Supplementary Material (ESI) for Chemical Communications

Electronic Supporting Information

An Enzymatic Kinetics Investigation into the Significantly Enhanced Activity of Functionalized Gold Nanoparticles

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Chemicals. Crystallized and lyophilized lipase (EC 3.1.1.3) from *Candida rugosa*, 4-nitrophenyl palmitate (*p*NPP), phosphate-buffered saline tablets (PBS, pH 7.4), and 3-mercaptopropyltrimethoxysilane (MPTMS) were purchased from Sigma. Trisodium citrate dehydrate was purchased from SHOWA (Chemical Co., Japan). Hydrogen tetrachloroaurate trihydrate ($HAuCl_4 \cdot 3H_2O$) was purchased from Sigma–Aldrich. Propan-2-ol (IPA), dimethyl sulfoxide (DMSO), and sodium chloride were obtained from TCI (Tokyo Chemical Industry Co.). Deionized (DI) water was purified (>18.2 MΩ cm) using a Milli-Q water system (Millipore).

Gold Nanoparticles (AuNPs). The AuNPs were prepared in aqueous solution using a previously reported chemical reduction method.[1] Aqueous solutions of 1 mM $HAuCl_4$ (10 mL) and 38.8 mM trisodium citrate dehydrate (1 mL) were mixed and then heated under reflux for 15 min with vigorous stirring. The color of the solution gradually changed from yellow to purplish-red. After cooling to room temperature, colloidal AuNPs were formed in the solution. SEM images and absorption spectra confirmed the size and shape of these AuNPs.

AuNPs-Lipase complex. Prior to labeling the AuNPs with the enzyme, the gold colloid was purified through centrifugation (13,000 rpm, 20 min)[2] to exchange the buffer solution while maintaining the temperature at 30 °C. Lipase was added to a solution of 13 (± 1)-nm AuNPs (1.5 μM enzyme per 1 mL of 2.2 nM colloidal gold) and the mixture incubated for 10 min. Lipase concentration was determined using

UV-Vis spectroscopy (molar extinction coefficient 3.7×10^4 M^{-1} cm^{-1} at 280 nm). In the experiment of determining the coverage of enzyme onto gold nanoparticles, the different concentrations of lipase was added into the colloidal gold to make the corresponding solution. After the addition of salt solution, we displayed the plots of changes in the time-dependent absorption ratio (A620/A520) in the presence of varying concentration of lipase.

Assaying the Free and AuNP-Bound Lipase. The reaction solution was prepared by mixing PBS solution (140 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer; pH 7.4; 30 °C; 1.3 mL), 0.5 mM 4-nitrophenyl palmitate in propan-2-ol (0.1 mL), and various concentrations of the free or AuNPs-lipase complex (0.1 mL). After mixing the catalytic substance with the reactants, the initial product release at the onset of the reaction was measured using a personal computer and a Hitachi UV-Vis-3310 enzyme reaction measurement system (a UV–Vis spectrophotometer possessing a temperature-controlled thermostatted cell holder; Hitachi, Tokyo, Japan) monitored at 405 nm.[3] In the experiments of varying the ratios of gold nanoparticles and enzyme concentration, we recorded the initial reaction rates from the slopes of the changes in absorption over time. The absorbance was converted to a concentration scale by a molar extinction coefficient of 12,800 M^{-1} cm^{-1} for 4-nitrophenol (*p*NP).

Michaelis–Menten Kinetics. The substrate was dissolved in the assay buffer (at concentrations of 5.55, 8.33, 11.11, 16.67, 22.22, 33.33, 44.45, 66.67, 88.89, and 133.33 μM). The free or immobilized enzyme (1.5 μM) was added to the various concentrations of the substrate in the assay buffer as described above. The initial reaction velocity of the change in absorbance at 405 nm was recorded. The plot of the initial velocity of the production of 4-nitrophenol (*p*NP) versus the *p*NPP concentration was fitted to a hyperbolic curve. The values of K_M and Vmax were obtained through nonlinear regression analysis using SigmaPlot 2001 (v. 7.0) and Enzyme Kinetics Module (v. 1.1, SPSS, Chicago, IL USA) software.[4] The assays were obtained in triplicate; average values are reported.

