Human Anti-gold Antibodies

BIOFUNCTIONALIZATION OF GOLD NANOPARTICLES AND SURFACES WITH ANTI-GOLD ANTIBODIES∗[1]

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The interface molecules designed to exhibit molecular recognitions between different species have become attractive tools for the bottom-up fabrication and hybridization of nanostructured units. Here, we focus on antibodies with high binding ability and specificity to construct a novel biomolecule interface for recognizing an inorganic material. Careful selection from a phage-displayed library of variable region heavy and light Fv chains of human antibodies using enzyme-linked immunosorbent assay and surface plasmon resonance assay resulted in the identification of an antibody fragment, A14P-b2, with high affinity (KD = 1.7 nM) and specificity for gold materials. Our results indicated the potential usefulness of human antibody libraries and the effectiveness of the antibody framework for recognizing bulk material surfaces. Construction of bivalent and bispecific antibodies on the A14P-b2 platform with high affinity by means of fusion technology enabled the functionalization of gold nanoparticles and allowed selective protein accumulation on gold spots patterned on a silicon substrate. This type of antibody engineering is potentially applicable to bio-inspired materials and nanobiosensing.

Molecular recognition, which plays a fundamental role in enzyme reactions and in the immune system, is indispensable not only for protein engineering (1) but also for medicinal chemistry (2) and nanotechnology (3). Molecules with sufficiently high recognition abilities can be used for tissue-targeted drug delivery (4), single-molecule imaging (5) and sensing (6), and bottom-up fabrication (3). Among molecular recognitions, protein recognizes the interfacial surface of corresponding protein molecule so that they bind to each other with high affinity. Many structural analyses of protein complexes have shown that highly specific mutual recognition of protein molecules is due to multiple-point interactions, such as hydrogen bonding and the formation of salt bridges (7), at the interface and that there are structural, electrical, and hydrophobic complementarities between protein-binding surfaces (8). Surface recognition by proteins has been also observed in the case of biopolymers in biological systems. Some enzymes, such as cellulase (9), chitinase (10), and polyhydroxybutyrate depolymerase (11), have independent binding and degradative domains; the binding domain binds to the surface of a solid material and then depolymerizes the material by using the degradative domain.

Artificial peptides with affinity for nonbiological inorganic materials have been identified by combinatorial library approaches (12, 13), and peptides with affinity for metals, metal oxides, and semiconductors have been used to synthesize crystalline nanometer- to micrometer-sized metal particles (14, 15). Peptides and proteins with excellent binding ability and specificity for an inorganic compound and its crystal face can be used to directly immobilize and pattern biomolecules on inorganic nanoparticles and substrates (12, 16) and to control crystal structure growth in inorganic synthesis processes (14, 17).

Here, we focused our attention on repertoire from a naturally occurring human antibody library. Advances in antibody engineering have enabled the fabrication of a number of antibodies specific not only for the biomolecules of antigens (18), receptors (19), low molecular mass haptens (20), but also for nonbiological organic molecules (21), metal ions (22), and the surfaces of organic matters (23, 24). In general, antibodies have high affinities for their target ligands and can be expected to be superior to low molecular mass peptides with relatively low affinities for their target surfaces. Previous studies have shown that the repetition of the sequence of an anti-material peptide can increase binding affinity (25, 26). In this study, we had an interest in antibody framework structure, and we obtained an antibody fragment with high affinity for gold surface materials by in vitro selection. One advantage of working with antibodies is the ability to use various fusion technologies. The construction of bivalent and bispecific antibodies with the selected anti-gold antibody with high affinity and specificity allowed us to functionalize gold nanoparticles and to permit highly ordered protein patterning and accumulation on a gold-patterned silicon substrate. We discuss the potential uses of naive antibody repertoire and the antibody engineering technique in nanobiotechnology.

EXPERIMENTAL PROCEDURES

Selection of Antibody Fragments Specific for a Gold Surface—Human antibody fragments against gold surfaces were

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selected on the basis of the stabilization of the variable fragment (Fv)² in the presence of the target antigen (27, 28). Variable regions of VL and VH chains were displayed on the filamentous bacteriophage M13. Approximately 10⁷ phages were mixed with 5 mg of gold particles (diameter, 3 μm; Sigma) in 1 ml of PBS and 0.5–1.0% Tween 20 detergent. The mixture was incubated for 1 h at room temperature with gentle agitation. Residual phages on the gold surface were amplified by directly adding the gold particles into Escherichia coli medium after exclusion of unbound phage from the phage-gold solution. After five rounds of screening, the amino acid sequences of VL and VH displayed on the isolated phage were analyzed in 48 and 192 phage clones, respectively, by enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR).

