Communication

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Minimization of synthetic polymer ligands for specific recognition and neutralization of a toxic peptide

Haejoo Lee†, Yu Hoshino†*, Yusuke Wada†, Yuka Arata†, Atsushi Maruyama‡, Yoshiko Miura†

†Department of Chemical Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

‡Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta 4259, Midori-ku, Yokohama 226-8501, Japan

Supporting Information Placeholder

ABSTRACT: Synthetic polymer ligands (PLs) that recognize and neutralize specific biomacromolecules have attracted attention as stable substitutes for ligands such as antibodies and aptamers. PLs have been reported to strongly interact with target proteins, and can be prepared by optimizing the combination and relative proportion of functional groups, by molecular imprinting polymerization, and/or by affinity purification. However, little has been reported about a strategy to prepare PLs capable of specifically recognizing a peptide from a group of targets with similar molecular weight and amino acid composition. In this study, we show that such PLs can be prepared by minimization of molecular weight and density of functional units. The resulting PLs recognize the target toxin exclusively and with 100-fold stronger affinity from a mixture of similar toxins. The target toxin is neutralized as a result. We believe that the minimization approach will become a valuable tool to prepare "plastic aptamers" with strong affinity for specific target peptides.

Synthetic polymer ligands (PLs) that recognize and neutralize target molecules have been evaluated as inexpensive and physicochemically stable substitutes for biomacromolecular ligands such as antibodies and aptamers.[1] Such PLs are prepared either by modification of the polymer backbone with a number of small ligands, and/or by copolymerizing a combination of a simple functional monomers to accumulate a number of weak interactions such as van der Waals, hydrophobic, electrostatic, hydrogen bonding, and pi-pi stacking forces.

For instance, *Schrader* and colleagues used the first approach to develop linear polyacrylamides functionalized with arginine receptors that target arginine-rich proteins.[2] Following the same strategy, *Haddleton* and colleagues prepared sequence-controlled multi-block glycopolymers modified by carbohydrates to inhibit the lectin DC-SIGN.[3] *Kiessling* and her group also investigated the effect of carbohydrate architecture in the ligand on the function and clustering of the lectin Con A.[4]

Using the second approach, *Haag* and coworkers developed a dendritic polyglycerol modified with sulfate groups to target selectin, even *in vivo*, by multipoint electrostatic interactions.[5] *Shea* and colleagues demonstrated that 3D nanoparticles (NPs) based on p-*N*-isopropylacrylamide targeted a specific peptide and protein through a combination of electrostatic, hydrophobic and pi-stacking interactions.[6,7] These NPs are fabricated through copolymerization of simple functional monomers, such as hydrophobic *N-tert*-butylacrylamide (TBAm), negatively charged acrylic acid (AAc), and aromatic *n*-phenyl acrylamide.[6,7] Affinity can be enhanced further by optimizing the volume density of functional groups[7], molecular imprinting[8], affinity purification[9], and tuning the flexibility and density of polymer chains[10].

However, a strategy has not been described to prepare PLs capable of recognizing a specific peptide from a pool of targets with similar molecular weight and amino acid composition. In this study, we demonstrate one such approach, in which the molecular weight of PLs and the relative proportion (density) of functional units are minimized. Thus, we were able to generate multifunctional PLs that specifically targeted and neutralized a peptide toxin in a pool of similar peptides.

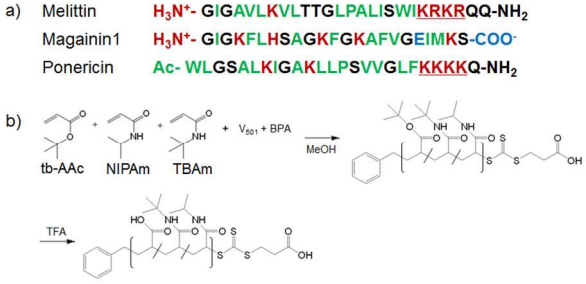

Figure 1. (A) Amino acid sequence of melittin, magainin 1 and ponericin. **(B)** Preparation of multifunctional PLs via RAFT living-radical polymerization.

