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An antimicrobial peptide-mimetic methacrylate random copolymer induces domain formation in a model bacterial membrane

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Corresponding Author:	Kazuma Yasuhara, Ph. D. Nara Institute of Science and Technology: Nara Sentan Kagaku Gijutsu Daigakuin Daigaku Ikoma, Nara JAPAN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Nara Institute of Science and Technology: Nara Sentan Kagaku Gijutsu Daigakuin Daigaku
Corresponding Author's Secondary Institution:	
First Author:	Kazuma Yasuhara
First Author Secondary Information:	
Order of Authors:	Kazuma Yasuhara
	Manami Tsukamoto
	Jun-ichi Kikuchi
	Kenichi Kuroda
Order of Authors Secondary Information:	
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	Japan Society for the Promotion of Science (21H05883) — Dr. Kazuma Yasuhara
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Abstract:	To address the emerging issue of drug-resistant bacteria, membrane-active synthetic polymers have been designed and developed to mimic host-defense antimicrobial peptides (AMPs) as antibiotic alternatives. In this study, we investigated the domain formation induced by synthetic polymer mimics of AMPs using model membranes to elucidate the biophysical principles that govern their membrane-active mechanisms. To that end, lipid vesicles mimicking E. coli membrane were prepared using an 8:2 (molar ratio) mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol), sodium salt (POPG). Our studies using differential scanning calorimetry (DSC) and fluorescence microscopy indicated that cationic amphiphilic methacrylate random copolymers induced the phase separation to form POPE- or POPG-rich domains. A rhodamine-labeled polymer also showed the binding to separated domains in the membrane. Based on these results, we propose the mechanism that the copolymers induce domain formation by clustering of anionic POPG lipids similar to natural AMPs. In addition, the time course of polymer binding to the GUV membrane was sigmoidal, suggesting the positive feedback loop in the membrane binding. We also hypothesize that this cooperative binding of the polymer is driven by the domain formation. This study demonstrates the potential of the

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	amphiphilic copolymers to modulate the lipid organization of cell membranes, which may provide a new strategy to design membrane-active antibiotic agents.
Keywords:	Antimicrobial agent; Polymethacrylate random copolymer; Lipid domain
Manuscript Classifications:	450: antimicrobial peptide; 460: antimicrobial peptides; 470: antimicrobial polymers; 5170: lipid domain; 5780: membrane microdomain
Response to Reviewers:	Response to the comments of reviewer 1 We appreciate the careful reading and useful comments by reviewer 1. The manuscript is revised according to the reviewers' comments as follows. Comment 1: One question that this reviewer has is in regard to Fig. 5, where fluorescence intensity is used to show saturation of polymer chains on the GUV membranes. Is the intensity of fluorescence from such membranes high enough to show this saturation curve? Has this been reported before? If so, please add the reference for that. Response to comment 1: We have acquired all images using the optimized condition of the intensity of the excitation light and the exposure time of the camera where all pixels show enough high intensity but none of them exceed the dynamic range of the camera for their quantitative analysis. We also added a reference that describes an intensity-based analysis of the binding to a GUV using a microscope (Moghal et al., 2020). To clarify this point, we have added the following description to the manuscript. P9L2: For the quantitative analysis of the fluorescence intensity, we have acquired all images using the optimized condition of the intensity of the excitation light and the exposure time of the camera where all pixels show enough high intensity but none of them exceed the linear range of the camera's sensitivity. P14L10: It should be noted that the fluorescence intensity was measured within the linear range of the camera installed in the microscope. Therefore, the fluorescence intensity in the images reflects the amount of polymer chains bound to the membrane, and the saturation of fluorescence intensity is not due to the saturation in the signal detection by the camera. Comment 2: It would be much clearer if colored images are supplied for Fig. 3 and Fig. 4 to show localization of the polymers on the membrane surfaces. Response to comment 2: According to the reviewer's suggestion, we provide color images for Figs 3 and 4. Additionally, the graphical abstract was updated to include the color image. It should note that all fluorescent images are in red pseudo-color because we have used a monochrome camera for the image acquisition. The following sentence was added to clearly mention this point. P9L6: For clarification, the fluorescent images acquired by a monochrome camera were converted to red pseudo-color images using the same software. Response to the comments of reviewer 2 We appreciate the careful reading and useful comments by reviewer 2. The manuscript is revised according to the reviewers' comments as follows. Comment 1: Under the authors and above the abstract, no C) Response to comment 1: We have corrected the affiliation as follows. Thank you. P1L13: c) Department of Biologic and Materials Sciences, University of Michigan School of Dentistry Comment 2: Abstract, line 15: vesicles were.. Response to comment 2: We have corrected the error as follows. Thank you. P2L6: To that end, lipid vesicles mimicking E. coli membrane were prepared Comment 3: Page 5, line 22, "ide" should be side

	Response to comment 3: We have corrected the error as follows. Thank you. P4L9: hydrophobic side chains Comment 4: GUV is not explained, only GV Response to comment 4: We have consistently used only 'GUV' in the revised manuscript. Additionally, we have added the explanation of GUV as follows. Thank you. P4L7: giant unilamellar vesicles (GUVs) Comment 5: Page 9, line 3: Confusing sentence: "The experiments were repeated three times from bacterial growth." Response to comment 5: We have modified the sentence to improve the clarity of the explanation. P8L1: To confirm the reproducibility, MIC measurements were repeated three times independently from bacterial growth. Comment 6: Figure 1 caption: where is R'? Response to comment 6: We have added the explanation of 'R' in the figure caption. Also, we have substituted incorrectly written R' in the figure to R Figure 1 caption: R represents the substituted groups at the polymer end: methyl (PE44) or rhodamine (RPE42) All reviewers: We have made other corrections to the errors found in the first manuscript as follows. These corrections do not affect the discussion and conclusion made in this manuscript. P4L2: the opposite side of P4L8: the mode of action P4L16: do polymer chains change P12L4: the binding of the methacrylate copolymers induces the formation of laterally separated domains P14L3: domain increased with time P14L5: the domain enriched in anionic PG lipids preferentially binds P15L3: between the POPG cluster and POPE domain P16L21: neurological diseases, and cardiovascular diseases P16L3: Acknowledgment

