



ELSEVIER

Biomaterials 22 (2001) 3035–3044

Biomaterials

www.elsevier.com/locate/biomaterials

Amphiphilic poly-*N*-vinylpyrrolidones: synthesis, properties and liposome surface modification

V.P. Torchilin^{a,*}, T.S. Levchenko^a, K.R. Whiteman^a, A.A. Yaroslavov^b, A.M. Tsatsakis^c,
A.K. Rizos^b, E.V. Michailova^d, M.I. Shtilman^d

^aDepartment of Pharmaceutical Sciences, Bouve College of Health Sciences, Northeastern University, Mugar Building, Room 312, 360 Huntington Avenue, Boston, MA 02115, USA

^bDepartment of Polymers, M.V. Lomonosov Moscow State University, Moscow 119899, Russia

^cDepartment of Medicine, University of Crete, Crete 117813, Greece

^dDepartment of Polymers, D.I. Mendeleev University of Chemical Technology, 9 Miusskaya Square, Moscow 125047, Russia

Received 1 November 2000; accepted 25 January 2001

Abstract

Certain amphiphilic water-soluble polymers including amphiphilic derivatives of polyvinyl pyrrolidone (PVP) were found to be efficient steric protectors for liposomes *in vivo*. In this study, we have tried to develop synthetic pathways for preparing amphiphilic PVP and to investigate the influence of the hydrophilic/hydrophobic blocks on some properties of resulting polymers and polymer-coated liposomes. To prepare amphiphilic PVP with the end stearyl (S) or palmityl (P) residues, amino- and carboxy-terminated PVP derivatives were first synthesized by the free-radical polymerization of vinyl pyrrolidone in the presence of amino- or carboxy-mercaptans as chain transfer agents, and then modified by interaction of amino-PVP with stearyl chloride or palmitoyl chloride, or by dicyclohexyl carbodiimide coupling of stearylamine with carboxy-PVP. ESR-spectra of the hydrophobic spin-probe, nitroxyl radical *N*-oxyl-2-hexyl-2-(10-methoxycarbonyl)decyl-4,4'-dimethyl oxazoline, in the presence of amphiphilic PVP demonstrated good accessibility of terminal P- and S-groups for the interaction with other hydrophobic ligands. Spontaneous micellization and low CMC values (in a low μ molar range) were found for amphiphilic PVP derivatives using the pyrene method. In general, S-PVP forms more stable micelles than P-PVP (at similar MW, CMC values for S-PVP are lower than for P-PVP). It was found that amphiphilic PVP incorporated into negatively charged liposomes effectively prevents polycation (poly-ethylpyridinium-4-vinylchloride)-induced liposome aggregation, completely abolishing it at ca. 10 mol% polymer content in liposomes. Additionally, the liposome-incorporated PVP prevents the fluorescence quenching of the membrane-incorporated hydrophobic fluorescent label [*N*-(4-fluoresceinthiocarbonyl)dipalmitoyl-PE] by the free polycation. PVP-modified liposomes were loaded with a self-quenching concentration of carboxyfluorescein, and their destabilization in the presence of mouse serum was investigated following the release of free dye. Amphiphilic PVP with MW between 1500 and 8000 provides good steric protection for liposomes. The degree of this protection depends on both polymer concentration and molecular size of the PVP block. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Liposomes; Steric protection; Amphiphilic poly(vinyl pyrrolidone); Micellization; Fluorescence quenching; Liposome stability in serum

1. Introduction

Long-circulating pharmaceutical carriers currently represent a fast growing area of biomedical research [1–3]. Long-circulating drug carriers may be used to maintain a required level of a pharmaceutical agent in

the blood for extended time intervals for better drug bioavailability. They can slowly accumulate (the so-called “enhanced permeability and retention, EPR, effect” or “passive” targeting) in pathological sites with affected and leaky vasculature (such as tumors, inflammations, and infarcted areas) and enhance drug delivery in those areas [4–6]. This approach led to a clear success in clinical cancer treatment with doxorubicin-loaded long-circulating liposomes [7–9]. Prolonged circulation also results in an improved targeting effect for targeted (specific ligand-modified) drug carriers, especially in

* Corresponding author. Tel.: + 1-617-373-3206; fax: + 1-617-373-8886.

E-mail address: vtorchil@lynx.neu.edu (V.P. Torchilin).

cases when the target has diminished blood supply (ischemic or necrotic areas) and/or low concentration of the target antigen [10,11].

Liposomes (artificial phospholipid vesicles with varying size) have been widely considered as promising drug carriers for many years [12,13]. Surface modification of liposomes is used to improve various physico-chemical properties of liposomes and to make them more stable in the biological milieu. To impart *in vivo* longevity to liposomes, chemical modification with certain synthetic polymers is usually applied [14,15]. Polymers have been shown to protect individual molecules and solid particulates from interaction with different solutes. This phenomenon relates to the stability of various aqueous dispersions [16]. In particular, hydrophilic fragments of liposome-attached polymers are exposed in the solution and protect liposomes from interactions with plasma proteins in the blood [17]. The term “steric stabilization” has been introduced to describe the phenomenon of polymer-mediated protection [18].

One of the most popular and successful methods to obtain long-circulating biologically stable liposomes is their coating with poly(ethylene glycol), or PEG [19–23]. To make PEG capable of incorporation into the liposomal membrane, the reactive derivative of hydrophilic PEG is single terminus-modified with a hydrophobic moiety (usually, the residue of PE or long chain fatty acid) [19,24]. The mechanism of PEG protective action was thoroughly investigated [25–28]. On the biological level, coating liposomes with PEG sterically hinders interactions of blood components with the liposome surface [26,29]. This prevents adsorption of opsonins onto liposomes and fast capture of liposomes by the RES (reticulo-endothelial system) [30]. The reduced binding of plasma proteins with PEG-liposomes was demonstrated in [31,32]. From the properties of colloids, it is known that repulsive interactions between colloidal particles can be enhanced by coating these particles with soluble, well hydrated, and chemically inert polymers [18]. Such modification might also decrease surface hydrophobicity and interaction of particles with the RES [33,34].

It was also demonstrated both theoretically and experimentally, that the important feature of protective polymers is their flexibility (short segment length and free rotation of individual polymer units around inter-unit linkages). The molecular mechanism of polymer protective action is determined by the properties of a flexible polymer molecule in solution and includes the formation of a polymeric layer over the liposome (particle, molecule) surface which is impermeable to other solutes even at relatively low polymer concentrations [35–37]. The more flexible the polymer, the larger the total number of its possible conformations and the higher the transition rate from one conformation to another. As a result, a water-soluble flexible polymer statistically exists as a distribu-

tion (“cloud”) of probable conformations. Thus, relatively small number of very flexible and water-soluble and polymer molecules can create sufficient density of conformational “clouds” over the liposome surface to prevent opsonization.

