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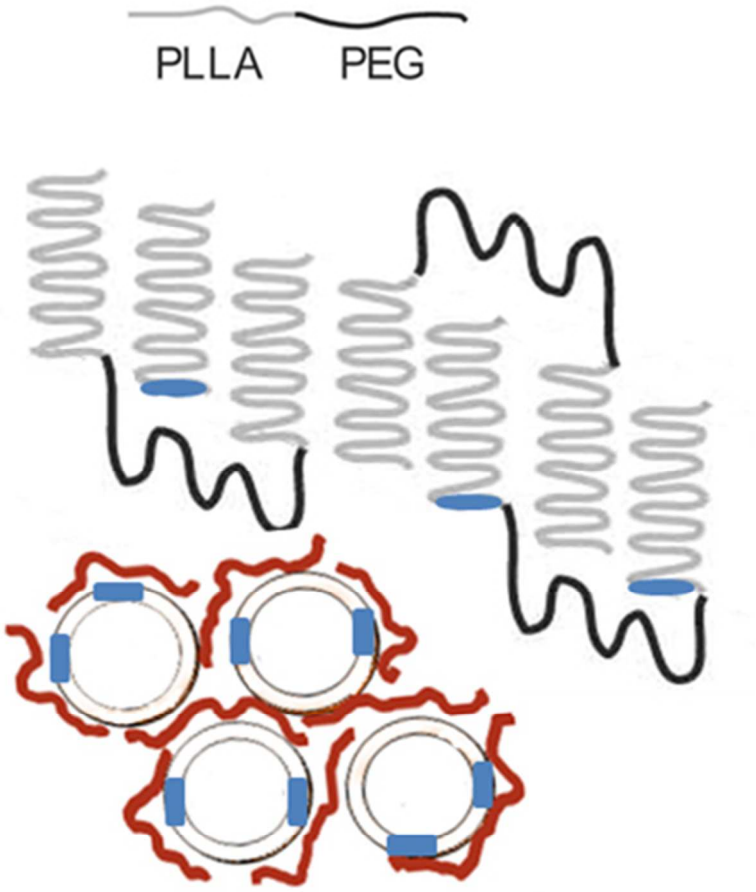

Polycation-liposomes complex

Structure of multiliposomal nanocontainer on base of anionic liposomes, polycation and polylactide
150x192mm (72 x 72 DPI)

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Paper

Biodegradable multi-liposomal containers

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Biodegradable multi-liposomal core-shell containers are described with a core from poly-L-lactide-polyethyleneoxyde-poly-L-lactide triblock-copolymer nary covered by electrostatically adsorbed liposomes. The liposomes retain their integrity after adsorption that allows their use for encapsulation of bioactive compounds. The multi-liposomal containers eventually decompose being attacked a hydrolytic enzyme that makes them promising in the drug delivery field.

Introduction

Bilayer lipid vesicles (liposomes) are currently used for delivery of biologically active substances: drugs[1-3], polypeptides and proteins,[4-6] nucleic acids.[7-9] Multi-liposome assembly, for example via immobilization of several liposomes on a nano-sized colloid, can increase the efficacy of liposome uptake by cells and therapeutic effect of a liposomal drug. However, attachment of liposomes on a solid carrier (glass, ceramic, metal, polymer, etc.) is usually accompanied by their destruction and uncontrolled release of encapsulated drugs.[10] A few successful examples of intact liposome immobilization, described in the literature, include pre-modification both of liposomes and the surface. [11] A need thus arises for carriers deprive of above mentioned disadvantages.

Recently we have proposed to adsorb electrostatically anionic liposomes on the surface of polystyrene microspheres with grafted polycationic chains, "spherical polycationic brushes".[12] It was shown that a mixture of liposomes with different contents could be immobilized on the brush surface.[13] This method allows concentration of dozens of liposomes within a rather small volume.[14] Hydrophilic layer of grafted macromolecules prevents direct contact of immobilized liposomes with the solid polystyrene core and ensures the integrity of immobilized liposomes.[12] Thus prepared multi-liposomal containers (MLC) can be used for constructing *in vitro* working catalysts, fluorescent markers, analytical systems. Their *in vivo* application is strongly restricted by non-biodegradability of the polysterene core of the brushes.

