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Selection and Characterization of Peptides Specifically Binding to TiO_2 Nanoparticles

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Abstract We have screened phage display peptide libraries to select for peptides binding to various sized TiO_2 nanoparticles. Phage libraries displaying random 7mer, 12mer, and C-7-Cmer peptides were used for screening. The size of target TiO_2 particles used were 7 nm, 15 nm, and 25 nm in diameter. We could select peptides binding each nanoparticles from all 3 libraries. Their binding was confirmed by transmission electron microscopy (TEM). Each peptide investigated was also shown to bind the other sized particles, meaning that the binding was specific for the nature of the particle rather than for the size of it. One of the 7mer peptides (PEP9, SVSPISH) was chosen for further analysis. The binding was shown to be in a dose-dependent manner, suggesting a specific interaction.

Key words: Nanoparticle, phage display, peptide

Phage display is a powerful tool for studying various protein-protein interactions [1, 12, 14–16]. Bacteriophages are grown in culture very fast with minimal cost. The proliferation is rapid and a high titer is usually obtained, representing the diversity of displayed molecules repertoir to a library size. One can display various sized peptides on the surface of a phage. The displayed molecules extend to proteins, including antibody fragments and cDNA products [2, 13, 17]. Bacteriophage M13 was the first developed phage for displaying peptides, since it could be manipulated genetically with ease, thanks to the use of a phagemid vector [16, 18]. However, a certain limitation existed owing to the size of displayed molecule. Usually, proteins with molecular weight less than 30 kDa were displayed. To overcome this limitation, bacteriophage lambda and T7 were later developed for phage display. These phages are much larger in size, with more genetic capacities [5, 7]. These are now used to display cDNA products. Phage display has recently also been used for gene delivery to animal cells [3].

Peptides are organic molecules [6]. They have never had a chance to interact with inorganic molecules bearing semiconductor and magnetic properties during their long evolutionary period. A group of researchers reported the ordering of quantum dots using genetically engineered viruses [8–10]. The core technology in the report was to use a phage display peptide library for selecting peptides binding to nanoparticles. In this study, we further extended the target to TiO_2 nanoparticles. TiO_2 is a material routinely used for paint and cosmetics. At the size less than 20 nm in diameter in the presence of UV light, it shows an bactericidal effect. Photoactivation of this nanoparticle is known to induce reactive oxygen species that eventually kill bacteria. In this study, blocking light for TiO_2 nanoparticles enabled selection of peptides binding specifically to this target, with minimum loss of phage infectivity.

Table 1. Amino acid sequences of selected peptides.

Target size	Peptide library	Amino acid sequence	Name
7 nm	12mer	A E T V E S C L A K S H	PEP-6
		L P S P P R I P G H K L	PEP-11
		G T Y I T P P L S S P R	PEP-12
15 nm	12mer	A C N Q S S K A L C G G	PEP-1
		G S M S P T V R W Y T P	PEP-8
	7mer	L P L S H A D	PEP-13
		S V S P I S H	PEP-9
	C7C	C N Y L S T H S C	PEP-7
		C L N S S N T I C	PEP-14
		C T S Q S Q H M C	PEP-10
25 nm	12mer	A C N Q S S K A L C G G	PEP-2
		N F M Q S L P R L G M H	PEP-5
	7mer	A C N Q S S K	PEP-3
	C7C	C S V S P I S H C	PEP-4

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Phage display peptide libraries displaying 7mer, C-7-Cmer, 12mer random peptides were purchased from New England Biolabs (U.S.A.). The sizes of TiO_2 particles used as targets were 7 nm, 15 nm, and 25 nm in diameter. Commercial chemicals, titanium isopropoxide, $Ti(OCH(CH_3)_2)_4$, and ethanol were supplied by Aldrich Chemicals Co. (U.S.A.) and used as received without further purification. Titanium isopropoxide (0.25–1.00 g) was added to 7.5–30 ml of ethanol, and the solution pH was adjusted by adding 0.7–3.0 M nitric acid. After several hours of stirring, the resulting solution was transferred to a glass-lined Parr Bomb, and then heated at 240°C for 1–72 h. Nanoparticles of titanium oxide were suspended in the resulting solution, and powdered TiO_2 particles were obtained after vacuum drying of the suspension at room temperature. The nanoparticles were characterized by X-ray powder patterns and transmission electron microscopy (TEM). The size of the nanoparticles can be controlled to 7, 15, and 25 nm by adjusting reaction parameters such as concentration of reagents and reaction time.