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An antimicrobial peptide-mimetic methacrylate random copolymer induces domain formation in a model bacterial membrane

Kazuma Yasuhara*[a, b], Manami Tsukamoto[a], Jun-ichi Kikuchi[a], Kenichi Kuroda[c]

a) Division of Materials Science, Graduate School of Science and Technology, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 6300192, Japan. E-mail: yasuhara@ms.naist.jp; Fax: +81-743-72-6099; Tel: +81-743-72-6091

b) Center for Digital Green-innovation, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 6300192, Japan.

c) Department of Biologic and Materials Sciences, University of Michigan School of Dentistry, 1011 N. University Ave., Ann Arbor, MI 48109, USA. E-mail: kkuroda@umich.edu; Fax: +1-734-647-2110; Tel: +1-734-936-1440

ORCID

Kazuma Yasuhara: 0000-0003-0701-6884

Kenichi Kuroda: 0000-0002-2935-8351

Keywords

Antimicrobial agent, Polymethacrylate random copolymer, Lipid domain, Differential scanning calorimetry, Fluorescence microscopy

Abstract

To address the emerging issue of drug-resistant bacteria, membrane-active synthetic polymers have been designed and developed to mimic host-defense antimicrobial peptides (AMPs) as antibiotic alternatives. In this study, we investigated the domain formation induced by synthetic polymer mimics of AMPs using model membranes to elucidate the biophysical principles that govern their membrane-active mechanisms. To that end, lipid vesicles mimicking *E. coli* membrane were prepared using an 8:2 (molar ratio) mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol), sodium salt (POPG). Our studies using differential scanning calorimetry (DSC) and fluorescence microscopy indicated that cationic amphiphilic methacrylate random copolymers induced the phase separation to form POPE- or POPG-rich domains. A rhodamine-labeled polymer also showed the binding to separated domains in the membrane. Based on these results, we propose the mechanism that the copolymers induce domain formation by clustering of anionic POPG lipids similar to natural AMPs. In addition, the time course of polymer binding to the GUV membrane was sigmoidal, suggesting the positive feedback loop in the membrane binding. We also hypothesize that this cooperative binding of the polymer is driven by the domain formation. This study demonstrates the potential of the amphiphilic copolymers to modulate the lipid organization of cell membranes, which may provide a new strategy to design membrane-active antibiotic agents.

Introduction

In recent years, there is a growing concern about infectious diseases caused by pathogens resistant against antibiotics (Aslam et al., 2018; Larsson & Flach, 2021). To overcome this issue of drug-resistant bacteria, novel antimicrobial agents immune to the resistance mechanisms are desired. Naturally occurring host-defense antimicrobial peptides (AMPs) are attracting attention as a promising candidate for antibiotic alternatives owing to low propensity for resistance development (Hancock & Sahl, 2006; Mahlapuu et al., 2016; Zasloff, 2002). AMPs act by disrupting bacterial cell membranes or targeting cytoplasmic components. The cationic nature of AMPs contributes to selective binding to bacterial cell membranes over mammalian cell membranes through the electrostatic interactions with negatively charged lipids enriched in the bacterial membrane. AMPs adopt membrane-active amphiphilic conformations by folding into α-helix or β-sheet upon the interaction of the bacterial membrane, leading to the pore formation (Brogden, 2005) or non-specific permeabilization (carpet mechanism) (Shai & Oren, 2001), depending on AMPs.

Much efforts have been dedicated to the development of synthetic mimics of AMPs using polymeric molecular frameworks with cationic amphiphilicity. AMP-mimetic polymers have been synthesized using various linear polymer backbones such as polyacrylates (Judzewitsch et al., 2020), polyacrylamides (Schaefer et al., 2021), polyoxetanes (King et al., 2014), polyurethanes (Mankoci et al., 2019), polynorbornenes (Colak et al., 2009), and nylon-3polymers (Liu et al., 2021; Mowery et al., 2007). We have previously developed antimicrobial methacrylate copolymers with random sequences of binary compositions of cationic and hydrophobic groups and investigated their structure-activity relationships. These copolymers showed potent antimicrobial activity with low toxicity to mammalian cells through the optimization of structural parameters including the composition of hydrophobic/cationic monomers (Kuroda, Caputo & DeGrado, 2009), molecular weights (Kuroda & DeGrado, 2005), and chemical structures of cationic (Palermo & Kuroda, 2009; Palermo et al., 2011) and hydrophobic groups (Kuroda et al., 2009). Although these random copolymers are not programmed to have defined conformations like peptides, computational simulations suggested that the polymers adopt facially amphiphilic structures in which the cationic and hydrophobic side chains were segregated to the opposite side of the polymer backbone (Palermo, Vemparala & Kuroda, 2012). This capitulates AMP folding to amphiphilic helices on bacteria cell membranes. Therefore, the antimicrobial methacrylate copolymers mimic not only the AMP's antimicrobial activity, but also the biophysical traits of AMP's mechanism.

We have recently demonstrated that the methacrylate copolymers permeabilize model membranes by pore formation or cause a burst of giant unilamellar vesicles (GUVs) that mimic AMP's mechanisms of action (Tsukamoto et al., 2021). Interestingly, the mode of action can be selected by altering the chemical structure of the hydrophobic side chains (methyl or butyl) in their random sequences. In addition, our study also demonstrated that the burst of GUVs could be described as the autocatalytic reaction in which the rate of GUV burst was increased with time. We proposed the mechanism that the polymer chain bound to the membrane recruited a free polymer chain from solution. These results suggest that the copolymers are capable of re-organizing the membrane structures, resulting in membrane permeabilization or pore formation. While many studies have been focused on the antimicrobial activity of polymers, the physical principles that drive the membrane reorganization by the syntenic polymers remains unclear. In short, how do polymer chains change the membrane structures?

