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Design of Multi-functional Linear Polymers that Capture and Neutralize a Toxic Peptide: A Comparison with Cross-linked Nanoparticles

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Y. Wada[a], H. Lee[a], Y. Hoshino[a*], S. Kotani[b], K. J. Shea[c], and Y. Miura[a]

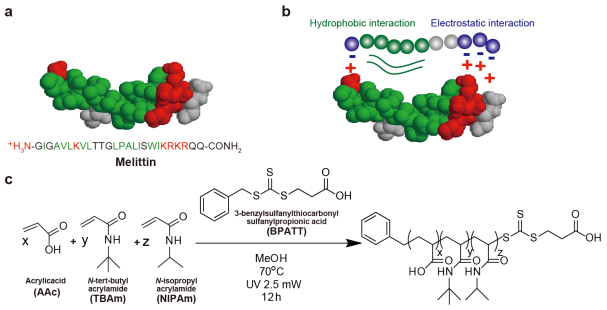
In this paper, a library of multi-functional linear poly-*N*-isopropylacrylamide (pNIPAm) polymers having a range of molecular weights and functional groups were synthesized and their interaction with the hemolytic peptide, melittin, was examined. The linear pNIPAm (LPs) containing both *tert*-butyl group and carboxylic acids bound with the peptide by a combination of hydrophobic and electrostatic interactions and neutralized its toxicity. The melittin binding capacity and affinity of each LP was quantified and further compared with cross-linked multi-functional nanogel particles (NPs) having same combination of functional groups. The binding capacity of the LPs (weight of captured melittin / weight of LP) was independent of their molecular weight and was three times higher than that of previously reported NPs. The binding constant depended on the molecular weight of the LPs, showing the highest value of 1.1 x 10^8 (M^{-1}) for a ~1000 mer linear polymer with 40% *tert*-butyl group and 20% carboxylic acid. Comparison of the interactions of the LPs and NPs suggested the importance of the flexibility of the polymer chain in order to achieve high binding capacity and affinity.

1. Introduction

The strong and specific interactions between biomacromolecules such as proteins and peptides are achieved through a combination of van der Waals, electrostatic and hydrophobic interactions on complementary three-dimensional binding surfaces.[1] As such, protein-mimicking materials with affinity for specific peptides or proteins might be achieved through the incorporation of an optimized combination and/or the ratio of functional groups on the surface of the nanomaterial.[2] For example, gold nanoparticles[3] and dendrimers[4] that express the appropriate combination and/or density of charged and/or hydrophobic functional groups on their surface are capable of recognizing proteins. Schrader and co-workers have designed molecular ligands that interact strongly with arginines and lysins.[5] Then, displayed the molecular ligands on the side-chain of linear copolymers and dendrimers together with hydrophobic, hydrophilic and charged functional group to recognize target proteins thru combination of electrostatic, hydrophobic and hydrogen bonding interaction.[6]

It has been reported that pNIPAm NPs, randomly copolymerized with a 2~10 mol% cross-linker and combinations of functional monomers were capable of binding peptides and proteins via a combination of hydrogen bonding, hydrophobic, aromatic and/or electrostatic interactions.[7] Dawson, Linse and their co-workers have shown that NPs with optimized size and hydrophobicity interact with proteins and peptides and accelerated or inhibit nucleation of fibrillation.[8] The affinity to the target can be enhanced by optimization of the volume density of functional groups in the NPs,[9,10,11] molecular imprinting,[12] affinity purification[13] and tuning the flexibility[14] and density[15] of polymer chains in NPs. NPs designed in this manner have been shown to neutralize target toxins *in vitro* and *in vivo*, and have been termed "plastic antibodies".[10,16] These polymers could potentially be used as stable and inexpensive materials for the purification of proteins and protein complexes.[17,18] However, in the design of multifunctional polymers, the influence of the molecular weight and higher-order structure of the polymer chains on their ability to recognize and neutralize biomolecules is not clear. Physical and chemical crosslinks inside the gel particles may limit the access of target molecules to the internal portion of the NPs or cause a reduction of the flexibility of the polymer chain, inhibiting the formation of stable target-polymer complex.