The phage stock was preincubated in an empty 1.5-ml polystyrene tube for 90 min to discard any nonspecific binding phages. A 200 μl of pretreated phage stock solution (1.5×10^{10} pfu/ml) was mixed with 50 μl of each nanoparticles (1.25 mg/ml) and incubated at room temperature for 90 min with occasional gentle shaking, in the dark. After centrifugation at 14,000 ×*g* for 1 min, supernatant was discarded. The precipitant was washed with wash buffer (TBST, 0.05% Tween 20 in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) 3 times. The phages were separated from the particle by incubating in 100 μl of elution buffer (0.2 M

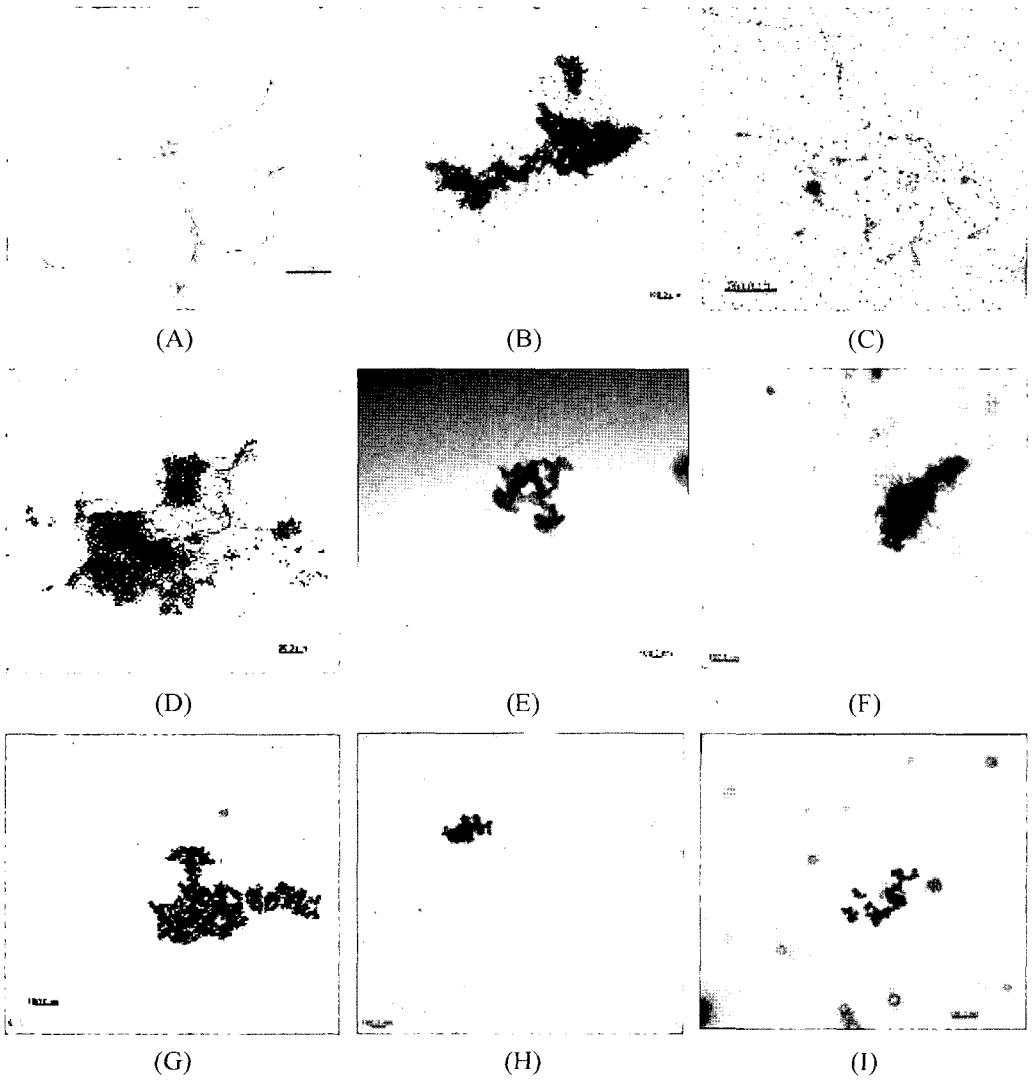

Fig. 1. Binding of peptide-displaying phages to target nanoparticles.
Six phages displaying different peptides were selected for TEM analysis. **A**. Unbound phage. **B**. PEP4 binding to 25 nm particles. **C**. PEP5 binding to 25 nm particles. **D**. PEP11 binding to 7 nm particles. **E**. PEP7 binding to 15 nm particles. **F**. PEP8 binding to 15 nm particles. **G**. PEP9 binding to 15 nm particles. **H**. Library phage with 15 nm particles. **I**. PEP4 with WO_3 particles.