Schematic mechanism of lipase-catalyzed reactions:

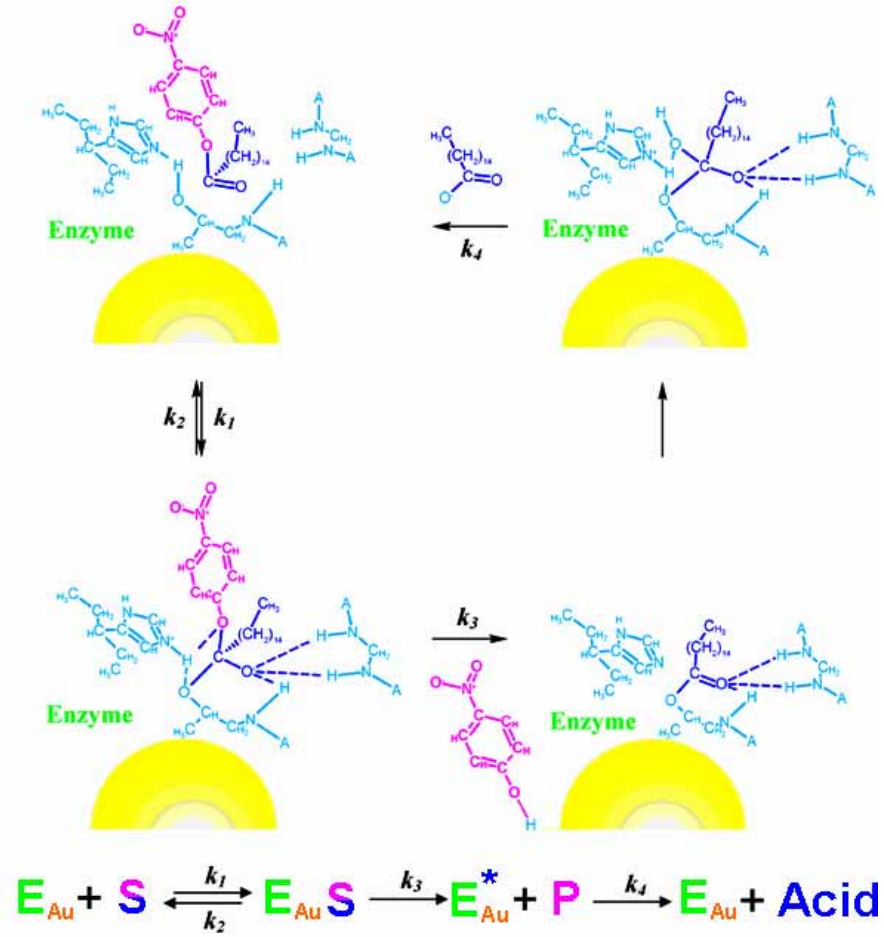


Figure S1. Mechanism of the catalytic reaction mediated by the enzyme-functionalized AuNPs.

A linker-free AuNP-bound lipase was developed to study the kinetics of the immobilized enzyme. To the best of our knowledge, the linker-free approach for the AuNP-lipase system has not been reported. Our kinetic and structural studies led us to propose the mechanism depicted in Fig. S1. There are four independent rate constants (k_1, k_2, k_3, and k_4) in this system.[5] Initially, the substrate (S) is bound to the active site of the enzyme (E) with a rate constant k_1, forming an intermediate complex (ES). Crossing over the transition state, the ES complex is converted into the acyl enzyme (E^*) with a rate constant k_3, releasing the product (P), in this case *p*NP. The acyl bond is then cleaved, with a rate constant k_4, upon nucleophilic attack, returning the enzyme to its initial state.