In this study, a library of multifunctional linear pNIPAm (LPs) was synthesized and the influence of the combinations and incorporation ratio of functional groups and molecular weight on the interaction with a model toxic peptide was analyzed. Melittin, a peptide composed of 26 amino acids, was selected as the target toxic peptide. Melittin is a hemolytic toxin from bee venom and has six positively charged amino acids and a large number of hydrophobic amino acids (**Scheme 1a**). It has been reported that melittin is captured by cross-linked pNIPAm NPs, which are copolymerized with negatively-charged acrylic acid (AAc) and hydrophobic *N*-tert-butylacrylamide (TBAm) through combination of electrostatic and hydrophobic interactions (**Scheme 1b**).[7,10] Therefore, in this study, AAc and TBAm were selected as the functional monomers. LPs with two different molecular weights (300 mer and 1000 mer; LP_{300} and LP_{1000} respectively) were synthesized by UV-initiated reversible addition fragmentation transfer polymerization in the presence of chain transfer agent (CTA) as reported. The binding capacity and affinity with melittin was analyzed by peptide neutralization assay and quartz crystal microbalance.[7,10] The results were further compared with the previously reported results of NPs.[7,10]



Scheme 1 (a) Crystal structure and amino acid sequence of melittin. Hydrophobic, and positively charged, amino acids are printed in green, and red respectively. The PDB ID is 2MLT. (b) Schematic of the binding between melittin and the ideal LP. Hydrophobic, and negatively charged functional groups are printed in green, and blue respectively. (c) Preparation of multifunctional LPs.

Since little have been reported about the influence of the molecular weight, higher-order structure and flexibility of synthetic polymers on their ability to recognize targets, this study may be useful in the future design of synthetic polymers capable of strongly binding to target biomacromolecules.

2. Results and Discussion

2.1. Synthesis of a Linear Polymer Library

It has been reported that poly-AAc can be synthesized in aqueous media by UV- or γ-ray initiated living radical polymerization by the reversible addition fragmentation chain transfer (RAFT) mechanism.[19] In this study, we modified the procedure to synthetize a library of multifunctional polymer, since the TBAm and chain transfer agent (benzylsulfanylthiocarbonylsulfanyl propionic acid; BPATT) was not soluble in water in the absence of AAc. We used methanol was as solvent to dissolve TBAm and BPATT, and polymerization reaction was carried out at 70 °C under UV-irradiation (**Scheme 1**).[19] LPs with two different molecular weights were prepared by tuning the stoichiometric ratio of the total monomer concentration to the chain transfer agent to be 1000 and 300, respectively (LP_{1000}, LP_{300}). NIPAm was used as the principle monomer and TBAm and AAc were used as functional monomers with a hydrophobic (*tert*-butyl) and a negatively charged (carboxylate anion) side chain. A total of 22 types of multifunctional polymers were prepared by changing the feed ratio of TBAm and AAc from 0~40 % and 0~20 %, respectively (**Table 1**). ^{1}H NMR confirmed that in a 90% or higher conversion of all LPs and an incorporation ratio in accordance with the feed was achieved. The PDI, apparent Mn and Mw were measured by GPC using polystyrene as standard. The GPC results showed that all LPs have mono-modal size distribution. The PDIs were approximately 1.50-2.25 for LP_{1000} and 1.38-1.82 for LP_{300}, indicating that polymerization process was not perfectly controlled comparing with the previous report.[19] It was difficult to obtain Mn and Mw that match up precisely with the designed value. Because there is significant interaction between carboxylic acids on LPs and stationary phase in the GPC column.[20] However, all NPs polymerized with small population of AAc showed that the addition of chain transfer agent allowed for the synthesis of LPs with controlled molecular weights. Although, PDI, Mn and Mw of some LPs were not perfectly controlled as designed, we decided to use the library for the further study, since the LPs in the library have well defined properties enough to compare melittin neutralization activity with NPs and to study the influence of the combinations and incorporation ratio of functional groups and molecular weight on the interaction with a model toxic peptide.

Table 1 Feed ratio of functional monomers, conversion of polymerization reaction, incorporated ratio of functional monomers, Mw, Mn and PDI of LPs.

