Nanoparticles with affinity for biopolymer: Bioassisted room-temperature selective multistacking of inorganic particles on biopolymer film

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Recently, we selected the antibody fragment with high affinity for the biopolymer film of polyhydroxybutyrate (PHB) from human antibody fragment libraries. In this study, we functionalized CdSe quantum dot (QD) nanoparticles by orderly conjugating the anti-PHB antibody fragments to perform spontaneous and selective stacking of inorganic particles on PHB-coated plates in neutral solutions at room temperature. Surface plasmon resonance analysis showed that the orderly clustering of anti-PHB antibody fragment on QD particles led to no dissociation of QD on PHB-coated plates, indicating the availability of avidity effect. The strong spontaneous immobilization using biomolecular recognition enabled stepwise stacking of inorganic particles on PHB-coated plates only by mixing operation in neutral solutions at room temperature. We show the potential of recombinant anti-material antibody fragments for the bottom-up stacking procedures for hybrid assembly.

I. INTRODUCTION

Downsized inorganic particles can have unique electric and photonic functions, and nanoparticles are applicable for the bottom-up procedures of nanoscale devices. Appropriate patterning and stacking of nanoparticles on substrate can lead the particles to fulfill their potentials. In studies on the patterning and stacking of nanoparticles on substrates, polymer-based materials have been attractive for the development of light flexible devices in the fields of photonics,[1] electronics,[2] and sensors.[3] However, general fabrication processes involve high processing temperature, which causes some damage on polymer-based materials due to lower thermal stability of organic compounds. The techniques of patterning and stacking inorganic nanoparticles at room temperature are potentially useful for the orientation of the nanoparticles in or on heat-labile organic compounds.

Recently, several biomimetic approaches have been studied for the assembly and patterning of nano-micro materials at room temperature.[4,5] Selective adsorption of inorganic particles on patterned substrates can be controlled by modifying surface functional groups of substrates, and for surface modification of bulk materials, the utilization of biomolecules such as deoxyribonucleic acid (DNA), peptides and antibodies is becoming attractive because of their strong selective binding ability. Highly selective base-pairing interactions between complementary single-strand DNA chains have been used for pinpoint deposition of nanoparticles between electrocodes,[6] and the peptides with affinity for gold surfaces enabled the patterning of protein and nanoparticles on gold substrates using microcontact printing technology.[7,8]

Peptides that have the function of molecular recognition with high specificity have been widely utilized in the fields of medical chemistry and sensing. Appropriate configuration of amino acid residues in the binding site of peptides generates a specific binding ability for antigens. Several peptides with affinity for non-biological materials have been identified by means of a combinatorial library approach,[9] and the material-binding peptides have been expected to be utilized for bottom-up fabrication procedures in the field of bio-nanotechnology. The spontaneous binding in mild solutions at room temperature is utilized for the direct immobilization and orientation of proteins and nanomaterials on inorganic materials.[4,7,8] Considering that the bottom-up process needs no heat treatment, the utilization of the peptide with affinity for organic compound is advantageous for direct immobilization and patterning of inorganic particles in or on heat-labile organic compounds if we can identify the peptides.

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In this study, we functionalized inorganic nanoparticles by orderly conjugating antibody fragments with affinity for the biopolymer film of polyhydroxybutyrate (PHB)[10] to perform spontaneous and selective stacking of inorganic particles on the organic-coated plate at room temperature. Antibodies also have high specific molecular recognition, but the binding affinity can be much stronger than peptides. Recently, antibodies with affinity for bulk material surfaces have been selected from human antibody libraries,[10,11] and further, material-binding functionalization of antibodies by peptide grafting was reported.[12] These results demonstrate the potential of antibodies for interface biomolecules. Here, we demonstrate that clustering high affinity anti-PHB antibody fragments on quantum dot (QD) nanoparticles enables strong and selective adsorption of QD particles on patterned PHB films due to the synergy of high affinity and avidity effect. We show the potential of recombinant anti-material antibody fragments for the bottom-up stacking of nanoparticles on heat-labile organic compounds at room temperature.