In this study, we extend our previous investigation to elucidate the biophysical principles that govern the membrane-disrupting mechanism of the AMP-mimetic copolymers. In particular, our focus is membrane reorganization. Some AMPs are known to induce the lateral phase separation or domain formation in the membrane (Arouri, Dathe & Blume, 2009; Epand et al., 2010; Jean-Francois et al., 2008; Kwon, Waring & Hong, 2013; Scheinpflug et al., 2017). Epand and co-workers extensively investigated the domain formation induced by various antimicrobial peptides using model membranes (Epand & Epand, 2009; Epand & Epand, 2011; Epand et al., 2010). They proposed that the binding of cationic groups of AMPs to anionic lipids of bacterial membranes induced the clustering of anionic lipids, resulting in the destabilization and permeabilization of the membrane. While the AMP-induced domain formation has been well studied, the investigation of polymer-based their synthetic mimics has been very limited (Epand et al., 2008a). In this study, we investigated polymer-induced domain formation using differential scanning calorimetry (DSC) and fluorescence microscopic observation of vesicles formed by 8:2 (molar ratio) mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol), sodium salt (POPG), which mimic the lipid composition of *E. coli* membrane (Raetz & Dowhan, 1990). The results indicated that, similar to natural AMPs, a methacrylate copolymer induced the clustering of a specific lipid to form domains in the bacterial model membrane. Furthermore, the polymer chains are bound to the membrane in a cooperative manner accompanied with the domain formation. We propose that the bound polymer chain creates more binding sites by the lateral reorganization of lipids in the membrane.

Experimental

Materials

Phospholipids, POPE and POPG, were purchased from NOF Co. (Tokyo, Japan). Texas Red® 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (TR-DHPE), *N, N, N′, N″*-pentamethyldiethylenetriamine (PMDETA), and magainin 2 were purchased from Molecular Probes (Eugene, OR, USA), Tokyo Chemical Industry (Tokyo, Japan), and AnaSpec (San Jose, CA, USA), respectively. All other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used without further purification.

Synthesis of polymers and characterization

General

Polymethacrylate derivatives were synthesized by atom transfer radical polymerization (ATRP). The ATRP initiators without (**1**) (Sun et al., 2011) and with (**2**) (Cai et al., 2011) rhodamine moiety were synthesized according to the literature procedures. The degree of polymerization (DP) and the mole percentage of alkyl methacrylate, f_{HB}, of polymers were determined by ^{1}H NMR analysis. We calculated these values by comparing the integrations of peaks from the methyl group (1.3 ppm, 3H) of the ethyl side chains of ethyl methacrylate and the methylene (3.0 ppm 2H) of the cationic sidechains relative to that of methoxy group at the polymer chain end (3.4 ppm, 3H) for **PE44**. For **RPE42**, the aromatic proton of rhodamine at the polymer chain end (8.3 ppm, 1H) was used for calculation as the end group reference.

*Synthesis of **PE44***

Copper (I) bromide (2.9 mg, 0.02 mmol) was introduced to a Schlenk tube under a nitrogen atmosphere. After the addition of PMDETA (6.3 µL, 0.03 mmol) and 2-propanol (400 µL), the solution was deoxygenated by nitrogen bubbling and then stirred for 30 minutes to form the Copper (I) / PMDETA complex. To this solution, an ATRP initiator 2-methoxyethyl-2-bromoisobutyrate (**1**, 4.5 mg, 0.02 mmol), 4-(tert-butoxycarbonylamino)butyl metacrylate (51.5 mg, 0.2 mmol), and ethyl methacrylate (16 µL, 0.2 mmol) were added. After the bubbling with nitrogen gas, the reaction mixture was stirred at 80ºC overnight. The reaction was stopped by flushing air that oxidizes the copper (I) in the complex. The complex in the crude mixture was removed by passing an alumina short column. Unreacted initiator and monomers were removed by a gel-filtration using Sephadex LH-20. After the concentration of the resultant solution under reduced pressure, the Boc-protecting group was cleaved in the mixture of trifluoroacetic acid (TFA, 500 µL) and methyl 3-mercaptopropionate (MMP, 500 µL) by stirring for 1 hour at room temperature. The excess cleavage solution was removed by a nitrogen flow and the product was dissolved in water. The resultant aqueous solution was lyophilized for two days to afford the target polymer **PE44** as a white powder. ^{1}H NMR (400 MHz, Methanol-d4, TMS) for **PE44**: δ4.3-4.0 (m, 40H), 3.6 (br, 2H), 3.4 (s, 3H), 3.0 (br, 21H), 2.1-1.7 (m, 86H), 1.3 (m, 25H), 0.9-1.1 (m, 57H)

Synthesis of ***RPE42***

Rhodamine-labeled **RPE42** polymer was polymerized in the slightly modified procedure to the synthesis of **PE44** as described above. We have used rhodamine-conjugated initiator **2** (19.2 mg, 0.03 mmol) instead of non-labeled 2-methoxyethyl-2-bromoisobutyrate. We have synthesized the polymer in different feeding conditions as follows: Copper (I) bromide (4.4 mg, 0.03 mmol), PMDETA (12.6 μL,0.06 mmol), 4-(tert-butoxycarbonylamino)butyl metacrylate (72.2 mg, 0.28 mmol), and ethyl methacrylate (9.7 μL, 0.12 mmol). The resultant polymer **RPE42** was obtained as a red solid. ^{1}H NMR (400 MHz, Methanol-d4, TMS) for **RPE42**: δ 8.3 (d, 1H), 7.9 (m, 2H), 7.5 (s, 1H), 7.0-7.1 (m, 6H), 4.3-4.0 (m, 35H), 3.7 (m, 8H), 3.0 (br, 18H), 2.1-1.7 (m, 72H), 1.3 (m, 31H), 0.9-1.1 (m, 46H)

