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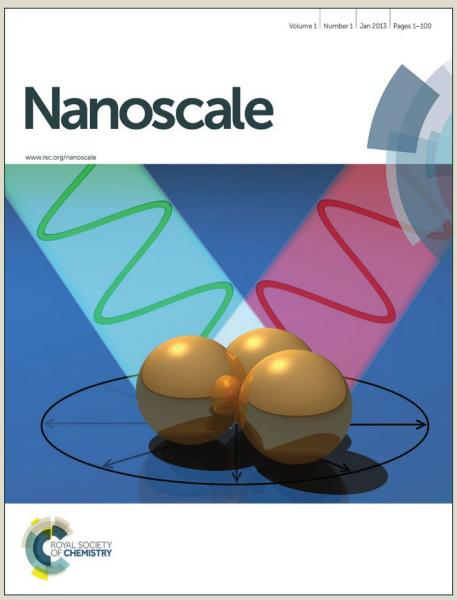

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A plasmonic nanosensor for lipase based on enzyme-controlled gold nanoparticles growth in situ

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A plasmonic nanosensor for lipase was developed based on one-pot nanoparticle growth. Tween 80 was selected not only as the substrate of lipase but also as the reducing and stabilizing agent in the sensor fabrication. The different molecular groups in Tween 80 could have different roles in fabricating procedure, the H_2O_2 produced by Tween 80 autooxidation in its ethylene oxide subunits could reduce the $AuCl_4^-$ to Au atoms, meanwhile, the lipase could hydrolyze its carboxyl ester bond, which could, in turn, control the rate of nucleation of gold nanoparticles (AuNPs) and tailor the localized surface plasmon resonance (LSPR) of AuNPs transducers. The color changes , which depending on the absence or presence of lipase, could be used to sense the lipase activity. A linear response ranging from 0.025 to 4 mg mL^{-1} and the detection limit of lipase as low as 3.47 μg mL^{-1} were achieved. This strategy circumvents the problems encountered by general enzyme assay that require sophisticated instruments and complicated assembling steps. And its methodology can benefit the assays of heterogeneous-catalyzed enzymes.

1. Introduction

Lipases have been widely applied as versatile biocatalysts for ester synthesis, hydrolysis, interesterification, which make the lipases a choice for potential applications in the food, cosmetic and pharmaceutical industries.[1-4] High-throughput lipase assays are essential tools in enzyme engineering and biological industries. One problem encountered in high throughput screening is to reliably assay many different samples at the lowest possible cost and with very good accuracy.[5]

In nanobiosensor fields of enzyme assays, the lipase assay is still virgin ground.[6,7] Unlike other hydrolases, such as acetylcholinesterase,[8,9] phosphatase[10,11] and pyrophosphatase[12, 13] that act at the aqueous phase, the biosensor could be constructed easily in the homogeneous environment by linking enzyme substrate on nanoparticles.[14] Lipases are unique as they perform at the oil-water interface.[15] The water-insoluble lipase substrates need to be emulsified, where surface active amphiphiles are required at the interface. The presence of the detergents can therefore complicate the assay environment and drastically influence authenticity of measurements.[2, 16, 17] Therefore, developing a reliable, convenient, and sensitive nanobiosensor to detect a true lipase activity is required.

Advances in nanotechnology have now made nanoparticle biosensors possible to enhance or supersede current analytical techniques.[18] Gold nanoparticles (AuNPs) are outstanding building blocks for the fabrication of biosensors because of their unique optical properties for localized surface plasmon resonance (LSPR) and easy synthesis.[19-22] Generally, the construction of AuNPs-based sensors for target analytes involved two steps: AuNPs were synthesized firstly and then decorated with recognition elements such as proteins, polysaccharides, antibodies, RNA and DNA aptamers and these steps required various combinations of washing, heat cycling and incubations.[18, 23] And some assay systems are also limited by high background signals caused by nonspecific adsorption of decorated AuNPs surfaces. These problems can be got round by generaling AuNPs nanostructures *in situ* that tailor the LSPR of AuNPs.[21, 24] The seminal researches were carried out by Willner and Stevens group separately, they presented the signal generating mechanisms by using enzymes to control the rates of metal nanocrystal nucleation on plasmonic transducers.[21, 25, 26] But the

scope of these enzymatic assays was limited to dehydrogenases, redox enzymes and oxidases.[27-33]

