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POLYMER BLENDS

Synthesis and Properties of Conjugates Involving Liposomes, a Linear Polymer, and the Micelle of a Polylactide–Poly(Ethylene Glycol) Block Copolymer

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Abstract—Multiliposome biodegradable conjugates have been obtained via electrostatic binding of a liposome–polylysine complex with micelles formed from a polylactide–poly(ethylene glycol) di- or triblock copolymer. It has been shown that the integrity of liposomes in conjugates is retained. Of special interest is a conjugate based on a polylactide–poly(ethylene glycol) diblock copolymer that exhibits stability in a physiological solution with 0.15 mol/L NaCl, but degrades in the presence of proteolytic enzymes. This fact allows the suggestion that the conjugate will be eliminated from the body after its transport function is completed. The results may be used to create biodegradable liposome containers for drug encapsulation and delivery.

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INTRODUCTION

Bilayer lipid vesicles (liposomes) are widely used for drug encapsulation and delivery [1–7]. The immobilization of liposomes on the surface of a colloid support accompanied by an increase in their local concentration leads to a gain in the efficiency of the liposome capture by cells and to improvement of the therapeutic effect of the immobilized drug.

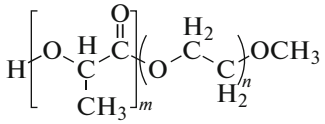
The electrostatic adsorption of cationic liposomes on the surfaces of negatively charged micelles formed from a triblock copolymer of poly-L-lactide (PLA) and poly(ethylene glycol) (PEG), PLA–PEG–PLA, was described in our recent work [8]. The resulting conjugate undergoes degradation in the presence of the hydrolytic enzyme esterase [9].

With the above approach, in this study, the formation of biodegradable conjugates of cationic liposomes with negatively charged micelles prepared from a PLA–PEG diblock copolymer was studied. As is known, micelles of diblock copolymers have a higher aggregation stability than that of triblock copolymer micelles [10]. This point may be of decisive importance to the search for carriers for liposome immobilization. Moreover, the stability of liposome conjugates with block copolymers of both types in a water–salt solution was analyzed and the rates of biodegradation of both conjugates in the presence of different proteolytic enzymes were compared.

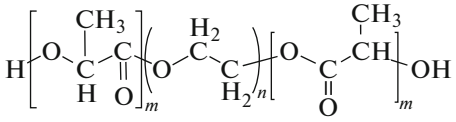
EXPERIMENTAL

Cardiolipin (CL^{2-}), phosphatidyl choline (PC), polylysine hydrobromide (a degree of polymerization of 340), and 5(6)-carboxyfluorescein (CF) (all reagents from Sigma, United States) were used without further purification.

The PLA–PEG block copolymers



PLA–PEG ($m = 64$, $n = 113$)



PLA–PEG–PLA ($m = 52$, $n = 91$)

were synthesized as described elsewhere [11].

The structures and purities of the products were confirmed via NMR spectroscopy, gel permeation chromatography, and IR spectroscopy. According to the GPC data, for PLA–PEG, $M_n = 9.6 \times 10^3$ and $M_w/M_n = 1.24$, while for PLA–PEG–PLA, $M_n = 11.4 \times 10^3$ and $M_w/M_n = 1.40$.

Block copolymers were dissolved in THF. After one day, distilled water was added dropwise to the solutions under intense stirring to make the water con-

tents as high as 10 vol %, and after another day, the water contents were brought to 20 vol %. Aqueous–organic mixtures were dialyzed for a week against water to remove the organic solvent. As a result, aqueous dispersions of block copolymers were obtained.

Liposomes from electroneutral PC and anionic CL^{2-} with a molar fraction of anionic groups of $\nu_{CL} = 0.1$ were prepared through the standard procedure via mixing of appropriate volumes of lipid solutions. During solvent removal under vacuum, the lipid film was dispersed in a 10^{-2} M Tris buffer solution with pH 7 and was additionally homogenized under ultrasonic treatment [12].

Liposomes with CF incorporated into the internal volume were prepared via the standard procedure described above, but the lipid film was dispersed in a 1 mg/mL CF solution in a Tris buffer. The resulting suspension was dialyzed for 12 h against a 10^{-3} M Tris buffer, which was changed each two hours.

In all experiments, freshly prepared liposomes with an average hydrodynamic diameter of 40–50 nm were used.

