced by mixed micelles [39]. Apparently, similar phenomena are the basis of the increased permeability of other physiological barriers caused by micellar enhancers.

Among the barriers reachable from systemic circulation, the blood–brain barrier also can be included into consideration as a potential penetration target for micelle-incorporated drugs. Being administered directly into systemic circulation, mixed micelles can substantially improve the performance of the sedative drug diazepam with target receptors in the brain. It has been reported that the micellar formulation of this drug has marked superiority over the regular non-micellar preparation in a number of psychometric and psychophysiological parameters in humans after intramuscular administration [40]. Another group has reported increased activity of the neuroleptic drug haloperidol in mice after intraperitoneal administration of its micellar form solubilized in polyol surfactant [41].

Amid other micelle-forming amphiphilic substances, low-molecular-weight oligoethyleneglycol-based surfactants are especially widely used in pharmaceutical technology as solubilizers for poorly water-soluble or water-insoluble drugs for parenteral and oral routes of drug delivery like, for example, Polysorbate 80 [42–44]. The main advantage of oligoethyleneglycol-based surfactants for pharmacological applications is their reported low toxicity [45–48]. The mechanism of the bioavailability enhancement is apparently close to the one for the biliary lipids/fatty acid mixed micelles earlier: direct disturbance of the absorbing membrane. For example, it has been reported that the presence of Polysorbate 80 at a concentration close to the surfactant's critical micelle concentration (CMC) might increase the polarity of the absorbing membrane and disrupt the stagnant diffusion layer surrounding the membrane [42].

As discussed above, micelles can be used for delivery of various pharmaceuticals, for example, diagnostic (imaging) agents and therapeutics, and they should meet certain specific requirements in each particular case. Thus, in the case of diagnostic micelles, achieving the highest possible target-to-nontarget ratio (relative accumulation) is of primary importance, whereas for micelles loaded with therapeutic agents, maximum possible accumulation in the target is usually required and we can tolerate even increased non-specific accumulation.

Summing up, in broad terms, the solubilization of drugs using micelle-forming surfactants (which results in formation of mixed micelles) might be advantageous for drug delivery purposes because of increased water solubility of sparingly soluble drug and its improved bioavailability, reduction of toxicity and other adverse effects, enhanced permeability across the physiological barriers, and substantial changes in drug biodistribution. The use of certain special amphiphilic molecules as surfactants can also introduce the property of micelle extended blood half-life upon intravenous administration. Besides, micelles may be targeted by chemical attachment of targeting moiety to their surface. In the latter case, local release of free drug from the 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 interactions with non-target organs. A very important property of micelles is their size, which normally varies between 5 and 50–100 nm and fills the gap between such drug carriers as individual macromolecules (antibodies, albumin, dextran) with the size below 5 nm and nanoparticulates (liposomes, microcapsules) with the size ca. 50 nm and up. The most usual size of a pharmaceutical micelle is between 10 and 80 nm, optimal CMC value should be in a low millimolar region, and the loading efficacy towards a hydrophobic drug should be between 5 and 25% wt.

However, the definite drawback of such systems is that mixed micelles composed of low molecular weight surfactants are thermodynamically not stable in aqueous media and are subject to dissociation upon dilution. Critical micellation concentrations of these surfactants are usually in a millimolar range. In vivo, it results in micelle collapse in the blood immediately upon administration, with subsequent precipitation of the incorporated drug or its transfer to plasma proteins because of instant dilution with the entire blood volume. Hence, in terms of drug delivery carrier development, there is a need to find a new class of surfactant molecules able to form more stable micelles with lower CMC values. One of the possible candidates for this role, a class of am-

phiphilic polymers known to form polymeric micelles in aqueous solutions, has been proposed as a drug carrier [49].

3. Polymeric micelles

3.1. Micelle formation by polymers

Polymeric micelles represent a separate class of micelles and are formed from copolymers consisting of both hydrophilic and hydrophobic monomer units. There exist several possible classifications of polymers and copolymers, however, a very simple classification presented in Fig. 4 will be sufficient for our purposes. Unlike homopolymers built of identical monomeric units, copolymers include two types of monomeric units differing in their solubility. Those two types of monomeric groups can be organized into a polymeric chain in different fashions providing random, block and graft copolymers. As follows from the structures presented in Fig. 4, di-block- and tri-block-copolymers can be prepared from the same hydrophilic and hydrophobic monomeric units.

It has repeatedly been shown that amphiphilic block AB-type copolymers with the length of a hydrophilic block exceeding to some extent that of a hydrophobic one can form spherical micelles in aqueous solutions. The particulates are composed of a core of hydrophobic blocks stabilized by a corona of hydrophilic polymeric chains. If the length of a hydrophilic block is too high, copolymers exist in water as unimers (individual molecules), while molecules with very long hydrophobic blocks form structures with non-micellar morphology, such as rods and lamellae [50]. Polymeric micelles are distinct from other particulate drug carriers by the following properties: the smaller size compared to liposomes and microparticles, the lack of an interior water compartment, the existence of an interior hydrophobic compartment, and the protective effect of an exterior polymer. Contrary to liposomes that have been shown to incorporate water-soluble drugs into the aqueous interior, polymeric micelles can be a carrier for hydrophobic and sparingly soluble pharmaceuticals. The structural resemblance of synthetic polymeric micelles and natural lipoproteins that also have been considered as drug carriers was noticed [51]. Moreover, polymeric amphiphiles reconstituted with hydrophobic drugs could represent a sound alternative to the reconstituted low density lipoproteins approach, since micelles possess the same hydrophobic interior capable of incorporating poorly soluble drugs, but they do not need to compete with any naturally occurring particulates or molecules for receptor binding.

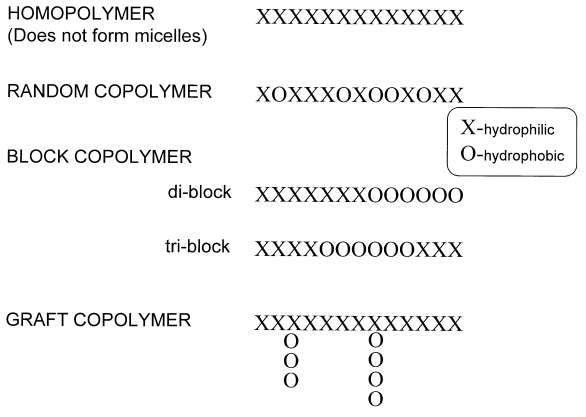


Fig. 4. Main structural types of copolymers.

Thermodynamic regularities underlying micelle formation from amphiphilic polymers are the same as for micellization of any low-molecular-weight amphiphiles. The major driving force behind self-association is the decrease of the free energy of the system, due to removal of hydrophobic fragments from the aqueous surroundings with the formation of a micelle core stabilized with hydrophilic blocks exposed to water [29,30]. Similar to low-molecular-weight surfactants, critical micellation concentration or CMC, the key parameter characterizing micelle formation, shows the concentration of an amphiphilic polymer in solution when micelles begin to form and exist in the equilibrium with unimers. At CMC and slightly above it, the micelles are still loose and contain some water in the core [52]. With further increase in amphiphile concentration in the medium, the unimer:micelle equilibrium shifts towards micelle formation, micelles become more tight and stable, lose the residual solvent from the core and decrease their size. The lower is the CMC value of a given amphiphilic polymer, the more stable are the micelles, even at a low concentration of an amphiphile in the medium. This is especially important from the

pharmacological point of view, since upon dilution with a large volume of the blood only micelles with low CMC value still exist, while micelles with high CMC value may dissociate into unimers and their content may precipitate in the blood. Another important issue is micelle stability against possible aggregation in solution. In general, micelles formed by amphiphilic graft-copolymers are more likely to form aggregates, since hydrophobic chains in such micelles are less mobile and more loosely packed than in micelles formed by block-copolymers so that they can interact with hydrophobic blocks from other micelles [49,52–56].

Numerous studies have been published developing a theoretical description of micelle formation and properties. Refs. [36,39,52,57] represent just a few examples. In general, micelles are considered as particles with a spherical core representing a molten liquid globule and a swollen hydrophilic corona surrounding this core. The liquid nature of the core permits effective mixing of hydrophobic blocks that leads to a thermodynamic equilibrium state. Strictly speaking, this is a quasi-equilibrium state, since simple dilution can shift micelle:unimer equilibrium towards unimers, i.e. causes micelle dissociation. However, even upon dilution, certain micelles demonstrate rather high kinetic stability, i.e. their dissociation into unimers proceeds very slowly. From the practical point of view, at a concentration above CMC micelles can exist indefinitely long, so that their storage stability should not be an issue. Certain data are available [57] demonstrating that in some cases the micelle core can exist in a glassy state or the molten core can undergo a liquid:glass phase transition. Different phases are characterized by different porosity and ability to entrap solvent molecules and/or drugs.

3.2. The structure and properties of micelle-forming co-polymers

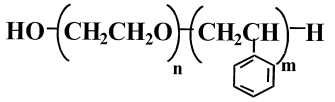
From the pharmacological point of view, micelle-forming di- and tri-block-copolymers are of particular interest [36,49,58–60]. As hydrophilic blocks, both di-block and tri-block-copolymers frequently contain poly(ethylene oxide) or PEO chains (this polymer is also commonly referred to as polyethylene glycol or PEG), since this polymer is known to be well soluble, highly hydrated and able to serve as an efficient steric protector for various microparticulates (such as micelles, liposomes, nanoparticles and nanocapsules) in biological media [4,60]. In di-block copolymers PEG chains are simply conjugated with various hydrophobic blocks, while in tri-block copolymers both termini of a hydrophilic or hydrophobic block may be coupled with the second component. Triple copolymers of hydrophilic ethylene oxide units with hydrophobic propylene oxide units (such polymers are known as Pluronics) are the most common examples of pharmaceutical tri-block copolymers. Some structures of pharmaceutical di- and tri-block copolymers are presented in Fig. 5. In the case of grafted co-polymers, multiple hydrophobic chains are distributed along the main chain composed of hydrophilic units [61,62].

Micelle-forming block co-polymers presented in Fig. 5 are synthesized by anionic polymerization, ring-opening polymerization, or by polymerization with the use of poly(ethylene oxide)-based initiators [47]. All these methods permit to synthesize amphiphilic co-polymers with different molecular mass (between 1000 and 10 000 Da) and different hydrophobic–lipophilic balance by controlling the lengths of hydrophobic/hydrophilic blocks. Various physicochemical and biological properties of micelle-forming polymers can be controlled by varying molecular size of different blocks and their molar ratio in the final copolymer. Types of micelles formed from different block co-polymers are shown in Fig. 6. Micelles formed by randomly modified grafted co-polymers are usually smaller in size than micelles formed by end-modified block co-polymers, since they can be formed within one polymeric chain providing micelles with a smaller aggregation number [53]. On the other hand, however, since randomly modified grafted co-polymers can form micelles where some of the hydrophobic fragments may be in close contact with water, such micelles can additionally aggregate into larger particles [52,55].

Whatever route of micelle formation is envisioned, still in the majority of cases the structure of amphiphilic unimers follows some simple regulations: PEG blocks with a molecular weight from 1 to 15 kDa are usual corona-forming blocks, and the length of a hydrophobic core-forming block is close or

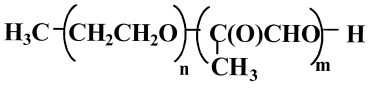
DI-BLOCK COPOLYMERS

1.



