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Distinct mechanisms of membrane permeation induced by two polymalic acid copolymers

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ABSTRACT

Anionic polymers are valuable components used in cosmetics and health sciences, especially in drug delivery, because of their chemical versatility and low toxicity. However, because of their highly negative charge they pose problems for penetration through hydrophobic barriers such as membranes. We have engineered anionic polymalic acid (PMLA) to penetrate biological membranes. PMLA copolymers of leucine ethyl ester (P/LOEt) or trileucine (P/LLL) show either pH-independent or pH-dependent activity for membrane penetration. We report here for the first time on the mechanisms which are different for those two copolymers. Formation of hydrophobic patches in either copolymer is detected by fluorescence techniques. The copolymers display distinctly different properties in solution and during membranolysis. P/LOEt copolymer binds to membrane as single molecules with high affinity, and induces leakage cooperatively through a mechanism known as “carpet” model, in which the polymer aligns at the surface throughout the entire process of membrane permeation. In contrast, P/LLL self-assembles to form an oligomer of 105 nm in a pH-dependent manner (pKa 5.5) and induces membrane leakage through a two-phase process: the concentration dependent first-phase of insertion of the oligomer into membrane followed by a concentration independent second-phase of rearrangement of the membrane–oligomer complex. The insertion of P/LLL is facilitated by hydrophobic interactions between trileucine side chains and lipids in the membrane core, resulting in transmembrane pores, through mechanism known as “barrel-stave” model. The understanding of the mechanism paves the way for future engineering of polymeric delivery systems with optimal cytoplasmic delivery efficiency and reduced systemic toxicity.

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1. Introduction

Hydrophobically modified polyanions comprise a group of membrane destabilizing polymers used for cytoplasmic delivery of nucleic acids and small molecular therapeutics [1–3]. Membrane permeation induced by these polymers usually involves events starting in solution at the polymer-membrane interface: the formation of an amphipathic polymer, the subsequent complexation with the membrane, membrane pore formation, and membrane leakage [1,4,5]. Mechanisms of membranolysis by polymers are, however, poorly understood, because of their variable compositions, structures, and random conformation unlike proteins and peptides whose interaction with membranes has been extensively studied [6–9]. With the booming of nanobiotechnology, more and more polymers are used in pharmaceutical applications such as drug delivery [10–12], and the understanding of their membrane permeation mechanism allows to optimize delivery efficiency and reduce systemic toxicity.

Of particular interest are pH-responsive drug delivery systems, which are membranolytic in the range pH 5.0 to pH 6.0 corresponding to the pH within maturating endosomes [13]. The membrane permeation activity allows the drug delivery system to escape from the endolysosome into cytoplasm, preventing its entrapment and degradation in the lysosome. pH-Responsiveness in this range ensures the occurrence of membrane permeation only at the endosome/cytoplasm interface after cellular uptake through endocytosis and avoids unspecific cytotoxic damage of the cellular membrane at pH 7.4. A commonly accepted mechanism of

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pH-responsive membrane permeation involves protonation of anionic polymers capable of forming an amphipathic structure for interaction with membranes [1]. Polymers with this activity depend on their hydrophobicity of their side chains [14,15]. While no general mechanism of membrane disruption applies to all kinds of polymers, several models are used to describe peptide–membrane interactions, notably the "carpet" and "barrel-stave" mechanisms [6,9,16,17], and similar mechanisms have been proposed for polymer-membrane interactions [5]. In the "carpet" model, polymers approach the membrane as single molecules and characteristically align with phospholipids head groups at the surface throughout the entire process of membranolysis [6,7]. Distinctively different, polymers in a transmembrane model (barrel-stave) start with polymer–polymer interactions localized next to the membrane surface. This assembly reaction is followed by perpendicular insertion of the formed oligomer into the core of the lipid membrane [6,7]. Thus, the decision of a polymer to follow one or the other mechanism reflects the degree of amphipathicity and the tendency for cooperative binding and/or oligomerization.

