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Distinct mechanisms of membrane permeation induced by two poly(malic 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 poly(malic 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 (pK_a 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.

1. INTRODUCTION

Hydrophobically modified polyanions comprise a group of membrane destabilizing polymers used for cytoplasmic delivery of nucleic acid based 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,

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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 maturing 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 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 group 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 ethylester (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 production 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 hydrophobic 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).

2. MATERIALS AND METHODS

2.1. Materials

Poly(β -L-malic acid) (PMLA) (100 kDa; polydispersity 1.3) was obtained from culture broth of *Physarum polycephalum* as described [23, 24]. Tripeptides H-Leu-Leu-Leu-OH (LLL), H-Leu-Leu-Leu-NH₂ (LLL-NH₂), and H-Leu-OEt (LOEt) were purchased from Bachem Americas Inc. (Torrance, CA, USA). N-(1-pyrene)-maleimide (Py) and rhodamine Red C₂-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 hr 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 μ L, 0.34 mmol) was added, followed with the sequential addition of triethylamine (49 μ L, 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 μ L). 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 μ g) was dissolved in 100 μ L 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. *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 hours. 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 5 μ L (lipid concentration 30 μ M) was added to the samples of P/LLL and P/LOEt in 100 μ L 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-minute 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-exponential kinetics equation using the software Graphpad Prism 3.02:

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

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 leakage of the second phase (%); k_1 is the first phase leakage rate constant and k_2 is the second phase leakage rate constant; T is the elapsed time before the first measurement.

The $Y1_{\max}$ values vs concentration dependence was further fit by a sigmoidal dose-response curve (Eq. 2) using GraphPad Prism (3.02) software.

$$Y = \text{Bottom} + \frac{\text{TOP} - \text{Bottom}}{1 + 10^{(\text{LogEC50} - X) \times \text{Hillslope}}} \quad (\text{Eq. 2})$$

Where, X is the logarithm of copolymer concentration, Y is the $Y1_{\max}$ from above fitting equation. $Y1_{\max}$ shows a sigmoidal relationship within the range between Top and Bottom with a slope factor, also called Hill-slope that describes the steepness of the curve as a function of polymer concentration. Hill-slope much larger than 1 indicates a strong positive cooperativity.

2.5. Preparation of giant unilamellar vesicle and fluorescent confocal microscopy study of its binding with copolymers

Giant unilamellar vesicles (GUV) were prepared by the evaporation method [18, 25]. The GUVs have the same lipid composition as the liposomes for the leakage assay with additional 1% (molar) green fluorescent L- α -Phosphatidylethanolamine-N-(4-nitrobenzo-2-oxa-1,3-diazole) (NBD-PE).

For confocal microscopy, the GUVs were incubated with 20 $\mu\text{g/mL}$ of rhodamine labeled P/LLL/Rh and P/LOEt/Rh in citrate buffer (10 mM, pH 5.0) for 30 min at room temperature. A TCS SP5 $\times$ spectral scanner (Leica Microsystems) was used with spectral settings for NBD, excitation 490 nm and emission 505–546 nm and for rhodamine, excitation 552 nm and emission 563–626 nm.

2.6. Fluorescence measurement

Conjugates, P/LLL/Py, P/LOEt/Py and P/Py (pyrene concentration 0.5 μM) were dissolved in phosphate buffer pH 7.4 and 5.0. The fluorescence spectra of each sample (0.6 ml) at different pH were measured with SPECTRAmax M2 (Molecular Devices) using the SoftMax Pro 4.7.1. The emission spectra of conjugates, alone or in the presence of liposomes (250 $\mu\text{g/mL}$) were recorded from 360 nm to 460 nm with excitation wavelength at 340 nm.

2.7. Hydrodynamic diameter

The copolymers were characterized with respect to their size (hydrodynamic diameter) using a Malvern Zetasizer Nano (Malvern Instruments, UK). The diameter that is measured in DLS (Dynamic Light Scattering) refers to the particle diffusion within a fluid and is referred to as the hydrodynamic diameter corresponding to the diameter of a sphere that has the same translational diffusion coefficient as the particle. All calculations were carried out by the Zetasizer 6.0 software. For the particle size measurements, the solutions were prepared in

phosphate buffers with a pH range (5.0–7.0) at the concentration of 40 $\mu\text{g/mL}$ or with varied concentration at pH 5.0, filtered through a 0.2 μm pore membrane. All the copolymer solutions were prepared immediately before analysis at 25 $^{\circ}\text{C}$. Data represent the mean value obtained from three independent measurements.

