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Design of Synthetic Polymer Nanoparticles that Capture and Neutralize a Toxic Peptide

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Abstract

Designed polymer nanoparticles (NPs) capable of binding and neutralizing a biomacromolecular toxin are prepared. A library of copolymer NPs is synthesized from combinations of functional monomers. The binding capacity and affinity of the NPs are individually analyzed. NPs with optimized composition are capable of neutralizing the toxin even in a complex biological milieu. It is anticipated that this strategy will be a starting point for the design of synthetic alternatives to antibodies.

Keywords

antibodies; biomolecules; nanoparticles; polymers; protein recognition

1. Introduction

The design of interactions between nanoparticles (NPs) and biomolecules is of significant interest in bionanotechnology.[1] To provide strong and specific binding, NPs are often conjugated with biomacromolecular ligands, such as peptides and antibodies.[2] Nonspecific protein binding to NPs can be ameliorated by attachment of polyethylene glycol to the NP. [3] The direct approach of designing NP affinity to specific biomacromolecules by controlling the chemical composition of the NP is less well known.[1] Synthetic polymer NPs capable of binding to specific biomacromolecules are of significant interest as substitutes for natural ligands, such as antibodies. Such particles may be utilized as inexpensive and stable functional materials for diagnostics, research tools in molecular biology, drug delivery, disease therapy, and as antidotes for toxins and viruses.

Herein, we describe the design of novel synthetic NPs that bind to and suppress the activity of a toxic peptide. These NPs are capable of capturing and neutralizing the target peptide in a complex biological milieu. Melittin was selected as a target peptide to develop the concept of neutralization of activity through designed NP–biomacromolecule interactions. Melittin, a 26 amino acid peptide isolated from bee venom, is a representative of membrane-damaging toxins, a number of which function as key virulence factors of infectious diseases (Scheme 1).[4] These toxins do not exert their biological activity by interacting with a specific binding site, but rather by a mechanism that involves direct association with cell membranes.[5] Due to the potential difficulty in targeting a specific mechanism of action, an effective strategy to neutralize the activity of such toxins is to inhibit their access to cell membranes during the solution transport of the toxin. We describe the synthesis and evaluation of NPs that neutralize a representative of these toxins.

2. Results and Discussion

2.1. Preparation of NPs

A precipitation polymerization method was employed for the synthesis of a library of water-soluble nanosized particles that incorporate a variety of functional monomers. Kokufuta et al. [6a] and Debord and Lion[6b] reported the synthesis of water-soluble random-copolymer NPs (<100 nm) by free-radical copolymerization of *N*-isopropylacrylamide (NIPAm) with small amounts of crosslinker (*N,N'*-methylenebisacrylamide; BIS) and hydrophobic and charged monomers. Linse's research group used NPs prepared by this method to evaluate the nonspecific binding of plasma proteins to NPs as a function of the amount of hydrophobic monomer in the particle.[1] For the current study, we modified this method to prepare a small library of NIPAm-based copolymers containing 2% cross-linker (BIS) and combinations of *N*-*tert*-butylacrylamide (TBAm; hydrophobic monomer), acrylamide (AAM; hydrophilic monomer), *N*-(3-aminopropyl)methacrylamide hydrochloride (3APM; positively charged monomer), and acrylic acid (AAc; negatively charged monomer) (Scheme 1; Table 1, NPs 1–7). Copolymers synthesized with 40% TBAm and 5% 3APM (4) aggregated during dialysis; however, all other polymers were prepared in reasonable yield. Copolymers prepared with 40% TBAm (2, 6, 7) were monomodal as determined by dynamic light scattering (DLS) and fell within a hydrodynamic size range of 50–70 nm (Table 1). The diameter of copolymers without TBAm could not be determined by DLS due to the wide size distribution and/or low scattering intensity.