In the present work we describe MLC, approx. 400 nm in diameter, based on biodegradable polylactide (PLA) particles stabilized by polyethyleneglycol (PEG) chains.[15] Liposomes have been adsorbed electrostatically on the PLA particle surface. Polylactide is destructed in the presence of hydrolytic enzymes (esterases),[16,17] the fact renders biodegradability to the entire MLC. Thus obtained MLC seem to be promising for "passive targeting" due to selective penetration of 200-400 nm particles in the capillaries of tumors and other inflammation areas.[18]

Experimental

Materials

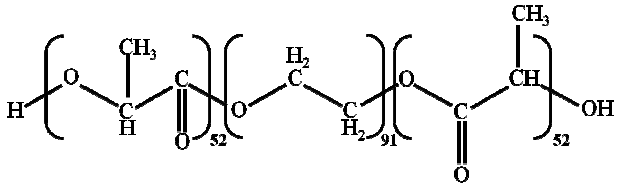

Fig.1 Triblock-copolymer of poly-L-lactide and poly (ethylene glycol).

Cardiolipin (CL^{2-}) and egg lecithin (EL) from Avanti and polylysine hydrobromide with a degree of polymerization equal to 340 from Sigma were used as received. Poly(N-ethyl-4-vinylpyridinium bromide) (PEVP) was synthesized via quaternization of poly(4-vinylpyridine) with degree of polymerization equal to 600 (Sigma) by ethyl bromide as described elsewhere.[19] The resulting product was actually a copolymer containing 95 mol.% of quaternized pyridinium and 5 mol.% of residual 4-vinylpyridine units as found by IR spectroscopy [19]. Concentrations of cationic polymers are given in moles of amino-groups (for polylysine) and quaternized units (for PEVP) per liter.

In order to hydrolyze ester bonds in PLA, proteolytic complex Morikrase from hepatopancreas of the Kamchatka crab Paralithodes camchatica (JSC RPE Trinita, Russia) was used.[20,21] Moricrase is actually a mixture of enzymes including serine proteinases, collagenases, metalloproteinases, etc., capable of cleaving ester and peptide/amide bonds. Moricrase shows enzymatic activity in a pH region from 6.0 to 9.0 with pH optimum at pH 7.5.

Triblock-copolymer of poly-L-lactide and poly (ethylene glycol) (**Figure 1**) was synthesized via ring opened polymerization of lactide in the presence of PEG, catalyzed by stannous octanoate.[22] The structure and purity of the copolymer was proved by NMR, gel chromatography and IR spectroscopy

(see details in Supporting information). Molecular mass of the copolymer was found to be 11.380 with poly dispersity index equal to 1.4. To prepare an aqueous solution of copolymer, a standard procedure was applied.[23] Briefly, the copolymer was dissolved in tetrahydrofuran so that 5×10^{-5} M copolymer solution was prepared. A day after distilled water was added under intensive stirring up to 10 vol% water content. Another day after distilled water was added again under intensive stirring up to 20 vol% water content. Thus prepared water-tetrahydrofuran mixture was dialyzed against distilled water for one week for organic solvent to be removed. Copolymer concentration is given in moles of copolymer macromolecules per liter.

Small unilamellar anionic liposomes, 50-60 nm in diameter, were prepared by the standard sonication procedure.[24] Briefly, a mixed EL/CL^{2-} chloroform solution was evaporated under vacuum, the resulting thin lipid film was dispersed in a TRIS buffer (pH 7, 10^{-2} M) for 400 s with a 4700 Cole-Parmer ultrasonic homogenizer. Liposome samples were separated from titanium dust by centrifugation for 5 min at 10,000 rpm, and used within one day. Mixed EL/CL^{2-} liposomes with a molar fraction of anionic CL^{2-} head-groups $\nu = 2[CL^{2-}]/(2[CL^{2-}] + [EL]) = 0.1$ were thus obtained.

To prepare liposomes loaded by a NaCl solution, we followed the procedure described in ref. 25. The lipid film was suspended in a mixed 1 M NaCl/10^{-3} M TRIS buffer solution and extruded through polycarbonate membranes with diameter 50 nm, an Avanti`s mini-extruder was used. Then the liposome suspension was separated from the excess of NaCl by dialysis for 4.5 hours, the outer 10^{-3} M TRIS buffer solution was changed every 1.5 hour. Integrity of NaCl-loaded liposomes was controlled conductometrically as described in ref. 12.

Double-distilled water was used for making solutions after additionally treating it with a Milli-Q Millipore system composed of ion-exchange and adsorption columns as well as a filter to remove large particles. All experiments were done at 20 ºC.