2.8. Fluorescence resonance energy transfer (FRET)

Liposome containing 1% NBD-PE was prepared using extrusion method [19] as previously described (50 $\mu\text{g/mL}$) was incubated with different concentration of rhodamine-P/LLL and rhodamine-P/LOEt, 0, 0.1, 0.2, and 0.4 μM (concentration of rhodamine). The fluorescence intensity of the mixture was recorded from wavelength 500 – 620 nm (excitation wavelength of 460 nm) on SPECTRAmax M2 (Molecular Devices) using the SoftMax Pro 4.7.1.

3. RESULTS

3.1. Cooperativity of membranolysis indicated by liposome leakage kinetics

Previous membranolysis study on PMLA copolymers indicated that copolymers P/LOEt and P/LLL disrupt liposome membranes by virtue of the hydrophobic side chain [19]. P/LLL was active only after pH-dependent protonation of the LLL-terminal carboxylate whereas P/LOEt was active independent of pH, which suggested that the intrusion of hydrophobic LOEt and LLL residues into the liposome membrane triggered the membranolysis. In addition, cooperative membranolysis by P/LOEt was indicated by Hill-slope much larger than 1 [19], which implies their different mechanisms of membrane interaction.

To substantiate their difference, we measured the kinetics of membrane permeation by P/LLL (Fig. 2A) and P/LOEt (Fig. 2B) using conditions of the liposome leakage assay [18, 19]. The kinetics data of both copolymers were subjected to curve-fitting with a two-phase exponential equation (Eq. 1), a fast concentration dependent first phase with $t_{1/2}$ less than 2 min and a much slower concentration-independent second phase with $t_{1/2}$ around 10 min. The maximum leakage caused by the first phase ($Y_{1\text{max}}$) was plotted against concentration and fitted with sigmoidal dose-response curve Eq. 2 (Fig. 2C). The concentration range of the first phase for P/LOEt (fitting Hill-slope = 5.8) was lower than the one for P/LLL (fitting Hill-slope = 1). Hill-slope of P/LOEt ($\gg 1$) and indicated a cooperative action of P/LOEt molecules to induce leakage.

According to the fitting results, the first and second phases of P/LLL contributed almost equally to the total liposome leakage at all experimental concentrations. In contrast, the first leakage phase for P/LOEt contributed almost exclusively to the leakage. The difference between the kinetics for P/LLL and P/LOEt suggested that their mode of action differed greatly. The slower second phase of leakage is considered as a result of a structural rearrangement after membrane-copolymer complexation.

3.2. Liposome concentration dependent leakage implies different membranolysis efficiency of copolymers

Obviously, both copolymers were consumed in the process of their membranolysis eventually, but their membranolysis efficiency might be different at different lipid to polymer ratio. As shown in Fig. 2D, the leakage was indicated by the absolute fluorescence intensity of leaking calcein with varied lipid to polymer concentration at pH 5.0. A maximum calcein leakage by P/LOEt was observed and its leakage remained constant or even slightly decreased as the ratio further increased. In contrast, for P/LLL the fluorescence intensity of leaked calcein increased steadily indicating that P/LLL was more efficient in membrane permeation especially at high lipid-to-polymer ratios. This behavior of the two copolymers was consistent with the accumulation of all the available P/LOEt molecules on

liposomes without redistribution. Differently, P/LLL was able to achieve higher leakage by encountering more liposomes. This phenomenon suggested irreversible binding of P/LOEt to membrane followed with cooperative membranolysis, which restricted the redistribution of P/LOEt on membrane thus limiting its efficiency of membranolysis.