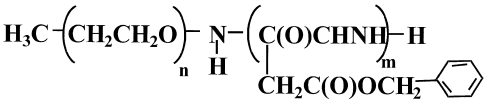
poly(ethylene oxide)-*b*-poly(styrene) block copolymer

2.



poly(ethylene oxide)-*b*-poly(D,L-lactide) di-block copolymer

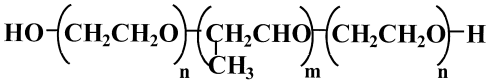
3.



poly(ethylene oxide)-*b*-poly(β-benzyl L-aspartate) di-block copolymer

TRI-BLOCK COPOLYMERS

4.



poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) tri-block copolymer

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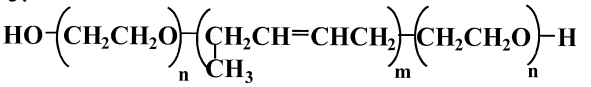


poly(ethylene oxide)-*b*-poly(isoprene)-*b*-poly(ethylene oxide) tri-block copolymer

Fig. 5. Some examples of micelle-forming di-block and tri-block co-polymers.

somewhat lower than that of a hydrophilic block [63]. Though some other hydrophilic polymers may be used to make corona blocks [12,64], still PEG remains the hydrophilic block of choice. At the same time, a variety of polymers may be used to build hydrophobic core-forming blocks. The list includes polymers of propylene oxide [41,65], L-lysine

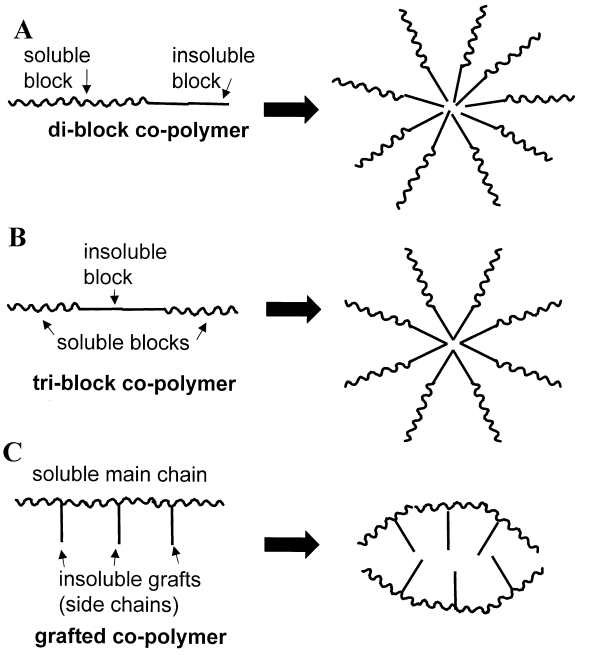


Fig. 6. The mechanisms of micelle formation from different types of amphiphilic co-polymers.

[66,67], aspartic acid [68,69], β-benzoyl-L-aspartate [55,70], γ-benzyl-L-glutamate [71], caprolactone [72,73], D,L-lactic acid [74,75], spermine [76]. In some cases, phospholipid residues — short, however, extremely hydrophobic due to the presence of two long-chain fatty acyl groups — can also be successfully used as hydrophobic core-forming groups [35]. In certain 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 taxol, cisplatin, antracyclin antibiotics, hydrophobic diagnostic units, etc.) yielding amphiphilic micelle-forming copolymer [36,67,77]. Table 1 presents some examples of various amphiphilic micelle-forming polymers that were used for the preparation of micellar drugs. The size and composition distribution of micelles is usually quite narrow and from the practical point of view it is reasonable to assume that all micelles in a given preparation are of a single size and composition [78].

Amphiphilic block co-polymers themselves (first of all, Pluronics®) were extensively studied as components of blood substitutes [79], adjuvants

Table 1
Some examples of the block copolymers used to prepare drug-loaded micelles; modified from [85]

Block co-polymers	Drugs incorporated
Pluronics®	doxorubicin, cisplatin [122] doxorubicin, epirubicin [117] doxorubicin [123] haloperidol [41] ATP [121]
Polycaprolactone-b-PEG	FK506, L-685,818 [73]
Polycaprolactone-b-methoxy-PEG	indomethacin [189]
Poly(*N*-isopropylacrylamide)-b-PEG	miscellaneous [56,63,126]
Poly(aspartic acid)-b-PEG	doxorubicin [54,68,115,116] cisplatin [190] lysozyme [69]
Poly(γ-benzyl-L-glutamate)-b-PEG	clonazepam [71]
Poly(D,L-lactide)-b-methoxy-PEG	pacitaxel [74] pacitaxel [111] testosterone [75]
Poly(β-benzyl-L-aspartate)-b-poly(α-hydroxy-ethylene oxide)	doxorubicin [63]
Poly(β-benzyl-L-aspartate)-b-PEG	doxorubicin [70,98,191] indomethacin [55] KRN [115,116] amphotericin B [192,193]
Poly(L-lysine)-b-PEG	DNA [66,133]
Oligo(methyl methacrylate)-b-poly(acrylic acid)	doxorubicin [86]
PEG-PE	dequalinium [161] soya bean trypsin inhibitor [166]

[80,81], antitumor/antimetastatic agents [82], etc. Some of these polymers (more hydrophilic ones) were shown to reduce blood viscosity and vascular resistance [83,84] increasing thus the efficacy of the microvascular transport.

Keeping in mind the future pharmaceutical application of polymeric micelles, one can put together a set of requirements towards properties of such micelles as a whole, as well as towards properties of corona- and core-forming blocks. According to [85], the 'ideal' pharmaceutical micelle should possess a suitable size (from 10 to 100 nm), demonstrate sufficiently high stability both in vitro and in vivo (i.e. have a good combination of reasonably low CMC value and reasonably high kinetic stability), be able to stay in the body long enough and still eventually disintegrate into bioinert and non-toxic unimers that should be easily cleared from the body, and carry a substantial quantity of a micelle-incorporated pharmaceutical agent. To meet these 'net' requirements, the core compartment should demonstrate high loading capacity, controlled release profile for the incorporated drug, and good compatibility between the core-forming block and incorporated drug. The micelle corona should build an effective steric protection for the micelle providing sufficient density and thickness of hydrophilic blocks. In more general terms, the micelle corona should determine for the micelle hydrophilicity, charge, the length of hydrophilic blocks, surface density of these blocks, and the presence of reactive groups suitable for further micelle derivatization, such as an attachment of targeting moieties [18,19,41,75,86,87]. These properties, in turn, control such important biological

characteristics of a micellar carrier as pharmacokinetics, biodistribution, biocompatibility, longevity, surface adsorption of biomacromolecules, adhesion to biosurfaces and targetability [1,18,19,41,75,88–90]. Similarly, the core compartment defines the drug compatibility, the size of a 'cargo' space for a hydrophobic drug, and the hydrophobic/hydrophilic ratio. These parameters, in turn, determine the efficacy of drug loading into micelle, drug release profile, and micelle stability [85].

3.3. Micelle preparation methods and micelle morphology

All individual methods of micelle preparation can be divided into two large groups — the direct dissolution method and the dialysis method [85], see Fig. 7. In each particular case, the choice of the method is usually determined by the extent of the solubility of a micelle-forming block co-polymer in an aqueous medium. In case of direct dissolution, co-polymer is simply dissolved in an aqueous medium at normal or elevated temperature and at a concentration well above its CMC value. This method is frequently applied for micelle preparation from Pluronics® and similar block co-polymers possessing a certain degree of solubility in water. Micelle-forming co-polymers with very low water solubility are converted into micelles by dissolution in a water miscible organic solvent, such as dimethylsulfoxide, dimethylformamide, acetonitryl, tetrahydrofuran, etc., and subsequent dialysis against water.

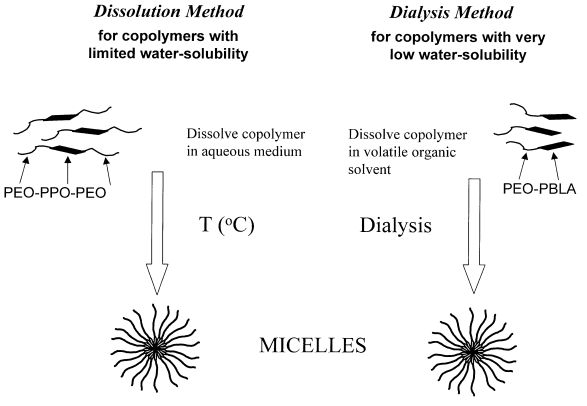


Fig. 7. Two main methods of micelle preparation — dissolution and dialysis.

The morphology of supermolecular aggregates that can form from amphiphilic block co-polymers is currently a subject of intensive research [73,85,91]. Usually, it is accepted that micelles are spherical particles with a clear distinction between core and corona compartments. However, as was shown in numerous studies from Eisenberg and his group [50,73,77,85,91–93], block copolymer aggregates can exist in many different morphologies, such as spheres, rods, large compound rods, branched short rods, discontinuous rods, various vesicles, tubules, branched tubules, baroclinic tubes, needles, large compound micelles, lamellae, hexagonally packed hollow hoops, various mixed and combined morphologies and many more. Different structures are formed at equilibrium, near-equilibrium and non-equilibrium conditions. Usually, non-spherical structures are formed from asymmetric block copolymers in which the length of the hydrophobic core-forming block is significantly shorter than the length of the hydrophilic corona-forming block. The formation of these crew-cut aggregates was explained [50,92] by a force balance effect between the degree of stretching of the core-forming blocks, the interfacial energy between the micelle core and the solvent, and the interaction between corona-forming hydrophilic chains [94]. Copolymer composition (which influences the degree of stretching of core-forming block and the interactions between corona-forming blocks), copolymer concentration (which increases the aggregation number of the micelles), and common organic solvent used for micelle preparation can be listed among the key morphogenic factors [85]. One should, however, admit that the usefulness of non-spherical aggregates as drug delivery vehicles is still to be established.

3.4. Stability of polymeric micelles

From general considerations, it is evident that pharmaceutical drug-loaded micelles should be stable enough to provide a sufficient time for drug delivery and accumulation in the target zone, and simultaneously be able to slowly dissociate into micelle-forming unimers to provide their easy and problem-free elimination from the body. Stability of micelles

both in vitro and in vivo, as well as their clearance from the body, depends on their CMC values. It is, however, important to differentiate between thermodynamic and kinetic stability. If the first one just shows below which concentration unimer:micelle equilibrium is shifted towards unimer formation, the latter one provides information on the actual time of micelle dissociation into unimers, since even upon dilution to a concentration below CMC preformed micelles can still exist long enough to perform their carrier function. The kinetic stability (the actual rate of micelle dissociation below CMC) depends on many factors including the physical state of the micelle core, contents of a solvent inside the core, the size of a hydrophobic block, and the hydrophobic/hydrophilic ratio [95–97].

From the practical point of view, the size of unimers formed upon micelle dissociation plays an important physiological role in the efficacy of its kidney filtration. In an optimal case, the unimer molecular size should not exceed 20 to 30 kDa, roughly corresponding to the renal filtration limit [63]. Though both hydrophilic and hydrophobic blocks influence the micelle CMC value, the hydrophobic block plays a more crucial role [98,99]. The following general regularities can be applied to characterize the role of different blocks in micelle stability: (a) the increase in the length of a hydrophobic block at a given length of a hydrophilic block causes a noticeable decrease in CMC value and increase in micelle stability; (b) the increase in the length of a hydrophilic block at a given length of a hydrophobic block results in only a small rise of the CMC value; (c) the increase in the molecular weight of the unimer at a given hydrophilic/hydrophobic ratio causes some decrease in the CMC value; (d) in general, the CMC value for tri-block copolymers is higher than for di-block copolymers at the same molecular weight and hydrophilic/hydrophobic ratio [78,98–101]. The stability of a micelle is also strongly influenced by such properties of the micelle core as its microviscosity, which can be experimentally determined using fluorescent probes [53,62] or proton magnetic resonance [102].

