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An electrostatic conjugate composed of liposomes, polylysine and a polylactide micelle: a biodegradability–cytotoxicity relationship

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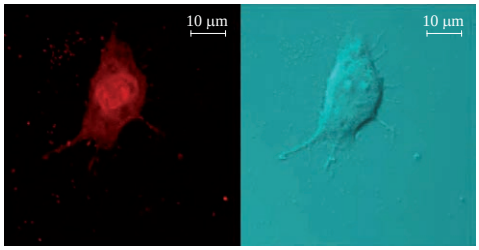
A low-toxic ternary complex composed of anionic liposomes, cationic polylysine and slightly negative micelles from a polylactide–polyethylene glycol diblock copolymer is described. Liposomes with the encapsulated antitumor antibiotic Doxorubicin (Dox) retain their integrity after incorporation into the ternary complex. The incubation of cells with the Dox-loaded ternary complex leads to its penetration into the cells, the release of Dox from liposomes and predominant drug accumulation in cell nuclei. The ternary complex degrades to nanoparticles in the presence of proteolytic enzymes to produce low-toxic digestion products.



The design of systems for the transport of biologically active compounds to target cells has been intensively developed.[1–4] Bilayer lipid vesicles (liposomes) are currently used for the encapsulation, delivery and controllable release of drugs, proteins and nucleic acids.[5–8] Generally, individual liposomes with a single type of bioactive contents inside are applied in the biomedical practice.[9–11] A multiliposome assembly loaded with therapeutic drugs can be used for the controlled delivery of medicines. In this context, the electrostatic adsorption of anionic liposomes on the surface of polystyrene microspheres with grafted polycationic chains (spherical polycationic brushes) for concentrating dozens of liposomes within a small volume has been described.[12,13] However, the *in vivo* application of liposome–brush complexes is strongly restricted by the non-biodegradability of a polystyrene core of brushes.

To overcome this obstacle, anionic liposomes were complexed with biodegradable colloidal particles: polypeptide vesicles or polylactide micelles.[14] The resulting complexes eventually degraded in the presence of proteolytic enzymes[15] to small (15–50 nm) particles, which can be removed from the bloodstream upon completing their transport function.

The above biodegradable liposome–colloid complexes contain the polycationic component polylysine (PL), which is characterized by high cytotoxicity.[16] It can manifest itself after the enzymatic destruction of the outer layer of adsorbed liposomes and the extrication of partially degraded cationic colloid. Considering this, we focused attention on the study of a relationship between the degree of enzymatic cleavage and the cytotoxicity of the liposome–PL–polylactide micelle ternary complex. Additionally, the interaction of the ternary complex with cells was visualized by confocal fluorescence microscopy using liposomes loaded with the antitumor antibiotic Doxorubicin (Dox).[†]

The first step was to estimate the cytotoxicity of the individual components of the ternary complex (EL/CL^{2-} liposomes, PLA–PEG diblock copolymer micelles and PL) towards breast adenocarcinoma cells (MCF-7/R). The cytotoxicity was defined as an additive concentration at which a 50% viability of cells was detected (LC_{50}): a lower value of LC_{50} corresponded to a higher cytotoxicity (Figure 1). The liposomes and copolymer micelles showed very low cytotoxicity: in both cases, LC_{50} was 0.5 mg ml^{-1}, the maximum sample concentrations available in our experiments (*1* and *2*, respectively). In contrast to this, PL was much more cytotoxic with LC_{50} = 0.005 mg ml^{-1} (*3*) obviously due to multiple cationic groups capable of binding to a negative cell surface thus affecting the cell functioning.

[†] Liposomes were prepared from zwitterionic egg yolk lecithin (EL) and doubly anionic diphosphatidylglycerol (cardiolipin, CL^{2-}) (both from Avanti); their structures are shown in Figure S1, Online Supplementary Materials. Dox (Figure S1) was purchased from VeroPharm. Small unilamellar liposomes, including those loaded by Dox, were prepared by a standard sonication technique (Procedure S1). The molar ratio of CL^{2-} (ν_{CL}) was 0.05. Freshly prepared liposomes with an average hydrodynamic diameter of 40–50 nm were used in the experiments. Polylysine hydrobromide with the degree of polymerization of 340 was purchased from Sigma. The polylactide–polyethylene glycol (PLA–PEG) diblock copolymer (Figure S1) was synthesized as described elsewhere[17] (see Procedure S2 for the preparation of micelles).

