An enzymatic kinetics investigation into the significantly enhanced activity of functionalized gold nanoparticles†

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Received (in Cambridge, UK) 26th June 2008, Accepted 1st August 2008
First published as an Advance Article on the web 17th September 2008
DOI: 10.1039/b810889g

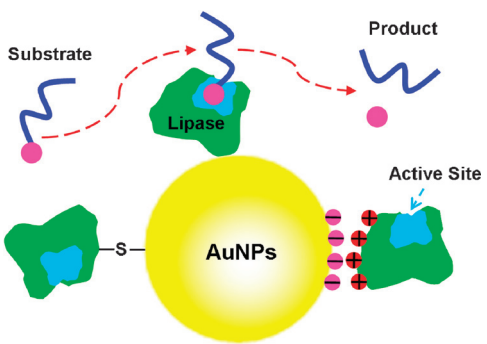
Kinetic and thermodynamic studies reveal that the property of significantly enhanced catalytic activity with colloidal stability is attributed to an efficacious means of tuning enzyme–substrate association by varying with the rate constants in the presence of functionalized gold nanoparticles.

Nanoparticles are attracting substantial interest in the rapidly developing area of nanobiotechnology.[1] Gold nanoparticles (AuNPs), which have high affinity for biomolecules, have been used as biosensors,[2] immunoassays,[3] therapeutic agents,[4] and vectors for drug delivery;[5] thus, the conjugation of AuNPs and biomolecules has become a major area of research for advancing the use of nanotechnology in biomedical applications.[6] Indeed, proteins, enzymes, DNA, and oligonucleotides have all been immobilized onto AuNPs; the physicochemical characteristics of these functionalized AuNPs have been investigated in a variety of academic studies.[7] Several techniques have been used to immobilize enzymes on various nanostructures in attempts to improve the enzymatic activity and stability.[8] Although some enzyme-functionalized AuNPs exhibit enhanced catalytic activity, which has been supposed to favorable conformational changes and electrostatic interactions,[9] there have been no detailed studies aimed at quantifying the differences between the enzymatic behavior of AuNPs-immobilized and free enzymes. Besides, it ought to evolve the regulable enzyme-functionalized nanoparticle with optimal efficiency and indicate the ability of the AuNPs to act as a factor for enhancing activity.

Enzymes immobilized onto AuNPs in the absence of a linker, using rapid and uncomplicated processes, generally possess higher activity bound to the surfaces through chemical modification. In previous reports, catalytic activity of enzyme-functionalized AuNPs has been investigated with the surface modification of linkers.[8,9] However, most of these kinetic investigations need steps such as modified biomolecules onto the AuNPs surface and separating the modified AuNPs from the unmodified AuNPs or surplus molecules. These steps, firstly, led to complication and relatively high cost of the experiments. In addition, long-time course (covalent bond) also led to activation loss of enzyme. What's more, the target binding sites and conformational changes of the enzyme after binding were not all known precisely, so labeling sites were not only difficult to design, but also could weaken the affinity between the reactant and the enzyme.[10] Therefore, developing modification-free AuNPs kinetic assays to simplify the detection process would be important and attractive. In this work, we have systematically investigated the interactions between the nanoparticle monolayer and the affected substrates by quantifying the kinetic parameters to understand the enhanced catalytic action of the AuNPs–lipase complex. We think that such fundamental research will be beneficial for the development of new nanobiotechnological applications.

Lipases are used industrially as detergent enzymes, in paper and food technology, in the preparation of specialty fats, and as biocatalysts for the synthesis of organic intermediates, and in various clinical studies and drug delivery.[11] The kinetic model of lipases is based on the so-called ping-pong mechanism, which also applies to many other enzymes, such as glucose oxidase, horseradish peroxidase, and alkaline phosphatase. As a representative esterase, lipase is an excellent model for studying the enhanced activity of the AuNPs-bound enzymes because of its well-defined structure, properties, and applications. Scheme 1 provides a schematic mechanism of lipase-catalysed reactions (see the ESI for a detailed discussion†).