II. EXPERIMENTAL PROCEDURES

A. Construction of expression vector for anti-PHB Fv fragment with biotin acceptor peptide

The DNA sequence coding the variable heavy (VH) chain fragment with C-myc tag (EQKLISEEDLN) in the variable fragment (Fv), IgA hinge linker (SPSTPPTPSPSTPP), biotin acceptor peptide (AviTag; GGLNDIFEAQKIEWH) and poly-histidine tag (HHHHHH) in this order at the C-terminus as reported by Cloutier et al.,[13] was generated by overlap polymerase chain reaction (PCR) from pRA2FHL plasmid containing the Fv fragment of human anti-PHB monoclonal antibody PH7-3d3 as described previously.[10] The amplified sequences for the VH domain with the peptide-tags were inserted into the pRA vectors in exchange for the corresponding VH domain of wild-type PH7-3d3 using the *Nco*I-*Sac*II digestion site to express the Fv fragments with AviTag in the C-terminus side of VH.

B. Preparation of anti-PHB Fv fragment with biotinylated tag

We first transformed *E. coli* BL21 (DE3) by the plasmid of pBIRAcm encoding biotin ligase (Avidity Inc., Aurora, CO) and then transformed the same cells by the plasmid encoding anti-PHB Fv fragments with AviTag. The transformed *E. coli* cells were incubated in 2 × YT medium at 28 °C. Anti-PHB Fv with AviTag and biotin ligase were induced by adding 1 mM IPTG in the presence of 50 μM of D-biotin (Sigma, St. Louis, MO). The harvested cells were centrifuged and suspended in a Tris-HCl solution (50 mM, pH 8.0) with 200 mM NaCl. After sonication, the suspension was centrifuged at 6300 × g for 30 min. The pellet was solubilized in the 50 mM Tris-HCl solution (pH 8.0) with 6 M GdnHCl and 200 mM NaCl, and the solubilized Fv fragments were refined by means of a metal-chelate chromatography column that interacted with poly-histidine tag in VH and VL domains. The anti-PHB Fv fragments were refolded by gradual removal of GdnHCl by means of stepwise dialysis from 6 M to 0 M through 3, 2, 1, and 0.5 M, as described previously,[14] and the refolded Fv fragments were fractionated by a gel filtration chromatography (GE Healthcare, Little Chalfont, UK). The fractions of the renatured Fv with biotinylated tag were collected after the presence of biotin was confirmed in VH with western-blotting analysis using Streptavidin-Horseradish Peroxidase Conjugate (GE Healthcare).

C. Stacking of inorganic particles on PHB film via anti-PHB Fv

PHB (ICI, Inc., Slough, UK) was dissolved in chloroform at a concentration of 1.5% (w/v), and 15 μL of the solvent was spin-coated onto the gold plate (10 × 10 mm) at 4500 rpm for 1 min. The PHB-coated gold plate was heated at 210 °C for 30 s and then incubated at 110 °C for 24 h before heat release to room temperature. The annealed plate was soaked in a PBS solution with 0.1% (w/v) skim milk to suppress nonspecific binding of proteins and particles.

For stacking CdSe QD nanoparticles (the size; 30 nm, Invitrogen, Carlsbad, CA) on the PHB-coated plate, 200 nM anti-PHB Fv with biotinylated tag and 6.6 nM QD with streptavidin conjugated were mixed in 2 ml Tris-HCl solution (pH 8.0, 200 mM NaCl), and the plate was soaked in the mixture solution for 30 min [Fig. 1(a)]. The soaked plate was washed several times with 50 mM Tris-HCl solution (pH 8.0, 200 mM NaCl) to exclude non-specifically bound QD.

In an alternative stacking process, the PHB-coated plate was soaked in 2 mL Tris-HCl solution (50 mM, 200 mM NaCl, pH 8.0) containing 200 nM anti-PHB Fv with biotinylated tag for 60 min at room temperature. After the soaked plate was washed by Tris-HCl solution (50 mM, 200 mM NaCl, pH 8.0), the plate was soaked in 2 ml 6.6 nM streptavidin-conjugated QD solution (50 mM Tris-HCl, 200 mM NaCl, pH 8.0) [Fig. 1(b)]. After washing, the plate was soaked in 2 mL Tris-HCl solution (50 mM, 200 mM NaCl, pH 8.0) containing 1 μM ZnO-binding peptide (EAHVMHKVAPRP[15]) with biotin at the N-terminus via SGGG sequence, and then in 2 ml fluorescent ZnO (size; ~1 μm, Kasei Optonix, Odawara, Japan) particle suspension. Finally, the plate was excited at 365 nm after washing operation was applied again.