Tween family (Tween 20, 40, 60, 80 and so on) are amphipathic, nonionic surfactants composed of fatty acid esters of polyoxyethylene sorbitan, which were used for substrates of lipase.[34-38] Among them, Tween 80 differs in moleculor structure, where the fatty acid ester side chain is unsaturated, containing a double bond. There was a report that the Tween 80 took a role of reductant *in situ* synthesis of AuNPs.[39] Autooxidation of the polysorbates occurs along the ethylene oxide moieties of Tween 80 and produces H_2O_2.[40-42] In addition, it is reported that the unsaturated fatty acid ester substituents not only participated in the autoxidation of Tween 80, but were twice as reactive as the ethylene oxide moieties.[43]

Inspired by the multifunctional properties of Tween 80, we here constructed a plasmonic nanosensor for lipase assay based on enzyme-controlled AuNPs growth *in situ*. In this procedure, the addition of lipase is a key step. The H_2O_2 produced by Tween 80 autooxidation could reduce the $AuCl_4^-$ to Au atoms[39-42], meanwhile in the presence of the lipase, the enzymatic hydrolysis of the carboxyl ester bond in Tween 80 could control the rate of nucleation of AuNPs and tailor , in turn, the LSPR of AuNPs transducers. The biocatalytic hydrolysis by lipase is linked to the growth of AuNPs to obtain purple or red-coloured solutions in the absence or presence of the lipase, respectively. The relationship between lipase concentration and LSPR signal hereby makes this approach highly suitable for fabricating a sensitive, simple and cheap sensor for lipase. Furthermore, one of the most important merits is that a one-pot construction process is indeed realized and the multiple steps involved modifications of AuNPs in assembling a sensor are omitted. This simple method might be preferable to sophisticated AuNPs-based enzyme assays which required complex attachement or linking procedures.

2. Materials and methods

2.1. Materials

Chloroauric acid tetrahydrate ($HAuCl_4{\cdot}4H_2O$),Tween 20, Tween 40, Tween 60, Tween 80, disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium hydroxide (NaOH), oleic acid were purchased from Sinopharm Chemical Reagent Company (Shanghai, China). Lipase Novozyme435 (immobilized on acrylic resin) was obtained from Novozymes (Beijing, China). Lipase from Rhizopus niveus (RNL), Pocine pancreas (PPL), Candida rugosa (CRL) were all ordered from Sigma (USA). Food grade lipase (SBE-01Li) was from Xia Sheng Industrial Group (Beijing, China). Lipase from *Bacillus subtilis* was fermented by National Engineering Research Center for Biotechnology in Nanjing Tech University. The 96-well polystyrene plate was purchased from R&D Systems (Shanghai, China). One unit of lipase hydrolyzes 1.0 μmol of Tween min^{-1} at pH 8.5 and 45 ℃. All other chemicals were at least analytical grade reagents and used without further purification. Unless otherwise pointed out, all experiments were carried out at room temperature.

2.2. Instruments

The absorbance of all solutions in 96-well plates was recorded by the SpectraMax Multi-Mode Microplate Reader M3 (Silicon Valley, USA) for quantitative analysis and determination of the optimal detection conditions. Zeta potential measurements were carried out on a Zetasizer Nano ZS90 zeta potential analyzer (Malvern, UK). Analysis of particle diameter was completed by the ZetaPals Particle-size analyzer (Brookhaven, USA).Transmission electron microscopy analysis were performed by using a JEOL JEM-1010 at an accelerating voltage of 100 kV (Japan). The samples for TEM characterization were prepared by placing a drop of colloidal solution on a 200 mesh carbon-coated copper grid and left to dry in the infrared lamp. Ultrapure water was obtained by the Millipore Milli-Q system (Sartorius, Germany).

2.3. Sensing Lipase Novozyme435 Activity

Colorimetric lipase Novozyme435 activity was performed under the following procedures. A volume of 100 μL of Tween 80 (1% v/v), 50 μL of phosphate (10 mM, pH 8.5) buffer (PB), and water were injected into a 2.0 mL plastic centrifugal tube, which made the final volume for 1000 μL, lipases range from 0.025 to 5 mg were added to it, the samples were incubated at 45 ℃ for 10 min, next, a centrifuge was used to separate the lipases, and 10 μL of $HAuCl_4$ (1.5 mM) was pipetted into supernatant. They were followed by incubation for another 3.5 h at ambient temperature. The final mixtures were then transferred separately into 96-well polystyrene plates, and their absorption spectra were recorded using the SpectraMax Multi-Mode Microplate Reader M3.

