High Affinity Anti-inorganic Material Antibody Generation by Integrating Graft and Evolution Technologies

POTENTIAL OF ANTIBODIES AS BIOINTERFACE MOLECULES

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Recent advances in molecular evolution technology enabled us to identify peptides and antibodies with affinity for inorganic materials. In the field of nanotechnology, the use of the functional peptides and antibodies should aid the construction of interface molecules designed to spontaneously link different nanomaterials; however, few material-binding antibodies, which have much higher affinity than short peptides, have been identified. Here, we generated high affinity antibodies from material-binding peptides by integrating peptide-grafting and phage-display techniques. A material-binding peptide sequence was first grafted into an appropriate loop of the complementarity-determining region (CDR) of a camel-type single variable antibody fragment to create a low affinity material-binding antibody. Application of a combinatorial library approach to another CDR loop in the low affinity antibody then clearly and steadily promoted affinity for a specific material surface. Thermodynamic analysis demonstrated that the enthalpy synergistic effect from grafted and selected CDR loops drastically increased the affinity for material surface, indicating the potential of antibody scaffold for creating high affinity small interface units. We show the availability of the construction of antibodies by integrating graft and evolution technology for various inorganic materials and the potential of high affinity material-binding antibodies in biointerface applications.

Peptides and proteins recognize the interfacial surfaces of their corresponding molecules with high affinity and selectivity because of the multiple-point interactions of hydrogen bonds and salt bridges and the surficial complementarities at the inter-

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3 The abbreviations used are: CDR, complementarity determining region; Fv, variable fragment of antibody; GdnHCl, guanidine hydrochloride; VHH, variable domain of heavy chain of heavy chain antibody; ZnO, ZnO-binding peptide; sZnOBP, selected ZnO-binding peptide; GFP, green fluorescent protein.
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chain camel antibody (VHH) to give a VHH fragment with the same affinity as the grafted peptide and without structural instability. Next, a nonrelated CDR loop in the peptide-grafted VHH was randomized by using an αββα motif sequence (see under “Results”) to screen for high affinity antibodies. Application of the single-domain VHH fragment as a framework prevented destabilization in the grafting of the alien peptide in the first step, and construction of a VHH library from the peptide-grafted VHH fragment by using the αββα motif sequence enabled us to bypass limitations on library diversity. We also demonstrate the enthalpy synergistic effect from grafted and selected CDR loops on the binding mechanism of antibodies onto material surfaces and the potential of antibody scaffold for creating high affinity small interface units.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors for VHH Fragment with Material-binding Peptide in CDRs—The DNA sequences coding the VHH fragments of camel anti-BcII β-lactamase antibody cAbBCII10 (20) were synthesized from five oligonucleotides and external primers (supplemental Table S1) by means of overlap extension PCR with LA-Taq DNA polymerase (21). The gene fragments produced were inserted into the Ncol-SacII site of pRA-FLAG vectors containing a FLAG peptide sequence, as constructed previously (22), to produce plasmids encoding the VHH fragment as a framework precluding destabilization in the grafting of the alien peptide in the first step, and construction of a VHH library from the peptide-grafted VHH fragment by using the αββα motif sequence enabled us to bypass limitations on library diversity. We also demonstrated the enthalpy synergistic effect from grafted and selected CDR loops on the binding mechanism of antibodies onto material surfaces and the potential of antibody scaffold for creating high affinity small interface units.

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The DNA sequences coding the VHH fragment where the CDR loops were replaced with ZnO-, Al2O3-, or CoO-binding peptides (11, 23, 24) were generated by means of overlap extension PCR from plasmid pRA-wtVHH-FLAG, using the oligonucleotides and external primers shown in supplemental Table S2. The amplified sequences for the VHH fragments were inserted into the Ncol-SacII sites of the pRA-FLAG vectors to express the encoding VHH fragments in E. coli.