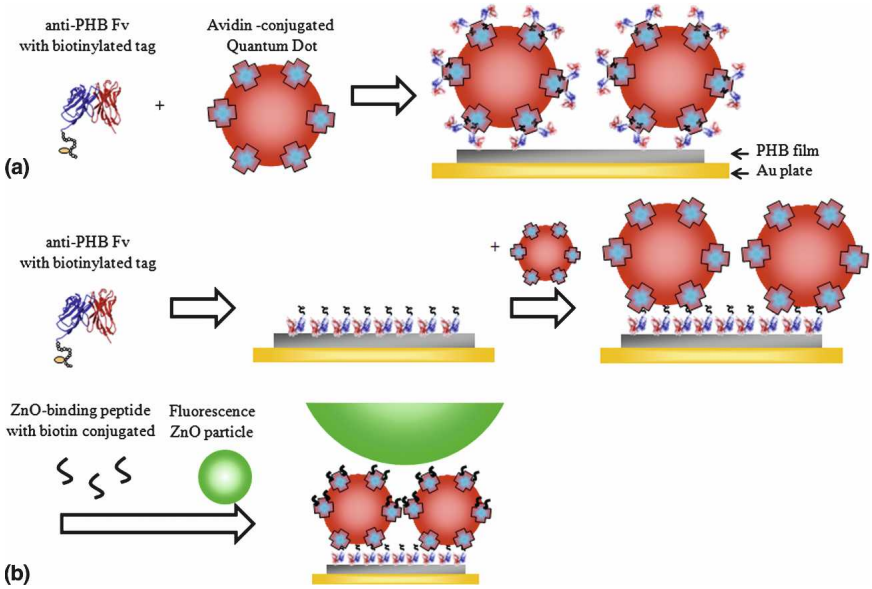

FIG. 1. Schemes for stacking inorganic particles on PHB-coated gold plate using anti-PHB Fv and ZnO-binding peptide.

D. SPR analysis

For SPR analysis, a sensor chip of Au (Biacore AB, Uppsala, Sweden) cleaned by ultraviolet-generated ozone gas, was spin-coated by PHB-chloroform solvent (0.5% w/v) at 6000 rpm. The PHB-coated plate was annealed with the same method as the gold plate for stacking experiment (see Sec. II. C). The SPR resonance angle was confirmed to be within the range needed to detect binding. SPR measurements were performed using Biacore 2000 (Biacore). The running solution for the experiments was PBS solution and it was run at the rate of 10 μl/min.

III. RESULTS

A. Selective immobilization of QD with anti-PHB Fv on PHB film

Figure 2 shows the immobilization experiment of QD particles via anti-PHB Fv. To make QD particles on which anti-PHB Fv fragments were orderly displayed, the streptavidin-conjugated QD particles were mixed with anti-PHB Fv with biotinylated tag at the C-terminus of VH. When we soaked the untreated and PHB-coated gold plates in the solution containing streptavidin-conjugated QD without antibodies, little fluorescence derived from QD was observed on both the plates [Fig. 2(a)]. In contrast, the use of the QD particles with anti-PHB Fv resulted in intense fluorescence from QD on the PHB-coated gold plate, but not on the untreated plate [Fig. 2(b)]. The Fv-displayed QD particles were specifically immobilized on PHB film. In addition, we orderly conjugated anti-lysozyme Fv fragments on QD using the same method as for anti-PHB Fv, and the immobilization of the Fv-displayed QD particles on PHB-coated plate was attempted [Fig. 2(c)]; consequently, no fluorescence derived from QD was observed on both the untreated and PHB-coated gold plates. The selective recognition ability of anti-PHB Fv enabled spontaneous immobilization of QD on PHB film.