Construction and Preparation of Fv and Single-chain Fv Dimers—The VH and VL genes were inserted into the secretory co-expression vector pRA2; the resulting plasmid was named pRA2FHL. In the plasmid, VL has a polyhistidine tag (HHHHHHH) at the C terminus, and VH has a c-Myc tag (EQKLI5EEDLNL) and a polyhistidine tag in this order at the C terminus. For single-chain Fv (scFv), expression vectors for scFv with a (Gly₄-Ser) linker between the VH and VL chains were constructed as described previously (29). The generated gene fragments, termed AH-AL (A14P-b2 VH-A14P-b2 VL), AH-10L (A14P-b2 VH-HyHEL10 VL), and 10H-AL (HyHEL10 VH-A14P-b2 VL), were separately introduced into the expression vector pRA. The A14P-b2 is the antibody fragment selected in this study that has affinity for gold surface (see “Results” and “Discussion”), and HyHEL10 is an anti-lysozyme antibody (30). Each gene product of Fv and scFv was expressed as inclusion bodies in E. coli BL21 (DE3) and then solubilized with 6 x guanidine hydrochloride to refold on the stepwise dialysis system as described previously (31). After refinement by means of a metal-chelate chromatography column, the solubilized AH-AL was refolded to the bivalent scFv homodimer, and the AH-10L and 10H-AL scFvs were also refolded to the scFv heterodimer (bispecific diabody) (32). The refolded solutions were fractionated by using a gel filtration chromatography column (Superdex 75 or 200), and the dimer fractions were obtained for experiments.

ELISA—A 96-well microtiter polystyrene plate was coated with gold by ion vapor deposition. Antibody fragments from each clone were expressed in E. coli on a small scale, and then the supernatant after centrifugation of the sonicated harvest cells (in PBS containing 0.1% Tween 20) was applied to the wells. The plate was incubated for 1 h at room temperature, and after five washings with PBS-Tween solution, binding of antibody fragments was detected with Fluoroskan Ascent FL (Labsystems) by using horseradish peroxidase-conjugated antibodies and ECL Western blotting detection reagent (Amersham Biosciences). Anti-histidine tag and anti-c-Myc tag antibodies were applied for the detection of VL and VH, respectively.

SPR Assay—SPR measurements were performed with BIAcore 2000 (Biacore AB), and BLAevaluation software (ver. 3.0) was used for analyzing and fitting the collected data. A Sensor Chip Au (Biacore AB) was soaked in piranha solution (H₂SO₄/H₂O₂, 3:1, v/v) after ultrasonic cleaning in acetone and was then rinsed with double-distilled water. The running buffer used for the experiments was PBS with 0.1% Tween 20.

Functionalization of Gold Nanoparticles—Gold colloid (diameter, 20 nm; Sigma) was dialyzed in 10 mM phosphate solution (pH 7.4) containing 0.1% Tween 20, using molecular porous membrane tubes with the molecular mass cut off of 6–8 kDa (Spectrum Laboratories, Inc.). After dialysis, some proteins (Fv, scFv homodimer, bovine serum albumin) were added to the gold colloid solution (1 nm) at a final protein concentration of 260 nm. The sizes of gold particles were analyzed using a Zetasizer Nano ZS dynamic light scattering spectrophotometer (Malvern Inst. Ltd.), and the change in the absorption spectra was observed with a U-3000 spectrophotometer (Hitachi). For aggregated gold particles, transmission electron microscopy images were obtained on a LEO 912 AB OMEGA electron microscope (Carl Zeiss) operating at 100 kV for aggregated gold nanoparticles.

Specificity of the Anti-gold Antibody for Other Noble Metals—Gold, platinum, palladium, and silver plates were purchased from Nilaco. The metal plates, each attached to a polypropylene plate, were soaked for 30 min in PBS (0.1% Tween 20) with A14P-b2 Fv fused with a c-Myc tag and then washed four times with PBS-Tween solution. Binding of Fv to the metals was detected by anti-c-Myc antibody horseradish peroxidase.

Fluorescence Microscopy—Hen egg white lysozyme was fluorescein-labeled by using a fluorescein labeling kit (Roche Applied Science) in accordance with the manufacturer’s protocol. A gold-patterned silicon crystal plate was soaked first in PBS (0.1% Tween 20) containing the bispecific diabody made from AH-10L and 10H-AL scFvs at a concentration of 1 μM and then in an FITC-labeled lysozyme solution after several washings with PBS-Tween solution.