2.2. Hemolytic Activity Neutralization Assay

Melittin is a hemolytic peptide that lyses red blood cell (RBC) membranes. We use this behavior as the diagnostic to evaluate the affinity of NPs to melittin. The ability of polymer NPs to neutralize the hemolytic toxicity of melittin is presented in Figure 1. Figure 1a shows

centrifuged RBC solutions incubated without (tube on far left) or with 1.8 μM of melittin (remaining tubes). The supernatant solution incubated with melittin (second tube from left) is red due to liberated hemoglobin from the RBCs. NPs **1–7** were preincubated with melittin then incubated with RBCs. The color of the supernatant of solutions incubated with melittin and NPs **1–3**, **5**, and **7** were the same as that without preincubation with NPs (within $\pm 5\%$ error in absorbance of 415 nm). However, the supernatant of solutions preincubated with NP **6** (40% hydrophobic (TBAm) and 5% anionic (AAc) monomers) was transparent due to neutralization of the hemolytic activity. In contrast, melittin preincubated with NP **4** comprising 40% TBAm and 5% 3APM (positively charged monomer) shows a higher hemolytic activity than in the absence of **4**. The results demonstrate that negatively charged hydrophobic polymer NPs can neutralize the hemolytic activity but positively charged hydrophobic polymer NPs enhance the hemolytic activity of melittin.

The neutralization results can be understood by an analysis of the amino acid sequence of melittin (Scheme 1). Melittin has 26 residues of which six are positively charged. The charged sites are from the N-terminal α -aminoglycine, three ϵ -amino groups from lysines at positions 7, 21, and 23, and two guanidinium groups from arginines at positions 22 and 24. The sequence is amphiphilic, since six amino acids at the C terminus of the peptide are hydrophilic whereas the remainder have a high proportion of apolar residues. The successful monomer combination for melittin neutralization contains 40% hydrophobic monomers (TBAm) and 5% negatively charged functional monomers (AAc). This copolymer composition is able to interact with melittin by both electrostatic and hydrophobic interactions that enable melittin to be captured by polymer NPs with high efficiency (Scheme 2a–d).

Melittin neutralization curves of NPs copolymerized with different feed ratios of AAc and TBAm are shown in Figure 1b and c. Here, the neutralization constants of polymers, an indication of neutralization efficiency, are defined by the initial slope of the neutralization curve and are plotted against AAc or TBAm feed ratio (Figure 1d–f). For polymers containing 40% TBAm, the neutralization constants are proportional to the feed ratio of AAc. The stoichiometrics of AAc/melittin are calculated to be approximately one from each neutralization constant, assuming that the incorporation of AAc in NIPAm polymers is about five times lower than the feed ratio.[6] This finding suggests that at least a single AAc residue is needed to bind a single melittin molecule, and the neutralization constants represent the amount of melittin that is captured by the NPs. Copolymers that are formed with 5% AAc and 10–20% TBAm show little neutralization activity (Figure 1f). For copolymers with 10% AAc, the neutralization activity was observed when the level of TBAm was $>20\%$ (Figure 1f). The plot of neutralization constant versus TBAm feed ratio shows that neutralization activity is proportional to the TBAm feed ratio at ratios $>10\%$.

NIPAm polymers and NIPAm–TBAm copolymers are thermoresponsive.[7] They are swollen, low-density hydrogels below their lower critical solution temperature (LCST) and collapse to form high-density particles above their LCST. It is known that at higher TBAm/NIPAm feed ratios the LCST is reduced.[6] Although NP **10** is above the LCST in the hemolysis test, NP **9** is below the LCST (Figure 2). This suggests that melittin binding is optimal with high loadings of TBAm, which results in a NP with a collapsed high-density hydrophobic core (Scheme 2e and f).

In general, hydrogen bonding as well as electrostatic and hydrophobic interactions are known to contribute to protein–protein interactions.[8] However, NPs copolymerized with hydrophilic monomers (AAm; **1**, **2**), which have a greater hydrogen-bond donor potential than other functional monomers, did not show any significant activity in the melittin toxicity assay. Furthermore, when 5% AAm was added to 40% TBAm and 5% AAc (**17**), a 25% reduction of the neutralization constant was observed relative to NPs prepared without AAm (**6**). This

indicates that the backbone monomer NIPAm, with its one potential hydrogen-bond donor group and a hydrophobic isopropyl group, contributes more to melittin capture than AAm.