To analyze the binding properties of the QD particles with anti-PHB Fv on PHB film, we measured the interaction of QD with PHB film via anti-PHB Fv by

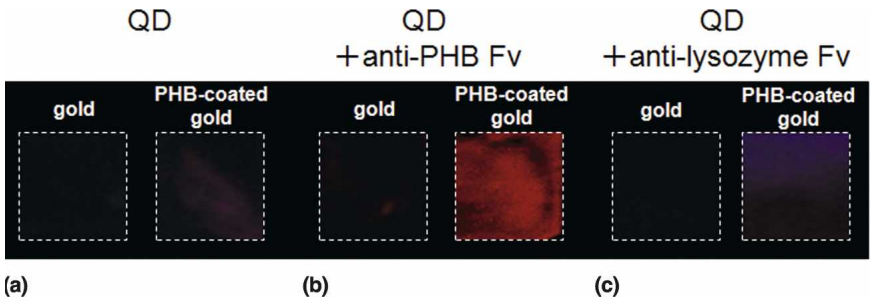

FIG. 2. Immobilization experiment of QD on untreated and PHB-coated gold plates: (a) streptavidin-conjugated QD particles, (b) streptavidin-conjugated QD particles with biotinylated anti-PHB Fv, and (c) streptavidin-conjugated QD particles with biotinylated anti-lysozyme Fv. All the plates were excited at 365 nm.

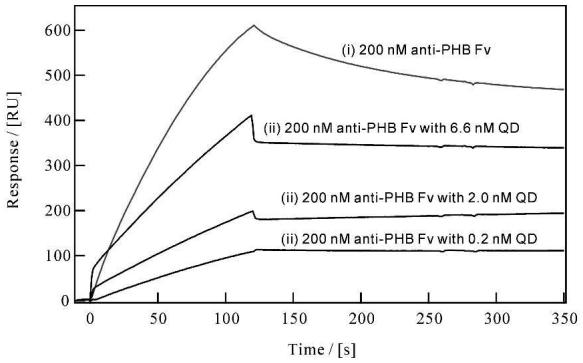

FIG. 3. SPR sensorgrams for the interaction of anti-PHB Fv with PHB-coated gold surface on sensor chip: (i) 200 nM biotinylated anti-PHB Fv solution was applied for 120 s (gray line), and (ii) 200 nM biotinylated anti-PHB Fv solutions after mixing with avidin-conjugated QD particles at concentrations of 0.2 nM, 2 nM, and 6.6 nM. QD particles were applied for 120 s (black line).

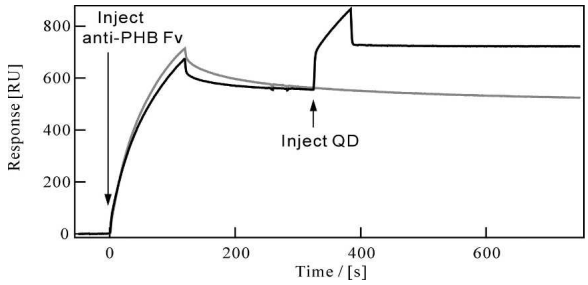

FIG. 4. SPR sensorgrams for the binding behavior of the stepwise immobilization of anti-PHB Fv and avidin-conjugated QD particles on PHB-coated gold surface on sensor chip. The 200 nM biotinylated anti-PHB Fv fragments were applied to PHB-coated gold surface, and then 0.2 nM avidin-conjugated QD particles was associated for 60 s, 320 after the start of dissociation process.

measuring SPR (Fig. 3). The binding of anti-PHB Fv with biotinylated tag to PHB film on a sensor chip was confirmed, and the binding behavior was the same as described previously (gray line in Fig. 3).[10] To reconstruct the immobilization experiment of QD via anti-PHB Fv in Fig. 2(b), the streptavidin-conjugated QD particles were injected on the PHB-coated sensor chip after the QD particles were mixed with biotinylated anti-PHB Fv (black lines in Fig. 3). The applied concentrations of QD were varied from 0.2 nM and 6.6 nM at the same Fv concentration of 200 nM; that is, the QD/Fv ratios were from 1/1000 to 1/33 (streptavidin-conjugated QD particles are considered to have about 30 binding sites for bitotin molecules). Although most anti-PHB Fv fragments were free at the QD/Fv ratio of 1/1000, the association and dissociation responses became slower than anti-PHB Fv without QD. In particular, the dissociation rates of anti-PHB Fv were drastically decreased by the conjugation with QD, indicating that few anti-PHB Fv were removed in the presence of QD. The Fv-displayed QD has much higher apparent affinity than an independent anti-PHB Fv.