Using one of the selected VHH genes (4F2 clone), we further prepared the DNA sequences of the selected VHH fragment with random CDR 1 loop exchanged for that of cAbBCII10 to evaluate the role of the CDR 3 sequence in selected VHH. The sequences coding the VHH fragment with CDR 1 of cAbBCII10 and CDR 3 of 4F2 VHH were generated by means of overlap extension PCR from plasmid pRA-FLAG vector with the 4F2 VHH gene, using the two oligonucleotides and external primers shown in supplemental Table S4, and the amplified sequences for the VHH fragments were inserted into the Ncol-SacII sites of the pRA-FLAG vectors to produce the pRA-VHH\textsubscript{ZnO}BP\textsubscript{3}-FLAG plasmids.

Expression and Purification of VHH Fragments—Transformed E. coli BL21 (DE3) cells harboring the expression plasmid encoding VH fragments were incubated in lysogeny broth medium at 28 °C, and expression of antibody fragments under the control of the T7 promoter was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside. VH fragments were extracted from the periplasm of the harvested cells by osmotic shock and purified by anion/cation exchange and gel filtration chromatographies after ammonium sulfate treatment.
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Analysis of Binding Affinities of Antibody Fragments to Material Surfaces—Material particles (0.2 mg) with a Brunauer-Emmett-Teller (BET) specific surface area (surface area estimated by nitrogen gas adsorption (26)) of 9.7 m²/g (ZnO), 12.4 m²/g (Al₂O₃), 30.9 m²/g (CoO), 33.9 m²/g (Fe₃O₄), C. I. Kasei Co., Ltd., Tokyo, Japan), 39.1 m²/g (TiO₂,C. I. Kasei Co., Ltd., Tokyo, Japan), or 39.8 m²/g (NiO, C.I. Kasei Co., Ltd.) were separately added to 300 μl of 10 mM phosphate solution (pH 7.5; 200 mM NaCl, 0.05% Tween 20) containing VHH, and the mixture was incubated for 30 min at several temperatures. After centrifugation at 20,000 × g for 10 min, the precipitated particles were added to 300 μl of 6 M guanidine hydrochloride (GdnHCl) solution (10 mM phosphate, 200 mM NaCl, pH 7.5) to elute the adsorbed proteins from the particles. The supernatants were analyzed by SDS-PAGE, and the proteins eluted separately added to 300 μl of 10 mM phosphate solution (pH 7.5; 200 mM NaCl, 0.05% Tween 20) containing VHH, and the mixture was incubated for 30 min at 4 °C. After centrifugation at 20,000 × g for 10 min, the precipitated ZnO particles were washed with 10 mM phosphate solution to remove nonspecifically adsorbed VHH. After removing the washing solution, the particles were suspended in 300 μl of 10 mM phosphate solutions containing the ZnO-binding peptides at 3 μM, and the suspension was centrifuged at 20,000 × g for 10 min. This inhibition procedure was repeated until the concentration of peptides was increased to 3 μM, and all the supernatants were analyzed by SDS-PAGE. Residual VHH on ZnO particle even after the inhibition procedure with 3 μM peptides were eluted with 6 M GdnHCl solution, and the supernatant was also analyzed by SDS-PAGE.

Competitive Inhibition Assay for the Binding of VHH onto ZnO with ZnO-binding Peptides—ZnO particles (0.2 mg) were added to 300 μl of a 10 mM phosphate solution containing 3 μM VHH (pH 7.5; 200 mM NaCl, 0.05% Tween 20), and the mixture was incubated for 30 min at 4 °C. After centrifugation at 20,000 × g for 10 min, the precipitated ZnO particles were washed with 10 mM phosphate solution to remove nonspecifically adsorbed VHH. After removing the washing solution, the particles were suspended in 300 μl of 10 mM phosphate solutions containing the ZnO-binding peptides at 3 μM, and the suspension was centrifuged at 20,000 × g for 10 min. This inhibition procedure was repeated until the concentration of peptides was increased to 3 μM, and all the supernatants were analyzed by SDS-PAGE. Residual VHH on ZnO particle even after the inhibition procedure with 3 μM peptides were eluted with 6 M GdnHCl solution, and the supernatant was also analyzed by SDS-PAGE.