The biodegradation of conjugates was initiated via addition of a Morikraza proteolytic complex prepared from the hepatopancreas of the Kamchatka crab *Paralithodes camchatica* (OAO Trinita, Russia) or from the enzyme lipase of *Rhizopus* sp. (Serva, Germany). Morikraza is a mixture of enzymes (serine proteinase, collagenase, metalloproteinase, etc.) capable of ester, peptide, and amide bond scission [13] that demonstrates enzyme activity in the pH range 6.0–9.0 with the maximum at pH 7.5. Lipase, which catalyzes the hydrolysis of ester bonds in lipid molecules, is active in the pH range from 4.5 to 8.0, with the optimum at pH 6.5–7.5.

Water was distilled twice and then passed through a Milli-Q system (Millipore, United States). The purified water had specific conductivity of 0.6 μS/cm.

Average hydrodynamic diameter d_{av} and the electrophoretic mobility (EPM) of particles were determined via the method of quasielastic scattering on a Brookhaven 90 Plus instrument (Brookhaven Instruments, United States). The measurements were performed in a thermally controlled cell at 20°C.

The fluorescence intensities of suspensions in buffer solutions were determined on an F-4000 spectrofluorimeter (Hitachi, Japan) in quartz cells 1 cm wide.

Solution pH was measured on a pH meter 210 (Hanna, Germany), with an HI 1131B glass electrode as a measuring electrode.

The morphologies of micelles and conjugates were studied via transmission electron microscopy on a LEO 912 AB OMEGA microscope (Carl Zeiss, Germany). The samples were deposited on a copper mesh with a Formvar–carbon film about 100 nm thick and were dried in air.

RESULTS AND DISCUSSION

In a buffer solution (10^{-2} M Tris, pH 7), the PLA–PEG diblock copolymer forms micelles with an average hydrodynamic diameter of 130 nm and an EPM of −0.4 (μm/s)/(V/cm) [8].

To impart an affinity toward negatively charged micelles to PC/CL^{2-} liposomes, cationic polylysine was adsorbed on the surfaces of liposomes. For this purpose, a 1.8×10^{-2} mg/mL Tris buffer solution of polylysine was added to a 1 mg/mL suspension of PC/CL^{2-} liposomes. As was shown in our previous work [14], at the above-mentioned concentration of components, all added polylysine is adsorbed on the liposome membrane. The resulting complex (modifying complex or MOCO), in which there are on average for each liposome four polylysine molecules, was used in further experiments. The average hydrodynamic diameter of the complex particles is 250 nm (at an initial size of liposomes of 40–50 nm), and the EPM is +0.5 (μm/s)/(V/cm).

The retention of the integrity of liposomes involved in the MOCO was observed via a fluorescence technique [15, 16]. In experiments, liposomes with the internal volumes filled with a fluorescent dye (DF) solution at a self-extinguishing concentration were employed. The disruption of the liposome-membrane integrity should be accompanied by the release of DF into the environmental solution; a decrease in its concentration; and, hence, an increase in the total intensity of the fluorescence of the suspension [17–19]. After the addition of the polylysine solution to the suspension of liposomes filled with the DF solution, no fluorescence was observed. Thus, after the binding of liposomes with polylysine and the MOCO formation, the integrity of liposomes is retained.

Positively charged MOCOs at various concentrations were added to a suspension of negatively charged micelles of the diblock copolymer. The binding of the MOCO with micelles was followed through changes in the particle size in the suspension. The results are illustrated in Fig. 1, where on the abscissa, for the sake of convenience, the polylysine concentration in the MOCO added to the micelles, $[\text{polylysine}]_{MOCO}$, is plotted. The gain in the MOCO concentration is accompanied by an increase in the particle size in the suspension, a result that suggests the formation of MOCO–micelle conjugates. At $[\text{polylysine}]_{MOCO} = 8.5 \times 10^{-3}$ mg/mL, the d_{av} value becomes constant, and it does not change later on.

The integrity of liposomes in the MOCO–diblock copolymer micelle conjugate was observed via the fluorescence method described above. To a suspension of micelles (9.6×10^{-2} mg/mL, 10^{-5} mol/L), MOCOs containing liposomes filled with a DF solution were added at various concentrations, and the fluorescence intensity was recorded for 3 h. It turned out that the intensity value remains the same if the concentration of polylysine in the