glycine-Hcl, pH 2.2) for 10 min followed by neutralization with 15 μl of 1 M Tris-HCl, pH 9.1. One μl of phage solution was saved for titration. The phage solution was used to infect 10 ml of *E. coli* ER2738 (from New England Biolabs, U.S.A.) culture. After 4.5-h incubation, the culture was subjected to centrifugation at 14,000 ×*g* for 10 min. The supernatant was collected and 2 ml of PEG-NaCl (20% polyethylene glycol 8000, 2.5 M NaCl) solution was added. The mixture was incubated at 4°C for 1 h for phage precipitation. After centrifugation at 14,000 ×*g* for 15 min, the supernatant was discarded. The pellet was resuspended in 2 ml of TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) buffer and 400 μl of PEG-NaCl was added. After centrifugation at 14,000 ×*g* for 15 min, the supernatant was discarded and the pellet was resuspended in 100 μl of TBS buffer with 0.2% sodium azide. This biopanning procedure was repeated 5 times for peptide sequence enrichment. The 5th eluate was used for infection of *E. coli* ER2738 and the cells were incubated on an IPTG/X-gal LB plate. The infected cells appeared as blue plaques and 10 plaques were picked to infect ER2738 in a liquid culture. The phages were isolated from each culture and phage DNAs were obtained using a HighPure M13 isolation kit (Roche, U.S.A.). The purified phage DNAs were subjected to DNA sequencing (Macrogen, Korea) to investigate the enriched peptide sequence.

Transmission electron microscopy (TEM, Seoul National University, Korea) was used for visual analysis of phage-nanoparticle binding. A grid was put on a glass plate and subjected to a hydrophilic coating for 40 sec. A mixture of phage and nanoparticle was incubated at room temperature for 90 min. Thirty μl of the mixture was applied to the grid for 15 sec, followed by application of 30 μl of 2% uranyl acetate solution for 15 sec.

The peptide sequences obtained after biopanning against various sized TiO_2 nanoparticles are shown in Table 1. Fourteen different peptides (PEP1-PEP14) were selected after enrichment by biopanning. Some of the sequences appeared more than once; e.g., PEP1 and PEP2 sequences were exactly the same, and PEP3 shared 7 amino acids with PEP1 and PEP2. This means "ACNQSSK" is a core sequence recognizing the nanoparticle and they cannot distinguish the size for binding since they bound either 15 or 25 nm particles. Similarly, PEP4 and PEP9 shared 7 amino acids. PEP4 is from a C-7-Cmer peptide library. It encodes a displayed peptide between two cysteine residues to confer conformational constraints to mimic a real biological molecule. In this study, the possible conformational constraints did not seem to play a critical role for binding of this peptide. The interaction of selected peptides (PEPs 4, 5, 7, 8, 9, and 11) with target nanoparticles was viewed with transmission electron microscopy (Fig. 1). TiO_2 particles were shown to aggregate to form a larger mass. Bacteriophage M13 is shown as a long and flexible filament. Binding phages are shown to attach to particle aggregate with a directional positioning toward particles, whereas non-binding phages are shown to be apart from the particles or co-localized