The fact that the absolute majority of research on long-circulating drugs and drug carriers was performed with the use of PEG as a sterically protecting polymer, can be explained by the very attractive combination of properties of PEG. The excellent solubility of PEG in aqueous solutions and its ability to bind a large number of water molecules, high flexibility of its polymer chain, very low toxicity, immunogenicity, and antigenicity, lack of accumulation in cells of the RES, minimal influence on specific biological properties of modified pharmaceuticals make PEG highly pharmacologically acceptable [38–40]. It is also important that PEG is not biodegradable and subsequently does not form any toxic metabolites. Currently, there are many chemical approaches to synthesize activated derivatives of PEG and to couple these derivatives with a variety of drugs and drug carriers including liposomes. The extensive review of these methods and their applicability towards different situations was done by Zalipsky [40,41].

Despite well developed chemistry of PEG coupling to pharmaceuticals, the search for alternative sterically protecting polymers is quite active. This might be explained by both the patent situation around PEG and its derivatives and the hope to get even better control over the properties of modified drugs and drug carriers. The suggested theoretical model for the protecting protein behavior and available experimental data (partially discussed above) permitted us to formulate some general requirements to polymers for steric protection of drugs and drug carriers. These polymers should be soluble, hydrophilic and have highly flexible main chain. Polymer biocompatibility has to be added to the list if polymer-liposomes are intended for medical use. Synthetic polymers of vinyl series, such as poly(vinyl pyrrolidone) (PVP) and poly(acryl amide) (PAA) may serve as the most evident examples of other potentially protective polymers [42,43]. Liposomes containing of distearoyl-PE covalently linked to poly(2-methyl-2-oxazoline) or poly(2-ethyl-2-oxazoline) also exhibit extended blood circulation time and decreased uptake by the liver and spleen [44]. A similar observation was made with phosphatidyl polyglycerols [45].

Similar to PEG, PVP has a long history of pharmaceutical application and demonstrates a high degree of biocompatibility [46,47]. Amphiphilic derivatives of PVP have been described containing phospholipid residues and long-chain acyls as hydrophobic groups and serving as efficient steric protectors for liposomes [42,43]. However, the choice of both the type of the hydrophobic group and MW of a polymer itself were usually quite arbitrary and based on the most general

consideration. In this study we made an attempt to develop various synthetic pathways for preparing amphiphilic PVP and to investigate the influence of hydrophobic and hydrophilic blocks on the properties of resulting polymers and their ability to provide steric protection to liposomes.

2. Experimental

2.1. Materials

N-Vinyl pyrrolidone (VP), mercaptoacetic acid, 2-mercaptoethylamine, *N,N'*-dicyclohexyl carbodiimide, stearoyl chloride, stearylamine, palmitoyl chloride, carboxyfluorescein (CF), octylglucoside (OG), Triton X-100, mouse serum, *N*-oxyl-2-hexyl-2-(10-methoxycarbonyl)decyl-4,4-dimethylloxazoline, azobisisobutyronitrile, pyrene, Sephadex G-25, and *N*-(4-fluoresceinthiocarbamoyl)-dipalmitoylphosphatidyl ethanolamine were obtained from Sigma-Aldrich (St. Louis, MO). Phosphatidyl choline (PC), cholesterol (Chol), and cardiolipin (CL) were from Avanti Polar Lipids Inc. (Alabaster, AL). All solvents and components of buffer solutions were analytical grade preparations.

2.2. Methods

2.2.1. Polymer synthesis

Similar to our previous experiments [43], amphiphilic poly(vinyl pyrrolidones) were synthesized in two steps. First, PVP with the terminal amino or carboxylic group was prepared, and then hydrophobic groups was attached to the reactive terminus of the PVP molecule.

2.2.1.1. PVP with single terminus amine or carboxylic group. The polymerization of VP was carried out under dry argon atmosphere in the dioxane solution (2.5 mol/l) in the presence of initiator, azobisisobutyronitrile, (0.06 mol/VP mol) for 1 h at 70°C. To prepare amino-terminated PVP (amino-PVP) with different MW, 2-mercaptoethylamine (0.03–0.1 mol/VP mol) was added to the system as a chain transferring agent. Alternatively, to prepare a carboxy-terminated PVP (carboxy-PVP) with different MW, variable quantities of mercaptoacetic acid (0.4–1.2 mol/VP mol) were used for chain transfer. The polymers formed were precipitated into diethyl ether, purified by repeated washing with ether, and dried in vacuum until a constant weight. The yield of different samples was between 70% and 90%.

MW values were determined by viscosimetry using an Ubbelodhe viscosimeter, and by HPLC with commercial PVP samples of known MW as standards. The carboxylic and amino groups in polymers were determined by potentiometric titration in alcoholic solutions.

2.2.1.2. Amphiphilic PVP with terminal stearyl or palmityl groups. To attach stearyl or palmityl groups to amino-PVP with the formation of S-PVP and P-PVP, respectively, ca. 1 g of a corresponding polymer was dissolved in 10 ml of dry dioxane, and then excessive amounts of triethylamine and stearyl chloride or palmitoyl chloride in the same solvent (5 ml) were added with stirring at 0°C. The mixture was allowed to stand at room temperature for 2 h. The mixture was then purified from any precipitate by filtration, and modified polymers were precipitated into diethyl ether, repeatedly washed with ether, and dried in vacuum until a constant weight. Alternatively, S-PVP may be prepared by the interaction of stearylamine with carboxy-PVP. For this purpose, the solution of carboxy-PVP in dry dioxane (1 mol/l) was supplemented with an excess of *N,N'*-dicyclohexyl carbodiimide in an equal volume of the same solvent. The mixture was stirred at 0°C for 1 h, and the excess of stearylamine dissolved in isopropanol was added. The mixture was incubated for 2 h at 60°C. The precipitated *N,N'*-dicyclohexylurea was filtered and the resulting polymer was precipitated into diethyl ether, washed with ether, and dried in vacuum. In all the experiments, the actual polymer yield was close to the theoretical yield.

2.2.2. ESR experiments

ESR measurements were performed with buffered aqueous solutions containing trace amounts of the spin label, *N*-oxyl-2-hexyl-2-(10-methoxycarbonyl)decyl-4,4-dimethylloxazoline, and a polymer. ESR spectra were obtained at room temperature with Bruker ELEXSYS X-band ESR spectrometer (Bruker, Holtsville, NY). The spectrometer was operated with a spin-ring resonator at about 9.8 GHz with 100 kHz field modulation.