2.4. Practical application of the strategy

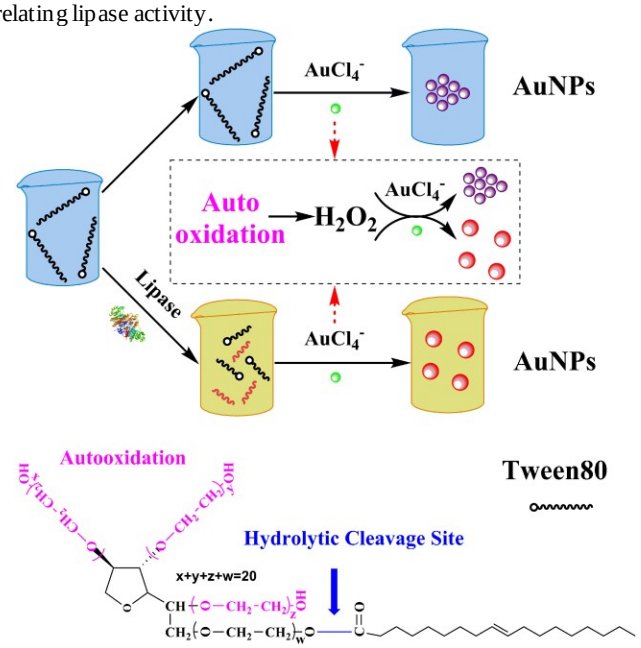
Four commercial purchased lipase samples (RNL, PPL, CRL, SBE-01Li) and the fermentation broth of *Bacillus subtilis* were used for verifying the practicality of the method. 500 μL of 20 mg mL^{-1} different commercial lipase aqueous solutions and 500 μL of fermentation broth were respectively injected into 10.0 mL plastic centrifugal tubes, which containing 500 μL of 10% v/v Tween 80, 250 μL of 0.2 M PB (pH 8.5), 3.75 mL of water and were kept for 10 min in a water bath (45 ℃), then 1mL supernatant in the sample was collected by centrifuging at 10000 rpm for 5 min. The enzyme activity was determined by using the procedure of sensing lipase activity. Furthermore, our controlled trial was conducted by adding 500 μL of inactivated fermentation broth which was previously boiled to aqueous solution containing 10% v/v tween 80 and 0.2 M PB (pH 8.5).

3. Results and discussion

3.1. Sensing Mechanism

Scheme 1 described the sensing mechanism employed in this study. The present system includes Tween 80 solution, the lipase and the

$HAuCl_4$. Tween 80 here was employed not only to act as substrate for lipase recognition, but as reducer to reduce $HAuCl_4$ *in situ* synthesizing AuNPs.[37, 39] The autooxidation in the position of Tween 80's ethylene oxide subunits generates hydrogen peroxide (H_2O_2), which, in turn, is able to reduce $AuCl_4^-$ into Au atoms without using any other reducing agents.[39-42] Furthermore, the nucleation and growth of Au nanocrystals can be directed by lipase activity in the hydrolysis reaction. Hydrolysis-triggered nanocrystal growth was exploited as a platform for plasmonic sensing. In the absence of lipase, the soluble Tween 80 maintained its original structure, the homogeneous phase could provide a fast nucleation environment and make the solution to show purple color; whereas in the presence of lipase, the hydrolytic cleavage of carboxyl ester bond could be catalyzed by lipase. The hydrolytic destruction of the Tween's amphipathic structure decreased the rate of nucleation and the solution color showed red. The changes in Au nucleation rate and particle growth size can be followed by UV-vis spectroscopy for relating lipase activity.



Scheme. 1 Schematic illustration of the colorimetric lipase assay based on enzyme-controlled AuNPs growth *in situ*.

A control experiment was performed to verify the sensing mechanism. Our main interest was to see how the lipase influenced the AuNPs growth. Under optimized conditions, the sample solutions with or without lipase were investigated by UV-vis spectra. As shown in Fig. 1 (vial a, b, d, e, curve a, b, d, e), the sample solutions individually containing Tween 80 or $HAuCl_4$ showed colorless and no spectra absorbance. The sample solution, which the Tween 80 and $HAuCl_4$ simutaneously existed, became purple and exhibited absorption at 530 nm (Fig.1 vial c, curve c), demonstrating that Tween 80 was able to reduce $HAuCl_4$ to AuNPs. When lipase was present, the sample solution appeared red in color and its broad SPR band occurred slight red shift at about 535 nm (Fig. 1, vial f, curve f). The inactivated lipase was used in this sensing assay to ensure the role of lipase in the hydrolysis of Tween 80. The color and absorbance of sample solution containing the inactivated lipase (Fig. 1, vial g, curve g) appeared same purple color and absorbance value as that without lipase, suggesting that the enzymatic hydrolysis of Tween 80 by lipase indeed controlled the growth of the AuNPs.