3.3. Confocal microscopy study of copolymers binding to membrane

Membranolysis induced by the copolymers involved their binding to membrane. Giant unilamellar vesicles (GUVs) of large-size membrane structure (~5 μm in diameter) were used for visualizing the accumulation of the copolymers on the membranes by confocal microscopy (Fig. 3). GUVs were prepared with solvent evaporation method which was more convenient than the methods of gentle hydration and electroformation, but with drawbacks of some degree of heterogeneity and multi-laminarity [25]. In this microscopy study, the membrane of GUV seemed intact without disintegration in presence of both copolymers at the experimented concentration. GUVs contained 1% phosphatidylethanolamine lipid fluorescently labeled with NBD (NBD-PE) at the head groups (in green, Fig. 3A, left). They co-localized with rhodamine labeled P/LOEt (in red, Fig 3A), suggesting P/LOEt irreversibly binds to membrane with high affinity. In a same setting, P/LLL didn't show detectable fluorescence on the GUV membrane (Fig. 3B), suggesting its low affinity binding to membrane. This agrees with the results in Fig. 2D that P/LLL was more efficient for liposome leakage in spite of its low binding affinity through different mechanisms. In addition, close inspection found a focal accumulation of copolymer P/LOEt along membrane (Fig. 3A), indicative of its cooperative binding to membrane.

3.4. Probing for hydrophobic regions in P/LLL and P/LOEt copolymers

Copolymers bind to liposomes through their hydrophobic side chains. The large number of LLL and LOEt residues on each copolymer molecule could form hydrophobic patches, which then bind strongly to lipophilic regions of membranes. In order to probe for such hydrophobic patches, copolymers are labeled with pyrene, a fluorophore responsive to enhanced hydrophobic environment indicated by an increase in fluorescence intensity [26]. Enhanced fluorescence intensity of pyrene was observed for copolymers P/LLL/Py and P/LOEt/Py at pH 5.0, in contrast to low fluorescence intensity in the case of free pyrene (Py) or for unsubstituted PMLA tagged with pyrene (P/Py) (Fig. 4B). Also, the fluorescence intensity of P/LLL/Py was low at pH 7.4, but increased dramatically as pH changed from 7.4 to 5.0 (Fig. 4A,B). This indicated a pH sensitive hydrophobic microenvironment within P/LLL molecules, thought as the result of conformational change induced by protonation of carboxylate in the LLL residues. In contrast, the hydrophobicity of P/LOEt was independent of pH and the fluorescence intensity of P/LOEt/Py remained the same regardless of pH change between 7.4 and 5.0. This result agrees well with previously found properties for pH-dependent P/LLL and pH-independent P/LOEt regarding, pKa, Zeta-potential and liposome leakage [18, 19].

Amphipathicity of copolymers may change due to their interaction with membrane in the presence of liposome, resulting in the fluorescence change of tagged pyrene. The fluorescence intensity of P/LLL/Py, however, didn't change in the presence of liposome at pH 7.4 (Fig. 4C), in agreement with the previous conclusion that at this pH the copolymer did neither form hydrophobic intra-molecular patches nor interact with membranes. In contrast, fluorescence intensities decreased when membranes were mixed with P/LOEt at both pH 7.4 and pH 5.0 (Fig. 4C and 4D), and with P/LLL at pH 5.0 (Fig. 4D) indicating a rearrangement of the pyrene microenvironments under these conditions. It should be mentioned that this observation is at variance with findings that the insertion of certain proteins into membrane enhanced the fluorescence of covalently bound pyrene [26, 27]. Thus the observed fluorescence decrease was consistent with the assumed relaxation of the

polymer structure around pyrene in favor of inserting side chains into the lipid membrane and leaving the environment of pyrene less hydrophobic. Overall, the interaction between copolymers and membrane correlated well with the assumed membrane binding of the hydrophobic patches during liposome leakage.

3.5. Probing the self-assembly of copolymers in solution

Hydrodynamic diameter of copolymers as a function of pH and concentration was investigated in order to understand whether oligomer formation was involved in the formation of hydrophobic patches and whether in membrane disruption followed the “barrel-stave” mechanism. The hydrodynamic diameter of P/LOEt was approximately 5.5 nm as measured by dynamic light scattering. The value remained unchanged within the range pH 5.0 – pH 7.4 and at concentrations < 250 µg/mL. This was in agreement with the diameter of dispersed molecules having the molecular weight of 150,000. P/LLL at pH > 6.0 had a similar hydrodynamic diameter of 6.5 nm (Fig. 5A) indicating fully dispersed molecules. However, as pH dropped below 6.0, P/LLL formed particles of 105 nm were formed as the result of oligomerization. The degree of oligomerization increased as pH decreased following a pKa of ~5.3 (Fig. 5A).