Methods

Mean hydrodynamic diameters of particles were determined by dynamic light scattering at the fixed scattering angle (90°) in a thermostatic cell with a Brookhaven Zeta Plus instrument. Software provided by the manufacturer was employed to calculate diameter values. In each experiment fresh liposomes were used whose size (hydrodynamic diameter) fluctuated from sample to sample but always fell into the 40-60 nm range with a mean value of 50 nm. Electrophoretic mobility (EPM) of particles was measured by laser microelectrophoresis in a thermostatic cell using a Brookhaven Zeta Plus instrument with the corresponding software.

The decomposition of PLA-polylysine-liposome ternary complex was initiated by addition of a 0.05 mg/ml proteolytic complex Morikrase. Then the particle size was measured by means of dynamic light scattering.

The pH values of solutions were measured using a pH meter 210 (Hanna, Germany); the conductivity of solutions using a CDM 83 conductometer (Radiometer, Denmark).

Results and discussion

Biodegradable PLA particles were used for constructing MLC. In a 10^{-2} M TRIS buffer aqueous solution, an average hydrodynamic diameter (size) of the particles was found to be equal to 160 nm; their EPM to -0.6 (μm/s)/(V/cm). A small negative charge was probably imparted due to dissociation of terminal OH-groups of the copolymer.

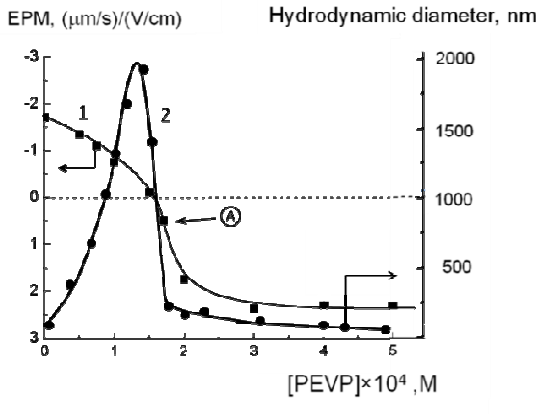

Fig.2 Electrophoretic mobility (EPM) (1) and hydrodynamic diameter (2) of PEVP-EL/CL^{2-} liposome complexes vs. PEVP concentration. ν_{CL} = 0.1; total lipid concentration 1mg/ml; TRIS buffer, 10^{-2}M; pH 7.0. Point Ⓐ corresponds to the PEVP concentration in MOCO used for complexation with PLA particles.

Liposomal containers were prepared from an EL/CL^{2-} mixture with a molar fraction of anionic CL^{2-} head-groups $\nu = 0.1$ as described in Experimental part. To make anionic EL/CL^{2-} liposomes capable of binding to the negative PLA particles, they were modified by a cationic polymer, PEVP or polylysine. **Figure 2** (curve 1) shows how EPM of EL/CL^{2-} liposomes varies when increasing concentration of cationic PEVP in the system. Addition of PEVP is accompanied by neutralization of the liposome surface charge and decrease of the EPM down to zero, then the liposome surface becomes positive and finally the EPM reaches an ultimate positive charge at [PEVP] = 2.5×10^{-4} M.

In parallel, size of EL/CL^{2-} liposomes in the presence of PEVP has been controlled by dynamic light scattering (**Figure 2**, curve 2). The liposome size increases with a rise in PEVP concentration, reaches maximum at EPM = 0 and goes down at higher PEVP concentrations. Both EPM and light scattering data prove electrostatic adsorption of positive PEVP on the surface of negative liposomes. These results are in agreement with earlier published data on aggregation of liposomes triggered by cationic polymers.[19,26,27]

In order to estimate an efficacy of PEVP-to-liposome binding, the liposomes with adsorbed PEVP were separated by centrifugation and the supernatants were analyzed spectrophotometrically (see details in Supporting Information). The dependence of PEVP concentration in the supernatant on the initial PEVP concentration (**Figure 3**) indicates a complete binding of PEVP to liposomes up to [PEVP] = 2.5×10^{-4} M. This point corresponds to the maximum positive charge of the PLA particles (**Figure 2**, curve 1) that leads to electrostatic barrier on the PLA particle surface and termination of further PEVP adsorption.