The specific role of hydrophilic PEG blocks in vivo is, as it has been already mentioned, the steric stabilization of a micelle preventing it from being opsonized and cleared through the RES. PEG chains exposed into the aqueous surroundings interfere with inter-particle attraction caused by van der Waals forces. In vitro, it results in the prevention of the aggregation process [103], while in vivo it prevents adsorption of blood proteins (including opsonins) onto the micelle surface. Since protein adsorption onto drug carriers not only facilitates their RES-mediated clearance, but can also influence such important characteristics as biodistribution, stability, drug release profile, etc., the protective capacity of the PEG corona is of primary importance. Naturally, the efficacy of protection depends on both the surface density of PEG blocks and the thickness of the protective layer, i.e. the size of the PEG block [55]. The mechanism of PEG-mediated steric protection is essentially the same as was discussed above for the case of PEG-modified sterically stabilized liposomes [11,21,104].

3.5. Solubilization with polymeric micelles. Preparation of drug-loaded micelles

The process of solubilization of water-insoluble drugs by micelle forming amphiphilic block-copolymers was investigated both as a theoretical and practical problem [78]. It was shown that the solubilization process strongly depends on the type and efficacy of the interactions between a solubilized drug and micelle core-forming hydrophobic block of a copolymer. However, the interactions between a drug to be solubilized and the hydrophilic corona-forming block as well as interfacial interactions between drug and solvent (water) may also influence the solubilization process. Recent mathematical simulation of the solubilization process [105] demonstrated that the initial solubilization proceeds via the 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 micelle size due to the expansion of its core with a solubilized drug.

The compatibility between the loaded drug and core-forming component determines the efficacy of drug incorporation [85]. This compatibility is based on such drug characteristics as polarity, hydrophobicity, and charge. Still, the structure of the hydro-

phobic block can be selected providing maximum compatibility with virtually any poorly soluble drug. To assess compatibility between the polymer and solubilized drug, the Flory–Huggins interaction parameter may be used [85]. This parameter, χ_{sp} is described as

$$\chi_{sp} = (\delta_s - \delta_p)^2 V_s / RT$$

where χ_{sp} is the interaction parameter between solubilized drug (s) and core-forming polymer block (p), δ is the Scatchard–Hildebrand solubility parameter of the core-forming polymer block and V_s is the molar volume of the solubilized drug [106,107]. The lower is the χ_{sp} parameter, the greater the compatibility between the drug and the micelle core.

Among other factors influencing the efficacy of drug loading into the micelle, one can name the size of both core-forming and corona-forming blocks [85]. In the first case, the larger the hydrophobic block the bigger core size and its ability to entrap hydrophobic drugs. In the second case, an 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 polymers in solution a smaller fraction of this polymer will be present in the micellar form and the quantity of the micelle-associated drug drops. The hydrophobic/hydrophilic balance of the drug molecule itself will also influence load efficacy, since depending on this balance, the drug molecule will be located in different micellar compartments (see Fig. 3) and, as a result, will have different strength of association with a micelle. Those molecules that are located within the corona area or close to it can be released from the micelle pretty fast, and mainly these molecules are kept responsible for the 'fast release' component of the net release curve [108]. The phase state of the drug can also be important for its association with a micelle, since in some cases the drug is not dissolved in the core compartment, but exists as a separate phase inside the core, which may hinder drug release from the micelle [109,110].

As noted in [59], the excessive stabilization of drug-bearing polymeric micelles may negatively influence drug efficacy and bioavailability, since the drug would not release from such micelles. Thus, the real optimization of micelle properties as drug carriers should aim at finding a proper balance between micelle stability and their ability to dissociate or degrade.

There are several types of micelles that can be formed by block co-polymers. According to the general scheme [85], micelles may be considered as microcontainers with physically entrapped drug inside, or may be formed by so-called polymeric drugs where the drug is chemically attached to a hydrophobic core-forming block, or may appear as associates of block ionomer complexes when charge-bearing drug molecules may interact with an opposite charge-bearing hydrophobic core-forming block. It means that the drug can be incorporated into the micelle by simple physical entrapment or via preliminary covalent or electrostatic binding with a hydrophobic block of a micelle-forming amphiphilic block co-polymer. Naturally, some mixed or intermediate cases are also possible. Since the hydrophobic interactions constitute the main driving force behind micellar solubilization of water-insoluble drugs, the increase in the hydrophobic block content within the polymer molecule was shown to enhance insoluble drug incorporation into micelles [111,112]. This was experimentally confirmed by much better micelle incorporation of a more hydrophobic compound [75]. Thus, the efficacy of the incorporation of Sudan Black into polylactide-PEG micelles was around 65%, while for testosterone this figure was well below 1%. The data become quite clear, if one takes into the account that the partition coefficient P (in an octanol/water system) for Sudan Black is much higher than for testosterone (log $P = 7.6$ and 3.35, respectively).

The successful incorporation of charged drugs into micelles requires the presence of the opposite charge on the hydrophobic block of a micelle-forming co-polymer. This is especially important for obtaining micellar forms of DNA.

If a drug is chemically or electrostatically attached to a hydrophobic block of a micelle-forming co-polymer, its incorporation into the micelle core proceeds simultaneously with micelle formation. However, if a drug is supposed to be physically entrapped into a micelle, different protocols are used for drug incorporation into micelles depending on the

method of micelle preparation (see Fig. 7). Thus, in the direct dissolution protocol, a co-polymer solution in water is added to a drug dried from a solution in an organic solvent, or, alternatively, drug dissolved in a volatile organic solvent is added to the water solution of preformed micelles with a subsequent solvent evaporation from the system. In the case of the dialysis method, a drug to be incorporated is simply dissolved together with a micelle-forming co-polymer in an organic solvent with further dialysis against water. The oil-in-water emulsion method is also frequently used to incorporate drugs into micelles.

Whatever exact protocol was used, Pluronic®-based micelles were shown to effectively solubilize such drugs as diazepam and indomethacin [113,114], adriamicin [54,68,115,116], anthracycline antibiotics [117], and polynucleotides [118,119]. Doxorubicin incorporated into Pluronic® micelles demonstrated superior properties as 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 [59]. In addition, the reduction of the side effects of the drug was observed in many cases. Thus, the toxicity decrease (smaller vascular damage and liver focal necrosis) was found for the polymeric micelle-incorporated anti-tumor drug KRN5500 [120].

A variety of different mechanisms are involved in polymeric micelle-mediated drug transport and delivery. Thus, Pluronic® micelles were found to produce a certain effect on specific membrane ATP transporters [121], reverse multiple drug resistance and cause the sensitization of resistant cells [122,123], increase drug transport across the blood–brain barrier by inhibiting efflux or stimulating a vesicular transport [65], and participate in the receptor-mediated transport of micellar drugs into cells [124].

Micelle-incorporated drugs may slowly release from an intact micelle, especially if those drugs are not too hydrophobic (log *P* value is not too high). Still, in some cases a micellar drug demonstrates lower activity than a free drug that is specifically attributed to retarded release of the drug from the micelle [115,116]. When the drug is covalently attached to a hydrophobic block, the drug-to-polymer bond should be cleaved for the subsequent drug release. The rate of drug release from the micelle can be controlled by a whole variety of parameters such as micelle structure, the size of a hydrophobic block, phase state of the micelle core, pH value of the external medium, and temperature. Thus, an accelerated release of indomethacin from micelles made of PEG-poly(β-benzyl-L-aspartate) co-polymer was shown when the pH value of the medium was increased from acidic to neutral [55]. Certain micelles (stimuli-responsive micelles) were specifically designed to release the incorporated drug in response to changes in external pH or temperature, see, for example Refs. [56,63,125,126], where pH-sensitive and temperature-sensitive micelles were described made of poly(*N*-isopropylacrylamide)-b-polystyrene or poly(alkylacrylic acid) co-polymers.

Early detailed studies of in vivo behavior and biodistribution of therapeutic polymeric micelles have been performed by Kataoka et al. using the micelles formed by copolymer of poly(ethylene glycol) and poly(aspartic acid) (PEG-b-PAA) with covalently bound adriamycin [PEG-b-PAA(ADR)] [36,68,98]. The approach involves the covalent attachment of a relatively hydrophobic drug to PAA of PEG-b-PAA copolymer, resulting in the formation of an amphiphilic AB-type copolymer. This copolymer is able to form polymeric micelles with the hydrophobic block making up the particle's core, while the PEG block forms a surrounding water-soluble shell. Micelles formed from this conjugate are rather stable in vivo and their disintegration takes hours. It has been found that upon intravenous injection, the circulation time and biodistribution of micelles depend on relative size of the copolymer blocks. PEG-b-PAA(ADR) micelles with PEG block of 12 kDa and PAA(ADR) with 20 aspartyl adriamycin units in mice have $t_{1/2}$ approx. 7 h, while micelles with 5 kDa PEG block have $t_{1/2}$ approx. 1.5 h, and micelles with 1 kDa block have circulation half-time substantially lower than 1 h. It seems that longer PEG blocks and shorter PAA segments favor longer circulation times and lower uptake by the reticuloendothelial system [127]. In addition, the presence of this polymer on the particle surface can contribute to its steric protection from interaction with some unwanted blood components and substantially prolong its circulation time. It is important to notice

that in this particular case it is not a polymer-conjugated drug that expresses anti-cancer activity, but rather a free drug solubilized in the micelle core in addition to adriamycin covalently attached to core-forming blocks [115,116].

The whole set of micelle-forming co-polymers of PEG with poly(L-aminoacids) was used to prepare drug-loaded micelles by direct entrapment of a drug into the micelle core and without covalent attachment of drug molecules to core-forming blocks [51,55,99,103,128]. The most vivid example is the micellar form of indomethacin [55]. A similar 'non-covalent' approach was used for a successful solubilization of taxol by micelle-forming co-polymers of PEG and poly(D,L-lactic acid) (PEG-b-PLA) [74,111]. The use of micelles permitted to increase taxol solubility from less than 0.1 to 20 mg/ml. In addition, micelles decrease taxol toxicity against normal organs and tissues. However, the final biodistribution (and, consequently, anti-tumor activity) of taxol administered in a micellar form does not differ much from that of free taxol, since micellar taxol is eventually transferred onto certain serum proteins and lipoproteins [74,111].

PEG-b-poly(caprolactone) co-polymer micelles were successfully used as delivery vehicles for dihydrotestosterone [129]. The loading capacity of such micelles for the drug was rather high — up to 1.3 mg of dihydrotestosterone per 0.1 ml volume of the micelle solution and the partition coefficient for the drug between micelle core phase and solution varied from approx. 1000 to 20 000 depending on conditions. The biological activity of micelle-incorporated hormone was fully retained.

An important area of polymeric micelle-mediated drug delivery is gene therapy, since both plasmid DNA and antisense oligonucleotides can assemble into micelle-like particles in the presence of various amphiphilic block co-polymers, such as PEG-b-poly-(L-lysine) (PEG-b-PLL) or PEG-b-polyspermine, primarily via electrostatic interactions [66,130–133]. This is an interesting example of a conversion of a soluble hydrophilic co-polymer (PEG-PLL) into micelle-forming co-polymer after interaction with a water-soluble drug (DNA). In this particular case, the formation of a tight electrostatic complex between the oppositely charged DNA and PLL block leads to the removal of water from the electrostatic complex formed with subsequent 'insolubilization'. The incorporation of DNA into the interior of such micelles was confirmed by the lack of DNA degradation under the action of nucleases in these conditions.