Figure 1A shows the primary structure of melittin, the peptide of interest, and of control peptides magainin 1 and ponericin. Melittin is an α-helical hemolytic toxin in bee venom, and is well studied as a model target molecule for synthetic PLs. Melittin, with MW 2846 Da, contains 50 % hydrophobic residues and six positive charges.[11] Magainin 1[12] and ponericin[13] were selected as control peptides, because they are also cell-lytic toxins, and have characteristics similar to melittin in terms of molecular weight (2409 Da and 2708 Da respectively), hydrophobicity (43% and 52% respectively), and number of positively charged amino acids (six positive charges each).

We hypothesized that the specificity of PLs can be improved by minimization of molecular weight. Thus, PL libraries containing 300-mer, 30-mer, and 15-mer monomers were synthesized by

heat-initiated reversible addition-fragmentation chain-transfer (RAFT) polymerization, using benzylsulfanylthiocarbonylsulfanyl propionic acid (BPA) as chain transfer agent and *N*-isopropylacrylamide (NIPAm) as main monomer (**Figure 1B**). Details of polymerization reactions and associated data are described in S1.

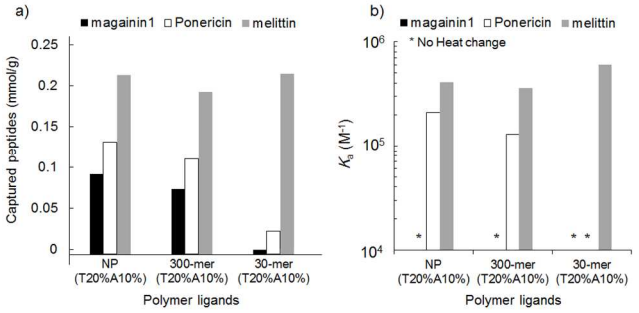

Figure 2. (A) Amount of peptides bound by solutions containing 1.9 mg/mL NP, 300-mer PL, and 30-mer PL from of a mixture of 0.1 mM each of magainin 1, ponericin, and melittin in PBS. Each synthetic polymer contained 20 mol% TBAm and 10 mol% AAc. Captured peptides were analyzed by HPLC. **(B)** Apparent binding constant (K_a) between peptides and synthetic PLs, as measured by ITC titration. Each 0.5 mM peptide was titrated at 37 °C into 0.38 mg/mL synthetic polymers in PBS.

Because the target peptide includes hydrophobic and cationic amino acids, PL libraries were prepared by controlling the relative proportion (density) of functional monomers, such as hydrophobic TBAm and negatively charged AAc, that could interact with the peptide. The average number of functional units incorporated into PLs was quantified by ^{1}H NMR. We found that the relative proportion of functional units incorporated into each PL was comparable to the ratio of functional monomers in the feed (± 5%). The polydispersity index of each PL was determined from GPC to be 1.1-1.4. For comparison, synthetic NPs were prepared as described[7] using the same density of functional units.

To investigate the effect of molecular weight on target specificity, 1.9 mg/mL NP, 300-, and 30-mer PLs, each containing 20 mol% TBAm and 10 mol% AAc, were incubated at 37 °C in PBS (35 mM phosphate buffer pH 7.3, 150 mM NaCl) with a mixture consisting of 0.1 mM each of magainin 1, ponericin and melittin. It was confirmed that all PLs were soluble in the buffer (S2). The amount of unbound peptide was quantified by HPLC after filtrating the PLs by a centrifugal filters (Milipore Co., Amicon Ultra-0.5, 8,000 G, 37 °C, 30 min, NMWL; 10 kDa) (S2).

Figure 2A summarizes the amount of peptides bound by synthetic PLs. Raw HPLC traces are collected in S2. Synthetic PLs bound similar amounts of melittin regardless of molecular weight because of multiple hydrophobic and electrostatic interactions. As expected, NP and 300-mer PL complexed magainin 1 and ponericin as well, because of the same interaction forces. However, 30-mer PL captured only a small amount of control toxins, indicating that this PL recognizes melittin specifically.