The biodegradation of the liposome–PL–polylactide micelle ternary complex was initiated by the addition of a Morikrase proteolytic complex[18] (Procedure S3). Cell survival (cytotoxicity) of the samples towards human breast adenocarcinoma MCF-7/R cells was evaluated with a methyltetrazolium blue[19] (Procedure S4).

The liposomes were loaded with Dox according to a modified procedure described previously[20] (Procedure S5). The intracellular localization of Dox was analyzed by laser scanning confocal microscopy (Procedure S6).

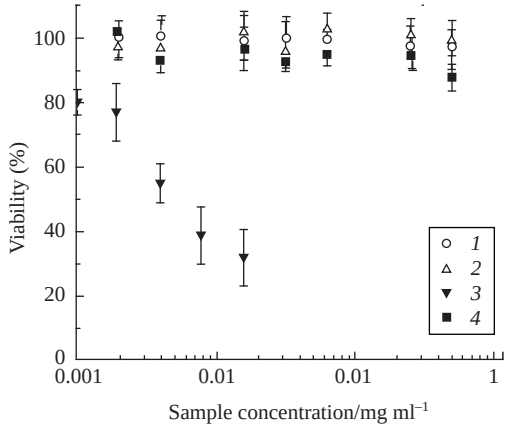


Figure 1 Viability of MCF-7/R cells *vs.* the concentrations of (*1*) EL/CL^{2-} liposomes, (*2*) PLA−PEG copolymer micelles, (*3*) PL and (*4*) the liposome–PL–micelle ternary complex. The initial sample of the ternary complex contained 0.5 mg ml^{-1} of total lipids, 8.5×10^{-3} mg ml^{-1} of PL and 9.6×10^{-2} mg ml^{-1} of PLA−PEG.

The ternary complex was prepared as described earlier.[15] The anionic EL/CL^{2-} liposomes were complexed with cationic PL followed by the binding of thus prepared positive liposome–PL complex to slightly negative PLA–PEG copolymer micelles. Based on the previous results, we found an optimal composition in which an undissociated liposome–PL complex was completely bound to PLA–PEG particles. Surprisingly, the cytotoxicity of the ternary complex with cationic PL did not exceed that of the initial liposomes (*cf. 4* and *1* in Figure 1). A significant decrease in the cytotoxicity of PL after its incorporation into the ternary complex was likely owing to the neutralization of PL positive charges by the negative charges of liposomes and micelles. Indeed, laser microelectrophoresis measurements showed a slightly positive electrophoretic mobility (EPM) of ternary complex particles, +0.04 (μm s^{-1}) (V cm^{-1})$^{-1}$, which was consistent with an assumption on the complete neutralization of PL in the ternary complex.

The biodegradation of the ternary complex was monitored by measuring the size of complex particles after the addition of a Morikrase proteolytic complex (Figure 2). In the absence of enzyme, no change in the particle size was detected within a longer period of time (curve *1*) due to negligible particle degradation. In contrast, the addition of Morikrase induced a decrease in the complex particle size (curve *2*) resulted from the joint action of Morikrase enzymes capable of cleaving ether bonds in lipids and polylactide block copolymer and amide bonds in PL.

The cytotoxicity of the product(s) of enzymatic degradation (PED) towards MCF-7/R was tested (Figure 3). We found that the cytotoxicity *vs.* concentration profiles for PEDs (*2*–*6*) coincide with the corresponding profile of the initial ternary complex untreated with Morikrase (*1*). These results indicate a low cyto-

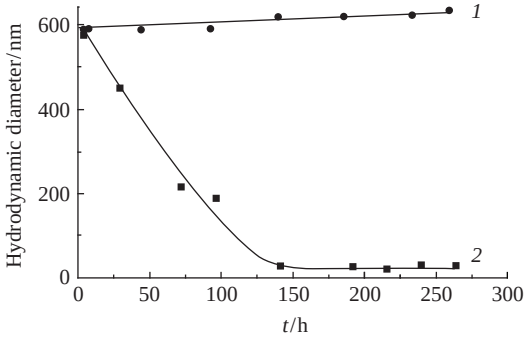


Figure 2 Time-dependent size of ternary complex particles (*1*) without and (*2*) after the addition of the Morikrase proteolytic complex, 5×10^{-2} mg ml^{-1}; EL/CL^{2-} liposomes; total lipids, 0.5 mg ml^{-1}; PL, 8.5×10^{-3} mg ml^{-1}; PLA–PEG, 9.6×10^{-2} mg ml^{-1}; 10^{-2} M TRIS buffer, pH 7.