B. Stacking of QD and ZnO particles on PHB film via anti-PHB Fv and ZnO-binding peptide

To examine multistacking inorganic particles on PHB film, we attempted stepwise immobilization of anti-PHB Fv and streptavidin-conjugated QD particles [Fig. 1(b)]. Figure 4 shows the SPR sensorgrams for the stepwise immobilization of anti-PHB Fv and QD. The biotinylated anti-PHB Fv was first injected, and then streptavidin-conjugated QD particles were applied to the PHB-coated sensor chip. At the injection of QD, vertical increase of SPR was observed due to bulk effect because the appearance of QD in solution caused the change of dielectric constant of solution phase; however, we confirmed the binding response of QD to the anti-PHB Fv bound on the PHB-coated sensor chip, which indicates the immobilization of QD particles via the biotin-avidin interaction. It should be noted that the dissociation of anti-PHB Fv was too slow to be observed after the QD particles were applied. The slow dissociation was also observed in the one-step immobilization of anti-PHB-Fv-displayed QD on PHB film (Fig. 3). The anti-PHB Fv fragments have the site for binding to PHB film at the opposite of the C-terminus of VH so that the biotin molecules conjugated on the AviTag peptide in the C-terminus side of anti-PHB-Fv are efficiently displayed on the surface of the sensor chip. The interactions of a streptavidin-conjugated QD particle with multiple anti-PHB Fv fragments via the biotin-avidin interactions created strong avidity effect.

The stepwise stacking of QD and ZnO particles on PHB film was performed by using anti-PHB Fv and ZnO-binding peptide (Fig. 5). At the step 2 in Fig. 5, where streptavidin-conjugated QD particles were stepwise immobilized on PHB film via biotinylated anti-PHB-Fv, the fluorescence from QD was observed on PHB film [step 2 in Fig. 5(a)]. After step 2, the QD-stacked plate was soaked in the solution containing the ZnO-binding peptides with a biotin molecule at the C-terminus and then soaked in fluorescent ZnO particle suspension (step 3 in Fig. 5); under ultraviolet irradiation, green fluorescence from ZnO was observed on the red fluorescence from QD [step 3 in Fig. 5(a)], while there was no fluorescence on PHB-coated plate without the addition of streptavidin-conjugated QD [step 3 in Fig. 5(b)]. This result indicates that ZnO particles were specifically bound to QD surface via biotin-conjugated ZnO-binding peptide.

IV. DISCUSSION

The discovery of peptide with affinity for non-biological materials, such as bulk metal, semiconductor compound, and ceramics matters, opened the way for