LPs	Feed ratio [Monomer] /[CTA]	Feed ratio (mol%)		Conversion (%)	Incorporated ratio (mol%)		PDI
		TBAm	AAc		TBAm	AAc	
$LP_{1000\text{-}1}$	1000	0	5	97	0	5	1.85
$LP_{1000\text{-}2}$	1000	0	20	98	0	21	1.50
$LP_{1000\text{-}3}$	1000	20	0	93	20	0	1.53
$LP_{1000\text{-}4}$	1000	20	1	96	20	1	1.85
$LP_{1000\text{-}5}$	1000	20	5	98	20	5	1.77
$LP_{1000\text{-}6}$	1000	20	20	96	20	20	1.54
$LP_{1000\text{-}7}$	1000	40	0	96	40	0	1.50
$LP_{1000\text{-}8}$	1000	40	1	99	40	1	1.65
$LP_{1000\text{-}9}$	1000	40	5	98	40	5	2.25
$LP_{1000\text{-}10}$	1000	40	10	95	40	10	1.71
$LP_{1000\text{-}11}$	1000	40	20	95	40	20	1.70
$LP_{300\text{-}1}$	300	0	5	98	0	5	1.50
$LP_{300\text{-}2}$	300	0	20	93	0	20	1.68
$LP_{300\text{-}3}$	300	20	0	97	20	0	1.38
$LP_{300\text{-}4}$	300	20	1	99	20	1	1.54
$LP_{300\text{-}5}$	300	20	5	97	20	5	1.67
$LP_{300\text{-}6}$	300	20	20	93	20	20	1.60
$LP_{300\text{-}7}$	300	40	0	99	40	0	1.38
$LP_{300\text{-}8}$	300	40	1	98	40	1	1.48
$LP_{300\text{-}9}$	300	40	5	98	40	5	1.59
$LP_{300\text{-}10}$	300	40	10	95	40	10	1.68
$LP_{300\text{-}11}$	300	40	20	83	38	22	1.82

2.2. LP Neutralization and Binding Capacity of Melittin

The neutralization of hemolytic toxicity of melittin by LPs was tested by the red blood cell lyses test as previously reported.[7] First, 100 μg/mL of polymer was mixed with 1.8 μM of melittin and red blood cells, and the mixture was incubated for 30 min. LPs with only AAc or TBAm were unable to neutralize the hemolytic activity of melittin substantially (inhibition ratio < 40 %) (**Figure 1a**, **b**). On the other hand, most LP_{1000} and LP_{300}, which contained both AAc and TBAm, showed a high inhibition ratio (> 60 %), indicating that melittin was captured by LP through combination of electrostatic and hydrophobic interaction regardless of their molecular weight (300 mer or 1000 mer).

Binding capacity of melittin to LPs was calculated from the relationship between concentration of LPs and inhibition ratio of red blood cell lyses as reported (**Figures 1c** and **1d**).[7,10] LPs containing 40% TBAm and 5% or higher of AAc showed the highest binding capacity of 1.5 g/g, and the binding capacity did not increase when the incorporation of AAc was increased from 5 % to 20 % (**Figure 1c**, **1d** and **2**). A comparison between LP_{300} and LP_{1000} showed that

the binding capacity did not depend on the molecular weight of the polymers (**Figure 1c**, **1d** and **2a**).

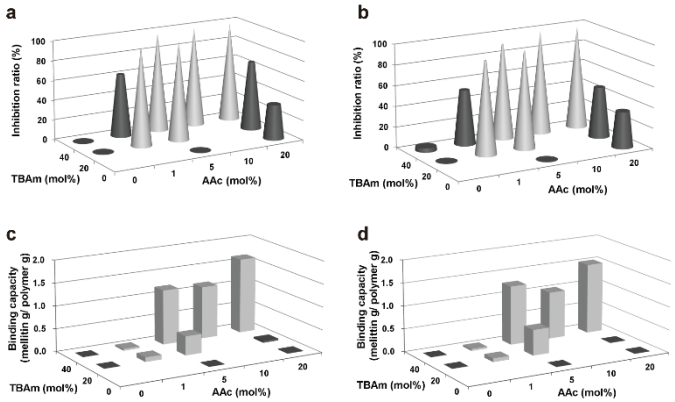


Figure 1 Hemolytic activity inhibition ratio with 100 μg/mL LP_{1000} (**a**) and LP_{300} (**b**). Gray cones indicate almost complete neutralization of melittin. Melittin-binding capacity of LP_{1000} (**c**) and LP_{300} (**d**). Binding capacity of LPs shown as black parallelogram was too small to be calculated.