$[E_0]$ is the total enzyme concentration; the kinetic parameters k_{cat} (turnover number), K_M (apparent dissociation constant), and k_{cat}/K_M (apparent specificity constant) are defined as follows:

$$k_{cat} = \frac{k_3 k_4}{k_3 + k_4}$$

$$K_M = \frac{k_2 + k_3}{k_1} \frac{k_4}{k_3 + k_4}$$

$$\frac{k_{cat}}{K_M} = \frac{k_1 k_3}{k_2 + k_3}$$

The rate constants in Fig. S1 are related to the experimental values of K_M and k_{cat} through the following equations:

$$v = \frac{k_{cat}[E_0][S]}{K_M + [S]}$$

$$V_{max} = k_{cat}[E_0]$$

The value of K_M for the kinetic studies represents the affinity of the enzyme toward the substrate; the rate-limiting step (k_{cat}), which occurred prior to formation of the acyl enzyme intermediate, was determined by monitoring the product's release.[6] According to these equations, we observed almost the same value of V_{max} for the reactions of the bound and free enzymes, indicating no significant difference in their rate-limiting steps (k_{cat}) at identical values of $[E_0]$. Therefore, the lower value of K_M for the AuNP-bound lipase reflected its increased activity (higher v) at each concentration of the substrate.

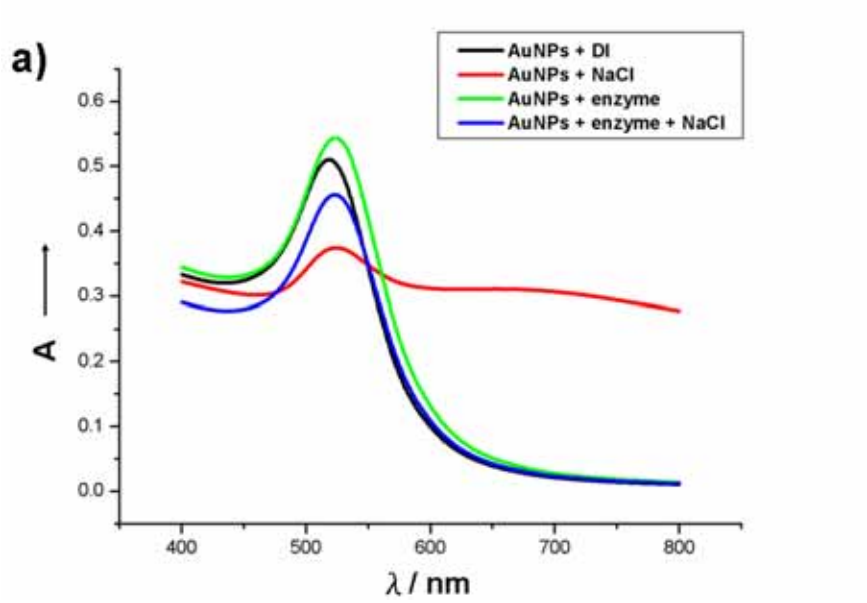


Figure S2. a) Determining the degree of enzyme immobilization on non-aggregated AuNPs. UV–Vis absorption spectra of AuNPs in DI water (black), AuNPs in salt solution (red), enzyme-capped AuNPs in DI water (green), and enzyme-capped AuNPs after adding salt solution (blue).