3.6. Micelles in immunology

A very interesting and promising area for the use of polymeric micelles is applied 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 immune response and preparation of new and effective vaccines [134]. 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 worth mentioning that Pluronics® themselves are able to provoke macrophage activation. Though the exact mechanism of this activation is still under investigation, there are data suggesting that actually Pluronics® activate the alternative complement pathway [135], and certain proteins belonging to the complement system, in turn, cause macrophage activation [136].

Pluronics® demonstrate 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 response [137,138]. Pluronics® with higher molecular weights (PPG blocks with MW around 10 kDa with attached from both sides shorter PEG blocks) form micelles able to incorporate various antigens. High adjuvant activity of such micelles was demonstrated with an influenza virus vaccine [134]. 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 using co-polymer with 11 kDa core PPG block and containing between 5 and 10% of attached PEG blocks. Naturally, the size of antigen-bearing polymeric micelles depends also on the size of micelle-incorporated protein antigen [139,140]. The mechanism of protein antigen inter-

action 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 [134].

As noted in [134], studies on cellular immune response provoked by ovalbumin in Pluronic® micelles demonstrated that 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 a Pluronic-based composition for vaccinations [141] permit to believe that polymeric micelles may find a real clinical future as adjuvants and vaccine components.

3.7. Targeted polymeric micelles

Three targeting mechanisms can be seen for micelles as for any other pharmaceutical long-circulating drug carrier. The first one is based on micelle spontaneous penetration into the interstitium through the leaky vasculature (EPR effect) and is considered as a 'passive targeting' [7–9]. Thus, it was repeatedly shown that micelle-incorporated anticancer drugs (such as adriamycin, see, for example, Ref. [127]) much better accumulate in tumors than in non-target tissues (such as the heart muscle) which minimizes undesired drug toxicity.

The second targeting mechanism is based on the fact that many pathological processes in various tissues and organs are accompanied with local temperature increase and/or acidosis [142,143], and micelles made of thermo- or pH-sensitive components, such as poly(*N*-isopropylacrylamide) and its co-polymers with poly(D,L-lactide) and other blocks, can disintegrate in such areas releasing the micelle-incorporated drug [29,56,63,144,145].

And, at last, specific ligands can be attached to the water-exposed termini of hydrophilic blocks, such as antibodies and/or certain sugar moieties [25,41,146]. In this case, in order to make 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 [22–25,147,148] and involving the use of a PEG component with the protein-reactive group on the distal tip of the hydrophilic PEG block.

4. Diacyllipid–PEG conjugates — a new class of micelle-forming polymers

4.1. In vitro properties of diacyllipid–PEG micelles

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.

Diacyllipid–PEG conjugates have been introduced into the area of controlled drug delivery as polymeric surface modifiers for liposomes [10]. Interestingly, the diacyllipid–PEG molecule itself represents a characteristic amphiphilic polymer with a bulky hydrophilic (PEG) portion and a very short but extremely hydrophobic diacyllipid part. Similar to other PEG-containing amphiphilic block-copolymers, diacyllipid–PEG conjugates were found to form micelles in an aqueous environment [149].

A series of PEG–phosphatidylethanolamine (PEG–PE) conjugates was synthesized using egg PE (transphosphotidylated) and *N*-hydroxysuccinimide esters of methoxy-PEG succinates (molecular weight of 2 kDa, 5 kDa and 12 kDa) [10]. HPLC-based gel permeation chromatography showed that these polymers form micelles of different sizes in an aqueous environment (see Fig. 8). The stability of the polymeric micelles was confirmed using the same method: no dissociation into individual polymeric chains was found following chromatography of the serially diluted samples of PEG(5 kDa)–PE up to a polymer concentration of ca. 1 μl/ml which corresponds to a micromolar CMC value (see Fig. 9), which was additionally confirmed using fluorescent dyes Orange T and pyrene methods.

PEG–PE micelles can efficiently incorporate some sparingly soluble and amphiphilic substances. We

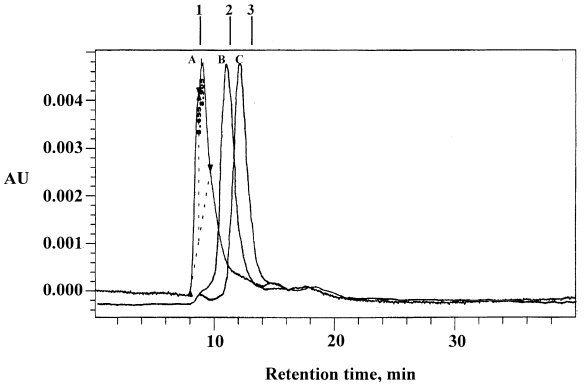


Fig. 8. HPLC-based gel permeation chromatography of micellar forms of PEG–PE conjugates with different molecular weights of PEG blocks. A: 12 kDa; B: 5 kDa; C: 2 kDa. Molecular weight standards; I: Blue Dextran (2000 kDa); II: thyroglobulin (669 kDa); III: amylase (200 kDa). Column: Shodex Protein KW-804; solvent PBS, pH 8.0; detection: 215 nm. From [182].

have developed a protocol for a prototype drug incorporation procedure involving sonication of PEG–lipid: drug: organic solvent: water mixture which allows the efficient solubilization of insoluble/amphiphilic substances into the core of PEG–lipid micelles [150,151]. HPLC-based GPC with diode-array UV/VIS detection allowed us to evaluate the nature of the resulting particles and the degree of

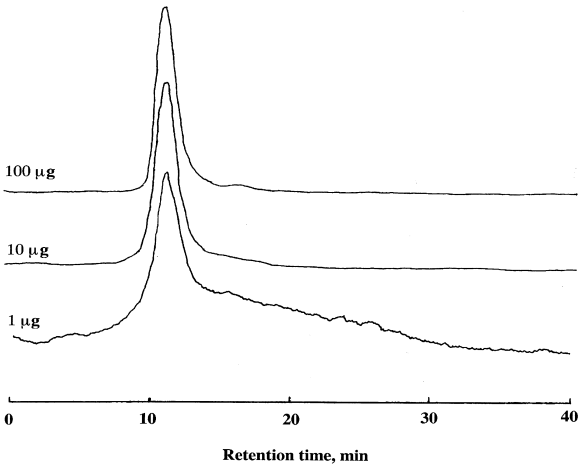


Fig. 9. HPLC-based gel permeation chromatography of serial dilutions of PEG(5 kDa)–PE micelles. Total amount of the polymer injected is indicated. Sample volume: 100 μl; Column: Shodex Protein KW-804; solvent PBS, pH 8.0; detection: 215 nm. From [182].

prototype drug incorporation (see contour chromatographs in Fig. 10). After running the protocol with several different water-insoluble/amphiphilic substances, we identified three basic types of interactions of prototype drug with the surfactant depending on the hydrophobicity of a drug: I. Incorporation of the drug into true stable micelles (for substances with a structural resemblance to micelle core-forming substances, like modified phospholipids, i.e. lipid derivative of rhodamine), Fig. 10B; II. Formation of stable larger aggregates (for non-polar hydrophobic particles, initially solubilized by PEG–lipid conjugates, e.g. diphenylhexatriene), Fig. 10C; III. Initial solubilization of the drug followed by its exclusion from the micelle core or formation of unstable aggregates (for more polar hydrophobic compounds, like topoisomerase inhibitor camptothecin), Fig. 10D.

An experiment on micelle incorporation of the topoisomerase II inhibitor ellipticine, a substance with water solubility as low as 1.5 μg/ml, demonstrated good ellipticine incorporation into PEG(5

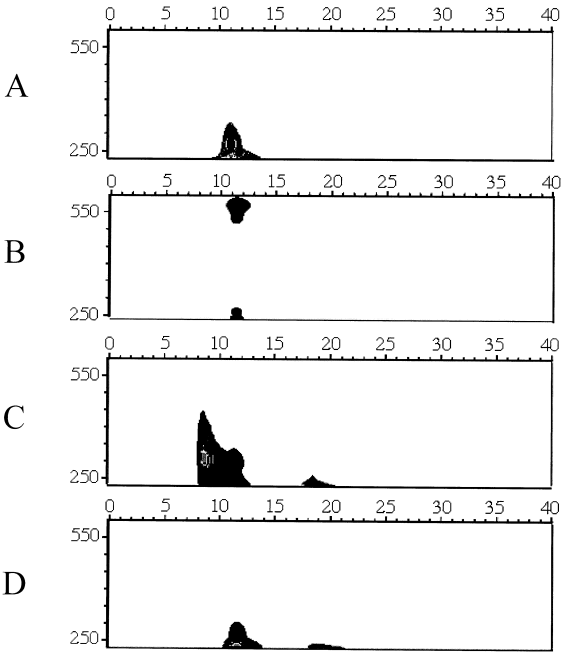


Fig. 10. Contour HPLC chromatograms (absorption wavelength vs. retention time) of different substances incorporated into PEG(5 kDa)–PE micelles. A: PEG–PE micelles (control); B: PEG–PE micelles + rhodamine–PE; C: PEG–PE micelles + diphenylhexatriene; D: PEG–PE micelles + camptothecin.

kDa)–PE micelles. The resulting mixed micelles contained up to 4% mol of an incorporated ellipticine. However, the most successful examples of hydrophobic substance incorporation into PEG–lipid micelles were different amphiphilic lipid derivatives. Unlike ellipticine, which can be successfully incorporated into PEG(5 kDa)–PE micelles only at the drug:PEO–PE molar ratio = 1:25, fluorescent lipid probe rhodamine–PE incorporates into the same micelles up to 2:1 drug:PEO–PE molar ratio.

One can suggest that the most successful therapeutic applications of PEO-lipid polymeric micelles as drug carriers would require the use of the appropriate hydrophobized prodrugs. The similar hydrophobized prodrug approach is not a new one in the area of drug delivery systems as it has been successfully used for increased permeation of peptides across intestinal membrane [151,152] and for increased loading of anti-cancer drug derivatives into reconstituted low density lipoproteins due to the covalent coupling of a lipophilic anchor directly to the drug [153].

By now, quite a few various amphiphilic polymers were shown to possess properties similar to that of PEG–PE [4,12,154,155]. Such polymers can incorporate into liposomes and provide them an efficient steric protection [4] and form micelles [35]. Some of these polymers are presented in Fig. 11. From the biological point of view, polymers of vinyl pyrrolidone (PVP) are of special interest because of their high biocompatibility. We have synthesized the whole set of various amphiphilic PVP derivatives using long chain acyls (such as palmityl, P, and stearyl, S) or phosphilipid residues as hydrophobic blocks [156,157]. Spontaneous micellization of different P-PVP and S-PVP samples and the corresponding CMC values were investigated by the pyrene method, HPLC and particle size measurements. It turned out that the ability of polymers to micellize depends on both the type of a hydrophobic residue and the MW of the PVP block. Fast spontaneous micellization and low CMC values (in a low μM range) were found for amphiphilic PVP with MW of a PVP block under 15 kDa. The determination of the CMC value for a typical representative of the amphiphilic PVP family using the pyrene method is shown in Fig. 12. One can see that the CMC value is rather low and close to that for

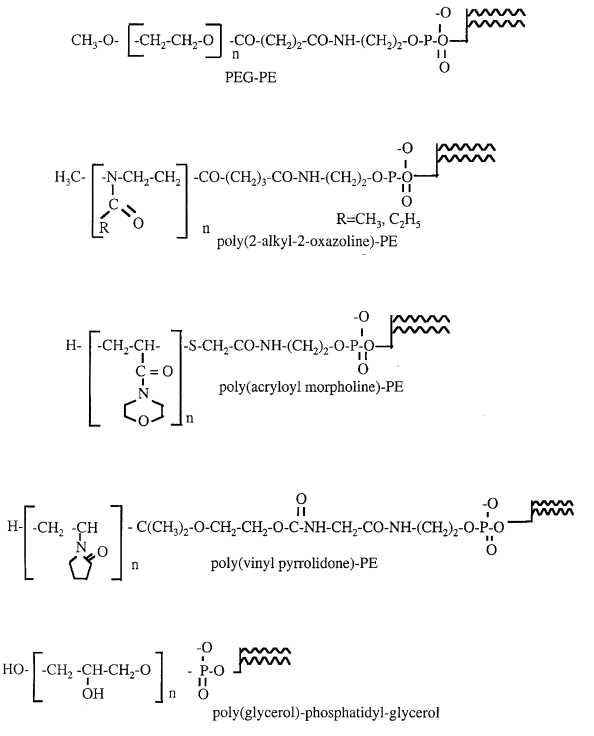


Fig. 11. Some of amphiphilic synthetic polymers that may be used for micelle preparation.