2.2.3. Micelle formation from PVP polymers and CMC estimation

To prepare micelles, 1–5 μmol of a corresponding PVP derivative was sonicated in 1 ml of HBS with 0.2% Na azide, pH 7.4, at 10 W for 15 min using a Sonic Desmembrator 60 (Fisher Scientific, Pittsburgh, PA); cycle was repeated three times with 1 min intervals. Alternatively, the same quantity of PVP derivative was dissolved in dimethylsulfoxide, and the solution obtained was dialyzed overnight using dialyzing bag with 12,000–14,000 MWCO (Spectrum Laboratories Inc., Rancho Domingues, CA) against 6l of HBS buffer, pH 7.4 at 4°C. The presence of micelles and their size were registered using a Coulter N4+ Submicron Particle Analyzer (Beckman-Coulter, Fullerton, FL).

To estimate CMC values for different PVP derivatives, the method was used based on the solubilization of water-insoluble fluorescent dye, pyrene, in micelles. For this purpose, aliquots of 100 μl of pyrene solution in methanol (10 mg/ml) in test tubes were dried under vacuum. Then, 1.8 ml of HBS with 0.2% Na azide was added

to each tube with the dried pyrene. Next, the tubes were supplemented with 200 μ l of serial dilutions (10^{-4} – 10^{-10} M) of various S-PVP and P-PVP samples, and shaken overnight at room temperature. The samples were filtered through 0.2 μ m Nuclepore filter (Spectrum Laboratories Inc., Rancho Domingues, CA) to remove the non-solubilized pyrene, and the fluorescence intensity of solubilized pyrene was measured at an excitation wavelength of 339 nm and emission wavelength of 390 nm using an F-2000 Fluorescence Spectrophotometer (Hitachi Instruments Inc., San Jose, CA).

2.2.4. Liposome preparation

Negatively charged liposomes were prepared by detergent (OG) removal from a mixture of PC:Chol:CL (7:3:1 molar ratio). The starting lipid mixture in chloroform was argon-dried, vacuumed, solubilized with OG in HEPES-buffered saline (HBS), pH 7.4 (final total lipid concentration may vary from 5 to 20 mg/ml), and dialyzed overnight against HBS at 4°C. Liposomes obtained were sized by passing through Nuclepore polycarbonate filters (Spectrum Laboratories Inc., Rancho Domingues, CA) with 0.6, 0.4 and 0.2 μ m pore size. When necessary, the initial lipid mixture was also supplemented with various quantities of different S-PVP samples. Additionally, 0.5 mol% of the membrane fluorescent label, *N*-(4-fluoresceinthiocarbonyl)-dipalmitoyl phosphatidyl ethanolamine, was added to the mixture for the fluorescence quenching experiments.

Liposomes for serum-provoked destabilization experiments were prepared by sonication. A solution of a mixture of PC:Chol (7:3 molar ratio) in chloroform and a required quantity of S-PVP of a certain MW was dried under argon and additionally freeze-dried using a Freezone 4.5 Freeze Dry System (Labconco, Kansas City, MO). The film obtained was hydrated with 0.5 ml of 45 mM CF in phosphate-buffered saline (PBS, pH 7.4). After sonication using a bath sonicator (Laboratory Supplies Co. Inc., Hicksville, NY) for 10–40 min, the liposomes obtained were separated from unbound CF using column chromatography (Sephadex G-25; 0.8 \times 5.2 cm). Fractions of liposomes were pooled and dialyzed overnight at 4°C against PBS, pH 7.4.

For all polymer-containing liposomes, the efficacy of polymer incorporation into the liposomal membrane was monitored by HPLC in order to check for possible formation and presence of micelles formed by free non-incorporated polymer. The separation is based on the fact that the size of the micelles is usually within the 15–60 nm limits, whereas liposomes are ca. 200 nm in size.

The size of liposomes and liposomal aggregates (in aggregation experiments) was measured using a Coulter N4 + Submicron Particle Analyzer (Beckman-Coulter, Fullerton, FL).

2.2.5. Liposome fluorescence experiments

The changes in the fluorescence intensity of liposomes with the membrane-incorporated fluorescent label under the influence of a polycation, poly(ethylpyridinium-4-vinyl chloride), in the presence of and absence of S-PVP were registered using a F-2000 Fluorescence Spectrophotometer (Hitachi Instruments Inc., San Jose, CA).

2.2.6. Liposome permeability experiments

Liposomes loaded with CF (10–30 μ g PC/ml) were incubated in the presence of 50% mouse serum. The fluorescence intensity of the released CF was measured at an excitation wavelength of 339 nm and emission wavelength of 390 nm in the absence of and presence of Triton X-100 at different time points.

3. Results and discussion

3.1. Synthesis of functional PVP derivatives and modification of terminal reactive groups

The starting amino- and carboxyl-containing PVP derivatives for this study were prepared by free-radical polymerization of VP in dioxane in the presence of 2-mercaptoethylamine and mercaptoacetic acid, respectively, as chain-transferring agents. In the synthesis of functional VP polymers, the ability of mercaptans to transfer the chain during the radical attack provides an opportunity to introduce a functional group onto a single terminus of a polymeric chain. In this particular case, the mercaptans perform the chain transfer due to the formation of free radical produced by elimination of a hydrogen atom from a SH-group. The above polymerization technique can produce polymers with a single functional end group corresponding to the structure of the mercaptan radical [48,49]. For all systems studied, the presence of mercaptans within the used concentration range resulted, according to the titration data, in the formation of functional VP polymers. MW of the polymers depended on the concentration of the corresponding chain-transferring agent and decreased with an increase in the amount of mercaptan added to the system. As a result, we have prepared a whole set of amino-PVP and carboxy-PVP with MW values varying from ca. 700 to 20,000 Da.

The hydrophobic stearyl end groups were introduced into these polymers by direct reaction of amino-PVP with stearyl chloride, or by treating carboxy-PVP with stearylamine after preliminary activation of terminal carboxy groups with *N,N'*-dicyclohexylcarbodiimide (see the reaction scheme on Fig. 1). The residue of palmitic acid was attached to the functional end of PVP only by reacting palmitoyl chloride with amino-PVP. No differences in solubility of amphiphilic PVP derivatives prepared following different protocols were found, and similar to starting functional PVP samples, all

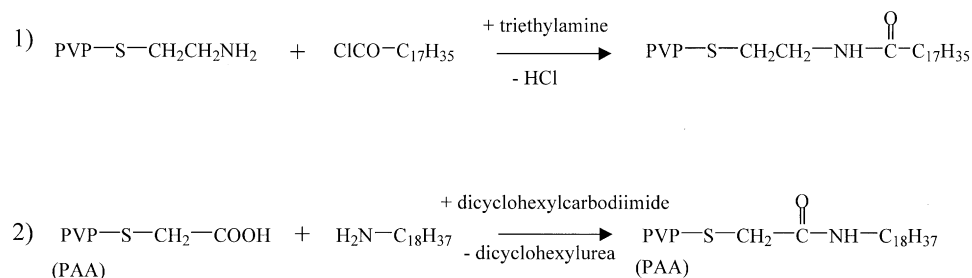


Fig. 1. Reaction schemes for the preparation of amphiphilic PVP from amino-PVP and carboxy-PVP. Stearyl derivatives are used as an example.