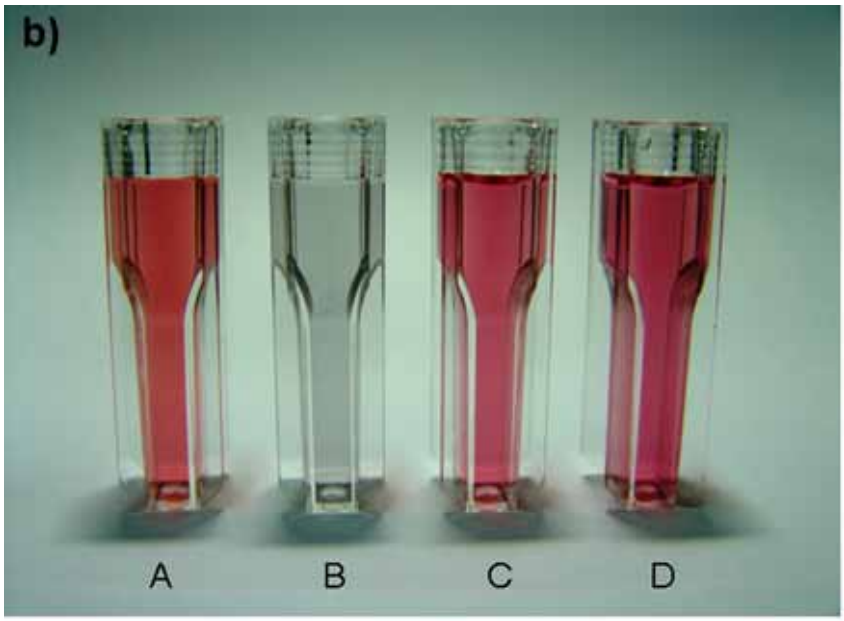


Figure S2. b) Photographic image of solutions of A) AuNPs in DI water, B) AuNPs in salt solution, C) AuNPs capped with lipase in DI water, and D) AuNP-bound lipase in DI water after adding salt solution. The variation in color allowed discrimination

between the aggregated and non-aggregated AuNPs in aqueous solution; i.e., a distinguishable color change from red (A, C, and D) to blue (B) occurred upon aggregation.

As for any conjugation procedure, determining the enzyme coverage on the surface of the AuNPs and the optimal ratio of AuNPs to enzyme are important for activity assays. There is a simple method to discriminate between the aggregated and the nonaggregated AuNPs in aqueous solution, based on distinguishable color changes from red to blue upon aggregation.

The UV–Vis absorption spectrum of the AuNPs solution (black trace) exhibited a strong surface plasmon resonance (SPR) at ca. 520 nm; adsorption of the enzyme onto the AuNPs caused a slight shift (green trace) in the SPR peak (Figure S2 (a)). The gold colloids aggregated at a high concentration of electrolytes in the absence of a protecting coating layer (red trace). After adding salt solution to the enzyme-capped AuNPs, the absorption signal remained sharp (blue trace) and the solution retained the color of the enzyme-capped AuNPs (Figure S2 (b)).