2.3. Quartz Crystal Microbalance Analysis

A 27-MHz quartz crystal microbalance (QCM) was used to quantify affinities between NPs and melittin (Figure 3).[9] The time courses of frequency changes after injection of NPs (**6,11,10,15**) into melittin-immobilized QCM cells are shown in Figure 3a. NPs that have a high neutralization constant (**6, 11**) show a frequency decrease due to binding; other formulations in the library had little affinity to melittin in the same concentration range ($\approx 200 \mu\text{g mL}^{-1}$). Although the neutralization activities of **10** and **15** were observed by the RBC test, the interaction between melittin and NPs **10** and **15** were not observed by the QCM experiment.

An average apparent binding constant of NPs for melittin is calculated from binding isotherms (Figure 3b), assuming that all particles in solution are homogeneous spheres and the polymer density is $0.08 < \rho < 0.27$ (Table 1).[6] NPs with 10% AAc (**11**) have binding constants three to four times greater than NPs with 5% AAc (**6**). It is interesting that the difference in the neutralization constants of **11** and **6** is less than 50 to 100%, although the binding constant difference is 200 to 300%. We believe this difference arises because the neutralization constant reflects the melittin-binding capacity per gram of NPs and the binding constant is a measure of the binding affinity for the surface of the NPs. It is important to note that by using both the RBC and QCM methods, we can estimate values of both binding capacity and affinity separately.

The binding specificity of designed NPs to melittin was revealed by two experiments. First, the interaction between NP **6** and serum proteins (bovine serum albumin (BSA) and γ -globulin) are not detected by QCM experiments. Nonspecific interactions of NPs with serum proteins are significantly lower than those for similar NIPAm-TBAm polymer NPs prepared by Linse et al.[1] The negative charges on our NPs seem to result in reduction of the protein-NP nonspecific interactions. Second, NP **6** did not show neutralization activity towards the hemolytic biotoxin α -hemolysin, a 34-kD protein.

2.4. Atomic Force Microscopy Imaging

Together with the DLS analysis, atomic force microscopy (AFM) images show that the NPs **6** are well dispersed over a wide area of the mica surface (Figure 4). The diameter of particles obtained from the height profile falls in the range of 10–60 nm (Figure 4, inset). The size is 100 times smaller than that of RBCs and comparable to that of immunoglobulin M (IgM), which suggests that the NPs may be capable of being transported by diffusion in viscous mucus as well as by forced convection in blood capillaries.

2.5. In vitro Assay

To demonstrate the melittin neutralization activity of NP **6** in a complex biological milieu, the results of a neutralization assay for human fibrosarcoma cells cultured in medium containing 10% serum are shown in Figure 5. Despite the fact that melittin was post-injected in a culture medium containing an excess of serum proteins, preinjected NPs were found to neutralize the activity of melittin for 24 h. These results demonstrate that rationally designed NPs with optimized composition can capture and neutralize melittin without interference by serum proteins. The captured melittin is not replaced by the presence of an abundance of serum proteins.

3. Conclusions

In summary, we have prepared polymer NPs with the capacity to bind and neutralize the hemolytic toxin melittin. A small library of NPs was prepared incorporating functional monomer combinations. The contribution of each functional monomer to the binding capacity and affinity were analyzed separately by suppression of the hemolytic function of melittin and by QCM analysis. Optimized NPs were able to neutralize the toxicity of melittin even in a complex biological milieu. The NPs are not biodegradable and are chemically more stable than protein antibodies. It is expected that they can remain longer in an enzymatic environment, such as the intestine, stomach, or mucosa, without being digested by proteases. Furthermore, due to their small size these polymer nanomaterials show enormous binding capacity. We propose that these NPs can serve as a new class of “polymer therapeutics” that can recognize and neutralize specific biomacromolecules without conjugation of targeting ligands.[2] The target molecule used in this study, melittin, is less complex than protein toxins. We anticipate that we will be able to apply our method to these more complicated targets by expanding the library of NPs with a greater diversity of functional monomers. In consideration of the comparable size of these NPs to a natural antibody (IgM), we anticipate that these results will be a starting point for synthetic polymer antibodies for a range of biomolecules.