Construction of Bispecific VHH Dimers with Affinity for ZnO Surface and GFP—The sequence of anti-GFP VHH (cAbGFP4) (27) was generated from five oligonucleotides and two external primers (supplemental Table S5) by means of overlap extension PCR with LA-Taq DNA polymerase, and the gene fragments were inserted into the NcoI-SacII fragment of the pRA-FLAG vector (4F2 cAbBCII10). The sequence of anti-GFP VHH (cAbGFP4) was fused at the C terminus of 4F2 VHH via a llama IgG2 upper hinge-linker (EPKIPQPQPKPEP) (28). The gene sequence was generated by overlap extension PCR with LA-Taq DNA polymerase, and the gene fragments were inserted into the NcoI-SacII site of the pRA-FLAG vectors (4F2 VHH) to product the plasmids (pRA-cAbGFP4-FLAG). To generate bispecific antibody with affinity for ZnO and GFP, the gene sequence of cAbGFP4 was fused at the C terminus of 4F2 VHH via a llama IgG2 upper hinge-linker (EPKIPQPQPKPEP). The gene sequence was generated by overlap extension PCR with LA-Taq DNA polymerase, and the gene fragments were inserted into the NcoI-SacII fragment of the pRA-FLAG vector (4F2 VHH) to product the plasmids (pRA-cAbGFP4-FLAG). To generate bispecific antibody with affinity for ZnO and GFP, the gene sequence of cAbGFP4 was fused at the C terminus of 4F2 VHH via a llama IgG2 upper hinge-linker (EPKIPQPQPKPEP). The bispecific antibody with affinity for ZnO and GFP (cAbBCII10) was used to measure the binding of VHH fragments to ZnO films and the bispecific VHH dimers. VHH monomer or bispecific VHH dimer (1 μM) in 10 mM phosphate buffer (pH 7.5; 200 mM NaCl) was incubated for 30 min at several temperatures. After centrifugation at 20,000 × g for 10 min, the precipitated ZnO particles were washed with 10 mM phosphate solution for 240 s. The change in wavelength with minimum reflection intensity was measured. For bispecific VHH dimers, 1 μM GFP was further injected after washing with 10 mM phosphate buffer for 240 s.

RESULTS

Design for Grafting ZnO-binding Peptide Sequences into CDR Loops of VHH Fragments—As a scaffold for the VHH fragment, we used the camel anti-BcII β-lactamase antibody cAbBCII10, which has an appropriate framework for CDR replacements (20). Table 1 shows the CDR amino acid sequences of VHH with ZnO-binding peptide (ZnOBP; EAHVMHKVAPRP) (11) in CDR 1 (VHH_ZnOBP1), CDR 2 (VHH_ZnOBP2), and CDR 3 (VHH_ZnOBP3). For grafting of ZnOBP into each CDR, we drew upon the crystal structure of the chimera of cAbBCII10 where the CDRs were replaced with those of cAbLys3 (20). In the crystal structure, the N-terminal side of CDR 2 and the C-terminal sides of CDRs 1 and 3 form β structures with the adjacent framework sequences; this has been observed in most reported VHH structures (29, 30). We preserved the edge sequences of the CDRs when the CDR loops were replaced with the ZnOBP sequence.

The crystal structure of ungrafted cAbBCII10 VHH was reported recently (31). It resembles that of the chimera cAbBCII10; consequently, we confirmed that our grafting design suited the crystal structure of ungrafted cAbBCII10 VHH.