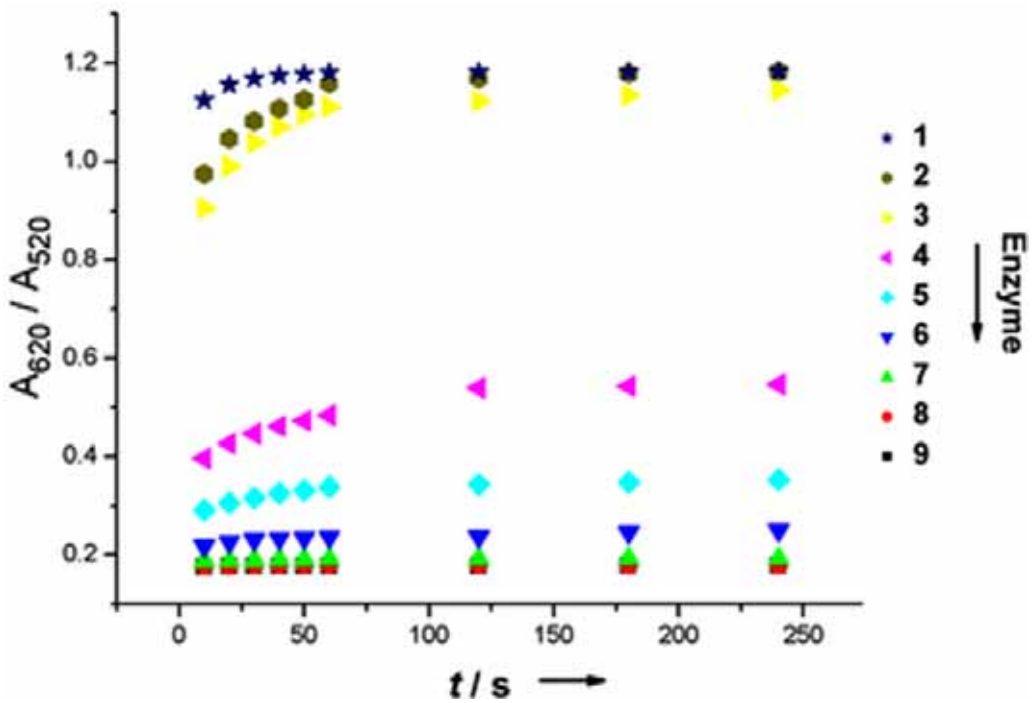


Figure S3. Plots of changes in the time-dependent absorption ratio (A620/A520) in the presence of varying concentration of lipase after the addition of salt solution: 1) 93.75 x 10^{-9} M, 2) 18.75 x 10^{-8} M, 3) 22.5 x 10^{-8} M, 4) 30 x 10^{-8} M, 5) 37.5 x 10^{-8} M, 6) 0.45 x 10^{-6} M, 7) 0.75 x 10^{-6} M, 8) 1.5 x 10^{-6} M, 9) 2.4 x 10^{-6} M.

The determination of the coverage of lipase enzyme onto the gold nanoparticles:

In general, the binding between two substances can be described by a simple equilibrium expression.

$$P + E \rightleftharpoons PE$$

The reaction is characterized by equilibrium constant, K_a, such as:

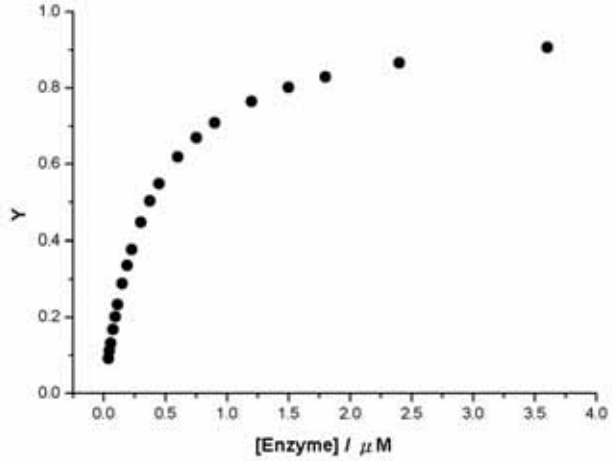
$$K_a = \frac{[PE]}{[P][E]}$$

Where
P: binding site of particles
E: enzyme
K_a: association constant
K_d: dissociation constant
So, $K_d = 1/K_a$

Define fraction, Y = the fractional occupancy of the gold nanoparticles-binding sites:

$$Y = \frac{\text{binding sites occupied}}{\text{total binding sites}} = \frac{[PE]}{[PE]+[P]} = \frac{[E]}{[E]+K_d}$$, At Y = 1/2, K_d = [E] = 0.37 μM

Y versus the concentration of enzyme [E] = enzyme dissociation curve:



The concentration of enzyme [E] that we prepared for kinetic assays was 1.5 μM. The degree of coverage can be determined from above hyperbolic curve.

In the simple equilibrium case, lipase was added to a solution of the AuNPs to form the AuNPs-enzyme complex. Actually, it was a system with mixture of particle-bound and unbound enzyme for the catalytic reaction in our manuscript. In fact, the AuNPs-enzyme complex system has reached chemical equilibrium, the state in which the concentration of the AuNPs and enzyme remained constant. According to Le Châtelier's principle, if the unbound enzyme was removed in the process of purifying nanoconjugates, the particle-bound enzyme desorbed from the surface of the AuNPs and the chemical equilibrium was broken. It would result in the aggregation of the AuNPs because of the insufficient amount of enzyme. Therefore, we developed a linker-free AuNPs kinetic assays system to product detection at equilibrium.