Linear polymers, as well as polymer chains in NPs, can map onto target proteins and peptides to form high-affinity complexes.[10,14] It has been reported that PLs with larger molecular weight shows stronger affinity to melittin than the smaller one, because the larger PLs has a higher degree of freedom in its structure and more easily map onto melittin to form high affinity binding sites.[14] Our results in this study indicate that large PLs interacted with all peptides, presumably because of the cumulative effects of multipoint electrostatic and hydrophobic interactions along the length of the flexible polymers. On the other hand, 30-mer PL has limited length and surface area with which to generate such interactions, even though it should be conformationally flexible enough to do so. Nevertheless, 30-mer PL binds melittin strongly because of specific features in the peptide sequence, including the motif KRKR instead of KKKK, as in ponericin: Presumably, the two guanidium ions on the KRKR sequence enabled selective affinity to melittin due to strong electrostatic interaction between guanidium cation and carboxylate anion supported by two parallel hydrogen bonds.[2,15] In addition, melittin contains 5 or more hydrophobic amino acids right next to the KRKR sequence that enable multipoint hydrophobic interactions simultaneously to the electrostatic and hydrogen bonding interactions.[6]

To confirm the interaction between peptide and PLs, we used isothermal titration calorimetry (ITC). A solution of 0.5 mM peptide in PBS was titrated into 0.38 mg/mL synthetic PL. Titration of magainin 1 into synthetic PLs and NP did not generate detectable changes in heat (S3), suggesting little interaction between the molecules. In contrast, endothermic titration curves were observed when NPs and 300-mer PLs were titrated with melittin and ponericin. This result suggest that there is interaction between the peptides and PLs which is entropically-driven presumably by dissociation of water and/or counter ions from hydrophobic and/or ionic functional groups on the peptides and PLs (S4). However, only melittin showed endothermic signal when 30-mer PLs was titrated by each peptide (S3, S4), indicating that 30-mer PLs interacted only to melittin as suggested by the competition filtration assay.

Table 1. Hemolysis neutralization (%) by (A) 30-mer and (B) 15-mer PLs containing with various ratios of functional units. PLs with negligible (< 10 %), moderate (20-80%), and almost complete (> 95%) neutralization are highlighted in gray, yellow, and green respectively.

a)

Incorporated ratio (incorporated No.)	TBAm 0% (0)	TBAm 10% (3)	TBAm 20% (6)	TBAm 40% (12)
AAc 0% (0)	* N.D	* N.N	N.N	N.N
AAc 5% (1)	N.N	N.D	35	41
AAc 10% (3)	N.N	N.N	97	98
AAc 20% (6)	N.N	N.D	98	98

b)

Incorporated ratio (incorporated No.)	TBAm 20% (3)	TBAm 40% (6)
AAc 0% (0)	N.N	N.N
AAc 5% (0)	26	25
AAc 10% (1)	58	60
AAc 20% (3)	79	100

* N.D ; No Data
* N.N ; No Neutralization (less than 10%)

To compare affinity of PLs to each peptide, apparent binding constants (K_a) were obtained by fitting the titration results to the Langmuir binding model (Figure 2B). Here, we approximated that all PLs has uniform molecular weight and structure and all binding event occurred in single site binding mode, although the molecular weight and structure of each PLs cannot be homogeneous.[9] All synthetic PLs bind melittin with high apparent binding constant around 5 x 10^{-5} M^{-1}. On the other hand, affinity to ponericin depended strongly on molecular weight: While NP bound ponericin (2 x 10^{-5} M^{-1}) with comparable affinity as melittin (4 x 10^{-5} M^{-1}), affinity got weaker as PLs got smaller. Indeed, 30-mer PL did not show significant affinity to ponericin. These data also confirm the specific affinity of 30-mer PL to melittin.

The ability of PLs to neutralize melittin toxicity was investigated by hemolysis neutralization assay (S5)[7,14]. A mixture of 1.8 μM melittin and red blood cells in PBS was incubated at 37 °C with 300 mg/mL 30- and 15-mer PLs. The amount of hemoglobin released from red blood cells was then measured after cells were pelleted by centrifugation. Hemolysis neutralization activity in % was calculated according to S6. **Table 1** lists hemolysis neutralization by 30-mer and 15-mer PL. Longer PLs containing at least 20 % TBAm and 5 % AAc showed significant neutralization activity. Those that contain at least 20 % TBAm and 10 % AAc neutralized > 97 % of melittin toxicity. Interestingly, 15-mer PL with the same density of functional units did not completely in-

hibit melittin (58 %). However, 15-mer PL with 40 % TBAm and 20 % AAc achieved almost complete neutralization (100%). Note that this PL has the same *number* (six TBAm and three AAc), but twice the *density*, of TBAm and AAc as the neutralizing 30-mer with 20% TBAm and 10 % AAc.