Structure and Binding Ability of VHH Fragments with ZnO-binding Peptide Sequence Grafted in Their CDR Loops—All the ZnOBP-grafted VHH fragments were expressed as soluble forms in E. coli, although the VHH_ZnOBP2 fragments were expressed mainly as insoluble aggregates (data not shown). Analysis of VHH_ZnOBP1 and VHH_ZnOBP3 by size-exclusion chromatography and examination of circular dichroism spectra demonstrated a monomeric form with the typical immunoglobulin structure of camel antibody (Fig. 2). However, VHH_ZnOBP2 formed oligomers with a random structure. Grafting of the peptide sequences into CDR 1 or CDR 3 resulted in little structural change in the framework, but grafting into CDR 2 led to deformation of the camel-type immunoglobulin structure. Reports of the conformational structures of VHH indicate

| Table 1 – Amino acid sequences of CDR loops of ZnOBP-grafted VHH fragments |
|------------------|------------------|------------------|------------------|
| Fragment         | Sequence of CDR 1 | Sequence of CDR 2 | Sequence of CDR 3 |
| cAbBCII10        | GQGEYS-TSTFGSL   | AIAGN-GDLTYYMEWV5-G | VRGYMPMLPFSNRY  |
| VHH_ZnOBP1       | GQGEYS-TSTFGSL   | AIAGN-GDLTYYMEWV5-G | VRGYMPMLPFSNRY  |
| VHH_ZnOBP2       | GQGEYS-TSTFGSL   | AIAGN-GDLTYYMEWV5-G | VRGYMPMLPFSNRY  |
| VHH_ZnOBP3       | GQGEYS-TSTFGSL   | AIAGN-GDLTYYMEWV5-G | VRGYMPMLPFSNRY  |

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that most VHH fragments have a β-structure in the center region of CDR 2 (20, 29–31). This β-structure in CDR 2 might be important for scaffold stability. We therefore analyzed the binding affinity of VHHZnOBP1 and VHHZnOBP3 for the ZnO surface.

To measure the ability of ZnOBP-grafted VHH fragments to bind to the ZnO surface, we measured the adsorption isotherms of VHHZnOBP1 and VHHZnOBP3 for ZnO particles (Fig. 3). In a phosphate solution, few cAbBCII10 VHH or VHHZnOBP3 fragments were adsorbed onto ZnO particles, but, critically, VHHZnOBP1 fragments were bound to ZnO particles with a dissociation equilibrium constant \( K_D \) comparable with that of VHH fragments with ZnOBP as a tag at the N terminus (VHHZnOBP1, 176 nM; VHHZnOBP3tag, 303 nM; see Table 2): ZnOBP was as functional in CDR 1 as at the N terminus.

In addition, we grafted the ZnOBP sequence into CDR 3 of VHHZnOBP1 (VHHZnOBP1,3) in an effort to improve the binding affinity for the ZnO surface; however, the \( K_D \) value of VHHZnOBP1,3 was the same as that of VHHZnOBP1 (Fig. 3 and Table 2). ZnOBP in the CDR 3 loop did not function effectively even in the presence of VHHZnOBP1.

**Generation of High Affinity Antibody Fragments from Peptide-grafted VHH**

To improve the binding affinity of ZnOBP-grafted VHH fragments, we employed a phage-display system whereby VHHZnOBP1 with the CDR 3 loop randomized was displayed on the filamentous bacteriophage M13.

Evaluation of statistics on the frequency of amino acids in reported material-binding peptides selected from a phage display peptide library (5) revealed the preferential selection of Arg, His, and Lys residues among polar amino acids, and Thai et al. (32) have implied the presence of the Arg-Xaa-Xaa-Arg sequence in metal oxide binding. Here, we made a CDR 3 library with an \( /H9251/H9252/H9251 \) -repeating sequence by utilizing degenerate codes; the \( /H9252 \) residues were randomized to Arg, Gly, Leu, or Val, and the \( /H9251 \) residues were randomized to Arg or His. Lys residues could not be included in the library because of the lack of flexibility of the codons encoding Lys.

Phages displaying VHHZnOBP1 with CDR 3 loop randomized was displayed on the filamentous bacteriophage M13.
VHH had a $K_D$ value of 9 nM, about 20 times the affinity of VHH (Table 3). The binding affinity of VHH was thus improved by optimization of the CDR 3 sequence by molecular evolutionary methods.