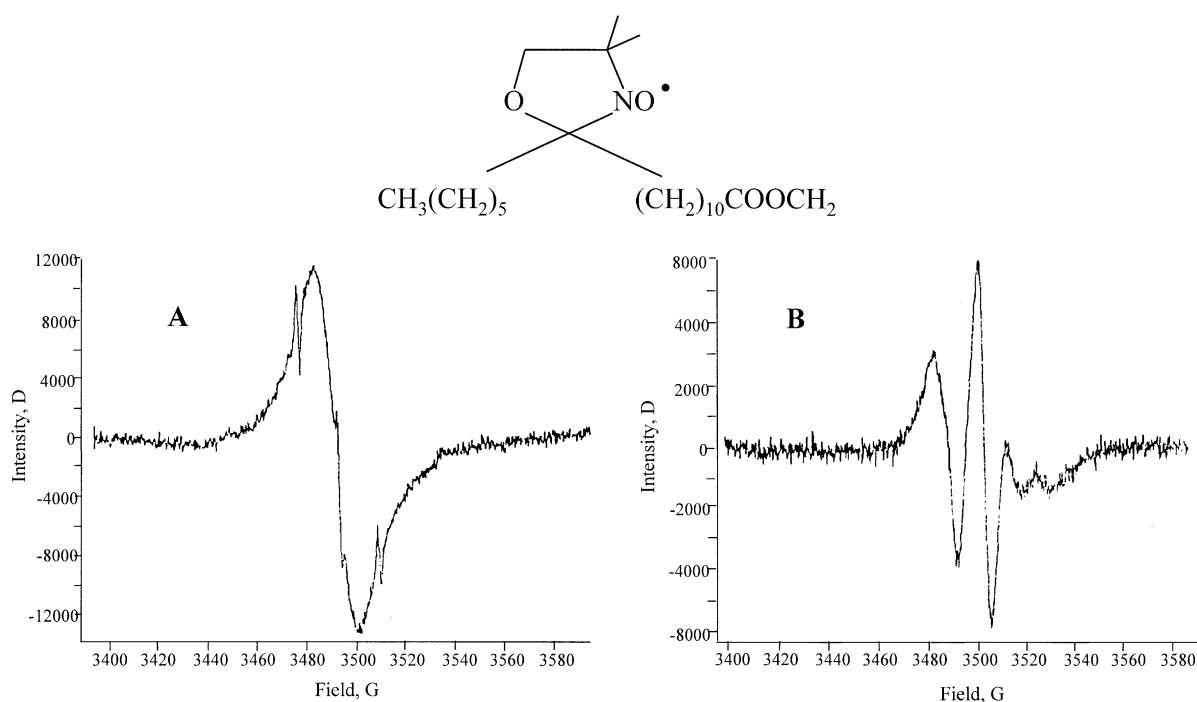


Fig. 2. Top panel — the structure of the spin probe; bottom panel — ESR-spectra of the spin probe in the presence of PVP with MW 3500 Da: (A) non-functionalized PVP; (B) S-PVP (4.0 wt% of PVP, 0.22 mM of spin probe). See Section 2.2 for details.

amphiphilic S-PVP and P-PVP samples were water-soluble, with the exception of polymers with the lowest MW values around 600 Da, which lost their solubility in water after the introduction of the hydrophobic end group.

3.2. Accessibility of the hydrophobic residue in amphiphilic PVP

Since amphiphilic PVP derivatives, especially those with higher MW values, contain significantly larger hydrophilic fragments than the hydrophobic ones, one can assume that hydrophobic fragments, minimizing their contacts with water, may “fold” themselves into the globule formed by the hydrophilic block. On the other hand, the accessibility of the hydrophobic unit for the interac-

tion with other hydrophobic molecules and groups is essential for the incorporation of amphiphilic PVP into the liposomal membrane. The accessibility of the hydrophobic fragments of amphiphilic PVP for other hydrophobic ligands was investigated following the interaction of various samples of amphiphilic PVP with the hydrophobic spin-probe, nitroxyl radical *N*-oxyl-2-hexyl-2-(10-methoxycarbonyl)decyl-4,4'-dimethyl oxazoline. Non-functional commercial PVP samples of similar MW were used as corresponding controls. ESR-spectra obtained with the use of the hydrophobic spin-probe demonstrated that, in all polymers, the terminal hydrophobic groups were accessible for interaction with this probe. The structure of the probe and typical ESR-spectra of this probe in the presence of S-PVP and non-functional PVP with MW ca. 3500 Da are shown on Fig. 2.

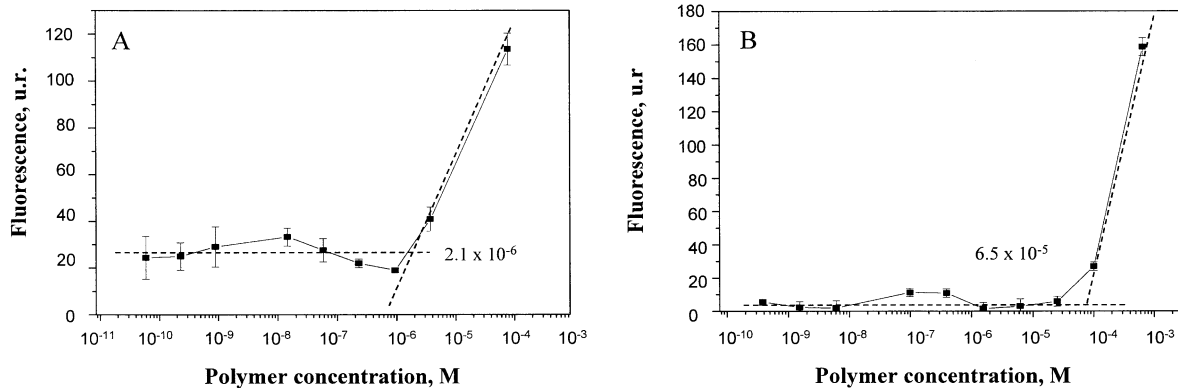


Fig. 3. Estimation of the CMC values for amphiphilic PVP (MW ca. 6000 Da) by pyrene method. (A) S-PVP; (B) P-PVP. See Section 2.2 for details.

It is clearly seen from these spectra that in the presence of non-functional PVP, the ESR-spectrum reflects the strong intermolecular interaction of nitroxyl radical molecules themselves that are present in the system as a separate phase distributed within the volume of the sample. In the presence of the amphiphilic PVP, however, the more complicated spectrum is observed, which can be attributed to the higher mobility of the spin-probe associated with the hydrophobic terminal group of the amphiphilic polymer. This result confirms the ability of amphiphilic derivatives of PVP to interact with other hydrophobic groups of lipids, and, consequently, to incorporate the lipid bilayer.