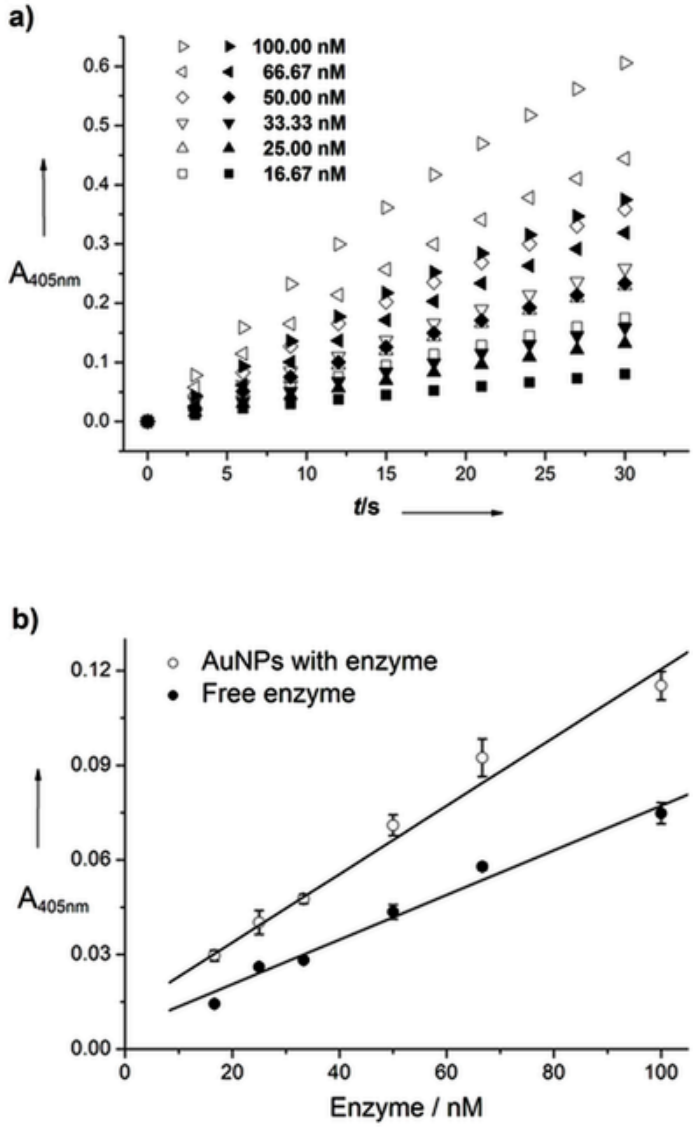


Figure S4. a) Product (*p*NP) formation over time in the catalytic reactions of various concentrations of enzyme-AuNPs complex (solid) and free lipase (hollow) solutions monitored at 405 nm; b) Initial velocities of *p*NP from *p*NPP plotted as a function of the concentration of the free enzyme (●) and the enzyme-capped AuNPs (○).