**Binding Properties of 4F2 VHH**—To evaluate the role of selected CDR 3 sequence in 4F2 VHH (sZnOBP, HLGHGLHRVH) for the binding onto the ZnO surface, we prepared the VHH fragment with CDR 1 of cAbBCII10 and CDR 3 of sZnOBP (VHHsZnOBP3), and the binding affinity of VHHsZnOBP3 for ZnO was measured (supplemental Fig. S1). The adsorption isotherm of VHHsZnOBP3 was adequately fitted by Langmuir adsorption isotherm equation, yielding a dissociation equilibrium constant $K_D$ of 168 nM which is similar to that of VHH (supplemental Fig. S1). Therefore, simultaneous binding of ZnOBP (EAHVMHKVAPRP) in CDR 1 and sZnOBP (HLGHGLHRVH) in CDR 3 is attributed to the high affinity of 4F2 VHH for the ZnO surface.

We further performed the competitive assay for the binding of VHH for ZnO surface by using the peptides of ZnOBP and sZnOBP (Fig. 6). In the competitive assay, VHH fragments were gradually dissociated from ZnO particles by increasing the concentration of the peptides in wash solution, and the dissociated VHH fragments were then analyzed by SDS-PAGE. In the mixture of ZnO particles and VHHZnOBPtag, the VHH fragments were dissociated from ZnO particles by the addition of ZnOBP and sZnOBP (HLGHGLHRVH) in CDR 3 is attributed to the high affinity of 4F2 VHH for the ZnO surface.

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4F2 VHH increased the negative enthalpy change implies that the improvement of binding affinity is due to the increase of electrostatic interaction between VHH and ZnO surface.

**Specificity of Anti-ZnO VHH Fragments for Other Ceramic Materials**—To analyze the affinity of ZnO-binding VHH fragments for other materials, we mixed VHH fragments with particles of each material. After centrifugation, the supernatant was removed, and the VHH fragments adsorbed onto particles were separated by using a 6 M GdnHCl elution solution. Fig. 8 shows the SDS-PAGE results for the supernatant and elute solution. Few peptide-ungrafted VHH fragments were adsorbed onto any ceramic particles except NiO, whereas VHH\textsubscript{ZnOBP1} bound to only ZnO and NiO particles. The finding that the amount of VHH\textsubscript{ZnOBP1} adsorbed onto NiO was comparable with that of peptide-ungrafted VHH indicates that the framework structure of VHH was apt to be adsorbed onto the NiO particles. Therefore, VHH\textsubscript{ZnOBP1} selectively bound to ZnO via the CDR 1 loop. In the case of 4F2 VHH, few VHH fragments were bound to CoO or Al\textsubscript{2}O\textsubscript{3} particles, but some were adsorbed onto Fe\textsubscript{2}O\textsubscript{3} and TiO\textsubscript{2}. However, the \(K_D\) values of 4F2 VHH for Fe\textsubscript{2}O\textsubscript{3} and TiO\textsubscript{2} were 745 and 286 nM, respectively, indicating much lower binding affinity than for ZnO (Table 3).

**TABLE 4**

| Fragment | \(\Delta H\) (kJ/mol) | \(\Delta S\) (kJ/mol) | \(-\Delta G\) (kJ/mol) |
|----------|-----------------------|-----------------------|------------------------|
| VHH\textsubscript{ZnOBPtag} | -32.6 | -3.6 | 36.2 |
| VHH\textsubscript{ZnOBP1} | -37.1 | 2.3 | 34.8 |
| VHH\textsubscript{ZnOBP1} | -37.3 | -0.5 | 37.8 |
| 4F2 VHH | -45.7 | -1.8 | 43.9 |

- ZnO-specific binding fragments to ZnO via the CDR 1 loop.
- The dissociation equilibrium constants \(K_D\) estimated from the adsorption isotherm at various temperatures were plotted to the van’t Hoff equation as follows: In \(K_D = \Delta H/RT - \Delta S/R\) (Fig. 7). The series of \(K_D\) values showed a good correlation to the van’t Hoff equation for all the VHH fragments, and enthalpies and entropies were obtained from the fitting (Table 4).