RESULTS AND DISCUSSION

Selection of a Human Antibody Fragment for Binding to Gold Surfaces—To select a fragment of the variable region in an antibody (Fv) for binding to gold surfaces, we employed phage-displayed libraries of the variable regions of human antibody VL and VH chains (33–35); here, we used the VL and VH library we prepared previously (27). Phages displaying VL chains were first selected from the VL chain library by using gold particles, and then the VH chain phages were selected and assayed in the presence of the selected VL chain to stabilize the VH chain (28). Unbound phages were removed by using a highly concentrated detergent solution (0.5–1.0% Tween 20) that was expected to prevent nonspecific adsorption of proteins, and the residual phages on the gold surface were amplified by directly adding the gold particles to E. coli medium, because some phages were not sep
arated from the gold particles by treatment with an acidic solution (0.2 M glycine-HCl at pH 2.2).

For the selection of VL chains, we measured ELISA for independent VL chains prepared from randomly 48 chosen clones with a gold-coated 96-well plate; consequently, the clone b2 was selected. The b2 VL chain showed weak binding to gold surface as shown in a SPR test (Fig. 1). For VH chains, we performed ELISA for randomly chosen VH chains in the presence of b2 VL chain. After we had selected the clones positive for ELISA, the selected VH chains were tested again by ELISA at various concentrations of Fv. The signals from three of the clones, 1B3, 2A8, and 2G9, depended strongly on the amount of Fv applied (Fig. 2).

For the positive clones, SPR sensorgrams for purified Fv were measured against the gold surface (Fig. 3). In PBS solution with 0.1% Tween 20, the 2G9-b2 Fv fragment showed no substantial interaction with the gold surface, and the 2A8-b2 Fv fragment showed only a weak interaction. In contrast, the 1B3-b2 Fv fragment showed critical binding curves that could be fitted globally to a 1:1 interaction model with a mass transport term, yielding a dissociation equilibrium constant \( (K_D) \) of 6.3 nM. Table 1 shows the sequences of the complementarity-determining region (CDR) in the 1B3-b2 Fv fragment. The usage of each amino acid in the CDRs showed no feature discriminative from those in general antibodies reported in the protein data bank, suggesting that 1B3-b2 Fv recognized the gold surface in the same manner as protein-protein interaction. When we compared the SPR sensorgrams between b2 VL chain and 1B3-b2 Fv, the binding affinity for gold surface was drastically increased by selecting VH chain. This suggests that the strong binding is mainly attributed to the interaction of VH chain with gold surface. The result from Fig. 3 that 2G9-b2 and 2A8-b2 Fv with the same VL as 1B3-b2 Fv have little affinity for gold surface supports little contribution of VL to the strong binding of 1B3-b2 Fv. The VL chain might only play the role in stabilizing the VH chain.

The selected 1B3-b2 Fv fragment had a high binding ability for the gold surface, but it was not so stable as to remain in a soluble form even for a week; consequently, the high affinity could not be maintained (data not shown). Comparison of the framework sequences of the 1B3-b2 Fv fragment and the germline-encoded V segments of human antibodies showed that the proline residue at the 14 position was mutated to alanine in the 1B3 VH chain. When we constructed a variant of 1B3-b2 Fv in which the alanine was replaced with proline (A14P-b2), the A14P-b2 Fv fragments were also expressed as insoluble aggregates in E. coli, as well as 1B3-b2; however, A14P-b2 Fv was efficiently prepared by the refolding from the insoluble aggregates on stepwise dialysis system. Little insoluble aggregates were formed in the refolding process, and the size exclusion chromatography for the refolded solution showed that most of refolded A14P-b2 formed heterodimer (supplemental Fig. S1). The fractionated A14P-b2 Fv fragment remained unaggregated.
A14P-b2 (93% sequence homology in their frameworks), the high affinity of A14P-b2 Fv for gold surface is attributed to the CDR area but not to the framework area.

We also estimated the thermodynamic properties of A14P-b2 Fv from van’t Hoff plots: \( \Delta H = -49.1 \text{ kJ/mol} \) and \( \Delta S = 3 \times 10^{-3} \text{ kJ/mol/K} \). Interestingly, the entropy change was so small that only the enthalpy change made a dominant contribution to the binding of A14P-b2 Fv to the gold surface. This thermodynamical result suggests that the conformation of A14P-b2 Fv is little changed by interaction with the gold surface or that few water molecules on the gold surface are dispersed by the binding of Fv.