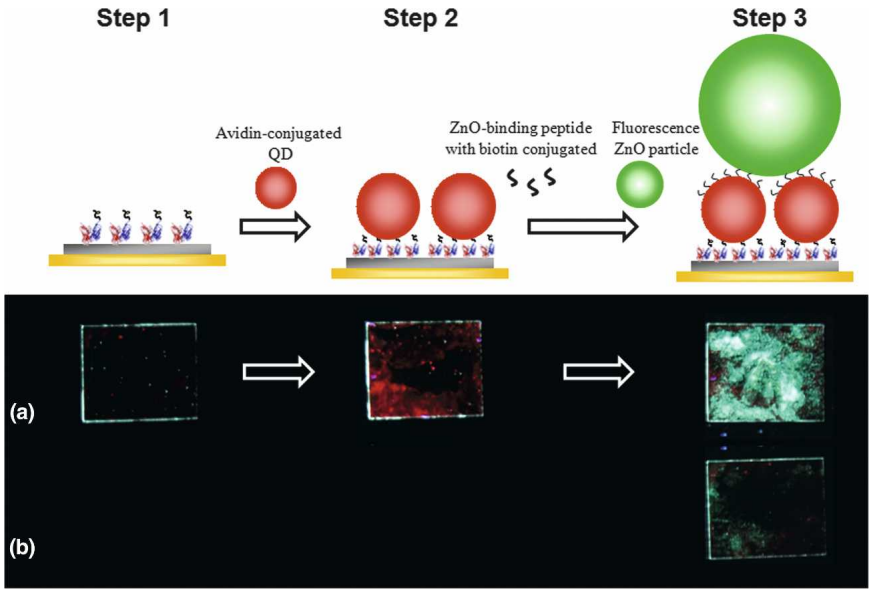

FIG. 5. Stepwise stacking of QD and ZnO particles on PHB-coated gold plate. (a) After the PHB-coated plate was soaked in 200 nM biotinylated anti-PHB Fv solution (Step 1), the plate was soaked in 6.6 nM avitin-conjugated QD solution (Step 2). After Step 2, the plate was soaked in 1 μM biotinylated ZnO-binding peptide and then in fluorescent ZnO particle suspension (Step 3). (b) After the PHB-coated plate was soaked in 200 nM biotinylated anti-PHB Fv solution (Step 1), the Step 2 process was skipped. The plate bearing anti-PHB Fv was soaked in 1 μM biotinylated ZnO-binding peptide and then in fluorescent ZnO particle suspension (Step 3). All the plates were excited at 365 nm.

utilizing the peptides as an affinity tag to enable the spontaneous and orientated immobilization of recombinant proteins on plane inorganic plates.[8] Besides the protein immobilization, recently, the patterning of gold nanoparticles via gold-binding peptide and the three-dimensional structualization by the peptides with the function of binding and mineralization have been reported.[7,15–17] A benefit of using a material-binding peptide is the application for the pattering on heat-labile substrate at room temperature. Sanghvi et al. functionalized conductive chlorine-doped polypyrrole (PPyCl) polymer using the PPyCl-binding peptide with a cell adhesive sequence at room temperature to promote cell attachment on PPyCl.[18] In this study, we applied the antibodies with affinity for the surface of organic polymer film to perform spontaneous stacking of QD particles in neutral solutions at room temperature. In the hybridization with organic and inorganic matter, the applicable conditions of temperature and solvent used are not variable in comparison with inorganic materials. The hybridization process in neutral solutions at room temperature will be potentially available for heat-labile or pH-sensitive organic compounds.

To make a strong interaction between nanoparticles and PHB films, we utilized the antibodies with high affinity for PHB film at the equilibrium dissociation constant of 14 nM.[10] The conjugation of anti-PHB Fv fragments on the surface of QD nanoparticles resulted in selective immobilization of QD on PHB film (Fig. 2). The SPR analysis implies that the anti-PHB Fv fragments conjugated on QD more strongly bind on PHB films than the Fv without QD; consequently, few anti-PHB Fv conjugated on QD were removed while running solution flowed [Fig. 3(b)]. In antibody engineering, the binding strength of antibody molecules can be promoted not only by increasing the affinity of a binding domain in an antibody, but also by increasing the number of the binding domain in an antibody. In general, the antibodies with several multiple binding domains (multivalent antibodies) generally show slow association and dissociation rates in SPR sensorgrams as the valency increases.[13,19] The change of association rate in SPR is determined by several factors, but the decrease of dissociation rate has been elucidated as avidity effect; with increased valency, the chance increases that at least one of the remaining arms will find a target before the monovalently bound complex dissociates. Therefore, the avidity effect by multidisplaying binding proteins on particles resulted in little dissociation of the Fv-conjugated QD on PHB films. Leggett et al. reported that the gold nanoparticles with multi anti-cotinine antibodies showed highly sensitive detection of the fingerprints from smokers.[20] Here, we confirmed the availability of avidity effect by orderly clustering anti-PHB antibody fragments on QD particles. Our SPR results in this study revealed clear evidence of the avidity effect by showing that the dissociation rate was drastically decreased by the use of QD. The quantitative kinetic and thermodynamic analyses are in progress.

In conclusion, we functionalized QD nanoparticles by

orderly conjugating anti-PHB antibody fragments for biopolymer PHB to perform spontaneous and selective stacking of inorganic nanoparticles on polymer-coated plate by mixing operation in neutral solutions at room temperature. SPR analysis clearly demonstrated the avidity effect causing the strong binding of the QD particles with multi antibodies. The multistacking techniques in neutral solutions at room temperature may have the potential for the bottom-up stacking procedures for hybrid assembly.

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