**FIGURE 6.** SDS-PAGE (18% acrylamide) results of competitive inhibition assay for the binding of VHH\textsubscript{ZnOBPtag} (A), VHH\textsubscript{ZnOBP1} (B), VHH\textsubscript{ZnOBP3} (C), and 4F2 VHH for ZnO particles with the peptides of ZnOBP and sZnOBP (D). Lanes 1–8 correspond to added VHH solution, unadsorbed fraction, wash fraction, fractions eluted with 3, 30, and 300 mM peptide solutions, and fraction eluted with 6 M GdnHCl, respectively.

**FIGURE 7.** van’t Hoff plot for the binding of VHH\textsubscript{ZnOBPtag} (open circles), VHH\textsubscript{ZnOBP1} (closed circles), VHH\textsubscript{ZnOBP3} (open squares), and 4F2 VHH (closed squares) to ZnO. Straight lines were obtained by fitting the four points to the linearized van’t Hoff equation as follows: In \(K_D = \Delta H/RT - \Delta S/R\), where \(\Delta H\) is van’t Hoff enthalpy; \(\Delta S\) is van’t Hoff entropy, and \(R\) is universal gas constant.

sZnOBP at less than 3 mM that was 1000-fold of VHH concentration (Fig. 6D), which supports the results from adsorption isotherm measurement that 4F2 VHH have higher affinity for ZnO than VHH\textsubscript{ZnOBP1} and VHH\textsubscript{ZnOBP3}. It should be noted that the addition of 3 mM resulted in the dissociation of 4F2 VHH. These might indicate that the peptide of sZnOBP has higher affinity for ZnO than ZnOBP.

**Thermodynamic Analysis of the Interaction between VHH and ZnO**—To study the thermodynamics for the interaction of ZnO-binding VHH with ZnO surface, we measured the adsorption isotherm of VHH fragments for ZnO at various temperatures. The dissociation equilibrium constants \(K_D\) estimated from the adsorption isotherm at various temperatures were plotted to the van’t Hoff representation (ln \(K_D = \Delta H/RT - \Delta S/R\), where \(\Delta H\) is van’t Hoff enthalpy; \(\Delta S\) is van’t Hoff entropy, and \(R\) is universal gas constant.)

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**FIGURE 8.** SDS-PAGE (18% acrylamide) analysis for the selectivity of VHH against ceramics. After the mixed solutions of each VHH (peptide-ungrafted VHH, VHH\textsubscript{ZnOBP1} or 4F2 VHH) and material particles (ZnO, Al\textsubscript{2}O\textsubscript{3}, CoO, Fe\textsubscript{2}O\textsubscript{3}, TiO\textsubscript{2}, or NiO) had been centrifuged, the supernatant was removed, and the VHH fragments adsorbed onto particles was eluted in 6 M GdnHCl solution. Lane 1, added VHH solution in particle suspension; lane 2, supernatant after centrifugation; lane 3, eluted fraction.
Availability of the Construction Method of Material-binding Antibodies for Other Inorganic Materials—We attempted to generate anti-Al₂O₃ VHH fragments from a reported Al₂O₃-binding peptide (KRHKQKTSRMGK) (23) and anti-CoO VHH fragments from a reported CoO-binding peptide (LGKDRPHFHR) (24). Grafting of the peptide into the CDR 1 loop functionalized the VHH fragments. Although the peptide grafting into CDR 3 showed no functionalization, the evolutionary approach in CDR 3 critically increased the affinity for the corresponding material surface (Table 5). VHH fragments with high affinity for gold have also been generated; hence, the construction of antibody by integrating grafting and evolution technology, we call CAnIGET, can widely generate high affinity antibodies against inorganic material surfaces.