Antimicrobial activity assay

The antimicrobial activity of the copolymers was evaluated by the minimum inhibitory concentration (MIC) in which the polymers inhibit the bacterial growth completely. The MIC values of polymers were determined by a turbidity-based microdilution assay (National Committee for Clinical Laboratory Standards, 2003). The overnight culture of *E. coli* ATCC 25922 in Muller-Hinton (MH) broth at pH 7.4 was diluted to OD of 0.1 and grown again to the mid-logarithmic phase (OD_{600} = 0.5-0.6). The bacterial culture was then diluted to give a stock suspension of OD_{600} = 0.001, which corresponds to ~ 2×10^5 cfu/mL. This bacterial stock suspension (90 μL) was then mixed with a polymer solution (10 μL) in two-fold serial dilutions in a sterile polypropylene 96-well plate (Corning #3359), which is not treated for tissue culture. The highest polymer concentration tested was 1,000 μg/mL. The plates were incubated at 37 °C for 18 hours without shaking. The OD_{600} of each well was then read by a microplate reader, and an increase in turbidity from the MH broth control was considered as *E. coli* growth. The MIC value of magainin 2 was also measured for comparison. To confirm the reproducibility, MIC measurements were repeated three times independently from bacterial growth.

Preparation of multi lamellar vesicles and DSC measurement

For the thermal analysis by differential scanning calorimetry (DSC), we have prepared multi-lamellar vesicles (MLVs) as follows. Chloroform solutions of the 8:2 (molar ratio) mixture of POPE / POPG were placed in a round-bottom test tube, and the solvent was evaporated *in vacuo* for 3 hours to form a thin lipid film. The obtained lipid film was hydrated with 10 mM HEPES buffer (pH=7.4) containing 100 mM of NaCl by vortex mixing at room temperature. The aqueous solution of the polymer was added after the preparation of MLVs if needed. The final concentrations of the lipid and polymer were set to 1 mM and 100 μM, respectively. The DSC measurement of MLVs samples was performed using a VP-DSC (MicroCal Inc., Northampton, MA, USA). The measurement was performed in the temperature range of 15 to 30°C with a scanning rate of 0.5°C·min^{-1}. MLVs samples were equilibrated at 15°C for 30 minutes in the calorimeter cell prior to the scan.

Preparation of giant vesicles and fluorescence microscopy

The giant unilamellar vesicles (GUVs) were prepared by the gentle hydration of a lipid film (Reeves & Dowben, 1969). Chloroform solutions of the 8:2 mixture of POPE / POPG (1 μmol in total) were placed in a round-bottom test tube and TR-DHPE (0.001 μmol) was added if necessary. The solvent was evaporated *in vacuo* for 3 hours to form a dry thin lipid film. The obtained lipid film was gently hydrated with 1 mL of Milli-Q water at room temperature overnight. The resultant suspension of GUVs was diluted with Milli-Q water to set the final lipid concentration to 40 μM. A series of microscopic images were acquired using an Olympus IX71 inverted optical microscope (Tokyo, Japan) equipped with x100 objective lens and 100 W mercury lamp in phase contrast and epifluorescence modes. For the fluorescence observation of TR-DHPE or a rhodamine-labeled polymer, a U-MWIY2 filter unit was used. Microscopic images were recorded using a Hamamatsu ORCA-Flash 2.8 CMOS camera (Hamamatsu, Japan). For the quantitative analysis of the fluorescence intensity, we have acquired all images using the optimized condition of the intensity of the excitation light and the exposure time of the camera where all pixels show enough high intensity but none of them exceed the linear range of the camera's sensitivity. Obtained images were analyzed using Image-J software. For clarification, the fluorescent images acquired by a monochrome camera were converted to red pseudo-color images using the same software. The fluorescence intensity of the polymers on the surface of the vesicle was calculated by subtracting the background fluorescence intensity from the apparent intensity of the membrane.

Results and discussion

Synthesis and Characterization of polymers

We synthesized amphiphilic methacrylate random copolymers having primary ammonium and ethyl groups in the side chains. This polymer structure was selected because this has previously shown potent antimicrobial activity, (Palermo et al., 2012). The polymers were synthesized by atom transfer radical polymerization (ATRP) (Figure 1). This is controlled radical polymerization enabling precise control of molecular weight as well as specific labeling of polymer terminal group. The polymers labeled without (**PE44)** and with rhodamine (**RPE42**) were prepared. The polymers have molecular weights of several thousand (Table 1), which are similar to natural antimicrobial peptides, and the fractions of the hydrophobic monomer unit if these copolymers were comparable. The minimum inhibitory concentration (MIC) against *E. coli* ATCC 25922 was 7.8 µg/mL for both polymers, which is in good agreement with the previous report (Palermo et al., 2012), indicating that modification of rhodamine did not alter the polymer’s antimicrobial activity. For comparison, the MIC of natural antimicrobial peptide magainin 2 was 125 µg・mL^{-1} under the same assay condition.