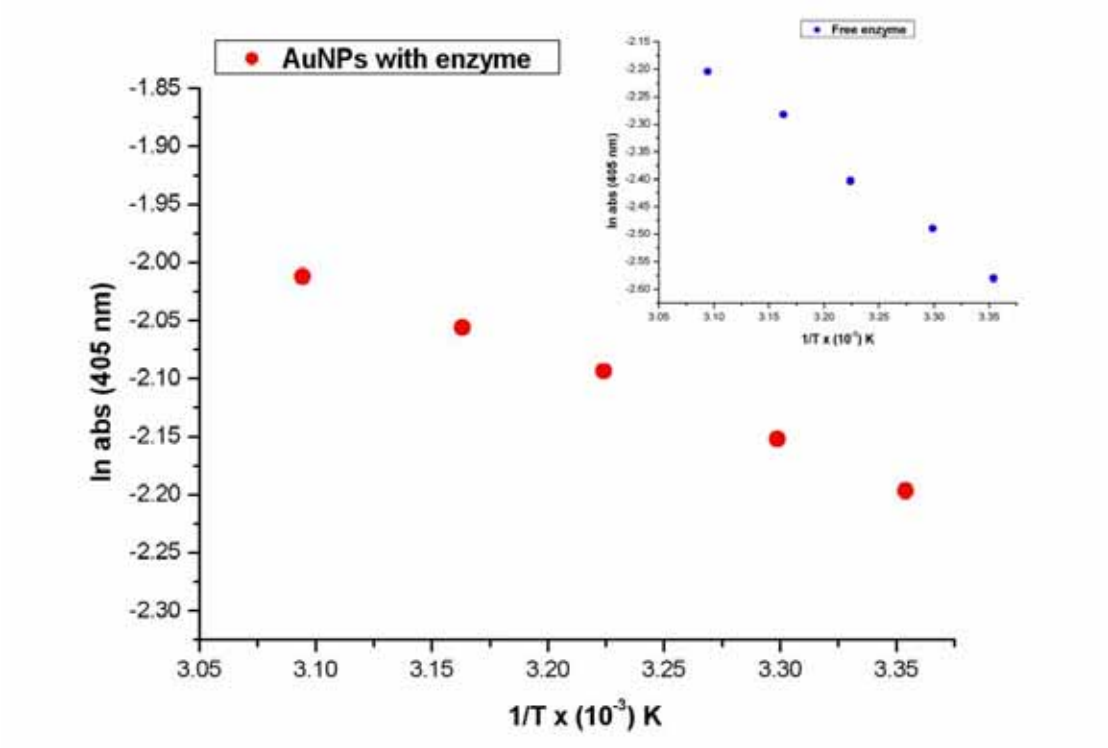


Figure S5. Arrhenius plots of ln(absorbance) versus the reciprocal of absolute temperature for the free (blue, inset) and enzyme–AuNPs complex (red) lipase. The activity was measured for reaction mixtures containing 0.1 μM enzyme at pH 7.4.