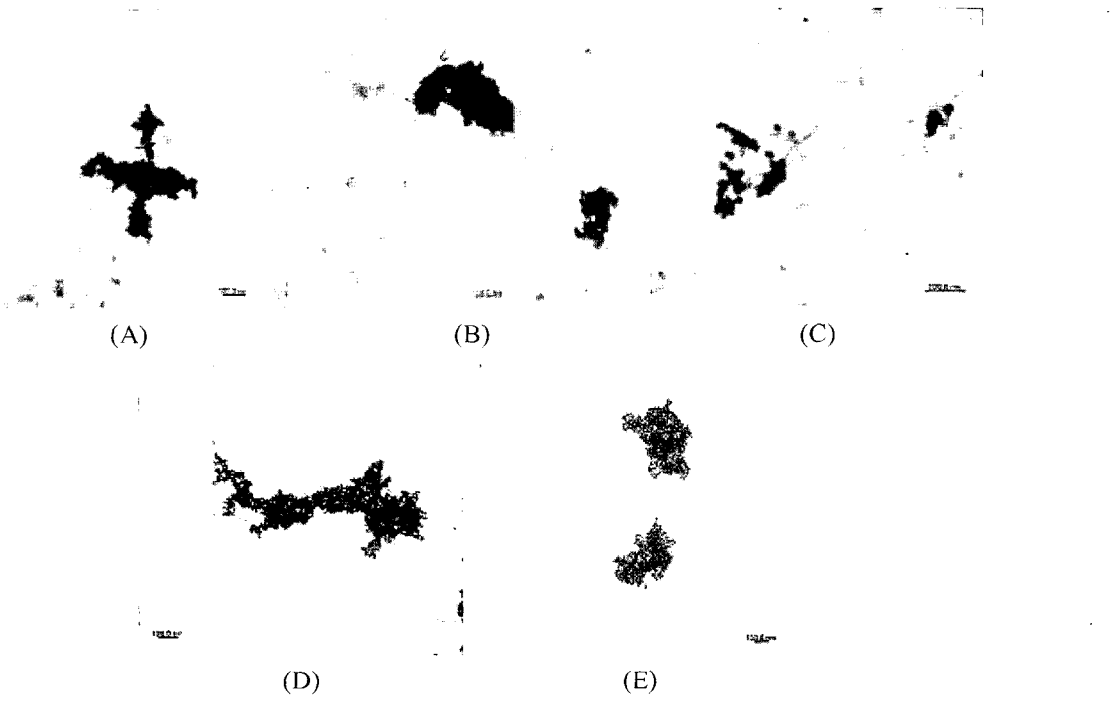

Fig. 2. Cross-recognition of peptides to particles with different sizes.
A. PEP4 binding to 7 nm particles. **B.** PEP11 binding to 15 nm particles. **C.** PEP11 binding to 25 nm particles. **D.** PEP8 binding to 7 nm particles. **E.** PEP8 binding to 25 nm particles. PEP4 binding to 15 nm particles is not shown owing to redundancy with PEP9 results.

without a directional positioning toward them. We then tested if the selected peptides (PEPs 4, 8, and 11) recognized and bound to each target, based on its size (Fig. 2). They were able to bind TiO_2 particles of any size. This cross-recognition suggests that, at least for the selected peptides tested, the binding to each particle is limited by the particle's nature but not by its size. We chose PEP 9 for further analysis to check if the interaction was specific [4]. In previously reported studies, the specificity of binding between selected peptides and target nanoparticles was never explored [8–10]. By increasing the amount of target particles (15 nm TiO_2), we tested whether the binding was in a dose-dependent manner (Fig. 3). Dose-dependency is good evidence for specificity of interaction; the binding was indeed shown to be in a dose-dependent manner.

For protein-protein interactions, a specific three-dimensional structure, a hydrophobic interaction, an interaction based on each amino acid's charge, and the van der Waal's interaction would be considered as major forces. In the case of interaction between a peptide and an inorganic nanoparticle, some of the above forces should also play a major role. However we could not determine what the most critical for the binding was.

TiO_2 is known to exhibit a bactericidal effect. It is mediated by production of reactive oxygen species in the presence of UV light. In our experiments, the light exposure was limited to a minimum to circumvent this problem. The reduction of the viability of the phages exposed to this nanoparticle was less than an order of magnitude (data not shown). Considering the large number of the library phages ($>10^{11}$ PFU), the phagecidal effect of TiO_2 should not be a major factor for a successful biopanning. Thus, we could extend the investigation of interaction between organic peptides and particles of titanium oxide.

Using the same methods, it would be possible to select peptides targeting any other nanoparicles with particular characteristics such as fluorescence, which has a potential for widespread use in biological experiments.

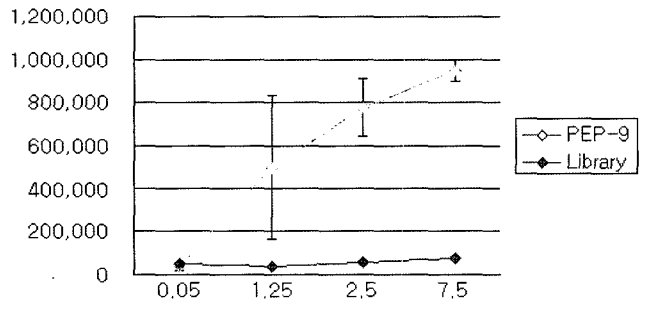

Fig. 3. Dose dependency test of binding between PEP9 phage and 15 nm TiO_2 nanoparticle.
X-axis, concentration of target particle added for binding (mg/ml); Y-axis, PFU. Naive pool of phage display library phages was used as a control. The values were obtained from three independent experiments.

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