4. Experimental Section

Materials

All chemicals were obtained from commercial sources: NIPAm, *N,N,N',N'*-tetramethylethylenediamine, melittin (from honey-bee venom), BSA, γ -globulin, and ammonium persulfate were from Sigma–Aldrich, Inc.; AAm, AAc, and sodium dodecyl sulfate (SDS) were from Aldrich Chemical Company, Inc.; BIS was from Fluka; TBAm was from Acras Organics; APM was from Polysciences, Inc.; EZ-Link NHS-PEO₄-biotin was from Pierce; avidin (from egg white) was from Nacalai Tesque, Inc.; bovine RBCs were from Innovative Research, Inc.; Dulbecco's Modified Eagle's Medium (DMEM) was from Wako Chemicals; fetal bovine serum (FBS) was from Japan Bioserum Inc.; and Alamar Blue was from Serotec Ltd. NIPAm was recrystallized from hexane before use. Other chemicals were used as received. Water used in polymerization and characterization was distilled and then purified by using a Barnstead Nanopure Diamond system.

Preparation of NPs

NIPAm (98–($W+X+Y+Z$) mol%), AAm (W mol%), AAc (X mol%), APM (Y mol%), TBAm (Z mol%), BIS (2 mol%), and SDS (10 mg) were dissolved in water (50 mL) and the resulting solutions were filtered through a no. 2 Whatman filter paper. TBAm (Z mol%) was dissolved in ethanol (1 mL) before addition to the monomer solution, which resulted in a total monomer concentration of 6.5 mM. The resulting solutions were degassed in a sonication bath under vacuum for 10 min and then nitrogen was bubbled through the reaction mixtures for 30 min. Following the addition of ammonium persulfate aqueous solution (30 mg per 500 μ L) and *N,N,N',N'*-tetramethylethylenediamine (15 μ L), the polymerization was carried out at 23–25°C for 15–20 h under a nitrogen atmosphere. The polymerized solutions were purified by dialysis against an excess amount of pure water (changed more than twice a day) for >4 days.

Characterization of NPs

The hydrodynamic diameter of the NPs was determined in aqueous solution by DLS (LB-550, Horiba Instruments Inc., CA, USA). The temperature of the NP samples was controlled by a Peltier device at (25 $\pm$ 0.1)°C. The yield of NPs was determined by measuring the weight of NPs after lyophilization. Here, a dilution factor due to dialysis was corrected. The apparent molarities of the NPs were calculated by using Equation (1):

$$[\text{NPs}] = \frac{6}{\pi N_A d^3 \rho} X \quad (1)$$

where N_A is Avogadro's constant, d is the hydrodynamic diameter of particles, ρ is the polymer density of particles, and X is the polymer weight concentration (mg mL^{-1}). The ρ values for NIPAm-based swollen particles were estimated by Ogawa et al. to be $\approx 0.01 \text{ g cm}^{-3}$. [6] The polymer density of deswollen particles was estimated to be 2^3 – 3^3 times higher than that of swollen particles ($0.08 < \rho < 0.27$). [6]

Hemolytic activity neutralization assay

Neutralization of the hemolytic activity of melittin by NPs was assayed by a modified standard hemolytic assay procedure. [10] RBCs were washed with phosphate-buffered saline (PBS; 35 mM phosphate buffer/0.15 M NaCl, pH 7.3), collected by centrifugation (10 min, 800 g), and then resuspended in PBS three times. Melittin (final concentration in RBC suspension was 1.8 μM) was preincubated with NPs for 30 min at 37°C in PBS. The melittin/NP mixture was then added to RBC solution (100 μL) to give a final volume of 200 μL (final erythrocyte concentration, 3% v/v). The resulting suspension was incubated at 37°C for 30 min. Samples were then centrifuged at 800 g for 10 min. Release of hemoglobin was monitored by measuring the absorbance (A_{sample}) of the supernatant at 415 nm. Controls for 0 and 100% neutralization of hemolytic activity consisted of RBCs incubated with 1.8 μM melittin without NPs ($A_{0\%}$) and a RBC suspension without melittin and NPs ($A_{100\%}$), respectively. The percentage of neutralization was calculated according to Equation (2):

$$\text{Neutralization} = \frac{A_{\text{sample}} - A_{0\%}}{A_{100\%} - A_{0\%}} \times 100 \quad (2)$$