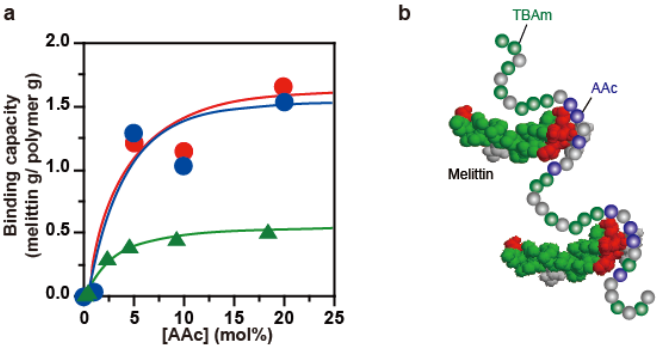


Figure 2 Effect of the amount of AAc on the melittin-binding capacity of NPs (green)[10], LP_{300} (blue), and LP_{1000} (red) containing 40% TBAm.

It has been reported that cross-linked NPs that were polymerized with the same combination of functional monomers as LPs captures melittin thru combination of electrostatic and hydrophobic interaction.[7,10] The binding capacity of NPs, which hydrodynamic diameter was 50-90 nm, was about 3 times smaller than LPs (**Figure 2a**).[10,18] This suggests the presence of a domain inside the NPs, which could not bind to melittin. Chemical crosslinks in the gel particles formed by crosslinking monomers and intra particle radical chain transfer[21] and/or physical crosslinks formed by hydrogen bonding of carboxylic acids and amides during the polymerization process [22] and/or hydrophobic interaction of *tert*-butyl groups and polymer main chain might cause the steric hindrance for the melittin to access the domain. Those intra-particle cross-links might be dramatically limited in the case of LPs, since LPs were polymerized without cross-linkers in the absence of strong intra- and inter-polymer interaction by using good solvent (methanol instead of water) for both monomers and polymers. It also suggested steric hindrance was eliminated by using linear polymers, thus allowing the binding of melittin on most parts of the polymer chain.

The binding capacity of 1.5 g/g suggests that one molecule of melittin was captured by 20-units of monomer on a LPs (including 8-units of TBAm and 1~4-units of AAc) on average. Molecular length of the 20-unit LP is shorter than melittin (26 amino acid peptide). Thus, the extremely high binding capacity of LPs observed in this study may suggest that the LPs recognized and captured limited sequence of melittin, presumably c-terminal cation-rich fragment, as NPs of the same composition did (**Figure 2b**). [18]

2.3. LP Binding Affinity of Melittin

The apparent binding constant between LPs and melittin was calculated by fitting binding curves obtained by the 27-MHz quartz crystal microbalance (QCM) to Langmuir isotherm as reported (Figure S1).[7,10] A high binding constant (K_a) greater than 10^6 M^{-1} for LP_{300} consisting of relatively high ratio of both TBAm and AAc was confirmed (**Figure 3a**). However, little interaction was observed by QCM for all LPs containing only AAc or TBAm.

Table 2 Binding constant of the interaction between LP_{1000}, LP_{300}, and NPs.