In this work we studied the mode of action of membrane permeation by two copolymers: poly(β-L-malic acid) conjugated with trileucine (P/LLL) and poly(β-L-malic acid) conjugated with leucine ethyl ester (P/LOEt) [18,19]. Both copolymers have been successfully used for drug delivery to treat brain and breast tumors [18,20–22]. The backbone poly(β-L-malic acid) (PMLA) prepared from *Physarum polycephalum* [23] is water-soluble, nontoxic, non-immunogenic and biodegradable (final degradation products CO_2 and H_2O [24]) but it cannot permeate membranes due to its hydrophilic nature. P/LLL and P/LOEt are the copolymers obtained by amidation of a fraction of the polymer carboxyl groups with ydrophobic trileucine or leucine ethyl ester (Fig. 1). pH-Independent P/LOEt and pH-dependent P/LLL exhibit distinct solution and membranolysis properties. Using unilamellar model membranes such as liposome and giant unilamellar vesicles, distinct mechanisms for membrane permeation by both copolymers were studied using liposome leakage analysis, dynamic light scattering, confocal microscopy, and fluorescence resonance energy transfer (FRET).

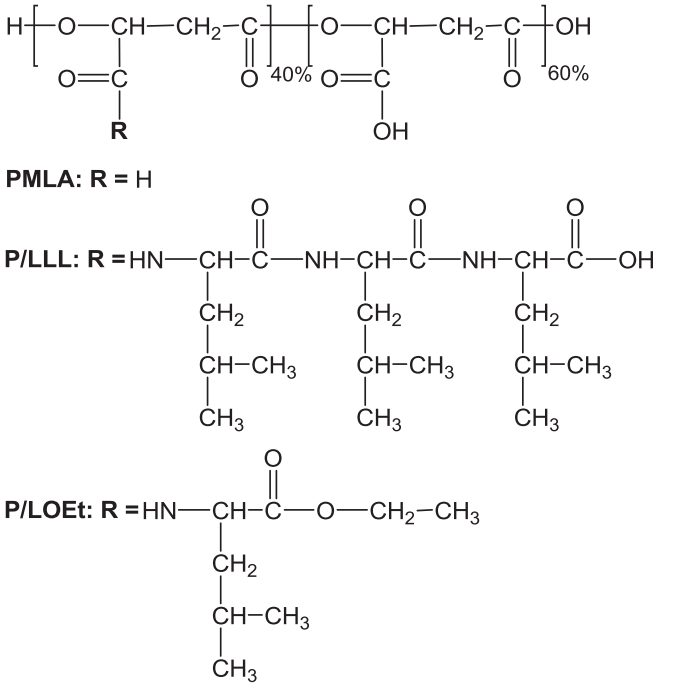

Fig. 1. Structure of polymalic acid grafted with 40% of trileucine (P/LLL) and 40% leucine ethyl ester (P/LOEt).

2. Materials and methods

2.1. Materials

Poly(β-L-malic acid) (PMLA) (100 kDa; polydispersity 1.3) was obtained from culture broth of *P. polycephalum* as described [23,24]. Tripeptides H-Leu-Leu-Leu-OH (LLL), H-Leu-Leu-Leu-NH_2 (LLL-NH_2), and H-Leu-OEt (LOEt) were purchased from Bachem Americas Inc. (Torrance, CA, USA). N-(1-pyrene)-maleimide (Py) and rhodamine Red C_2-maleimide (Rh) were purchased from Invitrogen (Carlsbad, CA, USA). L-α-Phosphatidylethanolamine-N-(4-nitrobenzo-2-oxa-1,3-diazole) (NBD-PE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Calcein and cholesterol were from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Synthesis of P/LLL and P/LOEt (structures see Fig. 1)