It is speculated that fatty acid (oleic acid) released by hydrolysis of Tween 80 played a critical role in AuNPs growth. Therefore, a set of control experiments were designed to verify the guess. Instead of lipase, we spiked oleic acid into the solutions, which just had Tween 80 and $HAuCl_4$ without lipase. Addition of the oleic acid indeed induced the color changes, and when oleic acid came up to 2 mM, the solution appeared similar color with the one that the lipase is present (Fig. 2). Zeta potential (ζ) of the AuNPs solution, which correlates with the surface charge and the local environment of AuNPs, was characterized to help understanding the mechanism of assay. The ζ values of the AuNPs are -3.79 mV and -17.1 mV depending on lipase is absent and present respectively (Fig. S1, Supporting information), demonstrating the increase in negative charge on the surface of the AuNPs due to adding lipase.These results demonstrate that the fatty acid formed by hydrolysis acts as a superior stabilizer for AuNPs compared to uncleaved Tween 80.

Further the morphology and size distribution of the AuNPs were characterized by Transmission electron microscopy (TEM) and Particle size distribution images (Fig. 3). The results showed that crystal AuNPs were indeed produced by autooxidative process and their size was associated with the presence of lipase.

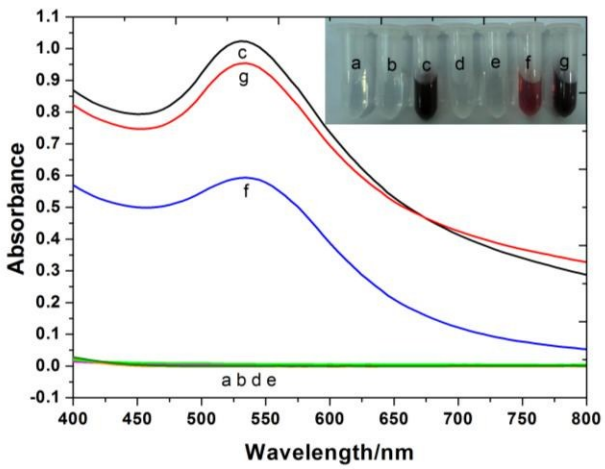


Fig. 1 Absorption spectra and photographs (inset) of solution: (a) Tween 80 (b) $HAuCl_4$ (c) Tween 80 + $HAuCl_4$ (d) Tween 80 + lipase (e) $HAuCl_4$ + lipase (f) Tween 80 + $HAuCl_4$ + lipase (g) Tween 80 + $HAuCl_4$ + inactivated lipase.

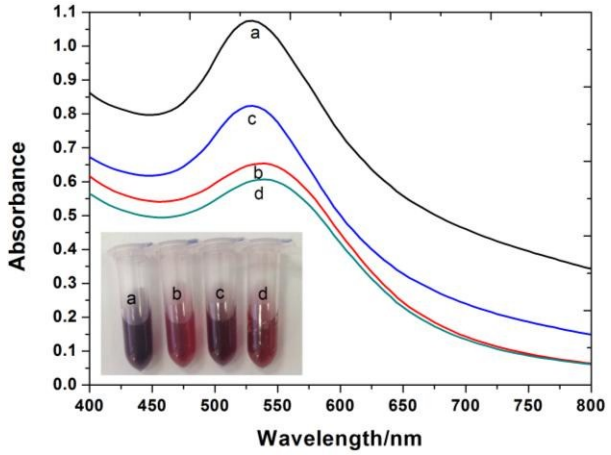


Fig. 2 Absorption spectra and photographs (inset) of solution: (a) Tweeen 80 + $HAuCl_4$ (b) Tween 80 + $HAuCl_4$ + lipase (c) Tween 80 + $HAuCl_4$ + 1 mM oleic acid (d) Tween 80 + $HAuCl_4$ + 2 mM oleic acid.