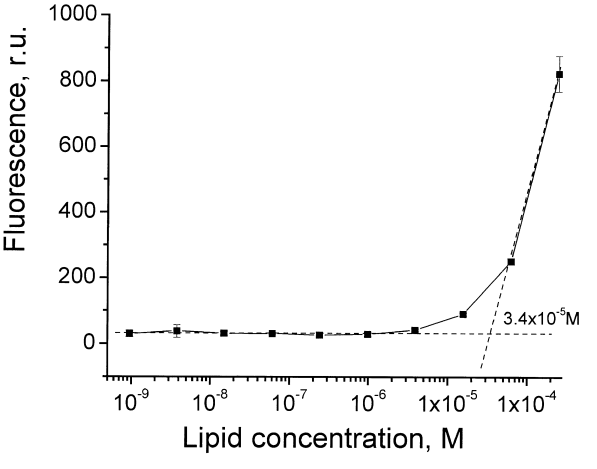


Fig. 12. CMC value for PVP(2.5 kDa)-palmityl as determined following pyrene fluorescence. Micelles were prepared by sonication of 5 mM PVP-PE dispersion in PBS, and micelle suspensions of different concentrations were prepared by serial dilutions. Each sample was incubated with an excess of pyrene for 20 h in darkness, the excessive pyrene was removed by filtration, and the fluorescence of the samples was measured at the excitation wavelength of 339 nm and the emission wavelength of 390 nm.

PEG–PE of a close molecular weight. This means that stable micelles may be prepared from a whole variety of different amphiphilic polymers, which broadens the possibility to prepare micelles with the required physico-chemical and biological properties for each particular purpose.

4.2. Interaction of diacyllipid–PEG micelles with blood plasma components. Implications for micelle stability in vivo

Some experiments have been performed on the interaction of a micelle-incorporated prototype drug with biological surroundings [151]. The stability of polymeric micelles in vivo represents a major issue in the development of these new drug carriers. Since poorly soluble drugs or hydrophobized prodrugs are the most likely candidates to be incorporated into polymeric micelles, the exchange of these pharmaceuticals with plasma components which possess an affinity to hydrophobic molecules should be thoroughly investigated. The studies showed that upon incubation of PEG(5 kDa)–PE micelles loaded with amphiphilic fluorescent rhodamine–PE with mouse plasma at 37°C, two rhodamine–PE fractions were detected after separation of the incubation mixture using NaBr gradient centrifugation: protein-rich fraction ($A_{570}/A_{280}=1.7$, density range 1.00–1.05 g/ml) and protein-poor fraction ($A_{570}/A_{280}=3.2$, density range 1.05–1.10 g/ml). Using spectral peak analysis, these fractions were identified as VLDL/LDL lipoprotein fraction and intact PEG–PE micelles. Approximately 50% of rhodamine–PE was transferred to lipoprotein from micelles after 2 h incubation. Some insignificant transfer of rhodamine–PE onto serum albumin was also shown (less than 5% after 2 h incubation). These results demonstrate the importance of experiments that can improve micelle stability in vivo or enhance the delivery of micellar drugs to target areas.

4.3. Some in vivo properties of diacyllipid–PEG micelles

During biodistribution experiments, ^{111}In-labeled PEG–PE micelles were found to have half-life times from 1.5 and up to 2.5 h in mice depending on the molecular size of PEG block (see the data for PEG(2 kDa)–PE micelles in Fig. 13). This is a long half-life compared with the majority of non-surface-modified particulates (though somewhat shorter than for PEG-containing liposomes). The shorter micelle half-life compared to PEG-liposomes may be accounted for by considering the following circumstances: first, micelles might extravasate from the vasculature due to their considerably smaller size when compared with liposomes; second, an exchange is possible of the amphiphilic label with the plasma proteins possessing affinity to lipidic moieties (such as albumin). The fact that the preincubation of micelles with the murine serum only to a small extent affects micelle longevity in the blood of experimental animals (see Fig. 13) may be considered as a certain evidence in the favor of assumption that namely extravasation and not opsonization plays the major role in the blood clearance of the micelles. Generally speaking, macrophage-evading properties of polymeric micelles are important not only for intravenous applications but also for other routes of drug carrier administration.

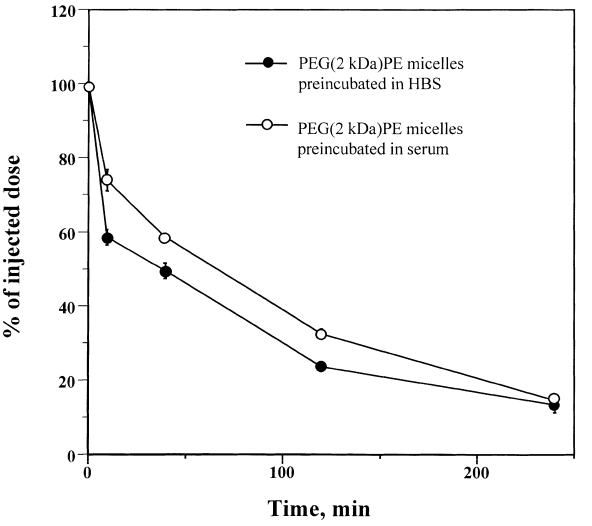


Fig. 13. Blood clearance of murine serum preincubated (●) and saline preincubated (○) PEG(2 kDa)–PE polymeric micelles labeled with traces of lipophilic 111-In-DTPA-PE after intravenous administration in mice. Dose: 1 mg of micelles (as polymer) per mouse; mean±S.E.M.; $n=4$.

Due to their size and macrophage avoiding properties, the use of PEG–lipid conjugates can be especially advantageous for some specific routes of

drug delivery. For example, it is known from experiments with ^{198}Au gold colloids that the optimal size for a particulate lymphatic absorption from the interstitial space after subcutaneous administration is rather small (approximately 5 nm) [158]. The structure of the particulate's surface is another factor that can regulate its disappearance from the injection site as well as uptake by the lymph node. The importance of PEG-coated particulates in percutaneous administration and lymph node delivery already has been demonstrated with poloxamer-coated polystyrene latex particles [159]. However, as others have pointed out [160], the potential of polystyrene nanospheres as vehicles for delivery of 'practical' bioactive agents remains to be established. At the same time, polymeric micelles already proved themselves as effective carriers for a variety of hydrophobic substances. Proper size, surface coating and ability to be loaded with sufficient quantities of drug or diagnostic agent makes PEG–lipid micelles a perfect choice for percutaneous lymphatic delivery.

4.4. Micellar form of dequalinium

In certain cases, micelles have been shown to serve as a better delivery system for some hydrophobic and/or amphiphilic drugs than the liposomes that are traditionally used for this purpose. One of the known examples of this kind is connected with a solubilization system for a 'bola'-like lipophilic cation dequalinium [161]. Dequalinium (DQA), a topical antimicrobial agent, drew considerable interest as a potential anticarcinoma drug. It was shown to be very effective in prolonging the survival of mice with intraperitoneally implanted carcinoma MB49 [162]. Other delocalized lipophilic cations were also shown to increase the sensitivity of carcinoma cells to radiation and photodynamic therapy [163,164]. However, sufficient bioavailability of these agents can hardly be achieved because of their poor solubility [165].

Thus, even at a very low salt concentration, the formation of large DQA aggregates is observed in a sonicated solution of DQA at 10 to 100 mM concentration range. To overcome solubility problems of DQA, an attempt was made to incorporate DQA into liposomes and micelles made of PEG [5000]–DSPE [161]. In the absence of NaCl, up to 50 mol% DQA could be incorporated into small 50–100 nm phosphatidylcholine (PC) liposomes made by probe sonication. However, other liposome preparation methods as well as the presence of salt preclude the formation of DQA-loaded liposomes. This indicates that the association of DQA with the bilayer membranes is not stable. In striking contrast to liposomes, micelles made from PEG(5 kDa)–PE effectively incorporate DQA in physiological NaCl solution, and the resulting micelles contain ca. 30 mol% DQA. Fig. 14 represents a hypothetical structure of PEG–PE micelles with incorporated DQA. DQA is a dicationic compound resembling bola-form electrolytes, that is, it has a symmetrical molecule with two charge centers separated at a relatively large distance. This bola-like molecule might be able to bend in the middle and incorporate into the micelle's core. So, in certain situations micelles and not liposomes may appear to be carriers of choice for some poorly soluble compounds. This might be possible due to more favorable orientation of a drug molecule within the carrier particle.

4.5. Accumulation of protein-loaded micelles in tumor

In other cases, however, it is the small size of micelles which makes them superior compared to other nanoparticulates including liposomes. Thus, the

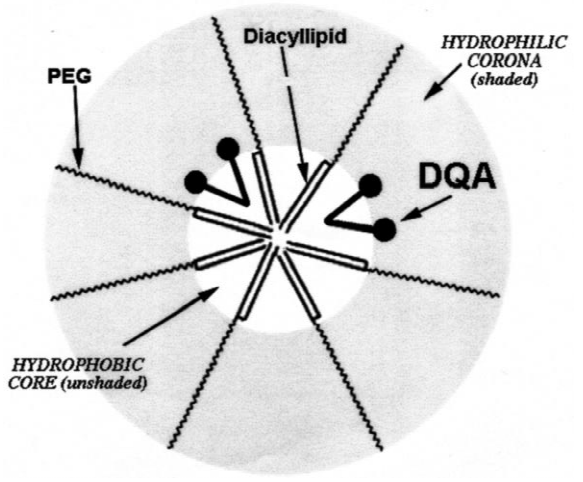


Fig. 14. Hypothetical structure of DQA incorporated into PEG-based micelles. From [161].

use of PEG–PE micelles for the effective delivery of a model protein drug to a solid tumor, Lewis lung carcinoma, in mice was recently reported [166]. 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 [167,168]. Diffusion and accumulation parameters were recently shown to be strongly dependent on the cutoff size of tumor blood vessel wall, and the cutoff size varies for different tumors [168–170].

In a general case, the biodistribution of a microparticulate carrier-associated anticancer drug depends on its circulation time in blood (summarized in Ref. [8], using liposomes as an example). Thus, it was repeatedly confirmed that long-circulating PEG-grafted liposomes demonstrate an increased accumulation in implanted tumors [171]. Later, however, it was found that in some cases even the use of long-circulating liposomes could not provide their sufficient accumulation in certain tumors. A recent report [172] has shown that coating 100 nm liposomes with PEG did not result in an increased accumulation of liposome-encapsulated drug in a subcutaneously established murine Lewis lung carcinoma. This phenomenon may be explained by the low vascular permeability (small cutoff size) of this as well as some other tumors. In those cases, drug carriers smaller in size than liposomes may provide more efficient drug delivery into tumors. 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 [166].