The synthesis of P/LLL and P/LOEt has been improved based on the method previously described [18,19]. To a 1 mL solution of PMLA (80 mg, 0.69 mmol equivalent of malic acid) in acetone was added a mixture of *N*-hydroxysuccinimide (NHS, 80 mg, 0.69 mmol) and dicyclohexylcarbodiimide (DCC, 142 mg, 0.69 mmol) in 1 mL DMF. After 2 h stirring at room temperature, H-Leu-Leu-Leu-OH (99 mg, 0.28 mmol, 40% equivalent to the total malyl groups) dissolved in DMF with the assistance of trifluoacetic acid (25 μL, 0.34 mmol) was added, followed with the sequential addition of triethylamine (49 μL, 0.35 mmol) in four aliquots at an interval of 0.5 h. The completion of conjugation was verified by TLC (ninhydrin test). Unreacted *N*-hydroxysuccinimidyl ester was hydrolysed by the addition of 3 mL phosphate buffer (100 mM pH 6.8). The dicyclohexylurea precipitate was removed by filtration. The product P/LLL was purified over PD-10 column to remove organic solvent and residual small molecules (GE Healthcare).

To prepare P/LLL/MEA, the remaining unreacted *N*-hydroxysuccinimidyl ester was used for conjugation of 2-mercaptoethylamine hydrochloride (MEA, 1.6 mg, 0.012 mmol, 2% equivalent to the total malyl groups) in the presence of triethylamine (2.4 μL). Reaction completion after 30 min was tested on TLC with ninhydrin. Remaining unreacted *N*-hydroxysuccinimidyl ester was hydrolysed by the addition of phosphate buffer pH 6.8. The product P/LLL/MEA was purified over PD-10 column (GE Healthcare). P/LOEt and P/LOEt/MEA were prepared similarly.

2.3. Synthesis of fluorescence labeled compounds

Pyrene labeled copolymers P/LLL/Py and P/LOEt/Py. N-(1-pyrene)-maleimide (50 μg) was dissolved in 100 μL of DMF, to which was slowly added P/LLL/MEA (1.5 mg dissolved in 0.3 mL of phosphate buffer 100 mM phosphate, pH 6.3) to avoid the precipitation. The mixture was incubated at 4 °C for 2 h. Unreacted N-(1-pyrene)-maleimide was removed by passing through a PD-10 column (GE Healthcare). Unreacted thiols on polymer were blocked with excess of 3-(2-pyridyldithio)-propionate (PDP) at room temperature. The product P/LLL/Py was purified over PD-10 column. The content of pyrene was estimated to be 0.4% of total malyl groups using UV spectrometry. P/LOEt/Py and P/Py were synthesized analogously and contained 0.4% and 0.2% pyrene respectively.

Rhodamine labeled copolymers P/LLL/Rh and P/LOEt/Rh. To the solution of P/LLL/MEA or P/LOEt/MEA (1.7 mg each) in phosphate buffer (100 mM pH 6.3) was added rhodamine Red C2 maleimide (1% equivalent to total malyl group) dissolved in DMF. The reaction was kept dark at room temperature with shaking for 2 h. Unreacted thiols on polymer were blocked with excess of 3-(2-pyridyldithio)-propionate (PDP) at room temperature. The product P/LLL/Rh was purified over PD-10 column. The content of rhodamine of P/LLL/Rh and P/LOEt/Rh was measured by UV spectrometry to be 1% and 0.7%.

2.4. Kinetics of liposome leakage

Kinetic experiments in triplicate were performed as following: Liposome prepared as previously described [18,19] 5 μL (lipid concentration 30 μM) was added to the samples of P/LLL and P/LOEt in 100 μL of 137 mM citrate buffer (pH 5.0) (96-well plate at different concentrations of copolymers). Immediately the plate was read by a Flexstation fluorescence spectrophotometer using the kinetics mode at 1 min intervals with excitation wavelength 488 nm and emission wavelength 535 nm at room temperature. The reading was ended after 30 min. Complete leakage of calcein was achieved in the presence of 0.25% (v/v) Triton-X 100. Leakage is reported as the fluorescence intensity over that in the presence of Triton-X 100. The kinetics data were fit by a two-phase exponential kinetics equation using the software Graphpad Prism 3.02:

$$Y = Y1_{max} \times \left(1 - e^{-k_1(X+T)}\right) + Y2_{max} \times \left(1 - e^{-k_2(X+T)}\right) \quad (1)$$

Where, Y is the liposome leakage (%); X is time in minutes; $Y1_{max}$ is the maximum liposome leakage of the first phase (%); $Y2_{max}$ is the maximum liposome