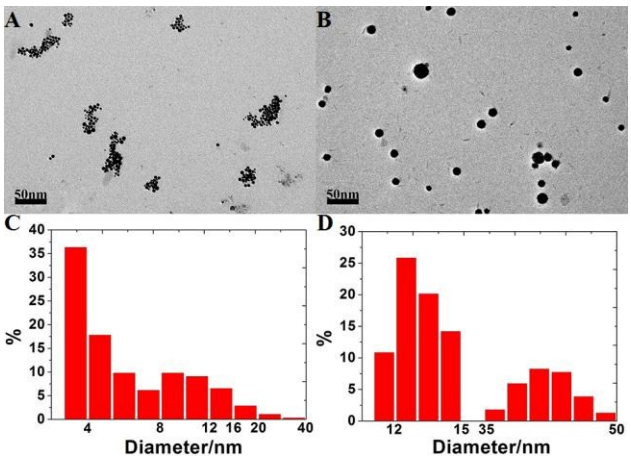


Fig. 3 TEM images: (A) AuNPs; (B) AuNPs + lipase; Particle size distribution (C) AuNPs, (D) AuNPs + lipase

3.2. Time effects on the AuNPs formation

In order to understand the AuNPs growth process, we first investigated the time effects on the AuNPs formation. Fig. 4 shows the time-dependent absorbance of the $HAuCl_4$ reduced AuNPs. It can be seen that the absorbance intensity increase with time whether if lipase is absent (Fig. 4A) or present (Fig. 4B), and the absorbance of the AuNPs tends to saturate after a reaction time of about 3.5 h, thus all biosensing experiments followed the formation of the AuNPs after a time interval of 3.5 h.

Fig. 4C shows the sample solution colors which just contain $HAuCl_4$ and Tween 80 in AuNPs crystal growth. After 30 min, the slight yellow solution turned to purple. But upon addition of the lipase (Fig. 4D), the system had to take 40 min to turn to red color. Comparing the corresponding absorption spectra curves (Fig. 4C and 4D) in the same time interval, the intensity of the AuNPs solution, where lipase is present, is lower. These observations agree with the proposed mechanism that lipase results in a slow rate of crystallization, which in turn favours the growth of the bigger AuNPs.

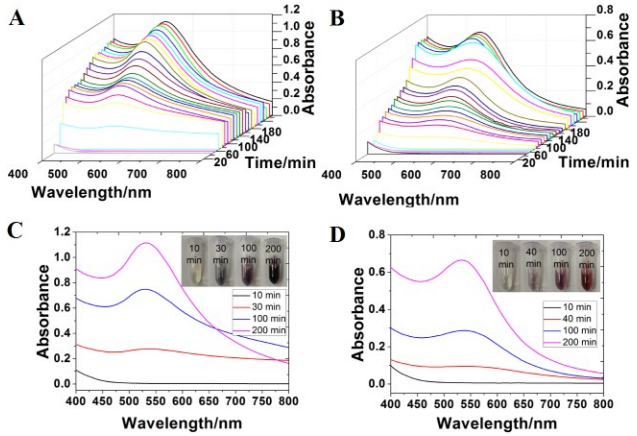


Fig. 4 *In situ* absorption spectra recording the growth process of the AuNPs (A) in the absence of lipase (B) in the presence of lipase. (time interval : 10 min.) Absorption spectra and the corresponding photographs (inset) of AuNPs obtained in different time interval (C) in the absence of lipase (D) in the presence of lipase.

3.3. Optimization of assay conditions

To define the optimal lipase hydrolysis time, several samples with Tween 80 and $HAuCl_4$ are incubated with different concentrations of lipase and the $|\Delta A_{530}|$ values, where $|\Delta A_{530}|$ is the difference between absorbances ($|A_{sample} - A_{blank}|$) at 530 nm, were determined at different reaction times. According to the results obtained (Fig. 5), $|\Delta A_{530}|$ increased continuously in the time interval 0 to 10 min, after 10 min the $|\Delta A_{530}|$ values have no significant differences. So 10 min was chosen as the optimal hydrolysis reaction time. And the rate of increase of $|\Delta A_{530}|$ is proportional to the concentration of enzyme. This suggests that the parameter $|\Delta A_{530}|$ can be used to determine the lipase concentration or activity.