As shown in Fig. 1, by exploiting interactions between the AuNPs and lipase, colorimetric changes of the AuNPs could sensitively differentiate the enzyme concentration after titrating with the salt solution. We monitored the stability of the AuNPs solution by its color and the absorbance spectra. If the enzyme did not cap the AuNPs completely, we obtained spectra with the aggregation of the AuNPs. As long as the solution changed dramatically with addition of electrolyte from pink red to violet



Scheme 1 Schematic representation of the mechanism of catalysis of enzyme-functionalized AuNPs. Color scheme: AuNPs, gold; enzyme, green; first product, pink; second product, blue.

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† Electronic supplementary information (ESI) available: Experimental details and additional figures. See DOI: 10.1039/b810889g

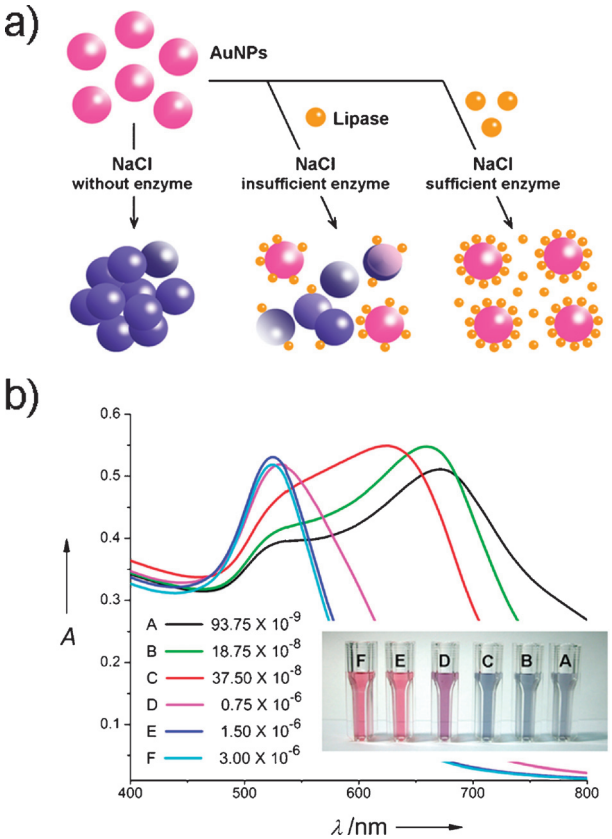

Fig. 1 (a) The diagram of determining the optimal immobilization of lipase-functionalized on gold nanoparticles; (b) variation in UV-Vis absorbance spectra of the AuNPs (2.2 nM) at different concentrations of enzyme after adding salt solution (0.1 mL 10% NaCl solution).

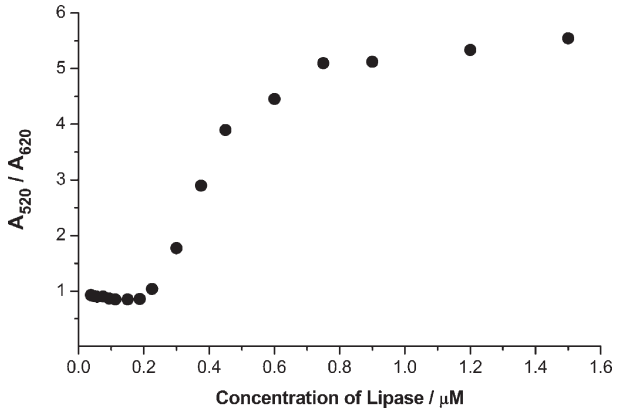

Fig. 2 Plot of the absorption ratio (A520/A620) in equilibrium *versus* the lipase concentration.

blue in seconds, the enzyme concentration is not sufficient to stabilize the suspension (A, B, C, and D traces). In contrast, when the enzyme concentration was sufficient to stabilize and protect the colloid, the solution color remained unchanged and the absorbance spectra exhibited a strong surface plasmon resonance (SPR) at *ca.* 520 nm (E and F traces).