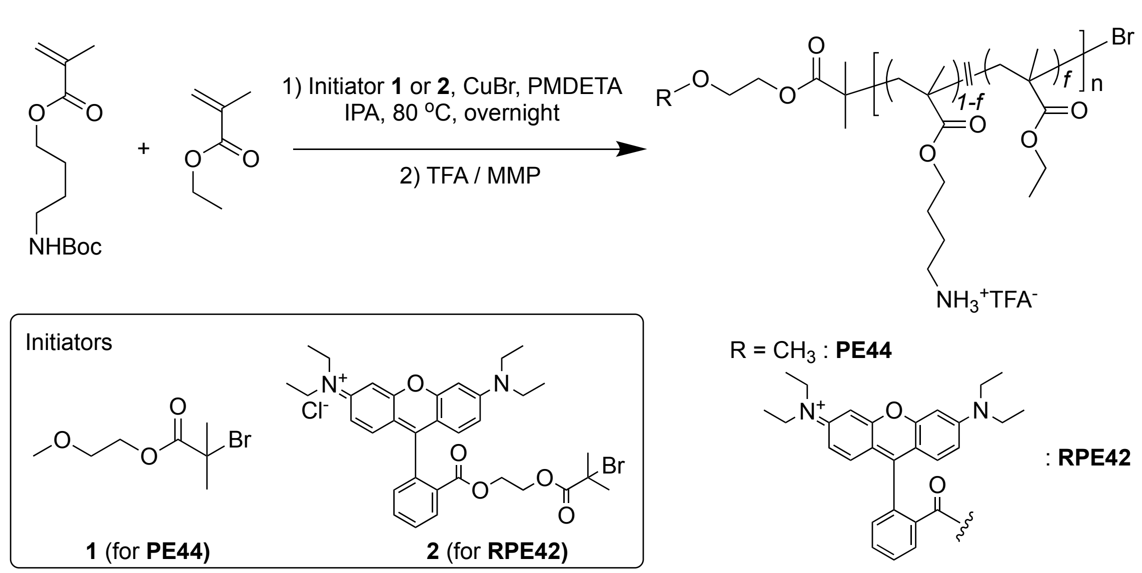

Figure 1. Synthesis of antimicrobial polymethacrylate random copolymer. R represents the substituted groups at the polymer end: methyl (PE44) or rhodamine (RPE42)

Table 1. Characterization of the polymers

Polymer	DP[a]	f_{HB}[b]	Mn ($g \cdot mol^{-1}$)[c]	MIC ($\mu g \cdot mL^{-1}$)[d]
PE44	18.9	0.44	5,600	7.8
RPE42	15.3	0.42	3,800	7.8

[a]Degree of polymerization determined by ^{1}H NMR analysis. [b]The mole fraction of hydrophobic monomer units in a polymer chain. [c]Number-averaged molecular weight calculated using the molecular weights of monomers, ATRP initiators, DP and f_{HB}. [d]Minimum inhibitory concentration against *E. coli* ATCC 25922.

DSC measurement

A thermal analysis by differential scanning calorimetry (DSC) was carried out to study the polymer-induced domain formation in a lipid membrane. The DSC thermograms indicated that POPE/POPG liposome showed a single endothermic peak at 22.0°C, which is in good agreement with a previous report, and the observed phase transition peak can be assigned to the phase transition from the gel to liquid-crystalline of the lipid bilayer (Navas et al., 2005). The addition of **PE44** caused the high-temperature shift of the endothermic peak to 24.9°C. Since the phase transition temperature (T_m) of pure POPE membrane is 25°C, the observed endothermic peak indicates the formation of POPE-rich domains. The other phase, the POPG-rich domain, is supposed to show another phase transition peak; however, the T_m of pure POPG is -5.3°C (Navas et al., 2005) that was below the detection limit of our experimental setup. A similar high-temperature shift of the T_m induced by domain formation was also reported for antimicrobial oligo-acyl-lysine (Epand et al., 2008b). The peak area also decreased as the endothermic peak shifted, indicating the decrease in the enthalpy of the phase transition. This probably reflects the decrease in the total amount of lipid involved in the phase transition due to the exclusion of POPG from the POPE-rich phase by the domain formation. Similarly, rhodamine-modified **RPE42** polymer showed a high-temperature shift of an endothermic peak to 24.5°C that also confirmed the formation of POPE-rich domains. These results suggest that the binding of the methacrylate copolymers induces the formation of laterally separated domains in the POPE/POPG membrane.

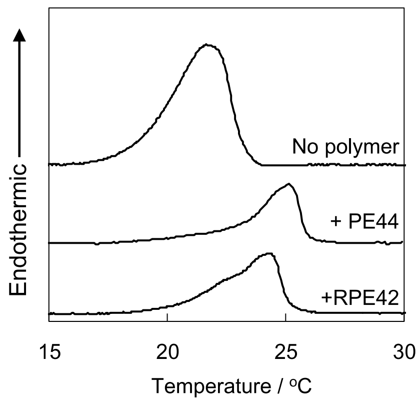

Figure 2. DSC thermograms of POPE / POPG (8:2) vesicles in the absence and presence of the polymers. [Lipid] = 1 mM, [Polymer] = 0.1 mM in 10 mM HEPES buffer (pH = 7.4, [NaCl] = 100 mM)

Polymer-induced domain formation observed by fluorescence microscope

Next, we examined the effect of the methacrylate copolymers on the membrane structure through direct visualization by *in situ* microscopic observation using cell-sized GUVs with fluorescent probe-labeled lipids (Dimova & Marques, 2020CRC Press, Boca Raton, FL). The phase-contrast image indicated that a spherical GUV produced in this lipid composition yielded a smooth surface (Figure 3). The fluorescence image indicated a uniform fluorescence signal over the entire surface of the GUV without visible phase-separated domains, suggesting both lipids were well co-mixed in the membrane. The good miscibility of POPE and POPG is consistent with the result of the DSC measurement. When **PE44** was added to the GUV, no significant change in the vesicle morphology was observed in the phase-contrast image. However, in the fluorescence image, the bright and dark regions were found coexisted in the same

GUV membrane. This heterogeneity in the fluorescence intensity reflected the differences in the fluorescent TR-DHPE concentration between the domain and surrounding region. According to the previous study on AMPs, one plausible mechanism is that the domain formation was driven by the specific binding of the cationic ammonium groups of the polymer to the anionic POPG lipid in the membrane, clustering the POPG lipids (Epand et al., 2008b). Together with the results of DSC measurements, it was demonstrated that **PE44** forms laterally separated domains in the *E. coli* mimetic POPE / POPG membrane.