Application of 4F2 VHH to Bio-interface Molecule—To utilize 4F2 VHH for spontaneous selective protein immobilization on a ZnO plate in a flow system, we developed a solution containing anti-ZnO VHH fragments on a ZnO film deposited on a silicon plate and detected immobilization of the protein by reflectometric interference spectroscopy (Fig. 10A). Under flow conditions, the time-dependent change in wavelength with minimum intensity in the reflected spectrum showed little adsorption of peptide-ungrafted VHH fragments on the ZnO film, whereas definite spontaneous immobilization of VHHZnOBP1, VHHZnOBPtag, and 4F2 VHH was observed, with the amounts of adsorbed protein comparable with those estimated under batch conditions (Tables 2 and 3). It should be noted that the dissociation of 4F2 VHH was too slow to be observed in the dissociation process.

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DISCUSSION

Single Domain Camel VHH Fragment as a Framework for Grafting Material-binding Peptide—The structural resemblance of human and mouse Fv frameworks enables the transfer of binding function to humanized antibodies (33); these replacement techniques are currently applied to the design of new functional antibody fragments by the grafting of alien proteins.
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motif peptide sequences (34–36). Previously, we functionalized an Fv fragment from mouse anti-lysozyme antibody HyHEL-10 by grafting material-binding peptides into the heavy chain CDR 2 region (22); however, the replacement caused dissociation of the Fv fragment. The framework used did not have enough structural versatility for the grafting of material-binding peptide sequences.

Here, we used the variable fragment from a camel antibody, cAbBCII10 VHH, as a new framework for the grafting of material-binding peptide. The cAbBCII10 VHH fragment is a single domain with a framework tolerant to various CDR loop structures (20). cAbBCII10 VHH was also an appropriate stable scaffold to give high binding affinity (38). In this study, our grafting design for CDR 3 might have yielded an improper conformation of ZnOBP, i.e. grafting into CDR 3 might have required more careful design. Our results demonstrate that CDR 3 should be functionalized by molecular evolution rather than by peptide grafting.

Affinity Maturation of ZBP-grafted VHH by Molecular Evolution in CDR 3—There have been a few studies of the selection of antibodies with affinity for material surfaces by using general methodologies with in vivo immune systems (15) or in vitro library methods (16–19). However, because of the low immunogenic potential of solid materials, far fewer antibodies with affinities for material surfaces have been selected than those with affinities for soluble molecules. Even with the use of in vitro selection methods, the limited library diversity and strong nonspecific interactions of coat proteins on phages with solid bulk surfaces make it difficult to select positive antibodies. We demonstrated here the possibility of steadily generating high affinity antibodies by coupling the grafting of peptides from a reported material-binding peptide repertoire and molecular evolution of the peptide-grafted variable fragment. The construction of a library from a low affinity antibody for which the binding site could be predicted allowed us to narrow down the segments that needed to be randomized, thus enabling us to bypass the limitations of library diversity.

Randomization of the CDR 3 loop in the format of the αββα repeating sequence can also decrease the necessary magnitude of library diversity. The αββα format was designed in consideration of the Arg-Xaa-Xaa-Arg format (32), the importance of His residues for inorganic material binding (39), and the frequent appearance of Arg, His, and Lys residues in material-binding peptides. Use of the αββα format with Arg/Gly/Leu/Val at the β positions and His/Arg at the α positions resulted in the preferential selection of Gly/Leu (β) and His (α) in the selection of VHH against ZnO. Notably, few Arg residues were selected at both the β and α positions. These results suggest that in CDR 3 in the VHH scaffold, Arg has less functional ability than His to bind to the ZnO surface.

Synergistic Effect of CDR Loops on Binding on the ZnO Surface—In this study, we focused the binding properties of the highest affinity 4F2 VHH fragment. The comparison with VHHZnOBP1 and VHHZnOBP3 indicates that the high binding affinity of 4F2 VHH resulted from synergistic effect from grafted CDR 1 and selected CDR 3 loops in the VHH framework (Fig. 6). The competitive binding assay by using the peptides of ZnOBP and ZnOBP implied that CDR 1 and CDR 3 recognized identical local surface structure of ZnO.