Preparation of biotinylated melittin

Biotinylation of melittin was carried out by standard procedures offered by Pierce. Melittin (5 mg) was dissolved in *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethane-sulfonic acid (HEPES) buffer (20 mM 2.5 mL, pH 7.4) then purified by a PD-10 desalting column (GE Healthcare, CT, USA). Eluted melittin fractions were collected and 0.64 mM melittin (2.5 mL) was incubated with NHS-PEO₄-biotin (1.9 mg per 0.2 mL) for 2 h then purified by a PD-10 column again. Modification of melittin by PEO₄-biotin was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

QCM analysis

An Affinix Q⁴ QCM instrument (Initium Co. Ltd., Tokyo, Japan) was used to quantify interactions between the NPs and melittin and control proteins. Avidin, BSA, and γ -globulin were covalently immobilized on the QCM electrode as follows. [9] Gold electrodes were cleaned with piranha solution for 5 min, twice. 3,3'-Dithiodipropionic acid (1 mM, 0.2 mL) was loaded in the QCM cells and incubated for more than 30 min. The resulting cells were washed with pure water and carboxylic acids on electrodes were activated by loading of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (100 mg mL^{-1}) and *N*-hydroxysuccinimide (100 mg mL^{-1}) 1:1 aqueous solution (0.1 mL) to form *N*-hydroxysuccinimidyl esters. Protein solution (1 mg mL^{-1}) was loaded on the cells to give protein-immobilized cells. Biotinylated melittin was immobilized on avidin-immobilized QCM electrodes in HEPES buffer (10 mM pH 7.4). Interactions between NPs and proteins/melittin were observed at $(37 \pm 0.1)^\circ\text{C}$ in PBS (35 mM phosphate buffer/0.15 M NaCl, pH 7.3). The apparent dissociation constant of NPs to melittin was calculated under the assumption that all particles have the same affinity to melittin. [11]

AFM imaging

The sample solution was dropped onto freshly cleaved mica. After evaporation of the solution on the surface, the topographic image was acquired in PBS (pH 7.3, 35 mM) by the tapping measurement mode of the atomic force microscope (Smena liquid head, NT-MDT, Russia).

In vitro neutralization assay

HT-1080 human fibrosarcoma cells were cultured in DMEM containing 10% FBS (Sigma–Aldrich, St. Louis, MO), penicillin (100 U mL⁻¹; MP Biomedicals, Irvine, CA), and streptomycin (100 µg mL⁻¹; MP Biomedicals) at 37°C in a 5% CO₂ atmosphere. HT-1080 cells (1 × 10⁴ cells well⁻¹) were seeded onto a 96-well plate (Beckton Dickinson Japan, Tokyo, Japan). After the cells had been cultured overnight, various concentrations of the NPs and then mellitin (30 µg mL⁻¹) in Hanks' Balanced Salted Solution (HBSS) were added continuously to the culture and the mixture was incubated for 24 h. Then, Alamar Blue (10 µL well⁻¹) was added and incubation was carried out for 4h. Viable cells were determined by the fluorescence (excitation/emission = 550/590 nm) measured with a fluorescence plate reader (ARVOsx, Perkin–Elmer Japan, Tokyo, Japan). The cytotoxicity was calculated as the percentage of control cell viability without exposure.

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Figure 1.