3.3. Micelle formation by amphiphilic PVP and corresponding CMC values

In an attempt to identify among the amphiphilic PVP derivatives those providing better (more firm) incorporation into the liposomal membrane, we have investigated the micelle-forming properties of the synthesized polymers and determined the corresponding CMC (critical micelle concentration) values for these polymers. The study was performed assuming that lower CMC values evidence stronger interaction of terminal hydrophobic groups with each other, i.e. the better potential ability to incorporate the liposomal membrane. With this in mind, the spontaneous micellization of different P-PVP and S-PVP samples in water solutions was investigated by particle size measurements, and the corresponding CMC values were determined by the pyrene method. Since the micellization of various PVP derivatives itself constitutes a rather interesting subject for discussion, here, we will concentrate only on those issues that are directly involved in the selection of small number of samples for the further liposome surface modification.

All tested S-PVP and P-PVP derivatives, form micelles in water solutions with the average size between 5 and 20 nm and narrow size distribution as follows from the particle size measurement. However, the CMC values

for various micelles depend on both the type of the hydrophobic residue and the molecular size of the hydrophilic PVP block. In general, the increase in the length of the PVP block increases the CMC value. The CMC values for amphiphilic PVP with PVP block size above 15,000 Da were around 10⁻⁴ M and did not differ much for S-PVP and P-PVP (in average, CMC values for S-PVP are 1.5–2-fold lower than CMC values for P-PVP). For very short PVP blocks (below 1500 Da), CMC values for amphiphilic PVP samples were around 10⁻⁶ M and were practically identical for both S-PVP and P-PVP. The biggest difference in CMC values was found for S-PVP and P-PVP samples with intermediate size of PVP blocks — between 1500 and 8000 Da. Fig. 3 shows the data on the CMC measurement by the pyrene method for S-PVP and P-PVP with MW of PVP block of about 6000 Da. It is clearly seen from Fig. 3 that the CMC value for S-PVP (2.1 × 10⁻⁶ M) is ca.30-fold lower than the CMC value for P-PVP of a similar MW (6.5 × 10⁻⁵ M), i.e. S-PVP produces more stable micelles.

One may interpret these results in terms of the relative intensity of hydrophobic interactions between fatty acyls of different lengths forming the micelle core and keeping the micelle together and the energy of the free motion of hydrophilic PVP chains forming the micelle shell in water and destabilizing the micelle. When PVP chains are short, both C16 palmityl and C18 stearyl provide effective micellization with low CMC values. When PVP chains are long, the energy of the free motion of the PVP block in water is higher than for short blocks, CMC value increases and the C2 difference between palmityl and stearyl residues begins to play a certain role. This role is, however, most pronounced at intermediate lengths of PVP blocks, when stearyl provides stronger hydrophobic interactions in the micelle core.

Similar findings were done by us earlier with polymer-coated liposomes, when it was shown that the extent of protective activity for different polymers towards liposomes in vivo depends on the length of hydrophobic

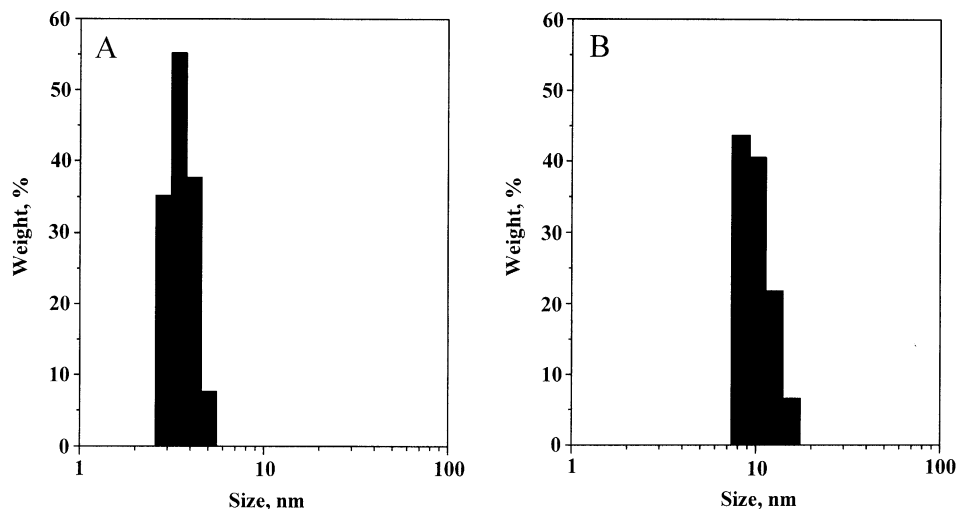


Fig. 4. Size and size distribution for the micelles formed by: (A) S-PVP (MW 1500); (B) S-PVP (MW 8000). See Section 2.2 for details.

anchor and polymer molecular weight [42,43]. When modified with the same long hydrophobic “anchor”, various soluble and flexible polymers (such as PEG, PVP, polyacryl amide) of similar molecular weight being used in similar concentration, all provide efficient steric protection for liposomes. The protective activity of polymers with shorter hydrophobic moiety or with higher MW was, however, much lower. From the thermodynamic point of view, it was assumed that the relatively short hydrophobic group is unable to keep a polymer molecule on the liposome surface. The energy of the polymeric chain motion in the solution might be higher than the energy of a short hydrophobic group interaction with phospholipid surroundings within the liposomal membrane. The longer anchor provides much firmer polymer binding with liposome (higher energy of interaction with hydrophobic membrane core due to the larger number of membrane-embedded CH_2 -groups), and thus much better liposome steric protection. On the other hand, even the length of a “long” anchor is insufficient to firmly fix a much larger polymer on the liposome surface because of the higher energy of polymer chain motion in water compared with that for the shorter polymers [50].

Based on our results with micellization studies, we have chosen S-PVP derivatives with intermediate size of PVP block (from 1500 to 8000 Da) for further experiments with liposomes. Typical sizes and size distributions for S-PVP (MW 1500) and S-PVP (MW 8000) micelles are presented in Fig. 4. One can see that the micelles have rather narrow size distribution with the mean size being smaller for S-PVP (MW 1500) micelles (ca. 5 nm) than for S-PVP (MW 8000) micelles (ca. 10 nm), which corresponds well to the difference in the molecular size of micelle-forming individual amphiphilic PVP unimers.

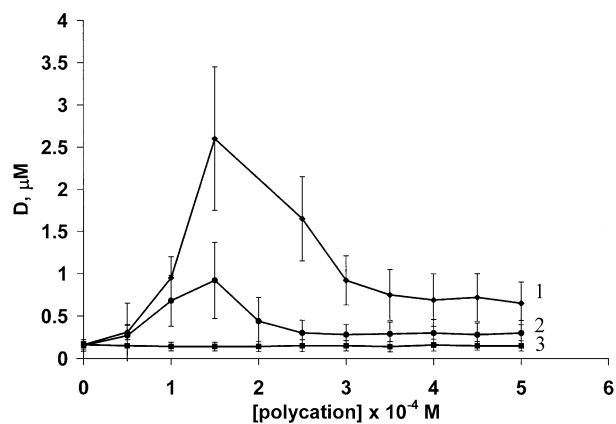


Fig. 5. Liposome aggregation by different concentrations of the polycation. (1) “Plain” negative liposomes; (2) liposomes with 3 mol% of S-PVP (MW 3000 Da); (3) liposomes with 10% of S-PVP; (D) aggregate size. See Section 2.2 for details.