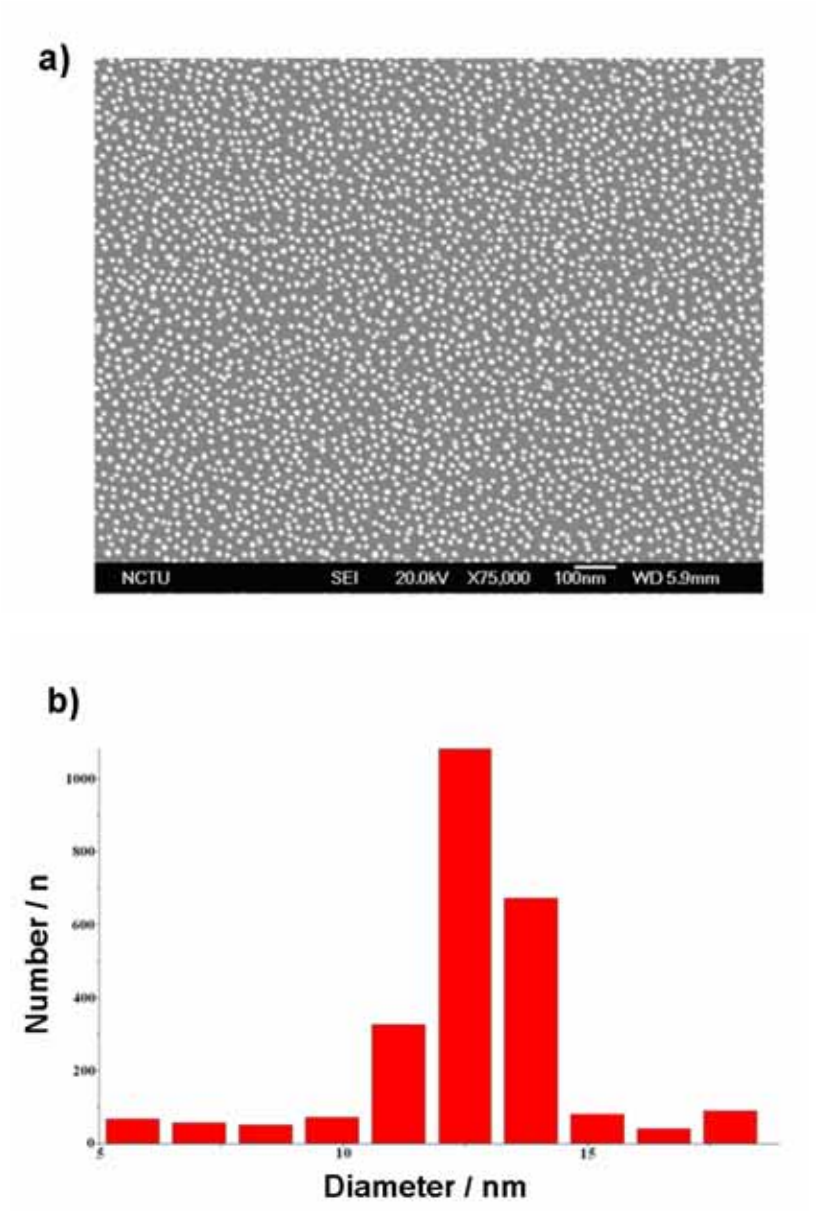


Figure S6. a) SEM image of AuNPs immobilized onto a silicon dioxide surface through an MPTMS linker. b) Particle size distribution of the AuNPs present in the SEM image, analyzed using a personal computer and Image-Pro Plus software.

The SEM image was recorded using a JEOL JSM-6700F electron microscope operated at 20 kV. The sample for SEM analysis was prepared by placing a few drops of the AuNPs solution onto a silicon wafer modified with the MPTMS linker. The sample was rinsed with DI water for 10 min and then dried prior to viewing under the electron microscope.

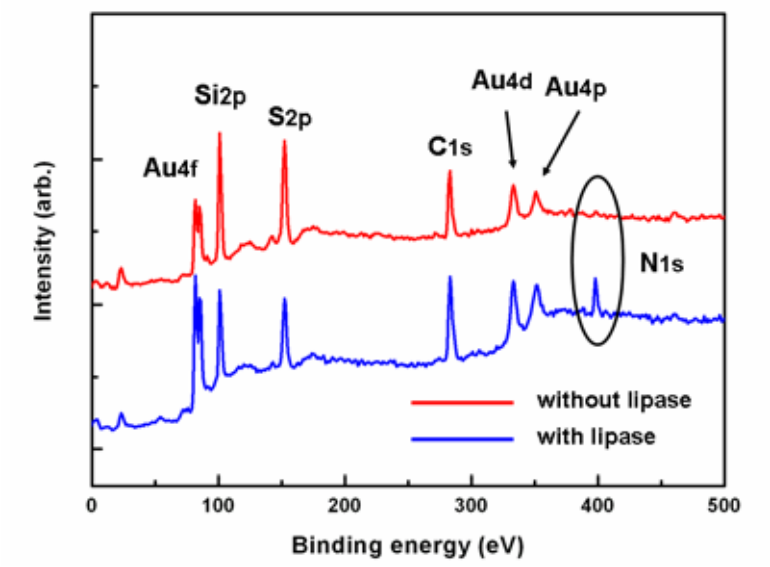


Figure S7. XPS spectra of AuNPs measured in the presence (blue) and absence (red) of the enzyme on the silicon dioxide surface.

The X-ray photoelectron spectroscopy (XPS, VG Scientific Microlab F310) was used to verify the attachment of the enzymes onto the surfaces of the AuNPs. For the AuNPs modified with lipase, the marked peak indicates the presence of NH_2 groups (N_{1s} binding energy = 399.6 eV) under X-ray irradiation initiated by the photoelectrons and secondary electrons emitted from the surface.[7] The result is in agreement with the existing literature for proteins and enzymes bound to the surface of AuNPs without surface modification.[8]

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