Polymer	Incorporated ratio (mol%)		K_a (M^{-1})
	TBAm	AAc	
$LP_{1000-11}$	40	20	1.1 x 10^8
LP_{300-11}	38	22	1.2 x 10^7
NP[10]	40	19	6.6 x 10^7

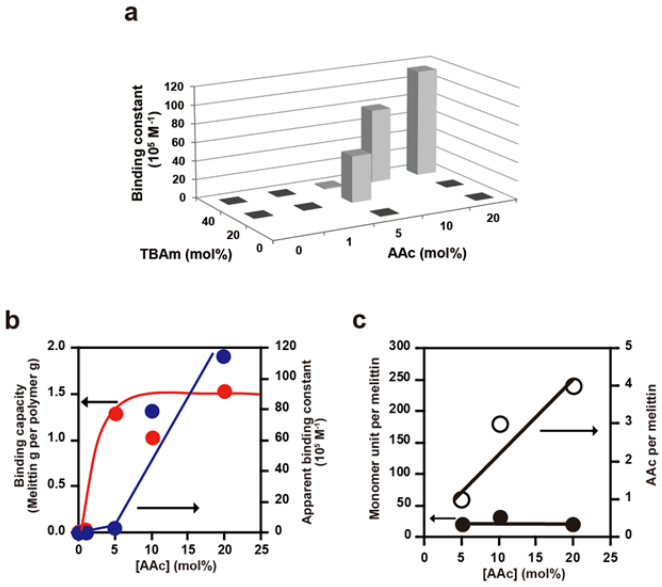


Figure 3 (a) The melittin-binding constant of LP_{300} of various compositions. (b) Influence of the amount of AAc on the melittin-binding capacity and binding constant of LP_{300} containing 40% TBAm. (c) Average number of monomer units and average number of AAc that are allowed for the LPs to capture one melittin.

The apparent binding constant increased dramatically by increasing amount of AAc in LP_{300} containing 40% TBAm (**Figure 3c**). LP_{300} containing 40% TBAm and 20% AAc had the strongest binding with a binding constant of K_a=1.2 x 10^7 M^{-1}. To explain the strong affinity, the average number of monomer unit and AAc, that can be used to capture one melittin, was calculated from the binding capacity of each LPs (**Figure 3c**). **Figure 3** indicates that LP_{300} containing 40% TBAm and 20% AAc are capable of capturing one melittin with 4 carboxylic acids in addition to 8 *tert*-butyl group thru multi-point and multi-modal interaction, enabling the strong affinity.

The binding constant of LP_{300}, LP_{1000}, and NPs[10] consisting of same ratio of functional monomers (40% TBAm and 20% AAc) were obtained and shown in Table 2. Interestingly, LP_{1000} (K_a=1.1×10^8 M^{-1}) showed even stronger affinity than NPs (6.6×10^7 M^{-1}), although, NPs showed stronger affinity than LP_{300} (K_a=1.2×10^7 M^{-1}). It has been reported that flexibility of polymer chain is important for the functionalized polymers to form stable polymer-protein complex through multipoint interaction.[14,15,23] Since LP_{1000} has a larger molecular weight than LP_{300}, it has a higher degree of freedom in its structure and more easily map onto melittin to form high affinity binding sites than LP_{300}. Although, NPs have a much higher molecular weight than LP_{1000}, NPs showed weaker affinity than LP_{1000} maybe because they are cross-linked and the flexibility of polymer chains are lower than those of LP_{1000}.

3. Conclusion

In summary, we prepared a multi-functional linear polymer library having a two different molecular weights and range of functional groups. Only LPs containing both TBAm and AAc were capable of neutralizing the toxicity of melittin. The maximum melittin-binding capacity was 1.5 g/g, which was approximately three times higher than that of the previously reported NPs. In addition, amount of melittin with can be captured and neutralized by a gram of LPs was independent of the molecular weight of LPs. Meanwhile, the binding constant strongly depended on the Mw of the polymers and the strongest affinity of K_a=1.1×10^8 (M^{-1}) was found with LPs with a flexible and relatively large molecular weight. Since, little has been reported about the influence of the Mw and structure of synthetic polymers on their binding capacity and affinity with peptides, this study may be useful for the design of synthetic polymers ligands capable of strongly binding with biological molecules.

4. Experimental Section

Materials: AAc (Wako Pure Chemical Industries) was used after purification by an alumina column (Al_2O_3 pH 6.8-7.8). NIPAm (Wako Pure Chemical Industries) was used after recrystallization from benzene and n-hexane. TBAm (Wako Pure Chemical Industries) was used as received. Preserved bovine blood (50% Alsever's solution) was purchased from Nippon Bio-Test Laboratories Inc. Melittin (from bee venom; molecular weight (MW): 2846 Da) was purchased from SERVA. Biotinylated melittin (sequence; Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-Lys(PEG4-Biotin)-NH_2, Mw; 3448.3 Da, purity; 98.2 %) was purchased from American Peptide Company. Other reagents were purchased from Wako Pure Chemical Industries or Tokyo Chemical Industry, Co., Ltd. and were used as received.