Inhibition of hemolytic activity of melittin by polymer NPs. a) Centrifuged RBCs after incubation for 30 min at 37 °C without melittin (tube 1), with 1.8 μM melittin (tube 2), or with 1.8 μM melittin and 0.12 mg mL^{-1} NPs (1–7). b) Neutralization of hemolytic activity of melittin by NPs polymerized with 40% TBAm and different feed ratios of AAc. White triangles: **11**; black squares: **6**; white squares: **15**; black circles: **16**; white circles: **7**. c) Neutralization of hemolytic activity of melittin by NPs polymerized with 10% AAc and different feed ratios of TBAm. Black triangles: **11**; black circles: **10**. d) Effect of AAc feed ratio on neutralization constants of 40% TBAm NPs. e) Effect of TBAm feed ratio on neutralization constants of 10% AAc NPs. f) 3D bar chart showing the effect of TBAm and AAc feed ratios on the neutralization constant.

AAC NPs. f) Summary of neutralization constants of NPs with different combinations of TBAm and AAC.

Figure 2.

Photographs of NP solutions (35 mM phosphate buffer/0.15 M NaCl, pH 7.3) at different temperatures: a) 25, b) 37, c) 45 °C. From left to right: NPs **12**, **9**, **13**, and **10**. Solutions of NPs **12**, **9**, and **13** are transparent at 25 and 37 °C but opaque at 45 °C, which indicates that the LCSTs of these NPs are between 37 and 45 °C. A solution of NP **10** is transparent at 25 °C but opaque at 37 and 45 °C, which indicates that the LCST of NP **10** is between 25 and 37 °C.

Figure 3.

Interaction between NPs and melittin monitored by a 27-MHz QCM. a) Time courses of frequency changes (ΔF) of the QCM. Solutions of NPs **6** (light shade), **11** (dark shade), **10**, and **15** (top thin lines, no change in ΔF) were injected into a melittin-immobilized QCM cell at 0 min. Inset: schematic illustration of QCM experiments. b) Adsorption isotherms of NPs **6** (light shade) and **11** (dark shade) on melittin.

Figure 4. AFM image of NPs **6** observed in phosphate buffer (pH 7.3). Inset: height profile of cross section (blue line).

Figure 5. Inhibition of cytotoxicity on cultured cells by NPs **6**. The data represent the mean $\pm$ standard deviation $n = 4$).

Scheme 1.

Monomers used for NP synthesis and the peptide sequence of melittin. Hydrophobic monomer and amino acids are the lightest shade. Positively charged monomer and amino acids are the darkest shade. Hydrogen-bond donor and acceptor monomers and their corresponding amino acid residues are an intermediate shade.

Scheme 2.

Schematic representation of melittin–NP interaction. Contributions of hydrophobic (light arrow) and electrostatic (dark arrow) interactions between melittin and NPs **7** (a), **6** (b), **2** (c), and **5** (d). Contributions of polymer density to interactions between melittin and NPs **9** (e) and **10** (f).

Table 1

Yield and diameter of polymer NPs.

Polymer NP	Functional monomer composition ratio [mol%]			Yield [%]	Hydrodynamic diameter [nm] ^[b]	Neutralization constant [% mL µg ⁻¹]	$K_a \times 10^5$ [M ⁻¹]
TBA _m	AA _m	3APM	AAc				
1	0	5	0	59	N.A. ^[c]	0	—
2	40	5	0	49	50	0	—
3	0	0	5	71	N.A. ^[c]	0	—
4	40	0	5	N.A. ^[a]	N.A. ^[a]	0	—
5	0	0	0	57	N.A. ^[c]	0	—
6	40	0	0	51	67	5.2	4.6
7	40	0	0	90	59	0	—
8	0	0	0	91	N.A. ^[c]	0	—
9	10	0	0	94	N.A. ^[c]	0	—
10	20	0	0	94	N.A. ^[c]	1.8	—
11	40	0	0	85	58	6.8	16
12	10	0	0	81	N.A. ^[c]	0	—
13	20	0	0	64	N.A. ^[c]	0	—
14	50	0	0	N.A. ^[a]	N.A. ^[a]	—	—
15	40	0	0	58	56	0.7	—
16	40	0	0	79	54	0.25	—
17	40	5	0	79	83	3.9	—

^[a] N.A. = not applicable. Polymers were aggregated during preparation.

^[b] Hydrodynamic diameters were determined by DLS.

^[c] Scattering intensities were too weak or particles too polydispersed to assign diameters.