To increase the affinity of the peptides with micromolar $K_D$ values, repetition and clustering of material-binding peptides are among the possible approaches to increasing the binding affinity of peptides that have micromolar $K_D$ values (3). One polypeptide with three repeats of a gold-binding peptide had a $K_D$ value of 89 nM (40), and clustering of 24 titania-binding peptides on a ferritin protein increased the binding affinity, giving a $K_D$ value of 11 nM (41). Our high affinity antibody fragments showed strong binding with only two loop structures (CDRs 1 and 3). Therefore, use of the antibody format has the
potential for efficient generation of high affinity interface molecules from material-binding peptides.

**Thermodynamic Analysis for the Binding of Anti-ZnO VHH to ZnO Surface**—The thermodynamic parameters estimated from van’t Hoff plots demonstrated dominant change of enthalpy in the absorption process of all the ZnO-binding VHH fragments (VHH$_{ZnOBrp}$, VHH$_{ZnOBp}$, VHH$_{ZnOBrp}$, and 4F$_2$ VHH) on the ZnO surface. This result is comparable with the binding properties of anti-gold antibody Fv fragment, A14P-b2 Fv (18). This implies that coulombic interaction dominates the binding process of anti-ZnO VHH fragments on ZnO. Considering that ZnO surface and the framework of VHH are positively charged at the pH value of 7.5, the grafted and selected CDR loops electrostatically interact with the ZnO surface. In general, one of major factors for protein adsorption on material surface is entropy changes that result from dehydration of the absorbent surface and from the conformational change of adsorbed proteins (42, 43). Therefore, our thermodynamic result implies that the conformation of anti-ZnO VHH fragments is little changed by interaction with the ZnO surface and that few water molecules on ZnO surface are dispersed by the binding of VHH. When the CD spectra were measured on 4F$_2$ VHH fragments with ZnO nanoparticles, we observed the CD spectra derived from the immunoglobulin structure of camel antibody (data not shown). Hence, 4F$_2$ VHH was not denatured on the ZnO surface.

**Application of High Affinity Antibody Fragment for Nanobiotechnology**—Antibody fragments are among the smallest units with naturally occurring binding domains, and an advantage of using them as high affinity interface molecules is the fact that various fusion technologies can be used. Artificial antibody formats constructed for therapeutic and imaging uses have been used to generate multivalent and multispecific material-binding antibodies to functionalize nanoparticles and to spontaneously and selectively accumulate proteins and nanoparticles on patterned spots on substrate (18, 44). Here, we show the potential of high affinity material-binding antibodies for selective immobilization of proteins on biosensor plates. The fusion of material-binding peptides and antibody fragments enables direct and oriented protein immobilization without the need for complicated processes (9, 45), and unmodified inorganic surfaces can directly receive electrons from immobilized redox proteins without mediators (46). Improving the binding strength of material-binding biomolecules will lead to more quantitative and reliable detection.

In conclusion, we generated antibody fragments with high affinity for inorganic material surfaces from low affinity material-binding peptides by the integration of peptide-grafting and phage-display techniques. The scaffold of the single-domain VHH fragment was so stable that VHH was directly functionalized by grafting of the material-binding peptide into CDR 1 without structural destabilization. Application of the αββα motif library to the CDR 3 of peptide-grafted VHH enabled us to bypass limitations on library diversity; consequently, this construction method allow us to generate high affinity antibodies against material surfaces from previously identified material-binding peptides. Quantitative thermodynamic analysis described the enthalpy synergetic effect from grafted and selected CDR loops in VHH, which shows the potential of antibody scaffold for creating high affinity small interface units with efficient binding mechanism. Use of the high affinity antibody fragments resulted in stable selective protein immobilization on material surfaces in flow systems. We expect to be able to use such material-binding antibodies as biointerface units for nanoscale quantitative biosensing and protein accumulation.

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