Therefore, this pH dependent oligomerization was a direct result of P/LLL side chain protonation and an efficient way for P/LLL to accommodate its enhanced hydrophobicity. In addition, the size and the degree of oligomerization increased with the increase of concentration (Fig. 5B) suggesting that the aggregate was gradually assembled to reach a hydrodynamic diameter of 105 nm at concentrations > 2 µg/mL.

The solution conformations of the copolymers were calculated using the software ChemBio3D ultra 11.0 (Fig. 7A). Note that side chain protonated P/LLL exposed hydrophobic leucine residues that allowing the formation of aggregates.

For comparison we investigated the oligomerization of P/LLL-NH₂, an analog of P/LLL with the side chain carboxylate masked by amidation. The insolution conformation, especially the exposures of hydrophobic side chains were similar as for P/LLL when all LLL residues were protonated (Fig. 7A). Different from P/LLL, which was only membranolytically active at pH 5.0, P/LLL-NH₂ has previously been found active at both pH 5.0 and pH 7.4 [19]. The conformation of P/LLL with side chain protonated at pH 5.0 was therefore thought comparable with that of P/LLL-NH₂. In fact, the hydrodynamic diameter of P/LLL-NH₂ was found to be 105 ± 10 nm and independent of pH and concentration, suggesting that this copolymer existed exclusively in the oligomerized form. As the side chain carboxylate of P/LLL-NH₂ was completely neutralized by amidation, it resembled P/LLL with full side chain protonation. The observation that complete oligomerization of P/LLL-NH₂ correlated with its pH-independence of membranolytic activity suggested that the oligomerization of P/LLL by protonation was the key to its membrane permeation. The dependence of P/LLL on oligomerization for membrane permeation markedly differed from that of P/LOEt. P/LOEt, incapable of oligomerization, bound to membranes individually and caused membranolysis through a cooperative action.

3.6. Fluorescence resonance energy transfer (FRET) between copolymers and membrane

Fluorescence resonance energy transfer (FRET) was used to compare the proximity and/or orientation of fluorophores in copolymers relative to fluorophores in the liposomal membrane. Membranes were prepared containing 1% phosphatidylethanolamine lipid fluorescently labeled with NBD (NBD-PE) as the energy donor at the lipid head groups. Copolymers P/LLL/Rh and P/LOEt/Rh contained rhodamine as the fluorescence acceptor covalently bound to PMLA backbone. Energy transfer for P/LOEt/Rh was observed

indicated by an increase in fluorescence intensity for rhodamine acceptor at 585 nm and a decrease for donor NBD at 535 nm (Fig. 6A). The higher the polymer concentration the more the intensity increased. This suggested that P/LOEt/Rh was in close proximity to lipid head groups as would be the case if the polymer backbone was aligned in parallel with the membrane surface. The intensity of the FRET signal was the same at pH 5.5 and pH 7.4 (results not shown) in agreement with the pH-independency of the liposome leakage. In contrast, the energy transfer between P/LLL/Rh and lipid bound fluorophores was barely detectable at pH 5.0 (Fig. 6B) even though this was the condition of high membranolytic activity. This suggested that polymer molecules were positioned distantly from lipid head groups and/or the polymer-bound rhodamine and the lipid-bound NBD were unfavorable oriented towards each other as would be the case if P/LLL/Rh inserted perpendicular into the lipid layer.

4. DISCUSSION

4.1 Amphipathic structure of P/LLL and P/LOEt

Hydrophobically modified anionic polymers were found to form amphipathic structures which could interact with phospholipid bilayer membranes [1, 2]. Their mode-of-action, however, was poorly understood. Anionic polymalic acid copolymers of leucine ethylester (P/LOEt) or trileucine (P/LLL) exhibited distinct membranolysis properties. A study on their mode-of-action of membrane permeation would contribute to the understanding of polymer-membrane interactions.