Once the salt is added, the ratio of A620 to A520 with insufficient enzyme displays an increasing trend as shown in Fig. S3,† which indicates that the AuNPs/lipase system gradually loses the stability protected by enzyme. The destabilization trend is obviously dependent on the concentration of lipase.[10] Fig. 2 displays a plot for the reciprocal of the ratio (A620/A520) of colloid solution stabilized by the different lipase concentrations after the addition of salt (0.1 mL 10% NaCl solution).[12] When the lipase concentration was enough to provide the stability for the AuNPs/lipase system, the absorption ratio remain constant with time. On the contrary, there is a significant decrease in A520/A620 ratio while insufficient enzyme was added. This result suggests that the enzyme coverage on the surface of the AuNPs can be defined by the ratio dependent on the lipase concentration. According to the observation, we obtained the dissociation constant K_d; and further, the enzyme coverage on the surface of the AuNPs can be elevated up to 80% (see the ESI for detail†).

We used the system of the direct binding onto AuNPs without surface modification for avoiding the congregation issue and determining the enzyme coverage. This linker-free approach has not been reported previously. The formation of lipase–AuNPs complex has been optimized with regard to its colloidal stability and retention of activity for the purpose of kinetic assays.

Prior to kinetic experiments, we dissolved 4-nitrophenyl palmitate (*p*NPP) in isopropyl alcohol solution, and then mix the reactants in reaction buffer.[13] We measured the activities of the free lipase and enzyme–AuNPs complex by monitoring (at 405 nm; Fig. S4(a)†) the initial velocity of 4-nitrophenol (*p*NP) production from substrate-*p*NPP at various concentrations of the enzyme solutions in 1 cm pathlength cuvettes and the changes in absorbance were recorded as a function of the reaction time (in real-time). We subtracted the background (the absorbance of AuNPs) to obtain the absorbance of product-*p*NP in the reaction. In Fig. S4(b),† we observed that the initial release of *p*NP was proportional to the concentrations of both the free enzyme and enzyme–AuNPs complex—with a significant increase in the catalytic activity in the presence of functionalized AuNPs. Moreover, this result suggested that the catalytic mechanism and the analytic method observed the enzymatic kinetics equations in both cases, allowing us to perform accurate kinetic assays.

To optimize the supreme efficiency of enhanced catalytic activity, the ratios of AuNPs to enzyme were studied under fixed enzyme and substrate concentration. Fig. 3 showed the quantitative relationship between the production rate change at 405 nm and the different concentrations of AuNPs. The non-aggregated enzyme–AuNPs complex mixed with assay buffer showed an increase in the absorbance. Moreover, the largest change in reaction rate, which was regarded as optimal condition from dispersed to aggregated nanoparticles, was observed when the concentration of AuNPs was approximately 2.2 nM in common with curve E in Fig. 1(b). It is noted that there was a significant increase in the production rate with addition of AuNPs as compared to the control (without AuNPs). Each increase in concentration of 25% resulted in an increase in reaction rate by about 0.14 μM s^{-1} and thereby demonstrated the ability of the AuNPs to act as a factor for enhancing activity.[9] Over optimal conditions, reaction rate decreased because the redundant AuNPs aggregated without sufficient coverage and lost their colloidal stability.[14] Therefore, it was supposed that catalytic activity can be manipulated by the ratios of AuNPs to enzyme.