For complexation with PLA particles, PEVP-liposome complex was prepared by mixing of 1.7×10^{-4} M PEVP solution and 1 mg/mL EL/CL^{2-} liposome suspension. This complex was

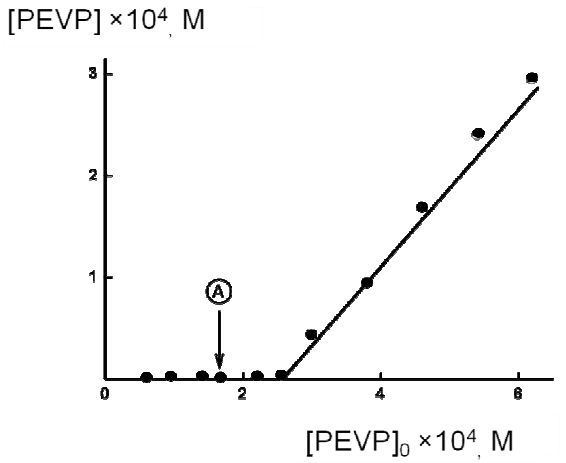

Fig.3 PEVP concentration in the supernatant after centrifugation of the PEVP- EL/CL^{2-} liposome complexes vs. initial PEVP concentration. ν_{CL} = 0.1; total lipid concentration 1mg/ml; TRIS buffer, 10^{-2}M; pH 7.0. Point Ⓐ corresponds to the PEVP concentration in MOCO used for complexation with PLA particles.

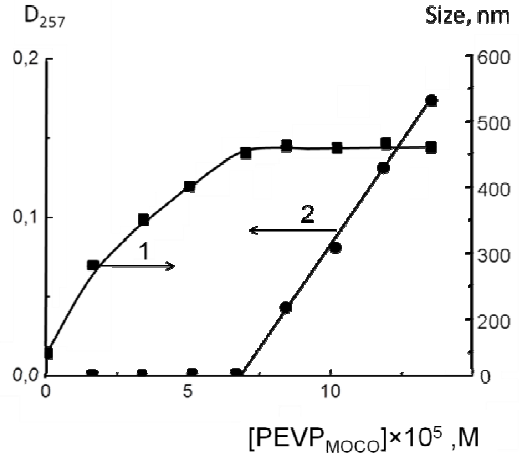

Fig.4 Hydrodynamic diameter (1) and optical density at 257 nm (2) of PLA-PEVP-liposome ternary particles vs. PEVP concentration in MOCO. EL/CL^{2-} liposomes, ν_{CL} = 0.1; TRIS buffer, 10^{-2}M; pH 7.0. $C_{copolymer}$ = 10^{-5} M

positively charged (point A on curve 1, **Figure 2**) and all added PEVP was involved in complexation with PLA particles (point A in **Figure 3**). Thus prepared modifying complex (MOCO) contained 4 PEVP chains per one liposome. A mean size of complex particles, measured by dynamic light scattering, was 170 nm.

The integrity of liposomes in MOCO was controlled conductometrically. Suspensions of liposomes loaded with a 1 M NaCl solution were prepared. A release of NaCl from liposomes into surrounding solution was accompanied by an increase the suspension conductivity (Ω). The results was compared with the conductivity of a suspension of NaCl-loaded liposomes completely destroyed in the presence of a surfactant (Triton X-100) excess and taken as 100% activity (Ω_{max}). It was found that the conductivity did not change (i.e. the liposome integrity retained) within 3 hours after MOCO formation.

MOCO was then added to a suspension of the PLA particles. Its binding to PLA was controlled by measuring size of particles in the system. The results are given in **Figure 4** (curve 1), on the X-axis is shown a molar concentration of PEVP in MOCO added to the PLA particles ($[PEVP]_{MOCO}$). An increase in PEVP concentration resulted in elevation of particle size that reflected formation of a PLA-PEVP-liposome ternary complex. The maximum particle size reached at $[PEVP]_{MOCO} = 6.8\times10^{-5}$ M.

Additionally, binding of MOCO to the PLA particles was analyzed spectrophotometrically as shown above for the PEVP-liposome binary system. After separation of the ternary complex, optical densities in the supernatants at 257 nm were measured that corresponds to maximum adsorption in the UV spectrum of PEVP. At the same wavelength, we had to register scattering of liposomes (or their complexes with PEVP) not included in complexation with the PLA particles. As follows from the data of **Figure 4** (curve 2), where "optical density vs. $[PEVP]_{MOCO}$" plot is presented, there is a rather long interval of polycation concentrations ($[PEVP]_{MOCO} \leq 6.8\times10^{-5}$ M) with zero optical density. In other words, all added MOCO is bound to the PLA particles up to $[PEVP]_{MOCO} = 6.8\times10^{-5}$ M. At this PEVP concentration, PLA-PEVP-liposome ternary particles reach their maximum size (curve 1 in **Figure 4**).