P/LLL and P/LOEt demonstrated distinct solution properties. The Zeta-potential of P/LLL is pH-dependent, in contrast to the pH-independent Zeta-potential of P/LOEt [18]. The pH-responsiveness of P/LLL (pKa of 5.5) is ascribed to the protonation of the terminal carboxyl groups of trileucine side chain at low pH that have been shown to involve changes in Zeta-potential as a function of pH [19]. Moreover, in the presence of lipid, the Zeta-potential of P/LOEt has been found to raise from -13 mV to -5 mV, indicating protonation of polymer pendant carboxyl groups. This result of polymer-membrane complex formation suggests the involvement of large surface contacts of P/LOEt with the membrane. In contrast, the Zeta-potential of P/LLL is independent of the presence the lipid [18], suggesting that backbone carboxyl groups of the copolymer are not in contact with membrane lipids.

On the basis of dynamic light scatter measurements, we find now that at physiological pH the amphipathicity of P/LOEt and P/LLL is well accommodated in solution by allowing single chain random structures without aggregation. When pH dropped below 6.0, P/LLL was accommodated its amphipathicity by oligomerization, forming aggregates of size ~ 105 nm as a result of side chain protonation (Figure 5A), similar to pH-independently formed aggregates of side chain neutralized P/LLL-NH₂. The increased hydrophobicity of P/LLL at pH 5 is observed as an enhanced fluorescence intensity of its attached pyrene (Figure 4). Although P/LOEt didn't oligomerize, its amphipathicity is accommodated when it is in contact with lipid membrane by burying its leucine ethyl ester side chains into the lipid bilayer and exposing the hydrophilic face of backbone.

Examples of other membranolytically active polymers, such as polyacrylic acids, are known for their membrane destabilization activity through involving a pH-dependent conformational shrinkage [3, 28]. However, shrinkage of hydrodynamic diameters of monomeric P/LLL and P/LOEt was not observed for our PMLA copolymers as a function of pH. Different to polyacrylic acids having a hydrophobic alkyl backbone and ionizable carboxyl side chains, the PMLA copolymers consisted of ionizable hydrophilic backbone with modified hydrophobic side chains. For both P/LLL and P/LOEt, the hydrophilicity of the backbone prevented structural shrinkage at low pH and was reinforced by intramolecular

electrostatic expulsion in the range $\text{pH} > 5.0$ [19]. The protonation of P/LLL side chains did not induce shrinkage but favored the formation of aggregates by intermolecular association between neutralized LLL groups (Figure 5).

4.2 The mechanistic models explaining membranolysis by P/LOEt and P/LLL

The mechanisms for membrane permeation by peptides has been well established [6, 7, 17, 27, 29] and may serve as a starting point to elucidate the mechanisms for polymers. Two general mechanisms have been proposed for peptides: (i) transmembrane channel formation via “barrel-stave” mechanism; and (ii) membrane destruction via “carpet” mechanism [6, 7]. Similarly, different modes of interaction were proposed for polymer-induced membrane permeation including “barrel-stave” and “carpet” mechanism [5], but predictions lack substantial data support. Here we present a correlation of our polymer-membrane interaction data with available mechanisms.

Distinct solution properties and membranolytic activity of P/LOEt and P/LLL copolymers suggested their different modes of membrane permeation. The mode of P/LOEt interaction with lipid membrane is interpreted in several aspects. (1) Without forming an aggregate in solution, it accommodated its amphipathicity by interacting with the lipid membrane independent of pH (Figure 4). (2) It bound to lipid membrane in high affinity as visualized by confocal microscopy of giant unilamellar vesicles (Figure 3). (3) Its binding to lipid membrane was oriented in close proximity to the lipid bilayer allowing a maximum of interactions of LOEt groups with lipids as indicated by the FRET result (Figure 6). (4) It induced liposome leakage in a cooperative manner (Figure 2). The overall evidence suggested the mechanism of membrane permeation by P/LOEt to be described by the “carpet” model (see schematic representation in (Figure 7H), a mechanism which involves an extensive membrane covering, close contacts with phospholipids throughout the entire process of membrane permeation [6, 7]. Our results indicated that membranolysis by P/LOEt occurred in the following sequences. First, copolymer bound to the membrane surface cooperatively forming a layer of polymer. When the layer reached a certain size, permanent pores were formed causing the observed leakage.