Synthesis of chain transfer agent: The chain transfer agent, 3-benzylsulfanylthiocarbonylsulfanyl propionic acid (BPATT), was synthesized as reported.[22,24] KOH (12.9 g, 230 mmol) was dissolved in water (125 mL), and 3-mercaptopropanoic acid (10.0 mL, 115 mmol) was added and the mixture was stirred for 4 h. Carbon disulfide (24.3 mL, 253 mmol) was added to the mixture, which was stirred at room temperature for 5 h. To the resulting solution, benzyl chloride (27.5 mL, 115 mmol) was added and warm to 80 °C. After stirring for 12 h, the reaction mixture was diluted with chloroform (ca. 30 mL), followed by addition of 1N HCl until the color of the solution changed from orange to yellow. The aqueous layer was extracted with chloroform (3 x 40 mL). The combined organic layer was washed with water (3 x 10 mL) and dried *in vacuo*. The obtained yellowish solid was dissolved in ethyl acetate, followed by addition of hexane to give a yellowish solid. After filtration, BPATT was obtained in 69% yield. ^{1}H NMR (JNM-ECP400) ($CDCl_3$): δ 2.84 (t, 2H, CH_2CO), 3.62 (t, 2H, CH_2S), 4.61 (s, 2H, CH_2-Ph), 7.27 (m, 5H, Ar-*H*).

Synthesis of linear polymers: BPATT, NIPAm, TBAm, and AAc were dissolved in methanol and mixed to obtain a total monomer concentration of 1.5 M and a monomer : chain transfer agent ratio of 300 : 1 or 1000 : 1. Next, 1.5 mL of the solution was added to a glass tube: Outer tube diameter was 10 mn: Glass thickness was 2 mm. A cycle of melting, vacuum, and freezing was repeated three times. The tube was sealed using a gas burner and irradiated with 2.5 mW/cm^2 ultraviolet light (High Power Xenon light source MAX-302:ASAHI SPECTRA). The polymerization reaction was conducted for 12 h at 70 °C. After the reaction, the tube was opened and the reaction was terminated by oxygen in the atmosphere. The reaction solution was added dropwise into an excess amount (> 30 mL) of diethyl ether or water. Polymers were recovered by centrifugation (H-103n KOKUSAN). The conversion and the incorporation ratio of monomers were quantified using ^{1}H-NMR (JNM-ECP400) (solvent: CD_3OD). Number average molecular weight: Mn, weight average molecular weight: MW, and polydispersity index (PDI = Mw/Mn) were confirmed by using gel permeation chromatography (GPC): Solvent; DMF / 10 mM LiBr, flow rate; 0.5 mL / min, column; LF-804 Shodex, RI detector; RI-2031 plus (Jasco), UV detector; UV-970 (Jasco), pump; Pu-2080 plus (Jasco), column oven; CO-965 (Jasco), auto sampler; AS-2057 (Jasco), degasser; DG-980-50 (Jasco), molecular weight standard; polystyrene).

Hemolysis inhibition experiments: The red blood cells were purified from preserved bovine blood, in accordance with the following procedure.[6,9] Preserved bovine blood (500 μL) was placed in a 1.5 mL microtube with 1 mL phosphate buffered saline solution (PBS; 35 mM phosphate, pH 7.3, 150 mM NaCl). The suspension was mixed and subsequently centrifuged at 800 G (KUBOTA 1120) for 10 min. After centrifugation, the supernatant was removed then 1 mL PBS was added again. This procedure was repeated five times. After the final centrifugation, 80 μL of the precipitated red blood cells were recovered slowly by pipet and diluted four-fold in PBS. This suspension was used as the red blood cell solution and was prepared prior to each experiment.