3.4. Amphiphilic PVP in liposomes

3.4.1. PVP-mediated protection against electrostatic agglutination of liposomes

To investigate the protective effect of liposome-incorporated S-PVP, negatively charged liposomes with an average size of about 170 nm were prepared from PC, Chol, and CL with various amounts of S-PVP with MW of PVP block of ca. 3000 Da. Then, a polycation-induced aggregation of negatively charged liposomes was studied following particle size measurements in the presence of and absence of liposome-incorporated S-PVP, using (poly-ethylpyridinium-4-vinylchloride) as an aggregating polycation. The results of this experiment are presented in Fig. 5. In the absence of attached S-PVP, the addition of the polycation to the suspension of negatively charged

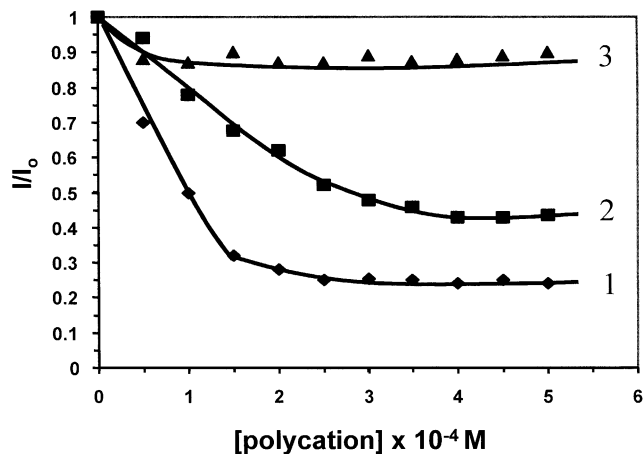


Fig. 6. Fluorescence quenching in negative liposomes by different concentrations of the polycation. (1) "Plain" negative liposomes; (2) liposomes with 3 mol% of S-PVP (MW 3000 Da); (3) liposomes with 10% of S-PVP. See Section 2.2 for details.

liposomes causes strong liposome aggregation, especially at low and intermediate concentration of the polycation, when it still performs the function of a cross-linking reagent. At the increased concentration of the polycation, the size of aggregates diminishes, probably, due to formation of liposome:polycation stoichiometric complexes, which include few liposomes, or even single liposomes, and are protected from further aggregation by electrostatic repulsion of polycation molecules associated with liposomes. Incorporation of just 3 mol% (of total lipid) of S-PVP into liposomes drastically inhibits the interaction of the polycation with negatively charged liposomes by steric hindrances for aggregation. As a result, the size of the formed aggregates is much smaller. The increase in the quantity of the liposome-attached S-PVP to 10 mol% completely abolishes liposome aggregation in the presence of the polycation, demonstrating an efficient steric protection of liposomes by PVP.

3.4.2. PVP-mediated protection of the liposome surface in fluorescence quenching experiments

Additionally, the ability of liposome-incorporated amphiphilic S-PVP (MW 3000 Da) to provide an effective steric protection to liposomes was investigated in experiments on the quenching of the fluorescence of the polycation-sensitive hydrophobic fluorescent label, [*N*-(4-fluoresceinthiocarbamoyl)dipalmitoyl-PE], incorporated into the membrane of negatively charged liposomes [51]. The data presented in Fig. 6 clearly demonstrate strong fluorescence quenching by the polycation in non-modified liposomes. The extent of quenching in this case is more than 50%, probably because of possible asymmetrical distribution of the label between inner and outer monolayers with predominant accumulation in the outer monolayer. The incorporation of 3 mol% of S-PVP into liposomes substantially decreases the overall quenching,

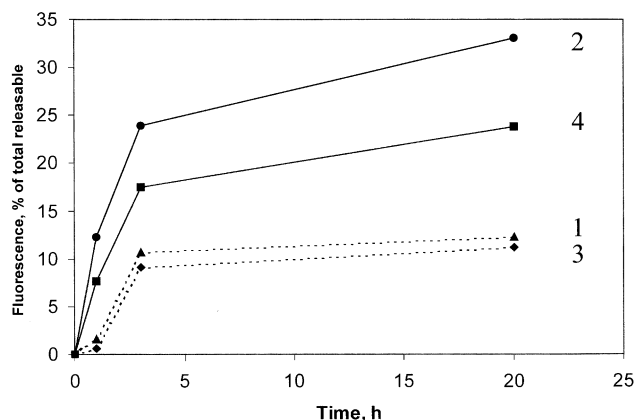


Fig. 7. Fluorescence release from PC:Chol liposomes containing S-PVP (MW 1500 Da). (1) 1.5 mol% of S-PVP; (2) 1.5 mol% of S-PVP in the presence of serum; (3) 6 mol% of S-PVP; (4) 6 mol% of S-PVP in the presence of serum. See Section 2.2 for details.

while the incorporation of 10 mol% of S-PVP almost completely prevents the quenching providing an additional evidence of PVP protecting activity.

3.4.3. Protective effect of grafted PVP against serum-induced liposome destabilization

Since the prevention of drug-carrying liposomes by blood components is one of the main reasons for developing sterically protected liposomes, we have performed a model study on the stability of PVP-coated liposomes in mouse serum *in vitro*. For this purpose, 200 nm liposomes were prepared from PC:Chol (7:3 mol) with the addition of 1.5 or 6 mol% of various S-PVP samples (MW 1500 and 6000 Da). Liposomes contained a self-quenching concentration (45 mM) of CF. Liposome destabilization in the presence of blood serum was studied following the fluorescence increase in the system due to the release of free CF from destabilized liposomes and its dilution in the medium to non-self-quenching concentration. First, it was shown that the efficacy of steric protection of CF-containing liposomes with S-PVP (MW 1500) depends on the quantity of liposome-attached PVP (see Fig. 7). While in serum-free medium liposomes containing 1.5% and 6% mol of S-PVP demonstrate insignificant and very similar release of CF (due to the presence of a certain fraction of "defective" liposomes in the system), the addition of 50% serum causes a pronounced liposome destabilization (CF release and fluorescence increase). Still, the release of CF from liposomes with 1.5 mol% of S-PVP is ca. 50% higher than from liposomes with 6 mol% of S-PVP, showing that the higher concentration of PVP on the liposome surface provides the better protection. Control CF-loaded plain liposomes demonstrate the CF release pattern practically coinciding with that for liposomes with 1.5% mol of S-PVP (MW 1500) both without and with serum (therefore data