P/LLL interacted with the membrane in different aspects. (1) It accommodated its amphipathicity by forming aggregates through oligomerization at low pH (Figure 5). (2) It bound to the membrane not detectably by confocal microscopy (Figure 3). (3) The interface area for its binding to membrane was small, as suggested by low FRET intensity, (Figure 6), explained by an alignment perpendicularly to the membrane layer illustrated in Figure 7G. (4) Its liposome leakage involved kinetics of two equal contributing phase, suggesting the formation of a primary pore by insertion of a polymer oligomer followed by a structural rearrangement of the oligomer-membrane pore complex. As a result of cooperative binding, large amounts of P/LOEt molecules deposit within focal regions seen by confocal microscopy, unlike P/LLL, which inserts only a few oligomerized molecules due to its different mode of interaction. The formation of these patches by P/LOEt consumed a large amount of polymer so that much less P/LOEt than by P/LLL detected liposome leakage at equal concentrations (Fig. 2D). Thus, P/LLL achieved liposome leakage more efficiently than P/LOEt that could be of advantage for the purpose of endosomolytic drug delivery.

The different mechanisms for P/LLL and P/LOEt are depicted in Fig. 7 assigned to the “barrel-stave” and the “carpe” model, respectively. Besides the arguments given above, the models account geometrically for the understanding of observed phenomena. For instance, according to the “barrel-stave” model only the exterior side chains of P/LLL in the “barrel-stave” are in contact with the hydrophobic core of membrane, while those inside the “barrel-stave” are without the contacts. This is much different from the side chains of P/LOEt in the “carpet” model, where intensive contacts of all copolymer molecules are indicated. These

different geometrical arrangements of copolymers and lipids can explain the strong FRET signal for P/LOEt as opposed to the weak signal for P/LLL.

We have recently reported on the engineering of several membranolytically active copolymers of polymeric acid mostly tripeptides, including P/LLL-NH₂, P/WWW, P/LWL, and P/FFF [19]. Because they share similar properties with P/LLL, their modes of interaction with membrane are likely to be similar with that of P/LLL, through “barrel-stave” mechanism. We have demonstrated this here for P/LLL-NH₂.

4.3 Biological impact of membrane permeation mechanisms

“Carpet” mechanism is considered a detergent-like mechanism which may cause permanent pores on membrane and eventually lead to membrane disintegration, an efficient mechanism for killing bacteria using antimicrobial peptides [7]. The “carpet” mechanism of P/LOEt and the formation of pores on cell membranes also explains its known negative effects on the viability of human cells [18]. P/LLL-NH₂, which also showed pH-independent membranolytic activity, followed the “barrel-stave” mechanism and caused only mild cytotoxicity [19]. Regarding membranolytic efficacy, P/LLL is the preferred copolymer [18]. Small amounts of this copolymer could provoke membrane leakage, while excess amounts of P/LOEt had to compensate for unproductive membrane binding in order to achieve the same amount of leakage (Fig. 2D and Fig. 3). As numerous targeted drug delivery systems are operative through the endosomolytic pathway, they are likely to use pH-responsive membrane disruption by the “barrel-stave” mechanism and thereby avoid damage of cytoplasmic membranes with undesired side effects on healthy tissue. According to our experience, delivery through pH-dependent P/LLL endosome escape system (“barrel-stave” mechanism) was less cytotoxic, more efficient for cytoplasmic delivery of antisense oligonucleotides, and more effective in inhibiting tumor growth than delivery by the P/LOEt system (“carpet” mechanism) [18].

5. CONCLUSION

Hydrophobically modified polymeric acid copolymers P/LLL and P/LOEt are membranolytically active. They demonstrated distinct properties in solution and in their interaction with membrane. P/LOEt was found to bind to membrane with high affinity and cause membranolysis in a cooperative manner. Its mode-of-action for membrane permeation is referred as “carpet” mechanism. In contrast, P/LLL forms aggregate first in solution followed by insertion into the membrane to form transmembranal pores causing membrane permeation, which fits the “barrel-stave” mechanism. Combined with our previously published data, the pH-responsive endosomolytic delivery of P/LLL through the “barrel-stave” mechanism was less cytotoxic and more efficient. As endosome disruption and cytoplasmic drug release is necessary for action of many targeted nanomedicines, the provided optimization of relevant disruption units is a significant step forward in the rational design of efficient anticancer drug delivery systems.