Recently, various material-binding peptides with micromolar \( K_D \) values have been found by in vitro selection (26, 38), and repetition of the material-binding peptides can increase their binding affinity for materials (25). Recently, Tamerler et al. (26) estimated the \( K_D \) value for a polypeptide with three repeats of a gold-binding peptide to be 89 nM. The result that we selected the antibodies with high affinity for gold surface from a human antibody library indicates the potential use of the antibody framework for fabricating the biomolecules that strongly bind to bulk material surfaces.

To date, Barbas et al. (22) attempted to select the antibodies using magnetite matter as an antigen, and Schnirman et al. (39) recently found the scFv fragments that can discriminate between crystalline face of gallium arsenide, although the scFv displayed on phage was used. To estimate the specificity of A14P-b2 Fv for the gold surface, we soaked plates of gold, platinum, palladium, and silver in solutions containing A14P-b2 Fv fused with a c-Myc tag. Residual fragments were then detected by the chemiluminescence from c-Myc antibody horseradish peroxidase (Fig. 5). A14P-b2 showed strong binding only on the gold plate and showed no affinity for the other noble metals (Fig. 5b). We also applied A14P-b2 Fv to silicon, aluminum, and tungsten and confirmed that the fragment did not bind to these materials (data not shown). In contrast, no significant responses were observed for the human antibody PH7-3d3 (Fig. 5c). Therefore, A14P-b2 Fv has both high affinity and specificity for gold surfaces, which are attributed to the binding of the CDR area on the gold surface.

Functionalization of Gold Nanoparticles—To apply the high affinity of A14P-b2 Fv to the stabilization of gold nanoparticles (Fig. 6a), we added A14P-b2 Fv into a gold nano-
particle suspension. The gold nanoparticles (1 nM) can be dispersed in 10 mM phosphate solution (pH 7.4) containing 0.1% Tween 20, but even small changes of ionic strength resulted in agglomeration of the nanoparticles (Fig. 6b, panel 1); in contrast, the gold nanoparticle suspension containing A14P-b2 Fv at a Fv concentration of 260 nM showed no color change at a high salt concentration of 2 M NaCl (Fig. 6b, panels 2 and 3). Bovine serum albumin can stabilize gold nanoparticles; however, gold nanoparticles with the same concentration of bovine serum albumin as A14P-b2 Fv were aggregated at 80 mM NaCl (Fig. 6b, panels 4 and 5). We observed stabilization with other proteins (lysozyme, green fluorescent protein, and PH7-3d3 Fv), but none could stabilize the gold nanoparticles over the same wide range of salt concentrations as A14P-b2 Fv (data not shown). The particle size estimated from dynamic light scattering measurement was about 8 nm greater than bare nanoparticles (Fig. 6c), demonstrating that the binding of A14P-b2 Fv stabilized the gold nanoparticles.

One advantage of working with antibodies is the ability to use various fusion technologies. Multivalent and multispecific antibodies have been fabricated from variable domains (40), and various proteins have been fused to these antibodies for therapeutic and imaging uses without decreases in binding activity (41). We evaluated the possibility of functionalization of gold nanoparticles with a bivalent antibody constructed from A14P-b2 Fv. The VH and VL chains of A14P-b2 Fv were linked by a five-residue linker (Gly-Ser), and the AH-AL scFv fragment was expressed as inclusion bodies. AH-AL scFv fragments were solubilized in 6 M guanidine HCl solution, and then they were stepwisely dialyzed to a guanidine HCl-free solution to form bivalent homodimer. Although insoluble aggregates were formed in the refolding process, a dimer form was dominant in the supernatant (supplemental Fig. S2). In this study, the A14P-b2 scFv homodimer fractionated by size exclusion chromatography was used for studies. When the purified A14P-b2 bivalent scFv homodimer was added to a 1 nM solution of gold nanoparticles at a final scFv homodimer concentration of 260 nM, the plasmon absorbance of the gold nanoparticles was slightly increased by the binding of the scFv homodimer, and the absorbance was gradually red-shifted to a wavelength of 560 nm over a period of 100 min (Fig. 7a). In general, bare gold nanoparticles are dispersed even after drying on carbon mesh. However, transmission electron microscopy showed that the gold nanoparticles that precipitated with the scFv homodimer were assembled with a space between them (Fig. 7b), in a manner similar to that seen in DNA-linked gold nanoparticle assemblies in which gold nanoparticles are assembled through the complementary binding of single-stranded DNA chains (42). Although some of the intervals between the nanoparticles seemed to be smaller than the scFv homodimer, probably because of multistacking of the nanoparticles, the interval distance in the monolayer area was ~5–8 nm, which is comparable with the size of the scFv homodimer. Considering that we used

![FIGURE 6. Stabilization of gold nanoparticles by A14P-b2 Fv.](https://example.com/figure6.jpg)