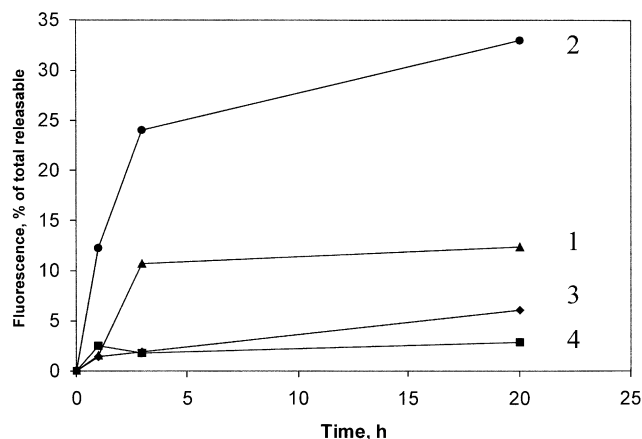


Fig. 8. Fluorescence release from PC:Chol liposomes containing 1.5 mol% of S-PVP (MW 1500 and 6000 Da). (1) 1500 Da S-PVP; (2) 1500 Da S-PVP in the presence of serum; (3) 6000 Da S-PVP; (4) 6000 Da S-PVP in the presence of serum. See Section 2.2 for details.

not shown). It means, that the incorporation of 1.5 mol% of S-PVP (MW 1500) into the liposomal membrane on one hand does not influence liposome permeability, however, on the other hand, this small quantity of liposome-attached PVP with a relatively low MW does not protect liposomes from serum-provoked permeabilization.

Similar, or even more pronounced, enhancement of the protective effect may be achieved by increasing the molecular size of the liposome-attached PVP (see Fig. 8). In this experiment, liposomes were modified with 1.5 mol% of S-PVP; however, one sample of liposomes contained S-PVP with MW 1500 Da, while the other liposomes were similarly grafted with S-PVP, MW 6000 Da. The data presented in Fig. 8 clearly show that S-PVP with the higher MW at the same molar concentration provides much better protection for liposomes both in the presence of and in the absence of serum. The leakage of CF from such liposomes under both conditions is only on a background level (ca. 5% after 20 h). These data are in a good agreement with earlier findings that certain minimal size of a liposome-grafted polymer molecule is required to form a protecting “cloud” of a sufficient thickness to prevent direct contact of the liposome surface with destabilizing molecules from the surrounding medium [11,17,20,35]. Thus, the efficacy of liposome protection can be controlled by MW of liposome-attached amphiphilic PVP and by its concentration in the liposomal membranes.

4. Concluding remarks

Amino- and carboxyl-containing PVP derivatives can be successfully prepared by free-radical polymerization of VP in the presence of various mercaptans that are able to perform the chain transfer due to the formation of free

radical produced by elimination of a hydrogen atom from a SH-group. The above polymerization technique can produce polymers with a single functional end group corresponding to the structure of the mercaptan radical.

The hydrophobic stearyl and palmityl end groups can be introduced into these polymers by direct reaction of amino-PVP with an acyl chloride, or by treating carboxy-PVP with a corresponding amine in the presence of *N,N'*-dicyclohexylcarbodiimide. According to the ESR data, terminal hydrophobic groups of amphiphilic PVP derivatives demonstrate a good accessibility for the interaction with other hydrophobic ligands. As a result, in aqueous solutions amphiphilic PVP can spontaneously micellize with low CMC values. In general, S-PVP forms more stable micelles than P-PVP.

Amphiphilic PVP easily incorporates into the liposomal membrane and provides good steric protection for liposomes. Thus, amphiphilic PVP incorporated into negatively charged liposomes effectively prevents polycation-induced liposome aggregation and polycation-mediated quenching of the membrane-associated fluorescent label. In addition, amphiphilic PVP effectively protect liposomes from blood serum-provoked destabilization, the degree of this protection being dependent of polymer concentration and MW of the PVP block.

Acknowledgements

This work was supported in part by the NIH grants HL55519-04 and GM60200-03 to V.P. Torchilin.

References

- [1] Gref R, Minamitake Y, Peracchia MT, Trubetsky VS, Torchilin VP, Langer R. Biodegradable long-circulating polymeric nanospheres. *Science* 1994;263:1600–3.
- [2] Cohen S, Bernstein H, editors. *Microparticulate systems for the delivery of proteins and vaccines*. New York: Marcel Dekker, 1996.
- [3] Torchilin VP. Polymer-coated long-circulating microparticulate pharmaceuticals. *J Microencapsulation* 1988;15:1–19.
- [4] Palmer TN, Caride VJ, Caldecourt MA, Twickler J, Abdullah V. The mechanism of liposome accumulation in infarction. *Biochim Biophys Acta* 1984;797:363–8.
- [5] Gabizon A, Catane R, Uziely B, et al. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. *Cancer Res* 1994;54:987–92.
- [6] Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J Control Rel* 2000;65:271–84.
- [7] Gabizon A. Liposome circulation time and targeting: implication for cancer chemotherapy. *Adv Drug Delivery Rev* 1995;16: 285–94.
- [8] Muggia FM. Clinical efficacy and prospects for use of pegylated liposomal doxorubicin in the treatment of ovarian and breast cancers. *Drugs* 1997;54(Suppl 4):22–9.