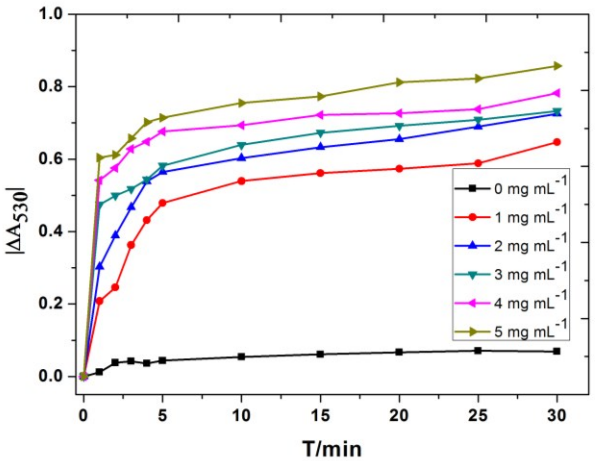


Fig. 5 Kinetic plots of time-dependent $|\Delta A_{530}|$ values versus those with different concentrations of lipase present.

To further verified the literature reports,[43] we employed the same concentration (1% v/v)Tween 20, 40, and 60 to replace 1% v/v Tween 80, reacting with 1.5 mM $HAuCl_4$ under the same conditions. The absorbance of samples contained 1% v/v Tween 20, Tween 40, and Tween 60 respectively is far less than sample contained 1% v/v Tween 80 (Fig. S2, Supporting information), demonstrating that Tween 80 is ideal candidate.

The effects of the Tween 80 concentration were invetigated in the range of 0.1%-3% v/v. (Fig. S3 A, Supporting information). 1% v/v Tween 80 was selected as optimal concentraion in the system. Too low or too high Tween 80 concentration were not good both for reducation of $HAuCl_4$ and for hydrolysis.

The influences of the concentration of $HAuCl_4$ were also explored from 0.1 to 2.0 mM $HAuCl_4$. (Fig. S3 B, Supporting information). To obtain a high signal, 1.5 mM $HAuCl_4$ was selected.

The lipase activity was significantly affected by pH and temperature. The investigations have been carried out to optimize pH and temperature. Results are shown in Fig. S3 C and S3 D (Supporting information). It implies that lipase has an activity maximum at pH 8.5 and 45 ℃, which are chosen as the optimal pH and temperature.

3.4. Sensitivity of assay

Under the optimized conditions, the absorbance of AuNPs solution was investigated as a function of lipase concentrations using UV-vis technique. As depicted in Fig. 6A, the absorbance of AuNPs got gradual decrease at 530 nm with the increasing concentration of lipase from 0 to 5 mg mL^{-1}, at the same time a gradual increase of $|\Delta A_{530}|$ was observed (Fig. 6B) and it increased linearly with lipase concentration over the range of 0.025 to 0.6 mg mL^{-1} and 0.6 to 4 mg mL^{-1}, with correlation coefficients of 0.988and 0.982, respectively. For each concentration of lipase, the measurement was repeated for three times. The limit of detection (LOD) of the lipase was as low as 3.47 μg mL^{-1} ,which is calculated by the equation LOD = 3s/slope, where s represents the standard deviation of the signal obtained from three independent blank measurements and its value is 5.9×10^{-4}.

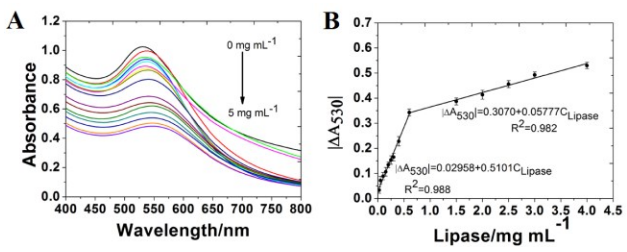


Fig. 6 (A) Absorption spectra of different concentrations of lipase; (B) calibration curves.

3.5. Practical application

A preliminary screening was carried out using this system to test the activities of four commercial purchased lipase samples (SBE-01Li,RNL,PPL,CRL) The color and the $|\Delta A_{530}|$ value of sample solutions were monitored, respectively, after optimal time interval. As shown in Fig. 7A, Tween 80 and $HAuCl_4$ incubated with SBE-01Li, RNL appeared purple color, while with PPL appeared dull-red and with CRL red color. The different color changes were attributed to the different lipase activities. We conclude that the lipase activity decreases in the following order: CRL > PPL > RNL > SBE-01Li. This order is consistent with the results detected with a pH-stat method (Table S1, Supporting information), demonstrating the possibility that the colorimetric assay protocol can be used as an alternative to conventional methods for a high-throughput assay of lipase activity.