Additionally, the MOCO-to-PLA complexation was controlled by the fluorescence. PEVP is known to be a strong fluorescence quencher.[28] PEVP binding to EL/CL^{2-} liposomes with the fluorescent label (N-fluorescein-*iso*-thiocyanyldipalmitoyl-phosphatidylethanolamine) incorporated into the lipid bilayer was accompanied by the decrease of the fluorescence. When MOCO with a quenched fluorescence was added to a micellar PLA solution, no change in the fluorescence intensity was detected thus indicating the binding of non-dissociated MOCO to the PLA particles.

The integrity of liposomes in the ternary complex was controlled using an earlier described conductometry method. MOCO with $[PEVP_{MOCO}]=3.4\times10^{-5}$ M and 0.2 mg/mL liposome concentration was added to the PLA suspension. The conductivity was monitored within 3 hours after mixing of components. It was found the suspensions of the ternary complexes did not change their conductivity within 3 hour interval that means the unchanged integrity of liposomes involved in the ternary complex formation.

Composition of the ternary complex can be rationalized as follows. According to Refs. 22, 29-30 PLA particles in water solution are micellar-like structures with a core composed of PLA blocks partly crystallized into unstable micro-crystallites and a PEG shell exposed into water thus ensuring the aggregation stability of the micelles in water surrounding. Each PLA block is assumed to form α-crystal (a=1.07 nm, b=0.615 nm, c=2.78 nm) consisting of 10/3 helical chains.[31] We have examined the PLA-PEG copolymer with use of calorimetry and atomic force microscopy. With the first, a melting peak at 145 °C was reveled for the copolymer that coincided with the melting point for homo-PLA polymer found earlier and thus confirmed a crystallyne structure of the PLA core in the PLA-PEG copolymer.[29] The second allowed the thickness of the PLA-PEG particles (micelles) immobilized on the mica surface of 10-15 nm. Bases on these data, two types of PLA chain packing can be regarded: extended or double folded with thickness of 14 nm or 7 nm, respectively

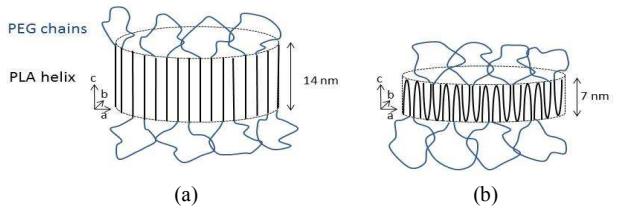

Fig.5 A chain-packing model of the PLA crystals with extended (a) and double folded (b) chains.

(**Figure 5**). In the case of more folded chains the size of primary nucleus would be too small. If so, an area per one the copolymer macromolecule with extended conformation in the micelle is 1.07 nm × 0.615 nm × 2 ≈ 1.3 nm^2, the macromolecule with double folded conformation occupies an area of 2.6 nm^2. A total area of the copolymer micelles with extended chains can be calculated as $S_m = 1.3\ nm^2 \times C_{copolymer} \times A = 0.8 \times 10^{19}\ nm^2$ and with double folded chains as $S_m = 1.6 \times 10^{19}\ nm^2$, where $C_{copolymer} = 10^{-5}$ M, A is Avogadro number.

Saturation of the copolymer micelles by MOCO is achieved at $[PEVP]_{MOCO} = 6.8 \times 10^{-5}$ M (**Figure 4**, curve 2) that corresponds to concentration of liposomes in MOCO equal to 0.4 g/L or 1.3×10^{16} liposomes per one liter. Assuming an area covered by each adsorbed liposome equal to 3.14×25 nm×25 nm = 1.9×10^3 nm^2, a total area that can be occupied by adsorbed liposomes $S_{lip} = 1.9 \times 10^3\ nm^2 \times 1.3 \times 10^{16} = 2.6 \times 10^{19}\ nm^2$.

We see therefore a good correlation between the copolymer micelle area available for liposome binding (S_m) and the area covered by liposomes at saturation (S_{lip}): $S_m \approx S_{lip}$. Actually, S_{lip} is less than S_m because of adsorption of liposome-PEVP (MOCO) aggregates but not individual liposomes.