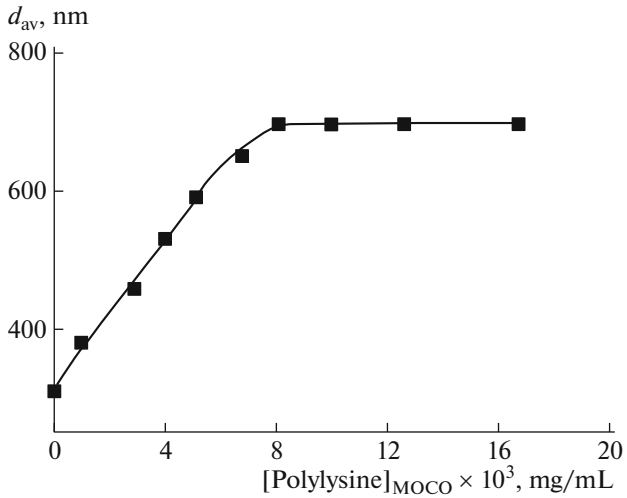


Fig. 1. Average hydrodynamic diameter d_{av} of particles of the MOCO–diblock copolymer micelle conjugate vs. polylysine concentration in MOCO. The PLA–PEG concentration is 9.6×10^{-2} mg/mL. From here on, the conditions are PC/CL^{2-} liposomes ($\nu_{CL} = 0.1$), 10^{-2} M Tris buffer, and pH 7.0.

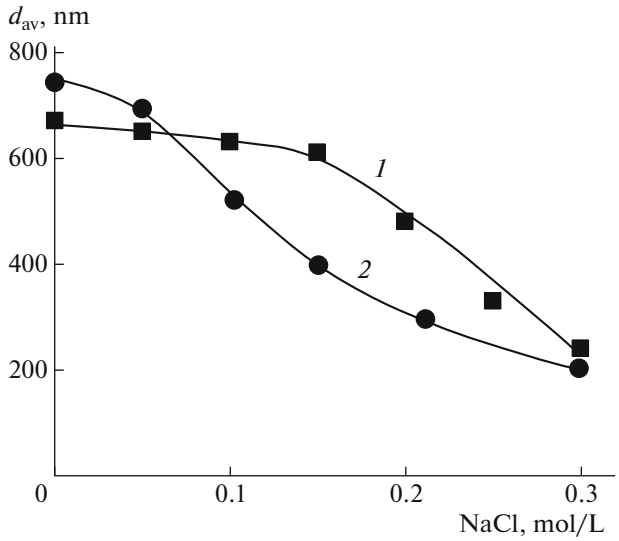


Fig. 2. Average hydrodynamic diameters d_{av} of particles of the conjugates (*1*) MOCO–diblock copolymer micelle and (*2*) MOCO–triblock copolymer micelle vs. NaCl concentration; [Polylysine]$_{MOCO} = 8.5 \times 10^{-3}$ mg/mL, the PLA–PEG concentration is 9.6×10^{-2} mg/mL, and the PLA–PEG–PLA concentration is 1.1×10^{-1} mg/mL.

MOCO does not exceed 8.5×10^{-3} mg/mL, which corresponds to a concentration of the added liposomes of 5×10^{-1} mg/mL. The result makes it possible to estimate the MOCO concentration range in which liposomes bound with diblock copolymer micelles retain their integrity.

Let us compare the behavior of a conjugate of the MOCO and a micelle of the PLA–PEG diblock copolymer to that of the conjugate obtained via the interaction of the MOCO with micelles of the PLA–PEG–PLA triblock copolymer described previously [9]. In our experiments, we used conjugates of the same composition (MOCO-to-micelle molar ratio): For each micelle there are four MOCO particles. The stability of both conjugates in water–salt solutions and their biodegradation in the presence of enzymes of two types, lipase and the proteolytic complex Morikraza, were studied.

The stability of conjugates in water–salt media was observed via measurements of particle size in suspensions. Figure 2 shows the dependences of the average hydrodynamic diameter for each conjugate on the salt (NaCl) concentration in the suspension. It is evident that the average size of the MOCO–diblock copolymer micelle conjugate remains practically the same up to a salt concentration of 0.15 mol/L (curve *1*). In other words, this conjugate retains stability in a physiological solution with [NaCl] = 0.15 mol/L. In contrast, the size of the MOCO–triblock copolymer micelle conjugate decreases successively as the NaCl concentration increases (curve *2*), a result that suggests its disruption into smaller fragments in a salt solution. The size of a conjugate in the physiological solution decreases two times relative to the initial size in the absence of added salt.