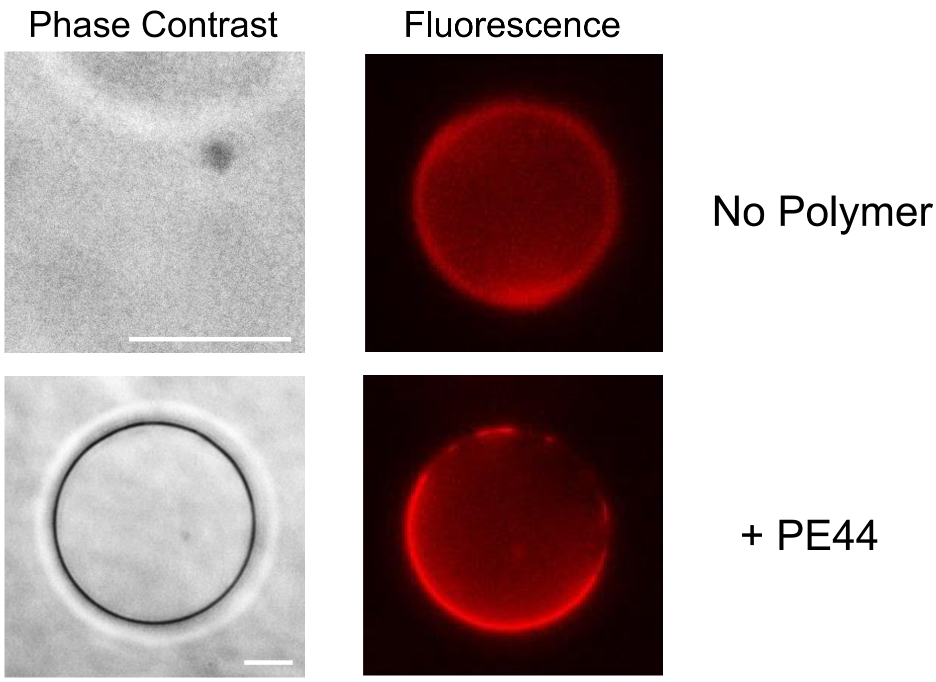

Figure 3. Microscopic observation of the domains formed on a GUV. The GUV was stained with 0.1 mol% of TR-DHPE. [Lipid] = 40 μM , [**PE44**] = 0.067 μg・mL^{-1}. Bar = 10 μm.

Next, we observed the binding and localization of the rhodamine-labeled **RPE42** to the non-labeled POPE / POPG membrane by fluorescence microscopy. A series of the time-lapse microscopic images of a GUV indicated that the fluorescence signal on the vesicular surface gradually became brighter over time (Figure 4), due to the increase in the amount of the polymer bound to the membrane. At the early stage of binding in the first 40 seconds, the fluorescence signal was distributed over the entire surface of the GUV (Figure 4). However, bright and dark domains developed on the surface, and the intensity of the bright domain increased with time, indicating the local accumulation of **RPE42**. Based on the results of DSC and lipid domain formation (Figures 2 and 3), we speculate that the polymer preferentially bound to a specific lipid domain. More specifically, the domain enriched in anionic PG lipids preferentially binds the cationic polymer chains by electrostatic interactions as compared to the other PE-rich domain. To investigate the relationship between the polymer binding and domain formation, the time-course of the fluorescence intensity of the membrane from sequential images was analyzed (Moghal et al., 2020). Interestingly, sigmoidal time dependence of the polymer binding was observed (Figure 5); the rate of polymer binding increased with time and eventually decreased as approaching saturation of binding. It should be noted that the fluorescence intensity was measured within the linear range of the camera installed in the microscope. Therefore, the fluorescence intensity in the images reflects the amount of polymer chains bound to the membrane, and the saturation of fluorescence intensity is not due to the saturation in the signal detection by the camera. This sigmoidal curve suggests a cooperative binding of the polymer chain to the GUV membrane where the initially bound polymer chains create a new binding site for another polymer. The cooperative interactions of several AMPs with membranes have been reported previously based on biophysical studies (Chen, Lee & Huang, 2002; Sani et al., 2014). We have also reported sigmoidal curves for the polymer-induced burst of GUVs and proposed that there was a positive feedback loop in the membrane binding mechanism of the polymers (Tsukamoto et al., 2021). Interestingly, the polymer binding steeply increased around 80 seconds, and at the same time, the localization of the polymer to a specific domain became apparent. The domain continued to grow as the polymer bound more, but after the saturation of the binding at 200 seconds, the further growth of the domain nor the appearance of new domains were not observed. These results suggest that the polymer-induced domain formation creates new binding sites in the GUV membrane to recruit free polymer chains. The molecular mechanism of the cooperative polymer binding remains unclear. However, our previous study using computational simulations showed that insertion of methacrylate copolymer chains induced clustering of POPG (Baul, Kuroda & Vemparala, 2014). The polymer chains were bound to the boundary between the POPG cluster and POPE domain. We speculate that the copolymer-induced POPG clustering creates hydrophobic pockets at the domain boundaries, which recruited another polymer chain into the bilayer, leading to the positive feedback loop in the polymer insertion.