Lipases are largely produced from microbes and the *Bacillus subtilis* is an important lipase-producing bacterial genera.[44, 45] The assay was also applied to fermentation broth of *Bacillus subtilis*. The supernatant of the crude sample without any pretreatment was incubated with Tween 80 for 10 min, and then with $HAuCl_4$ for another 3.5 h. The active sample appeared red color (Fig. 7B-b), but inactivated sample solution, which was deactivated by boiling, was purple color (Fig. 7B-a). It shows that the system provides a particularly useful probing approach for real samples that usually have significant background activities, which might cause false-positive signals during the measurements.

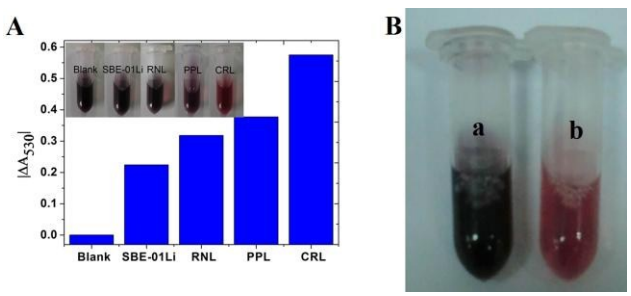


Fig. 7 (A) The plot of $|\Delta A_{530}|$ values of the formed AuNPs, inset: the corresponding photographs; (B) Photographs of the formed AuNPs upon adding (a) inactivated and (b) activated fermentation broth of *Bacillus subtilis*.

4. Conclusion

In conclusion, we have developed a new plasmonic assay for screening lipase activity. It circumvents the problems encountered by general enzyme assay that require sophisticated instruments and complicated experimental steps. The lipase enzyme activity controls growth of gold nanoparticles and generates coloured solutions with distinct tonality when the analyte is present. In bioengineering industries or in resources-constrained conditions, affordable methodologies for the enzyme assay can potentially improve the producing standard.

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†Electronic Supplementary Information (ESI) available: Zeta potential measurements, optimization of assay conditions, pH-stat method. See DOI: 10.1039/b000000x/

1 A. Houde, A. Kademi, D. Leblanc, *Appl. Biochem. Biotechnol.*, 2004, **118**, 155-170.

2 F. Hasan, A. A. Shah, A. Hameed, *Biotechnol. Adv.*, 2009, **27**, 782-798.

3 M. H. Sörensen, J. B. S. Ng, L. Bergström, P. C. A. Alberius, *J. Colloid Interface Sci.*, 2010, **343**, 359-365.

4 G. Yang, J. Wu, G. Xu, L. Yang, *Colloids Surf., B*, 2010, **78**, 351-356.

5 P. Babiak, J.-L. Reymond, *Anal. Chem.*, 2004, **77**, 373-377.

6 K. Saha, S. S. Agasti, C. Kim, X. Li, V. M. Rotello, *Chem. Rev.*,2012, **112**, 2739-2779.

7 E. Hutter, D. Maysinger, *Trends Pharmacol. Sci*, 2013, **34**, 497-507.

8 Y. Zhang, Y. Cai, Z. Qi, L. Lu, Y. Qian, *Anal. Chem.*, 2013, **85**, 8455-8461.

9 D. Liu, Z. Wang, A. Jin, X. Huang, X. Sun, F. Wang, Q. Yan, S. Ge, N. Xia, G. Niu, G. Liu, A. R. Hight Walker, X. Chen, *Angew. Chem. Int. Ed.*, 2013, **52**, 14065-14069.