All components of both conjugates (block copolymer micelles, polylysine and liposomes) are biodegradable, a circumstance that is indicative of the possibility of conjugate collapse in the biological medium. In the model experiment, the conjugate degradation was initiated by the enzyme addition. The process of degradation was followed with respect to changes in particle size in the suspension. In the absence of the enzyme, the conjugate size remains practically the same for a week (Figs. 3a, 3b, curves *1*). The addition of lipase to a suspension of the MOCO–diblock copolymer micelle conjugate results in its gradual degradation (Fig. 3a, curve *2*): d_{av} of particles decreases from 600 to 100–150 nm over 13 days after the enzyme addition. The presence of coarse "residual" particles is probably due to the selectivity of the action of the lipase, which initiates the ester bond scission in lipid molecules and does not interact with the rest of the conjugate components: the block copolymer and polylysine.

A different situation arises after the addition of the proteolytic complex Morikraza to a suspension of the same conjugate: As early as 6 days after, the particle size decreases to 10–15 nm, and it does not change later on (Fig. 3a, curve *3*). The above value is considerably lower than the size of any component of the complex: 130, 250, and 50 nm for micelles, the polylysine–liposome complex, and individual liposomes, respectively. It is evident that such prominent degradation of the conjugate may be a result of the combined action of the enzyme components of Morikraza,

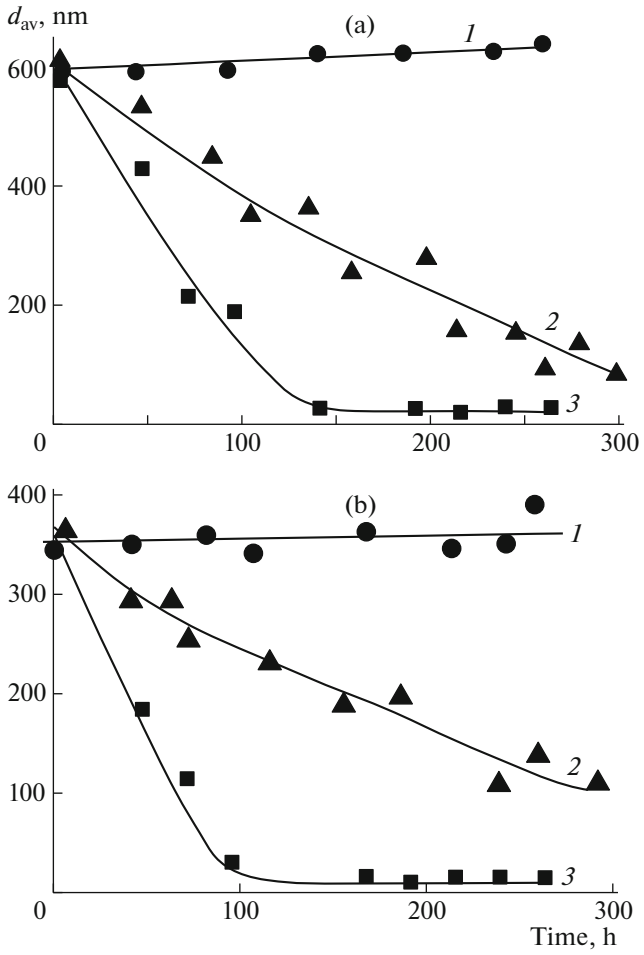


Fig. 3. Kinetics of change in particle size of the conjugates (a) MOCO–diblock copolymer micelle and (b) MOCO–triblock copolymer micelle (*1*) in the absence of enzymes and (*2, 3*) after the addition of (*2*) lipase and (*3*) Morikraza. $[Polylysine]_{MOCO} = 8.5 \times 10^{-3}$ mg/mL; concentrations of lipase and Morikraza of 5×10^{-2} mg/mL; and concentrations of PLA–PEG and PLA–PEG–PLA of 9.6×10^{-2} and 1.1×10^{-1} mg/mL, respectively.