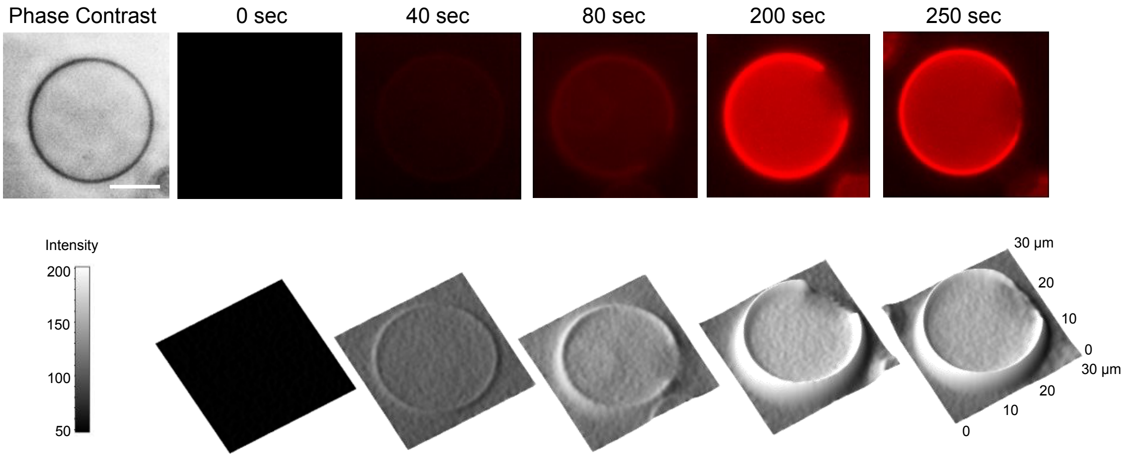

Figure 4. Microscopic observation of the polymer binding to a GUV and corresponding fluorescence intensity profiles. [Lipid] = 40 μM , [**RPE42**] = 0.067 μg•mL^{-1}. Bar = 10 μm.

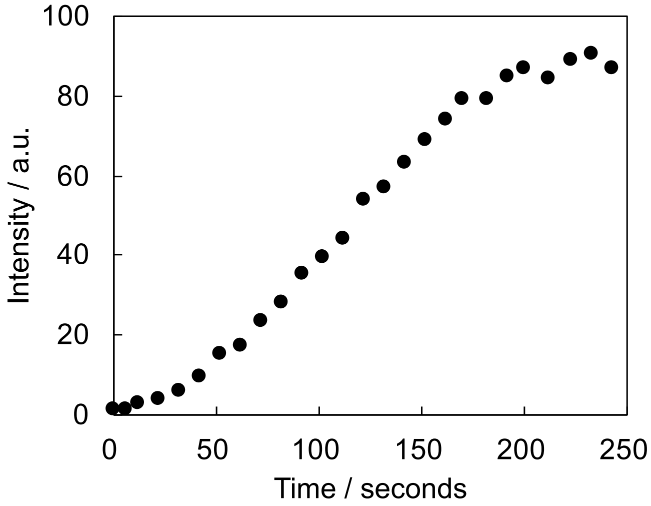

Figure 5. Time-course of the fluorescence intensity on the surface of GUV membrane.

Conclusion

We have demonstrated that the antimicrobial methacrylate copolymer induces the laterally separated domains using model membranes. The lipid membrane of miscible POPE and POPG formed phase-separated domains when the polymer bound. The domain formation and the membrane accumulation of the polymer are correlated, suggesting domain formation drives the cooperative interactions of the polymer with the membrane. In other words, the polymer chains catalyze their own membrane binding through lipid reorganization.

The polymers employed in this study do not have any specific monomer sequences and conformations. However, they displayed lipid domain formation similar to AMPs. This indicates that the AMP sequences may not be needed for the domain formation, but rather the physicochemical properties of polymer or peptide chains are essential. Although the relationship between polymer structures and domain formation in the bacterial membrane should be further investigated, this study demonstrated the potential of the amphiphilic polymers, which can regulate the lipid arrangement and organization in the membrane for biological activities. Domain formation in the bacterial membrane is known to play important roles

(Matsumoto et al., 2006) that can be targeted by the polymers to control the cell viability and bacterial physiology. In addition, eukaryotic cells also are known to have lipid domains, as proposed in the lipid raft hypothesis (Simons & Ikonen, 1997). Lipid raft domains are the clusters of specific lipids and membrane proteins that regulate signal transduction in a cell (Simons & Toomre, 2000). Furthermore, several diseases such as cancer, neurological diseases, and cardiovascular diseases have been found to be associated with lipid raft organization (Michel & Bakovic, 2007; Simons & Rajendran, 2009). Synthetic polymers that can manipulate the formation of lipid domains may provide new biophysical means to regulate various biological cellular activities through the organization of the microscopic assembly of the lipids in the cell membrane.

Acknowledgment

This work was supported in part by a Grant-in-aid for Scientific Research (B) (K. Y., No. 18H03533), Challenging Research (Exploratory) (K.Y., No. 21K19007), and Transformative Research Areas (A) Molecular cybernetics (K.Y., No. 21H05883) from the Japan Society for the Promotion of Science (JSPS), NSF (K. K., DMR-2004305, BMAT), and Support from Department of Biologic and Materials Sciences, University of Michigan School of Dentistry (K. K.).

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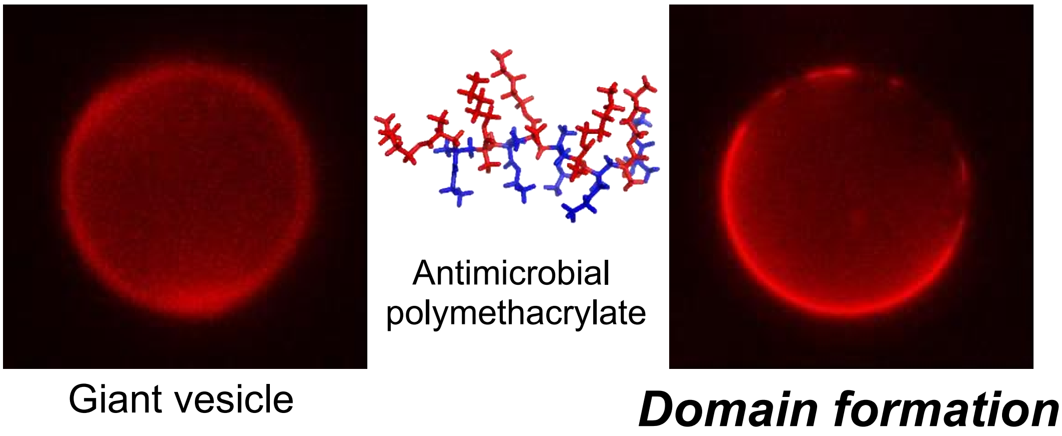
Graphical Abstract