To achieve STI incorporation into liposomes and micelles, the protein was made hydrophobic by modifying it with *N*-glutaryl-PE (NGPE). Animal experiments with radiolabeled STI-loaded liposomes and micelles in Lewis lung carcinoma-bearing mice revealed different biodistribution patterns for all tested preparations — free STI, STI-liposomes and STI-micelles. The native protein demonstrates the expected fastest disappearance from the blood and the maximum accumulation in the liver and spleen. Both micelles and liposomes demonstrate clear longevity in the blood, however, liposomes seem to stay in the circulation longer than micelles. Thus, after 1 h, blood concentration of liposomes is still ca. 75% dose/g, but only ca. 30% dose/g for micelles. The longevity of PEG-micelles and PEG-liposomes, together with their slow clearance via liver and spleen, naturally results in their improved accumulation in the tumor compared with the native STI. The most important finding, however, is that despite somewhat faster blood clearance, micelles still accumulate in the tumor significantly better than liposomes, delivering more protein there (see Fig. 15).

This result confirms that the efficacy of passive delivery to solid tumors is not only controlled by the exposure level around sites of extravasation but also by the more complex relationship between the tumor's microvascular permeability and the size of a drug carrier. The results discussed suggest that in certain tumors (such as subcutaneously established

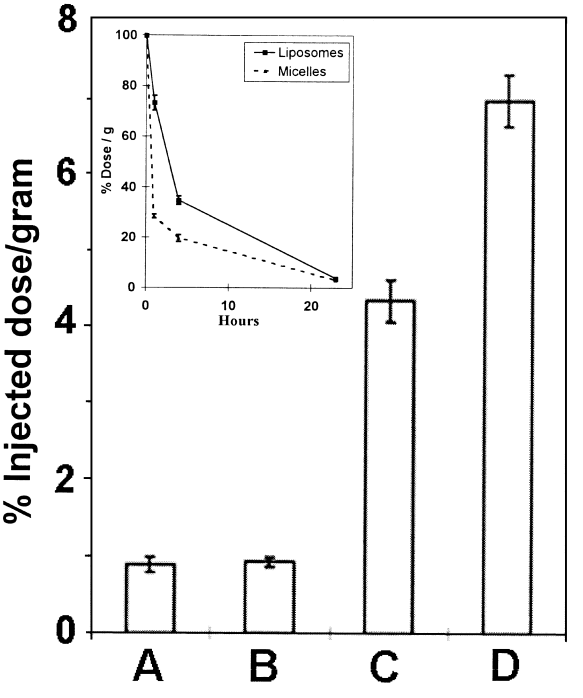


Fig. 15. Tumor accumulation 23 h after i.v. injection of native STI (A), NGPE-DTPA-STI (B), NGPE-DTPA-STI anchored in 100 nm long-circulating liposomes (C) and micellar-bound NGPE-DTPA-STI (D). Insert: Blood clearance of NGPE-DTPA-STI anchored in long circulating liposomes and PEG5000-DSPE micelles. From [166].

murine Lewis lung carcinoma) small-size micelles provide a better delivery of a drug (protein) than larger long-circulating liposomes.

5. Polymeric micelles in medical diagnostic imaging

5.1. Diagnostic imaging and imaging modalities

Another emerging area of using polymeric micelles as carriers for pharmaceuticals is medical diagnostic imaging. Currently used medical imaging modalities include: (a) Gamma-scintigraphy (based on the application of gamma-emitting radioactive materials); (b) Magnetic resonance (MR, phenomenon involving the transition between different energy levels of atomic nuclei under the action of radiofrequency signal); (c) Computed Tomography (CT, the modality utilizing ionizing radiation — X-rays — with the aid of computers to acquire cross-images of the body and three-dimensional images of areas of interest); (d) Ultra-sonography (the modality utilizes irradiation with ultrasound and is based on the different passage rate of ultrasound through different tissues). All four imaging modalities differ in their physical principles, sensitivity, resolution (both spatial and temporal), ability to provide images without contrast agent-mediated enhancement, and some other parameters, such as cost and safety.

Whatever imaging modality is used, medical diagnostic imaging requires that the sufficient intensity of a corresponding signal from an area of interest be achieved in order to differentiate this area from surrounding tissues. Usually, the imaging of different organs and tissues for early detection and localization of numerous pathologies cannot be successfully achieved without appropriate contrast agents (see further) in different imaging procedures. The contrast agents are specific for each imaging modality, and as a result of their accumulation in certain sites of interest, those sites may be easily visualized when the appropriate imaging modality is applied [173]. Different chemical nature of reporter moieties used in different modalities and different signal intensity (sensitivity and resolution) require various amounts of a diagnostic label to be delivered into the area of interest, and tissue concentrations that must be achieved for successful imaging vary between diagnostic moieties in broad limits (Table 2). Although rather low in the case of gamma-imaging, it is pretty high for MRI and CT.

To reach the required local concentration of a contrast agent, it was a natural progression to use microparticulate carriers for an efficient delivery of contrast agents selectively into the required areas. Fig. 16 demonstrates the principal scheme of micelle formation and its loading with various reporter moieties which might be incorporated into different compartments of this particulate carrier.

5.2. Loading of micelles with diagnostic agents for gamma and magnetic resonance imaging

The micellar transport of contrast agents is a relatively new field [35,174]. Apart from the practical value for diagnosis itself, this area has significant importance for delivery of drugs as it allows visualization of the exact sites of the drug carrier deposition within the body with extraordinary anatomical resolution. Chelated paramagnetic metal moieties (Gd, Mn or Dy aqueous ions) represent the major interest for the design of MR positive (T1) contrast agents. Different ways have been used to prepare contrast micelles for this purpose. Thus, for example, mixed micelles obtained from monoolein and taurocholate with Mn-mesoproporphyrin, were shown to be a potential oral hepatobiliary imaging agent for T1-weighted MRI [175]. Often, chelated metal ions

Table 2
Contrast agent concentration required for tissue attenuation in various imaging modalities

Modality	Reporter group	Required tissue concentration
Gamma-scintigraphy	radionuclide	10^{-10} M
MR imaging	paramagnetic metal	10^{-4} M
CT imaging	iodine	10^{-2} M

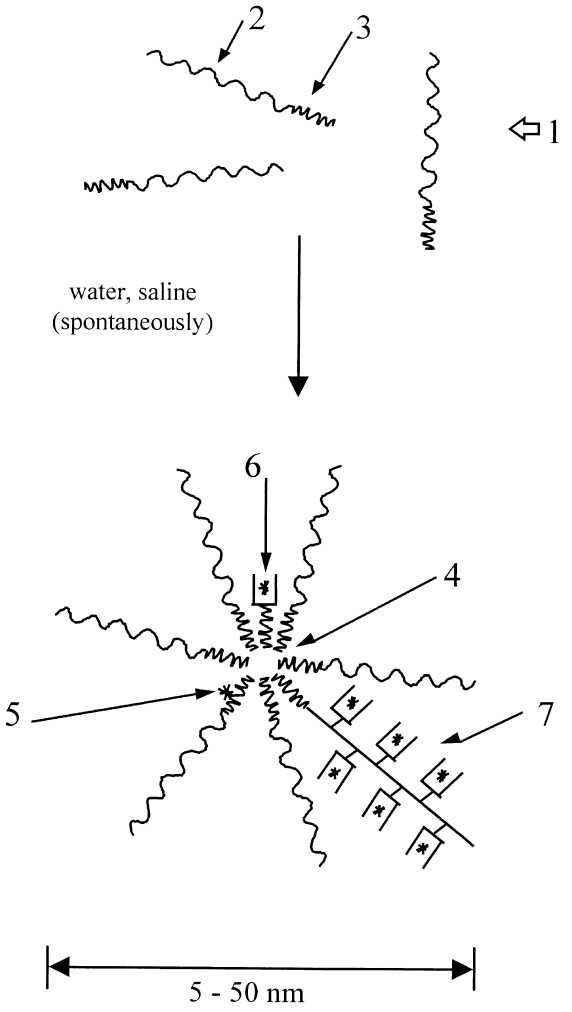


Fig. 16. Schematic structures of micelle formation and loading with a contrast agent. The micelle is formed spontaneously in aqueous media from amphiphilic compound (1) the molecule of which consists of distinct hydrophilic (2) and hydrophobic (3) moieties. Hydrophobic moieties form the micelle core (4). Contrast agent (*; gamma- or MR-active metal-loaded chelating group, or heavy element, such as iodine or bromine) can be directly coupled to the hydrophobic moiety within the micelle core (5), or incorporated into the micelle as an individual monomeric (6) or polymeric (7) amphiphilic unit. From [188].

possess a hydrophilic character: complex of diethylenetriamine pentaacetic acid (DTPA) with Gd^{3+}, for example, has a water molecule bound directly to the metal coordination sphere [176]. In order to be incorporated into micelles, such structures should acquire amphiphilic nature. Several chelating probes of this type have been developed earlier for liposome membrane incorporation studies, such as DTPA-PE [177], DTPA-stearylamine, DTPA-SA [178], and amphiphilic acylated paramagnetic complexes of Mn and Gd [179]. In these agents a hydrophilic chelating residue is covalently linked to a hydrophobic (lipid) chain. The lipid part of the molecule can be anchored either in the liposome bilayer or the micelle's hydrophobic core, while a more hydrophilic chelate is localized on the liposome surface or hydrophilic shell of the micelle (see Fig. 16). Same or similar chelates can be used for loading micelles with heavy radiometals (such as 111-In) for gamma-imaging.

A possible route to improve the efficacy of liposomes and micelles as contrast mediums is to increase the quantity of carrier-associated reporter metal (such as Gd or ^{111}In), and to enhance thus the signal intensity. We have tried to solve this task by using so-called polychelating amphiphilic polymers or PAPs [180]. The basic idea behind the new generation of microparticulate contrast agents was to increase the number of chelated metal atoms attached to a single lipid anchor, capable of being incorporated into the liposomal membrane or into the micelle core. Pursuing this way one might drastically increase the number of bound reporter metal atoms per vesicle which allows, in turn, to decrease the dosage of an administered lipid without compromising the image signal intensity.

To increase the loading of micelles (as well as liposomes that are also successfully used as carriers for imaging agents) with reporter metals, we have suggested and experimentally designed a new family of amphiphilic polymers containing multiple chelating groups and suitable for incorporation into liposomes and micelles. Polymer-attached chelating moieties provide high-affinity binding of the wide range of heavy metal ions, e.g., Fe, In, Tc, Re, Ga, Gd, Mn, Eu, Y, Bi, At, Sm, etc. The polymeric backbone for the attachment of multiple chelating moieties has to contain a sufficient number of reactive groups, such as amino-, carboxy-, aldehyde-, or SH-groups. Both natural and synthetic polymers can be used for this purpose, poly-L-lysine (PLL) containing multiple free amino-groups being the most frequent choice. To couple the chelator to a PLL, the reactive

intermediate of the chelator is usually used, containing mixed anhydride, cyclic anhydride, *N*-hydroxysuccinimide ester, tetrafluorophenyl ester, isothiocyanate, etc. The currently used optimized approach to synthesize polychelating polymers [181] is based on the use of CBZ-protected PLL with free terminal amino-group, which is derivatized into a reactive form with subsequent deprotection and incorporation of chelating DTPA residues, and provides an efficient heavy metal load on proteins and antibodies.