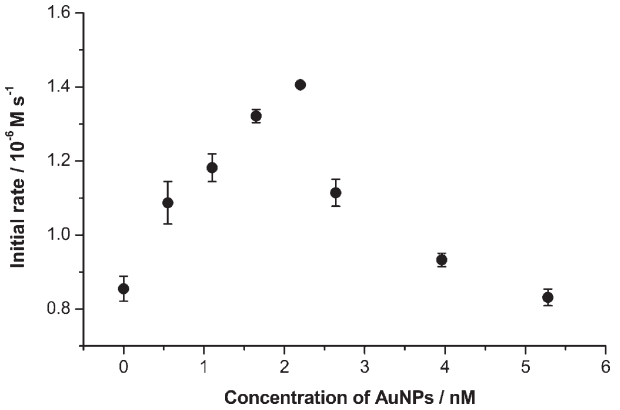

Fig. 3 Initial rate of *p*NP production *versus* the concentration of AuNPs. Conditions: pH 7.4, 30 °C, [enzyme] = 100 nM, [substrate] = 22.22 μM.

Fig. 4 displays Michaelis–Menten plots for the hydrolysis of *p*NPP (5.55–133.33 μM) by the free enzyme and enzyme-capped AuNPs at pH 7.4 and 30 °C. The value of the maximum velocity (V_{max}) was the same in the absence and presence of the AuNPs, but the Michaelis constant (K_M) was obviously smaller in the presence of functionalized AuNPs. We infered that the enzymes capped on the AuNPs retained their ability to perform nucleophilic attack *via* the formation of an acyl enzyme; *i.e.*, the presence of the AuNPs had no influence on the release of product in the rate-limiting step. The values of the Michaelis constant were 23.91 and 9.10 μM ($p < 0.05$) for the free and enzyme–AuNPs complex, respectively. A smaller value of K_M represented a higher affinity of an enzyme toward a substrate; *i.e.*, the presence of the AuNPs enhanced the selectivity of lipase toward the substrate. Because the binding of lipase on the surface of the AuNPs affected only the value of K_M, the addition of the AuNPs is an efficacious means of tuning the enzyme–substrate association. The active site of an enzyme not only recognized the substrate(s) to create an intermediate complex but also complemented and stabilized the transition state. The higher affinity of the substrate toward the enzyme-capped AuNPs resulted in a lower-energy transition state; indeed, the linear Arrhenius plots for the hydrolyses of *p*NPP mediated by the enzyme–AuNPs complex and free enzyme at 25–50 °C (Fig. S5 in the ESI†) provided activation energies of 5.9 and 12.2 kJ mol^{-1}, respectively. Thus, the enzymes immobilized onto the surfaces of the AuNPs exhibited higher

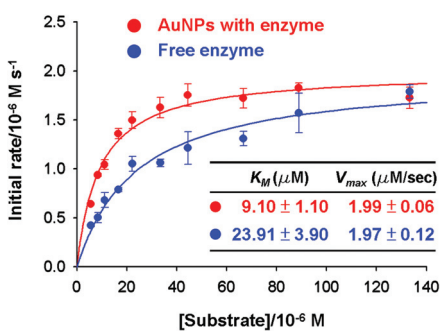

Fig. 4 Michaelis–Menten plots for the hydrolyses of *p*NPP mediated by the free enzyme (blue) and enzyme–AuNPs complex (red).

catalytic activity through more ready formation of the ES complex and reduction of the activation energy.

In summary, we have investigated the kinetics of the significantly enhanced activity in the presence of functionalized AuNPs. The immobilization of lipase onto the nanoparticles' surfaces, without any surface modification, provided colloidal stability that allowed us to determine the enzyme coverage on the surface of particles in our reaction system. In addition, the efficiency of enhanced catalytic activity with colloidal stability has been optimized by regularizing the ratios of AuNPs to enzyme. The lipase capping the AuNPs catalysed the hydrolysis of *p*NPP through the same kinetic model as that of the free enzyme, with the product's release being the rate-limiting step, but with increased catalytic activity, as evidenced by lower values of the activation energy and K_M. We ascribe this behavior to the enhanced substrate selectivity of the enzyme–AuNPs complex and thereby decrease the activation energy through enhancing the rate constants leading to the formation of the ES complex. In biological systems, regulation of enzyme activity plays a central role in governing cell behavior.[15] Our findings suggest that the ability of AuNPs to manipulate activity might become an important nanobiotechnological tool for optimizing clinical studies and improving drug delivery in the future.

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