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PMLA: R = H

Figure 1.

Structure of polymalic acid grafted with 40% of trileucine (P/LLL), trileucine amide (P/LLL-NH₂) and 40% leucine ethyl ester (P/LOEt).

Figure 2.

Membranolytic activity of copolymers at pH 5.0 measured by the liposome leakage assay at room temperature. Fluorescence intensity of calcein released from liposomes was measured at emission wavelength 535 nm (excitation wavelength 488 nm). Kinetics of liposome leakage (30 µM lipid) after mixing with indicated concentrations of P/LLL (panel A) and P/LOEt (panel B), which fitted with a two phase exponential equation. The fitting results of Y1max were again fitted with sigmoidal dose-response equation (panel C). Percentage of liposome leakage refers to total leakage induced by Triton-X 100 (100%). Panel D shows the liposome leakage as a function of lipid-to-polymer ratio with copolymers fixed at concentration of 0.2 nM. Liposome leakage refers to fluorescence intensity (arbitrary units).

Figure 3.

Binding of Rhodamine labeled P/LOEt/Rh and P/LLL/Rh to NBD-labeled GUVs at pH 5.0 at room temperature by confocal microscopy. Upper panels, GUV-NBD in green (left) and P/LOEt/Rh in red (middle) co-localized (right). Lower panels: no fluorescence of P/LLL/Rh (middle) was detected binding to GUV-NBD in green (left) and no colocalization (right). Notice the areas of concentrated P/LOEt binding to GUV in the upper right panel.

Figure 4. Fluorescence spectra of pyrene-labeled polymalic acid copolymers (pyrene concentration 5×10^{-7} M) in the absence of liposome at pH 7.4 (A) and pH 5.0 (B), and in the presence of liposome (lipid concentration 250 μ M) at pH 7.4 (C) and pH 5.0 (D). The emission spectra were recorded from 360 nm to 460 nm with excitation wavelength 340 nm.

Figure 5.

Hydrodynamic size of P/LLL and P/LLL-NH₂ as a function of pH and concentration. Panel A, hydrodynamic diameter of P-LLL aggregates and their relative number percentage as a function of pH at the concentration of 40 μg/mL. Panel B, hydrodynamic diameter of P-LLL aggregates at pH 5.0 and their relative number percentage as a function of total concentration of P/LLL. Hydrodynamic diameter was measured by the dynamic light scatter (DLS) method in the number modus.

Figure 6. Fluorescence resonance energy transfer (FRET) at pH 5.0 from NBD-phosphatidylethanolamine (NBD-PE) membrane to P/LOEt/Rh (A) or P/LLL/Rh (B). The arrow indicates the increase of FRET with the increase of concentration of P/LOEt/Rh copolymer.

Fig. 7.

Schematic representation of the mechanisms of membrane permeation by P/LLL and P/LOEt. Panels A-C, simplified P/LLL 3D models containing 8 malyl groups and three side chains were generated with ChemBio3D Ultra 11.0 and their favorable conformation was calculated using energy-minimization with the MMFF94 method. The ionized terminal carboxyl group of trileucine of P/LLL is distant from the backbone of PMLA (panel A). Instead the protonated terminal carboxyl group of trileucine is close to the backbone of PMLA after carboxylate neutralization in panel B, which is similar to the distance of terminal amide of trileucine in P/LLL-NH₂ in panel C. The membranolysis mechanism of P/LLL however is depicted in panels D-G. Panel D, schematically drawn structure of P/LLL in its unprotonated form at pH ≥ 6.0 . At lowering pH < 6.0 , the P/LLL is increasingly more protonated (panel E) resulting in enhanced hydrophobicity thus enabling assembly of the 105 nm aggregate (panel F). The P/LLL aggregate or its fragment inserts into membrane forming transmembrane pore through “barrel-stave” mechanism, different from the “carpet” mechanism by P/LOEt (panel H).