Developing the polylysine *N*-terminus modification chemistry further, we suggested a pathway for the synthesis of amphiphilic polychelator *N*,α-(DTPA-polylysyl)glutaryl phosphatidyl ethanolamine (DTPA-PLL-NGPE). This polychelator easily incorporates into the micelle core in the process of liposome of micelle preparation, and sharply increases the number of chelated Gd atoms attached to a single lipid anchor. The reaction scheme for the synthesis of the polychelating amphiphilic polymers is presented in Fig. 17.

After the saturation of polychelator with Gd^{3+} ions, the elemental analysis has revealed that Gd-DTPA-PLL-NGPE contains ca. 40% (w/w) Gd, which corresponds to 8–10 metal atoms per single lipid-modified polymer molecule assuming its molecular weight to be 3500–4000 Da. The higher Gd content leads to the better relaxivity and, consequently, to the greater MR signal intensity (assuming the Gd tissue concentration does not exceed millimolar range). Additionally, upon the incorporation into micelle core, the NGPE anchor grafted with a short-chain PAP keeps chelating groups within the micelle's corona. Metal atoms chelated into these groups are directly exposed to the exterior water environment and have better access to the adjacent tissue water protons. This might lead to the corresponding enhancement of the vesicle contrast properties. As a result, amphiphilic polychelator-containing micelles have a higher relaxation influence on water protons compared with conventional preparations at the same phospholipid content. In addition to the enhanced relaxivity, the micelle corona formed by PEG polymer can help in avoiding the contrast agent (both for gamma and MR imaging) uptake in the site of injection by resident phagocytic cells. The final preparations of contrast-loaded micelles are quite stable, and can serve as fast and efficient agents for scintigraphy or MR imaging.

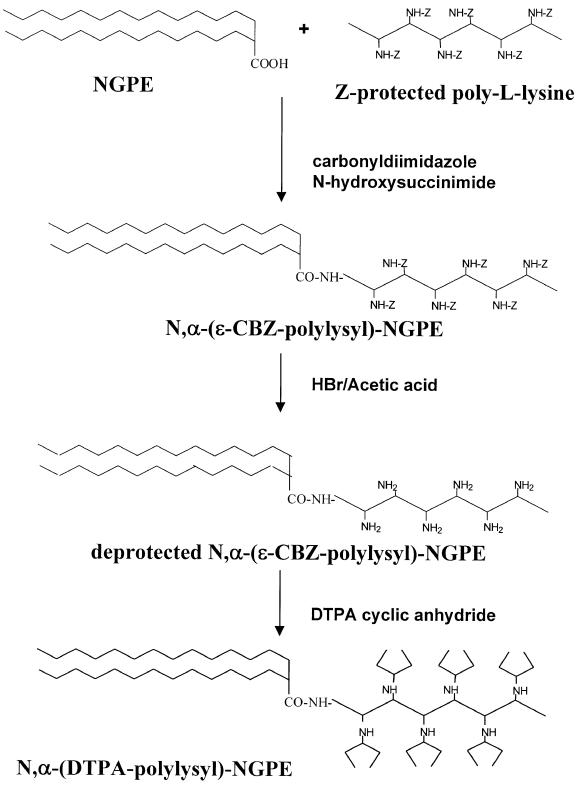


Fig. 17. Chemistry of polymeric chelates for loading micelles with multiple reporter metal atoms. Synthesis of amphiphilic DTPA-PLL-NGPE consisting of hydrophilic DTPA-polylysyl moiety and hydrophobic *N*-glutaryl phosphatidyl ethanolamine moiety.

5.3. In vivo gamma- and magnetic resonance visualization with micellar imaging agents

In experiments on lymphatic imaging [182], we have incorporated amphiphilic chelating probes Gd(111-In)-DTPA-PE and 111-In-DTPA-SA into PEG(5 kDa)–PE micelles (20 nm in diameter) and tried to use these particulate agents in experimental percutaneous lymphography using γ-scintigraphy and MR imaging in rabbits. As a result, we were able to demonstrate the localization of 111-In-labeled DTPA-SA/PEG(5 kDa)–PE micelles in local lymphatics after subcutaneous administration of a 20

μCi dose into the dorsum of a rabbit hindpaw. The popliteal lymph node can be visualized within seconds after injection. It was shown that the micelles as smaller particles exhibit higher accumulation in the primary lymph node.

As the above data demonstrates, the micellar particulates due to their size and surface properties can be moved with ease from the injection site along the lymphatics to the systemic circulation with the lymph flow. Fig. 18 shows T1-weighed transverse MR images of the axillary/subscapular lymph node area in rabbit obtained after subcutaneous injection of small doses of Gd-DTPA-PE/PEG(5 kDa)–PE micelles. The collecting lymph vessel (B) and axillary lymph node (C) become visible only 4 min after administration of Gd-containing micelles. Taking into account the ease and speed with which the primary lymph nodes may be visualized, one can suggest that unlike the other lymphotropic contrast media, polymeric micelles are lymphangiographic in nature. Their action is based on the visualization of lymph flowing through different elements of the lymphatics. The action of other lymphotropic contrast media is based primarily on their active uptake by the nodal macrophages (i.e. lymph nodes become visible only after the concentration of the medium in the lymph node has achieved a certain threshold value, which usually requires prolonged periods of time). Similar experiments with PEG–PE mixed micelles with a core-incorporated amphiphilic 111-In- or Gd-loaded PAP have also demonstrated fast and efficient gamma and MR visualization of different compartments of the lymphatic system. Micelles mostly stay within lymph fluid rather than accumu-

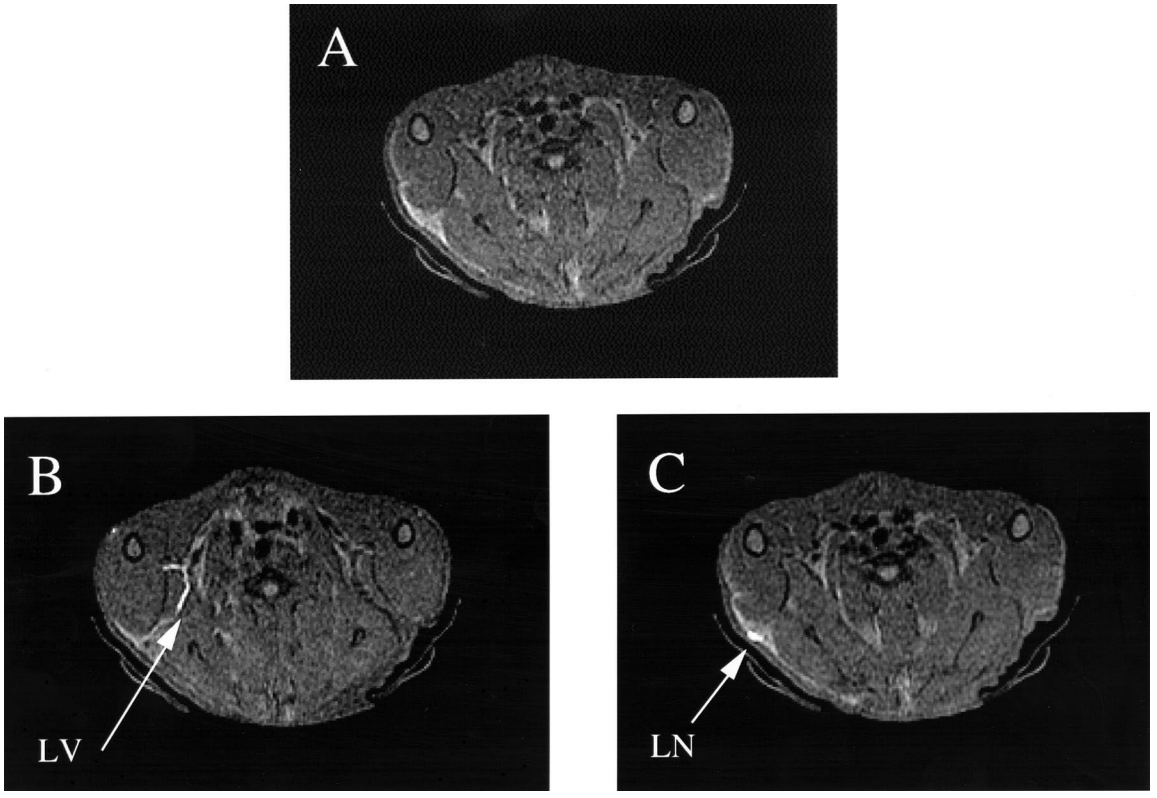
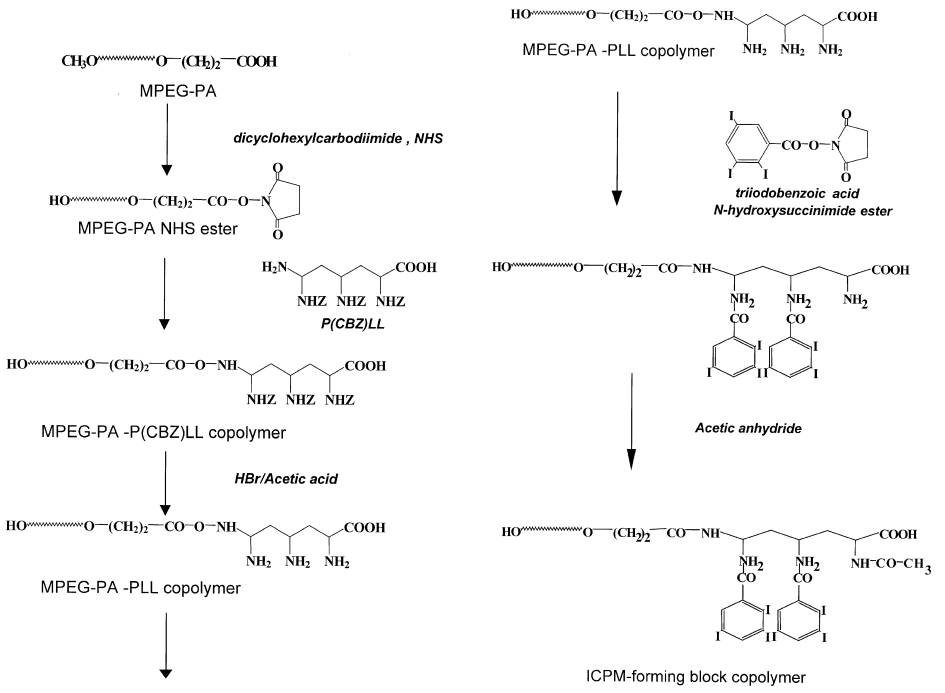
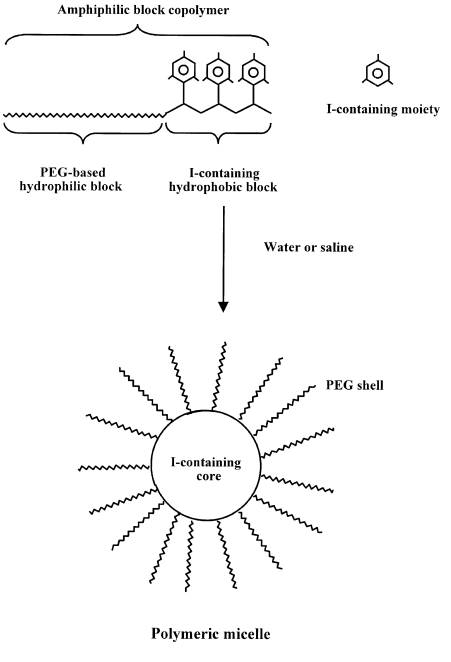


Fig. 18. Transverse MR images of axillary-subscapular lymph node area in the rabbit before (A) and 4 min after s.c. administration of PEG(5 kDa)–phosphatidyl ethanolamine micelles containing core-incorporated Gd-loaded amphiphilic chelate DTPA-phosphatidyl ethanolamine (B,C). The dose was 0.5 μmol Gd per injection site. Fast and clear visualization of both lymph vessel (LV) and lymph node (LN) can be seen. Images were acquired using 1.5 Tesla GE Sigma MRI scanner operated at fat suppression mode and T1-weighted pulse sequence. From [182].