- [9] Valero V, Buzdar AU, Theriault RL, et al. Phase II trial of liposome-encapsulated doxorubicin, cyclophosphamide, and fluorouracil as first-line therapy in patients with metastatic breast cancer. *J Clin Oncol* 1999;17:1425–34.
- [10] Torchilin VP, Klibanov AL, Huang L, O'Donnell S, Nossiff ND, Khaw BA. Targeted accumulation of polyethylene glycol-coated immunoliposomes in infarcted rabbit myocardium. *FASEB J* 1992;6:2716–9.
- [11] Torchilin VP, Narula J, Halpern E, Khaw BA. Poly(ethylene glycol)-coated anti-cardiac myosin immunoliposomes: factors influencing targeted accumulation in the infarcted myocardium. *Biochim Biophys Acta* 1996;1279:75–83.
- [12] Gregoriadis G, editor. *Liposomes as drug carriers*. Avon: Wiley, 1988.
- [13] Lasic DD, Papahadjopoulos D, editors. *Medical application of liposomes*. Amsterdam: Elsevier, 1998.
- [14] Lasic DD, Martin F, editors. *Stealth liposomes*. Boca Raton, FL: CRC Press, 1995.
- [15] Torchilin VP, Trubetskoy VS. Which polymers can make nanoparticulate drug carriers long-circulating? *Adv Drug Delivery Rev* 1995;16:141–55.
- [16] Molyneux P. *Water-soluble synthetic polymers: properties and behavior*, vol. 2. Boca Raton, FL: CRC Press, 1997.
- [17] Senior J, Delgado C, Fisher D, Tilcock C, Gregoriadis G. Influence of surface hydrophilicity of liposomes on their interaction with plasma proteins and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles. *Biochim Biophys Acta* 1991;1062:77–82.
- [18] Naper DH. *Polymeric stabilization of colloidal dispersions*. New York: Academic Press, 1983.
- [19] Klibanov AL, Maruyama K, Torchilin VP, Huang L. Amphiphatic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett* 1990;268:235–7.
- [20] Mori A, Klibanov AL, Torchilin VP, Huang L. Influence of steric barrier activity of amphiphatic poly(ethylene glycol) and ganglioside GM₁ on the circulation time of liposomes and on the target binding of immunoliposomes in vivo. *FEBS Lett* 1991;284:263–6.
- [21] Allen TM, Mehra T, Hansen C, Chin YC. Stealth liposomes: an improved sustained release system for 1- β -D-arabinofuranosylcytosine. *Cancer Res* 1992;52:2431–9.
- [22] Gabizon A, Papahadjopoulos D. The role of surface charge and hydrophilic groups on liposome clearance in vivo. *Biochim Biophys Acta* 1992;1103:94–100.
- [23] Blume G, Cevc G. Molecular mechanism of the lipid vesicle longevity in vivo. *Biochim Biophys Acta* 1993;1146:157–68.
- [24] Torchilin VP. How do polymers prolong circulation time of liposomes. *J Liposome Res* 1996;6:99–116.
- [25] Needham D, McIntosh TJ, Lasic DD. Repulsive interactions and mechanical stability of polymer-grafted lipid membranes. *Biochim Biophys Acta* 1992;1108:40–8.
- [26] Woodle MC. Surface-modified liposomes: assessment and characterization for increased stability and prolonged blood circulation. *Chem Phys Lip* 1993;64:249–62.
- [27] Yamaoka T, Tabata T, Ikada Y. Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration in mice. *J Pharm Sci* 1994;83:601–6.
- [28] Patel H. Serum opsonins and liposomes: their interaction and opsonophagocytosis. *Crit Rev Ther Drug Carriers Systems* 1992;9:39–90.
- [29] Allen TM, Hansen CB, de Menenez DEL. Pharmacokinetics of long-circulating liposomes. *Adv Drug Delivery Rev* 1995;16:267–84.
- [30] Senior JH. Fate and behavior of liposomes in vivo: a review of controlling factors. *CRC Crit Rev Ther Drug Carriers Systems* 1987;3:123–93.
- [31] Chonn A, Semple SC, Cullis PR. Separation of large unilamellar liposomes from blood components by a spin column procedure: towards identifying plasma proteins which mediate liposome clearance in vivo. *Biochim Biophys Acta* 1991;1070:215–22.
- [32] Chonn A, Semple SC, Cullis PR. Association of blood proteins with large unilamellar liposomes in vivo: relation to circulation lifetimes. *J Biol Chem* 1992;267:18759–65.
- [33] Harper GR, Davies MC, Davis SS, Tadros TF, Taylor DC, Irving MP, Waters JA. Steric stabilization of microspheres with grafted polyethylene oxide reduces phagocytosis by rat Kupffer cells in vitro. *Biomaterials* 1991;12:695–9.
- [34] Allen TM. The use of glycolipids and hydrophilic polymers in avoiding rapid uptake of liposomes by the mononuclear phagocyte system. *Adv Drug Deliv Rev* 1994;13:285–309.
- [35] Torchilin VP, Papisov MI. Why do polyethylene glycol-coated liposomes circulate so long? *J Liposome Res* 1994;4:725–39.
- [36] Torchilin VP, Omelyanenko VG, Papisov IM, Bogdanov Jr AA, Trubetskoy VS, Herron JN, Gentry CA. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. *Biochim Biophys Acta* 1994;1195:11–20.
- [37] Torchilin VP. Effect of polymers attached to lipid headgroups on properties of liposomes. In: Lasic DD, Barenholz Y, editors. *Handbook of nonmedical applications of liposomes*, vol. 1. Boca Raton, FL: CRC Press, 1995. p. 263–84.
- [38] Powell GM. Polyethylene glycol. In: Davidson RL, editor. *Handbook of water soluble gums and resins*. New York: McGraw-Hill, 1980, [Chapter 18].
- [39] Pang SNJ. Final report of the safety assessment of polyethylene glycols (PEGs)-6, -8, -32, -75, -150, -14 M, -20 M. *J Am Coll Toxicol* 1993;12:429–56.
- [40] Zalipsky S. Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates. *Bioconj Chem* 1995;6:150–65.
- [41] Zalipsky S. Chemistry of polyethylene glycol conjugates with biologically active molecules. *Adv Drug Delivery Rev* 1995;16:157–82.
- [42] Torchilin VP, Shtilman MI, Trubetskoy VS, Whiteman K, Milstein AM. Amphiphilic vinyl polymers effectively prolong liposome circulation time in vivo. *Biochim Biophys Acta* 1994;1195:181–4.
- [43] Torchilin VP, Trubetskoy VS, Whiteman KR, Caliceti P, Ferruti P, Veronese FM. New synthetic amphiphilic polymers for steric protection of liposomes in vivo. *J Pharm Sci* 1995;84:1049–53.
- [44] Woodle MC, Engbers CM, Zalipsky S. New amphiphatic polymer-lipid conjugates forming long-circulating reticuloendothelial system-evading liposomes. *Bioconj Chem* 1994;5:493–6.
- [45] Maruyama K, Okuizumi S, Ishida O, Yamauchi H, Kikuchi H, Iwatsuru M. Phosphatidyl polyglycerols prolong liposome circulation in vivo. *Int J Pharm* 1994;111:103–7.
- [46] Dreschler H. Indication to prescription of blood and blood substitutes in operative medicine. *Med Lab (Stuttg.)* 1974;27:115–21.
- [47] Kramer SA. Effect of povidone-iodine on wound healing: a review. *J Vasc Nurs* 1999;17:17–23.
- [48] Ryabov AV, Smirnova LA, Panova GD, Soldatov VM, Rudin AA. Kinetics of polymerization of methacrylates in the presence of chain transferring agent. *Vysokomol Soed Ser A* 1974;16:29–34 [in Russian].
- [49] Akashi M, Kirikihira I, Miyauchi N. Synthesis and polymerization of a styryl terminated oligovinylpyrrolidone macromonomer. *Angew Macromol Chem* 1985;132:81–9.
- [50] des Cloizeaux J, Jannink G. *Polymers in solution. Their modeling and structure*. Oxford: Clarendon Press, 1990.
- [51] Yaroslavova EG, Yaroslavov AA, Kabanov VA, Menger FM. Negatively charged vesicles coated by poly(oxyethylene) layer: interaction with polycations. *Polym Prepr* 1997;38:640–1.