Nara Institute of Science and Technology
8916-5 Takayama-cho, Ikoma, Nara, 630-0192, Japan

Dr. Alexey Ladokhin,
Editor-in-Chief,
The Journal of Membrane Biology

Feb. 3, 2022

Dear Dr. Ladokhin,
Thank you for your e-mail on the 25th January 2022. We appreciate the referees for their careful reading of our manuscript and valuable comments. We are herewith submitting a revised manuscript that addresses all the comments from the reviewers:

Manuscript number: JMBI-D-21-00110
Title: An antimicrobial peptide-mimetic methacrylate random copolymer induces domain formation in a model bacterial membrane
Authors: Kazuma Yasuhara, Manami Tsukamoto, Jun-ichi Kikuchi, Kenichi Kuroda

We have studied the reviewers' comments carefully and made corrections, which we hope meet with their approval. The revised parts of the manuscript are highlighted in red. Our responses to the reviewers' comments are also described in this letter. In addition, the revised supporting information is also attached.

We are looking forward to receiving your decision and publishing our study in *The Journal of Membrane Biology*.

Thank you.

Sincerely yours,

Kazuma Yasuhara, Ph. D.
Associate Professor
Division of Materials Science, Graduate School of Science and Technology
Nara Institute of Science and Technology
8916-5 Takayama, Ikoma, Nara 6300192, Japan
Tel: +81-743-72-6091
Email: yasuhara@ms.naist.jp

Nara Institute of Science and Technology
8916-5 Takayama-cho, Ikoma, Nara, 630-0192, Japan

Response to the comments of reviewer 1

We appreciate the careful reading and useful comments by reviewer 1. The manuscript is revised according to the reviewers' comments as follows.

Comment 1: One question that this reviewer has is in regard to Fig. 5, where fluorescence intensity is used to show saturation of polymer chains on the GUV membranes. Is the intensity of fluorescence from such membranes high enough to show this saturation curve? Has this been reported before? If so, please add the reference for that.

Response to comment 1:
We have acquired all images using the optimized condition of the intensity of the excitation light and the exposure time of the camera where all pixels show enough high intensity but none of them exceed the dynamic range of the camera for their quantitative analysis. We also added a reference that describes an intensity-based analysis of the binding to a GUV using a microscope (Moghal et al., 2020). To clarify this point, we have added the following description to the manuscript.

P9L2: For the quantitative analysis of the fluorescence intensity, we have acquired all images using the optimized condition of the intensity of the excitation light and the exposure time of the camera where all pixels show enough high intensity but none of them exceed the linear range of the camera's sensitivity.

P14L10: It should be noted that the fluorescence intensity was measured within the linear range of the camera installed in the microscope. Therefore, the fluorescence intensity in the images reflects the amount of polymer chains bound to the membrane, and the saturation of fluorescence intensity is not due to the saturation in the signal detection by the camera.

Comment 2: It would be much clearer if colored images are supplied for Fig. 3 and Fig. 4 to show localization of the polymers on the membrane surfaces.

Response to comment 2:
According to the reviewer's suggestion, we provide color images for Figs 3 and 4. Additionally, the graphical abstract was updated to include the color image. It should note that all fluorescent images are in red pseudo-color because we have used a monochrome camera for the image acquisition. The following sentence was added to clearly mention this point.
P9L6: For clarification, the fluorescent images acquired by a monochrome camera were converted to red pseudo-color images using the same software.

Response to the comments of reviewer 2

We appreciate the careful reading and useful comments by reviewer 2. The manuscript is revised according to the reviewers' comments as follows.

Comment 1: Under the authors and above the abstract, no C)
Response to comment 1: We have corrected the affiliation as follows. Thank you.
P1L13: c) Department of Biologic and Materials Sciences, University of Michigan School of Dentistry

Comment 2: Abstract, line 15: vesicles were..
Response to comment 2: We have corrected the error as follows. Thank you.
P2L6: To that end, lipid vesicles mimicking *E. coli* membrane were prepared

Comment 3: Page 5, line 22, "ide" should be side

Response to comment 3: We have corrected the error as follows. Thank you.
P4L9: hydrophobic side chains

Comment 4: GUV is not explained, only GV

Response to comment 4: We have consistently used only ‘GUV’ in the revised manuscript. Additionally, we have added the explanation of GUV as follows. Thank you.
P4L7: giant unilamellar vesicles (GUVs)

Comment 5: Page 9, line 3: Confusing sentence: "The experiments were repeated three times from bacterial growth."

Response to comment 5: We have modified the sentence to improve the clarity of the explanation.
P8L1: To confirm the reproducibility, MIC measurements were repeated three times independently from bacterial growth.

Comment 6: Figure 1 caption: where is R'?

Response to comment 6: We have added the explanation of ‘R’ in the figure caption. Also, we have substituted incorrectly written R’ in the figure to R
Figure 1 caption: R represents the substituted groups at the polymer end: methyl (PE44) or rhodamine (RPE42)

All reviewers:
We have made other corrections to the errors found in the first manuscript as follows. These corrections do not affect the discussion and conclusion made in this manuscript.

P4L2: the opposite side of
P4L8: the mode of action
P4L16: do polymer chains change
P12L4: the binding of the methacrylate copolymers induces the formation of laterally separated domains
P14L3: domain increased with time
P14L5: the domain enriched in anionic PG lipids preferentially binds
P15L3: between the POPG cluster and POPE domain
P16L21: neurological diseases, and cardiovascular diseases
P16L3: **Acknowledgment**

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