(a)



(b)

Fig. 19. Preparation of iodine-containing polymeric micelles (ICPM) for blood pool CT imaging. A: Synthesis of ICPM-forming block co-polymer from methoxy-PEG-propionic acid; MPEG-PA, and poly(CBZ)-L-lysine; P(CBZ)LL. B: Scheme of micelle formation. From [67].

late in the nodal macrophages (because of the protective effect of surface PEG fragments) and rapidly move via the lymphatic pathway. They can serve as fast and efficient lymphangiographic agents for scintigraphy or MR imaging.

5.4. Diagnostic iodine-containing polymeric micelles for computed tomography imaging of the blood pool

Blood pool imaging is of special interest for the evaluation of the current state of blood flow and for the discovery of irregularities caused by atherosclerotic lesions, thrombi or tumors. Blood pool imaging requires prolonged circulation of contrast agents and is usually based on the utilization of sterically-protected polymer-modified microparticulates with contrast properties.

Computed Tomography (CT) represents an imaging modality with high spatial and temporal resolution. The diagnostic value of CT might be further significantly increased when contrast agents (i.e. substances containing X-ray absorbing heavy elements, such as iodine) are used to attenuate tissues and organs of interest. Since, as was already told, providing diagnostically acceptable imaging requires the iodine concentration on the order of millimoles per ml of tissue [183], large doses of low-molecular-weight CT contrast agent, such as iodine-containing organic molecules, are normally administered to patients. In order to more specifically target contrast agents, attempts have been made to use contrast agent-loaded microparticulate carriers for CT imaging [184–186]. However, currently suggested particulate contrast agents possess relatively large particle size (between 0.25 and 3.5 μm) and are actively cleared by phagocytosis. Thus, in order to prepare a material whose distribution is limited to the blood pool, certain simple properties seem necessary to be met: a size larger than fenestrated capillaries (>10 nanometers), resistance to phagocytosis, and the radiopaque moiety structurally incorporated within the particulate.

Amphiphilic polymers which are able to spontaneously form stable and long-circulating 10 to 80 nm micelles in an aqueous media seem to be carriers of choice which meet all the above mentioned requirements [35]. Recently, we have described some results on the synthesis and in vivo properties of a block-copolymer of methoxy-poly(ethylene glycol) (MPEG) and iodine-substituted poly-L-lysine [67,187]. This copolymer easily micellizes in the solution forming stable and heavily iodine-loaded particles (up to 35% of iodine by weight) with a size well below 100 nm. The synthesis of MPEG-propionic acid(PA)-poly[ε,*N*-(2,3,5-triiodobenzoyl)]-L-lysine (MPEG-iodolysine) block-copolymer is shown in Fig. 19A. First, MPEG-PA NHS ester was prepared from MPEG-PA, NHS and dicyclohexylcarbodiimide, and than converted into MPEG-PA-poly-(CBZ)lysine diblock copolymer by reaction with poly(CBZ)lysine and triethylamine. The product of this reaction was 'deprotected' by removing CBZ-groups with hydrogen bromide in acetic acid. Then, 2,3,5-triiodobenzoic acid NHS ester was prepared by reacting 2,3,5-triiodobenzoic acid, dicyclohexylcarbodiimide and NHS. To prepare MPEG-PA-poly-[ε,*N*-(2,3,5-triiodobenzoyl)]-L-lysine, both synthesized components were conjugated in the presence of triethylamine. The formation of a polymeric micelle with the iodine-containing core and hydrophilic corona consisting of PEG chain is schematically shown in Fig. 19B. According to size measurement data, the prepared iodine-containing micelles had a size between 50 and 70 nm. The iodine content in these micelles is about 30 to 35% of total weight. Other amphiphilic polymeric micelles formed by

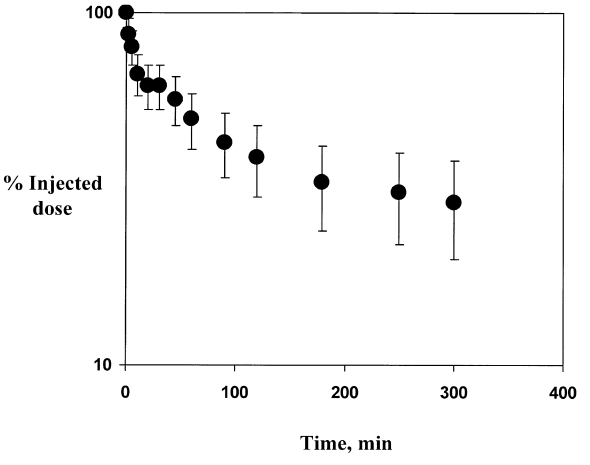


Fig. 20. The clearance of ICPM from the blood of experimental rats. Iodine-containing micelles labeled with 111-In-DTPA-SA were injected intravenously at a dose of 170 mg I/kg.

block copolymers have sizes around 10–50 nm [49,51]. The slightly increased particle size of MPEG-iodolysine micelles may be explained by non-covalent binding of triiodobenzoic acid to the core of the micelle.

In vivo, the iodine-containing polymeric micelles demonstrate prolonged circulation time (the data on their clearance from the blood of experimental rats are presented in Fig. 20). Since such micelles still are able to dissociate slowly into unimers, their components are eventually removed from the body via both the reticuloendothelial system and via the kidney with urine [182]. To prove the diagnostic utility of this family of polymeric micelles, the micellar CT contrast agent was injected intravenously into rats and rabbits, and the X-ray signal was

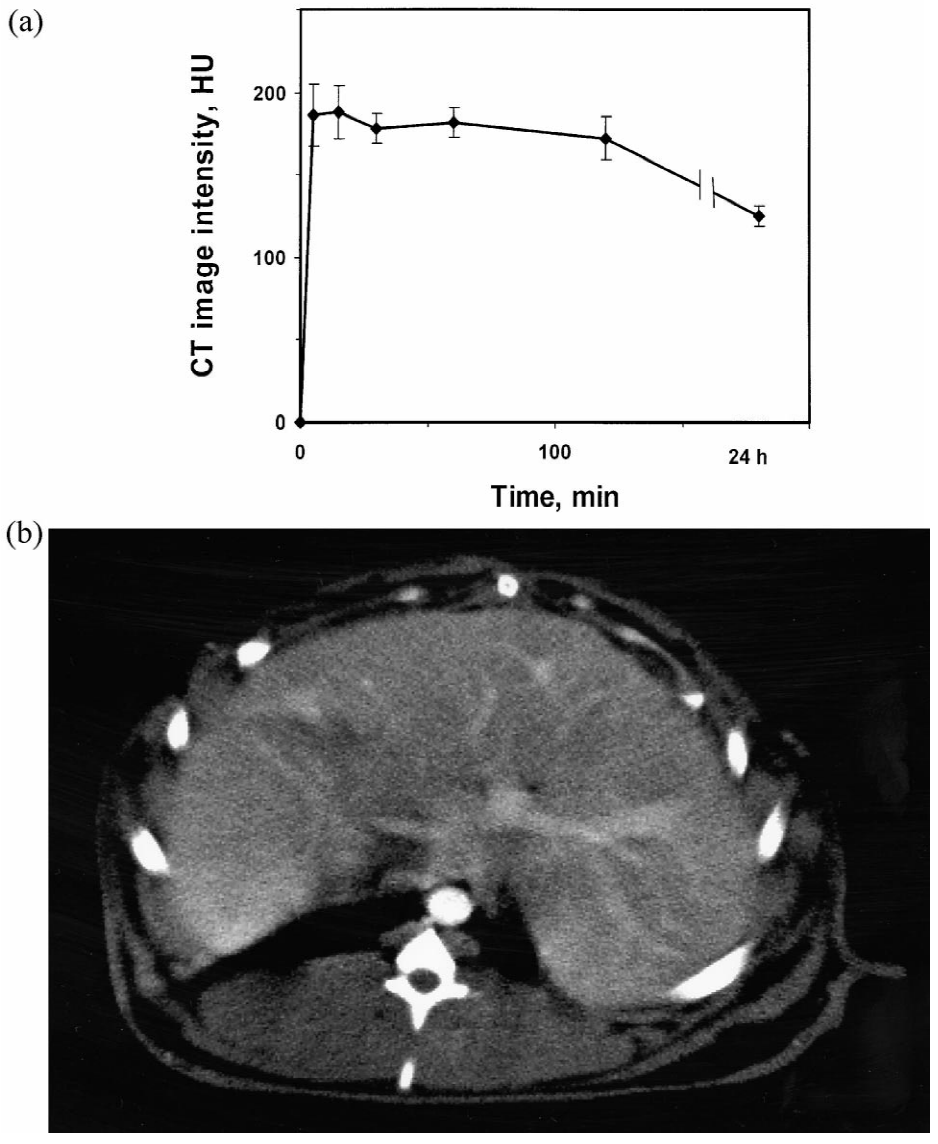


Fig. 21. A: CT signal intensity (Hounsfield units) from the rabbit blood pool (registered at the ventricular area) after i.v. administration of iodine-containing polymeric micelles (250 mg Iodine/kg). B: CT section of the rabbit liver 2 h after i.v. administration of ICPM; vascular network is still clearly visualized.

monitored from several organs using a CT scanner. A significant enhancement of 3 to 4-fold in the blood pool (aorta and heart) was visually observed in rats and rabbits for at least a period of 2 h following the injection. The quantification of some of these images with the help of image-processing software showed no sign of a decrease in the blood opacification during at least a 3-h period following the injection (see the data from the rabbit experiment in Fig. 21). A similar pattern was also observed in rats. Micelles with such properties might find a certain clinical utility for the diagnostic CT imaging of the blood pool. The clinical utility of selective blood-pool contrast agents may be variable: minimally invasive angiography, image guidance of minimally invasive procedures, oncologic imaging of angiogenesis, ascertaining organ blood volume tomographically, and identifying haemorrhage are applications that could benefit from a long-lived intravascular agent.

5.5. Concluding remarks on the diagnostic application of polymeric micelles

Perhaps the key benefit of micelle-based diagnostic is the ability to 'image' (i.e., to measure) the functional characteristic of a tissue or other region of interest. As long as an understanding of the characteristics of the microparticulate carrier and how it interacts with the biological milieu exists, it should be possible to design diagnostics that make these functional measurements possible. The data obtained so far lead to a definite conclusion that contrast-loaded micelles may be and will be successfully used for visualization of numerous organs, tissues, and disease sites in all imaging modalities. Gamma-imaging with radiometal-loaded micelles seems to be the most attractive because of relatively small quantities of both carrier and reporter metal needed for successful imaging, which greatly minimizes the problems of side-effects. By using polymeric chelates, micelles can be easily loaded with sufficient quantities of radioactive (for gamma-imaging) or MR-active (for MRI) agents. Additionally, PEG forming the corona of the micelle can serve as a capture-avoiding agent permitting effective accumulation of the diagnostic label in the target and also for improvement of MR contrast properties. The same properties make iodine-containing polymeric micelles promising candidates for CT imaging of the blood compartment.

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