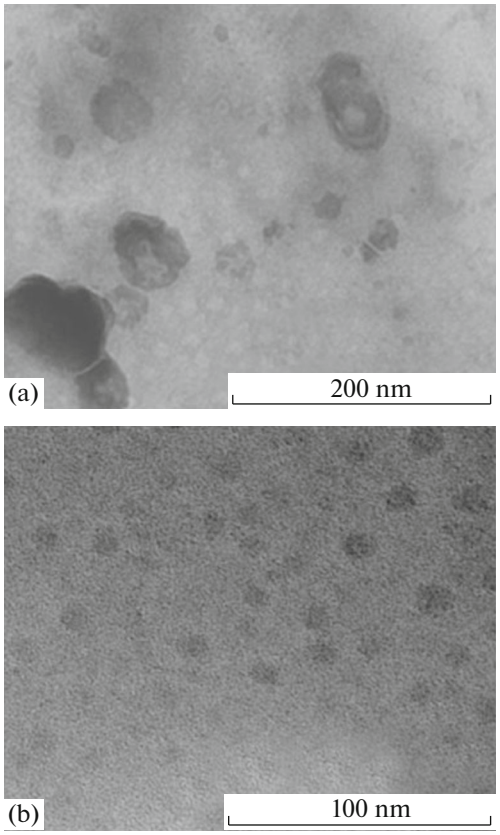


Fig. 4. SEM images of particles obtained (a) 13 days after the addition of lipase and (b) 6 days after the addition of Morikraza to a suspension of the MOCO–diblock copolymer micelle conjugate. $[Polylysine]_{MOCO} = 8.5 \times 10^{-3}$ mg/mL, concentrations of lipase and Morikraza of 5×10^{-2} mg/mL, and a PLA–PEG concentration of 9.6×10^{-2} mg/L.

which are capable of ester bond scission in lipids and the polylactide block of the copolymer as well as amide bond scission in polylysine.

The degradation of the MOCO–triblock copolymer micelle conjugate initiated by enzymes develops in a similar manner. Its size decreases from 350 to ~100 nm over 11 days after the lipase addition (Fig. 3b, curve *2*), while the introduction of Morikraza results in a decrease in the average hydrodynamic diameter of particles in the suspension to 10–15 nm after 4 days (Fig. 3b, curve *3*).

With the use of liposomes filled with the fluorescent dye solution, it was shown that the enzymatic degradation of the conjugate is accompanied by the quantitative release of the liposome contents into the surrounding solution.

Additional information on the system state after the enzyme addition was obtained with the use of scanning electron microscopy. Figure 4 shows SEM images of particles in a suspension of the MOCO–diblock copolymer micelle conjugate 13 days after the addition of lipase and 6 days after the addition of Morikraza. In the first image, particles about 100 nm in size and, in the second image, finer particles not exceeding 10–15 nm in size are distinguishable.

Similar results were obtained for the MOCO–triblock copolymer micelle conjugate (Fig. 5): particles about 100 nm and 10–15 nm in size after the addition of lipase and Morikraza, respectively.

Thus, in this study, the possibility of obtaining multiliposome conjugates based on a liposome–polylysine complex with micelles formed from a polylactide–poly(ethylene glycol) diblock copolymer was demonstrated. The stability of the above conjugate in water–salt media is considerably higher than that of similar conjugates based on a triblock copolymer. It

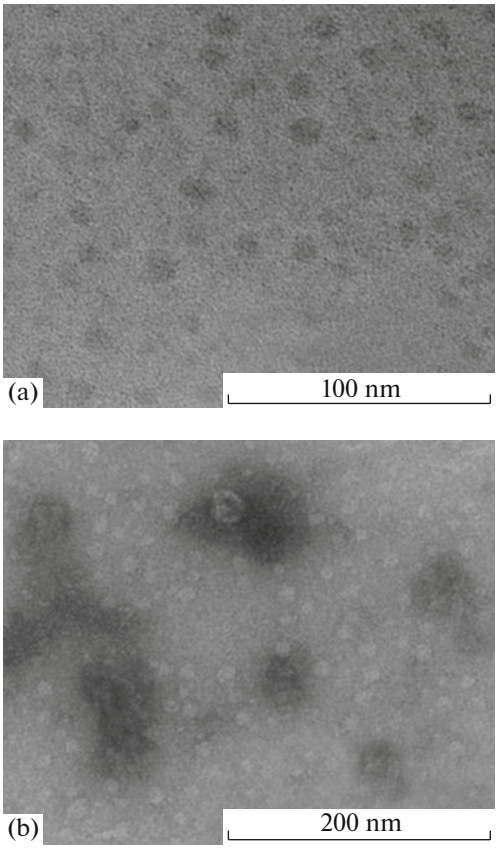


Fig. 5. SEM images of particles obtained (a) 11 days after the addition of lipase and (b) 5 days after the addition of Morikraza to a suspension of the MOCO–triblock copolymer micelle conjugate. $[\text{Polylysine}]_{\text{MOCO}} = 8.5 \times 10^{-3}$ mg/mL, concentrations of lipase and Morikraza of 5×10^{-2} mg/mL, and a PLA–PEG–PLA concentration of 1.1×10^{-1} mg/mL.

was shown that the integrity of liposomes in conjugates is retained. Degradation of the conjugate under the action of proteolytic enzymes occurs at a marked rate, an outcome that makes it possible to predict the removal of the conjugate from the body after completion of its transport function.

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