Based on this result, we conclude that there is a minimum *number* (not *density*) of functional units required to capture and neutralize melittin: Multi-point electrostatic interaction between at least three carboxylate anions on a polymer side chain and cations on melittin supported by several hydrogen bonds to guanidium groups *and* strong hydrophobic interaction given by at least six *tert*-butyl groups on a PLs are both required to capture melittin. This phenomenon is characteristic of low-molecular weight PLs. In 300- and 1000-mer PLs[14], as well as NPs[7], the *density* (not *number*) of incorporated functional units determine the affinity to the target because all of those large PLs has a number of functional units which is far greater than that of melittin, thus PLs with lower density can still form the multipoint interactions by mapping onto the sequence of melittin.[14] However, for the small PLs, such as 30-mer PLs, if density of AAc is lower than 10% and/or density of TBAm is lower than 20%, the PLs cannot form such multipoint binding structure because number of AAc and/or TBAm on a polymer side chain is less than three and/or six respectively.

To further characterize the influence of functional units on target specificity, the binding properties of 30- and 15-mer PLs, which showed almost complete melittin neutralization (neutralization > 97 %, green in table 1), were determined by the competition filtration assay using a mixture of target and control peptides (S7). Results indicate that all of the 30-mers, regardless of composition, captured similar amounts of melittin (**Figure 3**) as suggested by the hemolysis neutralization assay. However, 30-mers consisting of 20 % TBAm and 20 % AAc, and those containing 40 % TBAm and 10 % AAc also captured magainin 1 and ponericin to a significant extent. These results indicate that target specificity in 30-mer PLs decreases with increasing density of functional units. Although, the control peptides does not have guanidium cations to form the stable hydrogen-bonded salt bridges with PLs, PLs containing more than 10 % AAc or more than 20 % TBAm can capture control peptides thorough multipoint electrostatic or hydrophobic interaction respectively. Consistent with this observation, 15-mer PL consisting of 40 % TBAm and 20 % AAc also captured control peptides. Because the high density functional groups on the small PLs enabled multipoint electrostatic and hydrophobic interaction with the positively charged and hydrophobic domains on the control peptides even without drastic conformation change expected only for large PLs. Taken together, these results indicate that PLs, regardless of molecular weight, lose target specificity if the density of functional units is not minimized.

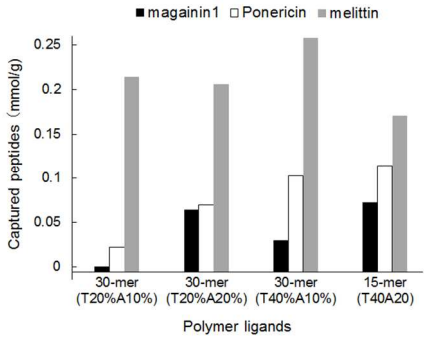

Figure 3. Amount of peptides bound by 0.38 mg/mL synthetic polymer ligands from of a pool consisting of 0.1 mM each of magainin 1, ponericin, and melittin in PBS. Complexed peptides were analyzed by HPLC.

Based on all data, we conclude that *molecular weight* must be minimized to achieve target specificity in multifunctional PLs. In addition, the *density* of functional units must also be minimized to prevent nonspecific interactions. However, as observed for melittin-binding PLs, a PL of minimal size must also contain a minimum *number* of functional units.

These results demonstrate for the first time the ability to recognize a specific target from a pool of similar peptides. We anticipate that this strategy of minimization will become a valuable tool, besides molecular imprinting and affinity purification, to generate inexpensive and physicochemically stable substitutes for biomacromolecular ligands like RNA, DNA, and peptide aptamers.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures and supporting data. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

yhoshino@chem-eng.kyushu-u.ac.jp

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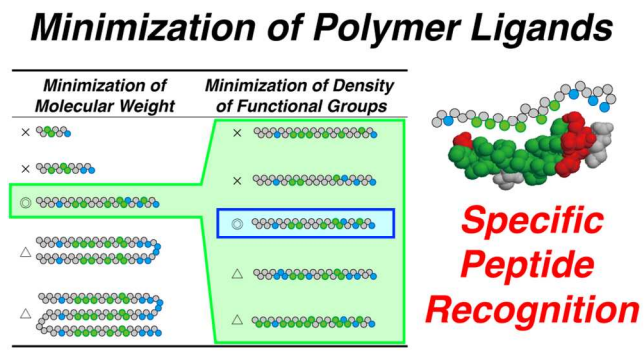
Minimization of Polymer Ligands
Minimization of Molecular Weight
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Specific Peptide Recognition