*a*, experimental strategy for the analysis of the stabilization of gold nanoparticles by A14P-b2 Fv. *b*, gold nanoparticles without A14P-b2 Fv (panel 1), with A14P-b2 Fv (panels 2 and 3), and with bovine serum albumin (panels 4 and 5) in a 10 mM phosphate buffer solution at various NaCl concentrations; 5 M NaCl solution was titrated in 1 ml of each gold nanoparticle-protein solution (1 nM gold nanoparticles, 260 nM protein). *c*, distribution of hydrodynamic diameter determined by dynamic light scattering for gold nanoparticles in an A14P-b2 Fv-free solution (top) and an A14P-b2 Fv solution (bottom).
the purified scFv homodimer without multimers, this result suggests that the gold nanoparticles were linked via the bivalent scFv homodimer.

We added SDS detergent to the solution containing precipitated gold nanoparticles. At an SDS concentration of 1% (w/v), the precipitates were dissociated, giving completely dispersed nanoparticles with a peak absorbance at 520 nm (Fig. 7c). In general, the direct binding between the surfaces of gold nanoparticles is too strong to separate nanoparticles even by SDS. The fact that the nanoparticles precipitated by the addition of A14P-b2 scFv homodimer were completely dispersed supports the idea that gold nanoparticles were linked via the bivalent scFv homodimer. Redispersion of the gold nanoparticles resulted from deactivation of the scFv dimer by SDS.

These bivalent scFv homodimer experiments demonstrate the functionalization of gold nanoparticles by means of the functional Fv-fused A14P-b2 Fv dimer. There have been reports of functionalization of nanoparticles by the immobilization of functional protein (43). In general, functionalization requires an appropriate surface design and a protein-immobilization technique (e.g., formation of covalent linkages), and the direction of the immobilized protein is difficult to control. Use of the fusion technique with A14P-b2 Fv enabled functionalization of gold nanoparticles without the need for a surface design or a protein immobilization procedure, and the technique also constrained the direction of the fused protein because of the rigid folded structure of Fv.

**Protein Patterning and Accumulation via the Anti-gold Antibody Fragment**—By using the high specificity of A14P-b2 Fv, we attempted specific patterning and accumulation of protein via the antibody on a gold-patterned substrate. A bispecific scFv het-
erodimer with two kinds of binding ability, called a bispecific diabody, was constructed from the anti-gold Fv (A14P-b2) and anti-hen egg white lysozyme Fv (HyHEL10 Fv). Here, we used the purified bispecific diabody by size exclusion chromatography after the refolding from inclusion bodies (supplemental Fig. S3). A gold-patterned silicon crystal plate was soaked in a solution containing the bispecific diabody composed of A14P-b2 and HyHEL10 Fv at a concentration of 1 μM and was then soaked in an FITC-labeled lysozyme solution after several washings. Another gold-patterned silicon plate was soaked only in a 1 μM FITC-labeled lysozyme solution. When the lysozyme patterning and the accumulation on the anti-gold antibody were imaged by fluorescence microscopy (Fig. 8a), no fluorescence from FITC-labeled lysozyme was observed on the silicon plate without the diabody. In contrast, the silicon plate soaked in the solution containing the bispecific diabody composed of A14P-b2 and HyHEL10 Fv specifically showed strong fluorescence from FITC-labeled lysozyme on the patterned gold spots. The bispecific diabody functioned as an interface molecule for protein patterning and accumulation (Fig. 8b). This result indicates that an immunoassay plate with oriented Fv can be prepared by patterning gold on the probe surface. Various immobilization methods have been reported for the evaluation of ligand-receptor interactions, because the immobilization of proteins on solid phase surfaces simplifies solution phase assays, enabling high throughput measurements (44). In many methods, the proteins are immobilized on the inorganic plate through several complicated steps to conserve their activity and to achieve a high degree of orientation. Use of anti-material antibodies enables single-step production of Fv-oriented immunoassay plates.

Conclusion—We selected and prepared a human antibody fragment with high affinity and selectivity for gold surface materials. The high affinity anti-gold antibody fragments significantly stabilized gold nanoparticles, and the construction of bivalent and bispecific scFv dimer from the anti-gold Fv with high affinity enabled the bio-functionalization of gold nanoparticles and the production of a highly oriented antibody immunoassay plate. Antibody fragments are among the smallest units with naturally occurring binding domains and are the most promising binders in materials engineering because of their high affinity and specificity. Artificial format construction based on anti-material antibodies with high affinity will provide opportunities for new applications of protein engineering in nanobiotechnology.

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