The PLA-PEVP-liposome ternary complex described above contains biodegradable core – PLA particles and shell – peripheral electrostatically bound liposomes. However the third "intermediate" component, PEVP, does not decompose down to smaller fragments in biological environment. In order to get round the difficulty, we changed PEVP for a biodegradable cationic polymer, polylysine. This polycation was found to complex quantitatively anionic EL/CL^{2-} liposomes similar to PEVP. In the excess of polylysine, the complex acquired a positive charge and adsorbed on the surface of anionic PLA particles. **Figure 6** shows how size of particles in the system changes when polylysine-liposome modifying complex (MOCO), with 4 polylysine chains per one liposome, binds to PLA particles. An increase of polylysine concentration results in elevation of particle size, an ultimate (maximum) particle size reaches at polylysine concentration in the binary complex $[polylysine]_{MOCO} = 4.3 \times 10^{-5}$ M. As follows from the conductometrical experiments with NaCl-loaded liposomes, their integrity remain unchanged after binding to polylysine and subsequent adsorption of the polylysine-liposome MOCO on the surface of PLA particles.

I In the PLA-polylysine-liposome ternary complex all components are biodegradable that allows its decomposition in biological environment. The decomposition was initiated by addition of a proteolytic complex Morikrase, capable of cleaving ester bonds in PLA and lipid molecules and amide bonds in polylysine, to a suspension of the ternary complex using dynamic

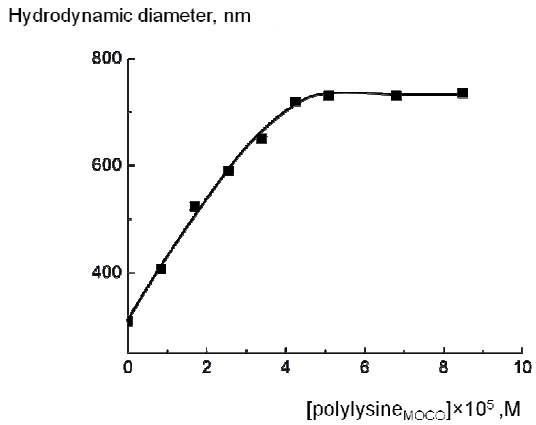

Fig.6 Hydrodynamic diameter of PLA-polylysine-liposome ternary particles vs. polylysine concentration in MOCO. EL/CL^{2-} liposomes, ν_{CL} = 0.1; TRIS buffer, 10^{-2}M; pH 7.0. $C_{copolymer} = 10^{-5}$ M

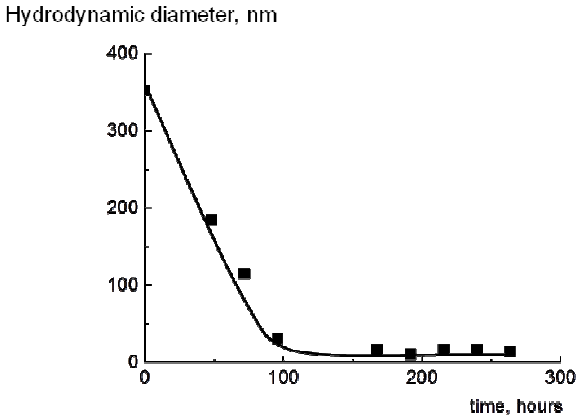

Fig.7 The kinetics of a decrease in hydrodynamic diameter of PLA-polylysine-liposome ternary particles after Morikrase addition. $[polylysine]_{MOCO} = 4.3 \times 10^{-5}$M. [Morikrase] = 0.05 mg/ml; EL/CL^{2-} liposomes, ν_{CL} = 0.1; TRIS buffer, 10^{-2}M; pH 7.0. $C_{copolymer} = 10^{-5}$ M

light scattering. A hydrolytic process was controlled by measuring a size of particles in the suspension. In a control experiment without Morikrase, no changes in size of the ternary complex particles were detected within a week after complex preparation. Contrastingly, addition of Morikrase to the ternary complex initiated a decrease in its size down to 10-15 nm within 100 hours (4 days) (**Figure 7**). This ultimate size was much lower than size of either initial component: 160 nm for PLA, 200 nm for polylysine/liposome binary complex, 60 nm for liposomes. This result definitely shows enzyme-induced biodegradation of the PLA-liposome complex.

Biodegradable multi-liposomal containers composed of PLA core and electrostatically adsorbed liposomes are described. The liposomes retain their integrity after adsorption that allows their use for encapsulation of bioactive compounds. The multi-liposomal containers eventually decompose being attacked hydrolytic enzymes that makes them promising in the drug delivery field.

Acknowledgments

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