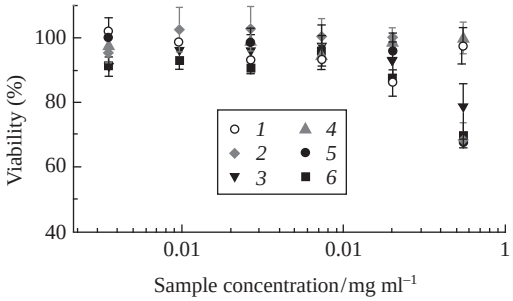


Figure 3 Viability of MCF-7/R cells *vs.* concentration of (*1*) the initial liposome–PL–micelle ternary complex and the complexes pretreated by Morikrase for (*2*) 1, (*3*) 15, (*4*) 39, (*5*) 63 and (*6*) 87 h. EL/CL^{2-} liposomes. The initial sample of the ternary complex contained 0.5 mg ml^{-1} of total lipids, 8.5×10^{-3} mg ml^{-1} of PL and 9.6×10^{-2} mg ml^{-1} of PLA–PEG. Morikrase, 5×10^{-2} mg ml^{-1}.

toxicity of both the initial ternary complex (comparable with that of the individual liposomes) and PEDs in the process of biodegradation. According to laser microelectrophoresis data, the EPM of PEDs slightly shifted from a positive to a negative region during the biodegradation: from +0.03 to –0.08 (μm s^{-1}) (V cm^{-1})$^{-1}$. The cytotoxic and electrophoretic data show that biodegradation affects all of the ternary complex components, including cationic PL; it finally allows one to keep the particle charge close to zero. These results indicate the prospects of biodegradable multiliposomal complexes as containers for the immobilization of biologically active substances.

As stated above, the biodegradable ternary complex is intended for drug encapsulation and delivery to cells. For this purpose, liposomes were loaded with the antibiotic Dox, which is characterized by bright fluorescence at λ_{em} = 557 nm, which was partially quenched after concentrating Dox inside liposomes. The release of Dox from the liposomes, if it occurred, was accompanied by fluorescence recovery.[21] In a control experiment, no change in the fluorescence intensity, *i.e.*, no Dox release from the individual liposomes, was found at least within 3 h. Similarly, no Dox release was detected within the same period from the Dox-loaded liposomes after their incorporation into the ternary complex. The ternary complex with Dox-loaded liposomes was visualized using confocal fluorescence microscopy (Figure S2, Online Supplementary Materials), which showed red fluorescent particles with an average size between 0.5 and 1 μm.

The capability of the ternary complex to deliver a drug in tumor cells was studied by confocal fluorescence microscopy. The incubation of MCF-7 cells with the Dox-loaded ternary complex resulted in bright red fluorescence in the cell nuclei and a weaker staining of cytoplasm (Figure 4). The picture definitely shows that, after the penetration of the ternary complex into cells, the Dox released from liposomes penetrated into the nucleus where inter-

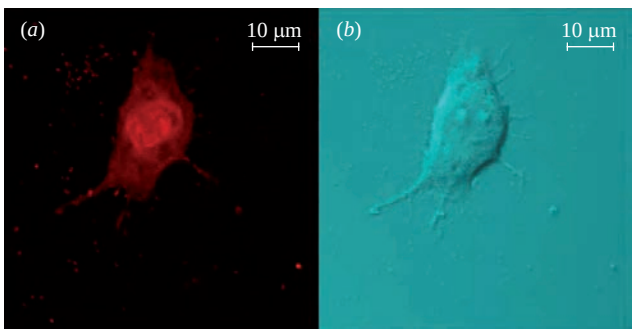


Figure 4 Confocal microscopy images of Dox fluorescence in MCF-7/R cells pretreated with a suspension of the Dox-loaded ternary complex for 1 h. Concentration: total lipid, 0.5 mg ml^{-1}; Dox, 25 μmol dm^{-3}; PLA–PEG, 9.6×10^{-2} mg ml^{-1}. (*a*) Fluorescent mode, (*b*) DIC.

calated into DNA, while a minor part of Dox was distributed in the cytoplasm.

Taking into account the degradation of the ternary complex in a model Morikrase solution, we expected that the ternary complex would be decomposed after entering the body (a cell medium) and, finally, excreted as nanosized particles. The above results offer a challenge for further development of these complexes as potential vehicles for the delivery of antitumor drugs.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mencom.2017.05.028.

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