10 A. Hayat, S. Andreescu, *Anal. Chem.*, 2013, **85**, 10028-10032.

11 D. Rasale, I. Maity, A. Das, *J. Cluster Sci.*, 2013, **24**, 1163-1170.

12 J. Deng, Q. Jiang, Y. Wang, L. Yang, P. Yu, L. Mao, *Anal. Chem.*, 2013, **85**, 9409-9415.

13 J. Sun, F. Yang, D. Zhao, X. Yang, *Anal. Chem.*, 2014, **86**, 7883-7889.

14 J. E. Ghadiali, M. M. Stevens, *Adv. Mater.*, 2008, **20**, 4359-4363.

15 R. Gupta, P. Rathi, N. Gupta, S. Bradoo, *Biotechnol. Appl. Biochem.*,2003, **37**, 63-71.

16 Y. Gargouri, G. Pieroni, C. Riviere, L. Sarda, R. Verger, *Biochemistry*, 1986, **25**, 1733-1738.

17 F. Beisson, A. Tiss, C. Rivière, R. Verger, *Eur. J. Lipid Sci. Technol.*, 2000, **102**, 133-153.

18 P. D. Howes, R. Chandrawati, M. M. Stevens, *Science*, 2014, **346**, 1247390-12473910.

19 W. Zhao, M. A. Brook, Y. Li, *Chembiochem*, 2008, **9**, 2363-2371.

20 X. Cao, Y. Ye, S. Liu, *Anal. Biochem.*, 2011, **417**, 1-16.

21 L. Rodríguez-Lorenzo, R. de la Rica, R. A. Álvarez-Puebla, L. M. Liz-Marzán, M. M. Stevens, *Nat. Mater.*, 2012, **11**, 604-607.

22 P. D. Howes, S. Rana, M. M. Stevens, *Chem. Soc. Rev.*, 2014, **43**, 3835-3853.

23 Z. Wang, L. Ma, *Coord. Chem. Rev.*, 2009, **253**, 1607-1618.

24 V. Pavlov, *Part. Part. Syst. Char.*, 2014, **31**, 36-45.

25 I. Willner, R. Baron, B. Willner, *Adv. Mater.*, 2006, **18**, 1109-1120.

26 R. de la Rica, M. M. Stevens, *Nat. Nano.*, 2012, **7**, 821-824.

27 Y. Xiao, V. Pavlov, S. Levine, T. Niazov, G. Markovitch, I. Willner, *Angew. Chem. Int. Ed.*, 2004, **116**, 4619-4622.

28 B. Shlyahovsky, E. Katz, Y. Xiao, V. Pavlov, I. Willner, *Small*, 2005, **1**, 213-216.

29 Y. Xiao, B. Shlyahovsky, I. Popov, V. Pavlov, I. Willner, *Langmuir*, 2005, **21**, 5659-5662.

30 M. Zayats, R. Baron, I. Popov, I. Willner, *Nano Lett.*, 2004, **5**, 21-25.

31 Y. Xiao, V. Pavlov, B. Shlyahovsky, I. Willner, *Chem. Eur. J.*, 2005, **11**, 2698-2704.

32 V. Pavlov, Y. Xiao, I. Willner, *Nano Lett.*, 2005, **5**, 649-653.

33 A. Virel, L. Saa, V. Pavlov, *Anal. Chem.*, 2008, **81**, 268-272.

34 Y. Sakai, M. Hayatsu, K. Hayano, *Soil Sci. Plant Nutr.*, 2002, **48**, 729-734.

35 L. A. Carson, M. S. Favero, W. W. Bond, N. J. Petersen, *Appl. Microbiol.*, 1973, **25**, 476-483.

36 G. Gomori, *Exp. Biol. Med.*, 1945, **58**, 362-364.

37 F. Plou, M. Ferrer, O. Nuero, M. Calvo, M. Alcalde, F. Reyes, A. Ballesteros, *Biotechnol. Tech.*, 1998, **12**, 183-186.

38 W. Zhang, Y. Tang, J. Liu, L. Jiang, W. Huang, F. Huo, D. Tian, *J. Agric. Food Chem.*, 2014, DOI: 10.1021/JF505339Q.

39 M. R. Hormozi-Nezhad, P. Karami, H. Robatjazi, *RSC Adv.*, 2013, **3**, 7726-7732.

40 M. Donbrow, E. Azaz, A. Pillersdorf, *J. Pharm. Sci.*, 1978, **67**, 1676-1681.

41 M. Donbrow, R. Hamburger, E. Azaz, A. Pillersdorf, *Analyst*, 1978, **103**, 400-402.

42 B. A. Kerwin, *J. Pharm Sci.*, 2008, **97**, 2924-2935.

43 J. Yao, D. K. Dokuru, M. Noestheden, S. S. Park, B.A. Kerwin, J. Jona, D. Ostovic, D. L. Reid, *Pharmaceut. Res.*, 2009, **26**, 2303-2313.

44 J. Ma, Z. Zhang, B. Wang, X. Kong, Y. Wang, S. Cao, Y. Feng, *Protein Expres. Purif.*, 2006, **45**, 22-29.

45 P. Gupta, N. Gupta, P. Rathi, *Appl. Microbiol. Biotechnol.*, 2004, **64**, 763-781.