A stock solution of the LPs (30 mg/mL) in DMSO was prepared and diluted in PBS to the desired concentration. An 18 μM stock solution of melittin was prepared by dissolution in milliQ water. Twenty microliters of melittin stock solution, 2 μL of an PBS solution of LPs, 30.4 μL of 5 times concentrated PBS, and 101.6 μL of milliQ water were added into a 1.5 mL microtube and the mixture was pipetted five times. Each sample was prepared in triplicate. A solution of red blood cells (48 μL) was added to each sample and pipetted five times. The mixture was incubated in a thermostat (MG-1200 EYELA) at 37 °C for 30 min. After incubation, the samples were centrifuged at 800 G (KUBOTA 1120) for 10 min. Immediately after centrifugation, 10 μL of the supernatants were recovered then diluted 20-fold in PBS in a 96-well plate. The absorbance (405 nm, A_{sample}) of each solution was measured by a

plate reader (MULTISKAN JX: Thermo electron). Samples using milliQ water instead of an aqueous solution of melittin ($A_{100\%}$), and samples using DMSO instead of the DMSO solution of LPs ($A_{0\%}$) were prepared as controls. The hemolytic activity inhibition ratio was calculated as follows:

$$Inhibition\ ratio\ (\%) = \frac{A_{sample} - A_{0\%}}{A_{100\%} - A_{0\%}} \times 100$$

Inhibition curves were achieved by plotting inhibition ratio against concentration of LPs. Binding capacity of LPs was calculated from the initial slope of the inhibition curves. [10]

Analysis of the binding constant by using a quartz crystal microbalance (QCM): An Affinix Q4 QCM instrument (Initium Co. Ltd., Tokyo, Japan) was used to quantify interactions between the NPs and melittin and control proteins.[7,10] A 1% sodium dodecyl sulfate (SDS) solution (500 μL) was added to a QCM cell and allowed to stand for 10 min or longer. Surface of the gold electrode (substrate) on the bottom of the cells, containing SDS solution, was wiped carefully by a cotton swab to remove dust. After washing the cell with water, 2.5 μL of a fresh piranha solution (hydrogen peroxide : concentrated sulfuric acid (1 : 3)) was mounted onto the gold substrate, allowed to stand for 10 min, and washed with water. This piranha washing process was performed three times to remove all organic matter from the gold surface. MilliQ water (200 μL) was added to the QCM cell soon after it was washed (note that the gold surface should not be dried after the piranha washing). Subsequently, ethanol solution (0.1 M, 2 μL) of 3,3'-dithiodipropionic acid (MW: 210.26) were also added and the mixture was allowed to stand for 30 min or longer to carboxylate the gold surface. The carboxylated QCM cell was washed with water. 50 μL of aqueous 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride (EDC, Mw: 192.70) solution (100 mg/mL) and 50 μL of aqueous *N*-hydroxysuccinimide (NHS, Mw: 115.09) solution (100 mg/mL) was added to the QCM cells, and was allowed to stand for 30 min at room temperature. After washing by milliQ water, 50 μL of avidin (from hen egg white) solution (PBS, 1 mg/mL) was added and was allowed to stand for 1 h at room temperature. After the avidin-immobilized substrate was washed with water, 500 μL of HEPES buffer (10 mM HEPES, pH 7.4) was added and the solution was set on the Affinix Q4 instruments at 25°C. 1 μL of water solution of biotinylated melittin (100 μM) was injected into the cell and the time-course of frequency changes were measured. One hour later, the melittin-immobilized QCM cell was washed with water, replaced with 500 μL PBS, and set on the Affinix Q4 instruments at 37 °C. After stabilizing the base line, PBS solution of LPs was injected consecutively into the cell and the time-course of frequency change were monitored. For each LPs concentration, amount of LPs bound on the melittin-immobilized sensor were plotted and the apparent binding constant K_a (M^{-1}) was calculated using the Langmuir isotherm.[7,10]

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[a] Department of Chemical Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan yhoshino@chem-eng.kyushu-u.ac.jp
[b] Priority Organization for Innovation and Excellence, Kumamoto University, Kumamoto 862-0973, Japan
[c] Department of Chemistry, University of California, Irvine, Irvine, CA 92697, USA

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