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Proteolysis-mediated protection of gold nanoparticles for sensitive activity assay of peptidases

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ABSTRACT

Rapid, sensitive and quantitative assays for peptide hydrolysis enzymes are of paramount importance for drug development and in the diagnosis of disease. Here, we proposed a novel biosensor for sensitive and selective active screening of peptidases. This strategy relies on the proteolysis-mediated protection of gold nanoparticles (AuNPs) that were decorated with biotin-labeled substrate peptides and can be aggregated by streptavidin. Enzyme-mediated protection of AuNPs offers this strategy high specificity, and the use of AuNPs additionally allows a visual and homogeneous assay format, thus permitting improved simplicity and throughput of the assays. As a model case, desirable selectivity and sensitivity in peptidase assay were achieved in the active assays of pancreatic elastase with a wide linear response range from 0.005 to 0.10 U/mL and a detection limit of 0.003 U/mL. The results indicated that this strategy can offer a simple, robust and convenient platform for visualized peptidase activity analysis and related biochemical studies with high sensitivity and selectivity.

1. Introduction

Peptidases are known to be abundant in almost all species [1]. Their behaviors may vary from the very broad and indiscriminate to the exceptionally specific, cleaving single peptide bonds in a single target protein [2]. This kind of enzymes is implicated in regulating a wide range of fundamental biological processes such as cell-cycle progression [3], development [4], wound healing [5] and apoptosis [6]. They have also been involved in various pathological states such as cancer, diseases of the central nervous system, cardiac diseases and numerous viral and infectious diseases [7–9].

To investigate the pathological and physiological functions of peptidases, the characterization and quantification of the activities of these peptidases are essential steps. Mass spectrometry usually plays a central role in peptidase assay, especially for the identification of proteins [10]. However, it often involves laborious and time-consuming steps for sample preparation [10]. Assays based on other sophisticated analytical instruments such as NMR or IR spectrometers have been developed as well [11]. The technique of fluorescence resonance energy transfer is also a common choice for peptidase detection [12,13]. Other methods for protease activity screening include assays based on colorimetric [14], amperometric [15], radioactive [16], and chemiluminescent [17] detection. Despite the successes of these methods, the use of costly label reagents, sophisticated instrumentation, or multistep washing and separation may limit their application. In the context, the development of rapid, easy and sensitive techniques for peptidase activity monitoring is still a topic of intensive interest in bioanalytical chemistry.

In the present study, we develop a novel homogeneous biosensing strategy for visual screening of the activities of peptidases on the basis of proteolysis-mediated protection of gold nanoparticles (AuNPs) decorated with biotin-labeled substrate peptides that can be aggregated by streptavidin. For its high sensitivity comparable with the fluorescence assay and exquisite capability in visual detection using "naked" eyes, AuNPs has been demonstrated to be a useful tool in analytical biochemistry [18,19], and employed for the detection of a variety of targets such as DNA [20,21], small molecules [22,23], proteins [24,25], and cancer cells [26]. Herein, with the combination of enzymatic reaction, we propose a novel biosensing strategy for visual and homogeneous screening of the activities of peptidases. Enzyme-mediated protection of AuNPs offers this strategy high specificity, while visual and homogeneous assay format affords improved simplicity and throughput. To demonstrate the performance of this strategy in the activity screening of peptidases, pancreatic elastase [27,28] was used as a model case. This new strategy may create a robust, convenient and visualized platform for screening the peptidase activities with high sensitivity and selectivity.

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2. Experimental

2.1. Materials and reagents

Porcine pancreatic elastase type IV (50–90% protein, lyophilized powder, ≥ 4.0 U/mg protein), Hydrogen tetrachloroaurate trihydrate ($HAuCl_4 \cdot 3H_2O$) and trisodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) were purchased from Sigma-Aldrich Co. Streptavidin was purchased from New England Biolabs (Ipswich, MA, USA). Other reagents that were of analytical grade and were used without further purification were all purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All solutions were prepared and diluted using ultra-pure water (with an electric resistance >18.3 mΩ) produced by a Millipore Milli Q system. The substrate peptide sequence used in the experiments was biotin-EEEEEAAEEC, and the sequence of the peptide used in the control experiment was biotin-EEEEEEEEEC. Both peptides were synthesized and purified by A'Peptide Co., Ltd. (Shanghai, China).

2.2. Preparation of peptide-decorated AuNPs

AuNPs were synthesized by citrate reduction of $HAuCl_4$ according to documented protocols [29,30], briefly described as follows: trisodium citrate (10 mL, 38.8 mM) was rapidly added to a stirred boiling solution of $HAuCl_4$ (100 mL, 1 mM). After several minutes the color of the solution changed from pale yellow to deep red. The solution was then heated under reflux for another 30 min to ensure complete reduction followed by slow cooling to room temperature. The average size of AuNPs was 13 ± 2 nm as calculated from the transmission electron microscopy (TEM) image. The concentration of these AuNPs was determined to be ~13 nM based on an extinction coefficient of 2.7×10^8 L mol^{-1} cm^{-1} at 520 nm for 13 nm AuNPs using a UV-2450 UV–vis absorption spectrophotometer (Shimadzu, Japan). The AuNPs solution was stored at 4 °C for future use.

The peptide-decorated AuNPs, which serve as artificial substrates for pancreatic elastase, were prepared according to the reported method [31] with some modifications. Briefly, the peptide-decorated AuNPs were prepared by adding 100 μL biotin-labeled peptide (250 μM) into 500 μL AuNPs solution under vigorous stirring and then incubating at room temperate for 12 h. The excessive peptides were removed via centrifugation at 15,000 rpm for 15 min followed by resuspension of the sediment in 1 mL 10 mM phosphate buffer (PB, pH 8.0). This step was repeated three times to sufficiently remove all excess peptides. Subsequently, the peptide-modified AuNPs were redispersed in 600 μL of PB (10 mM, pH 8.0) and stored at 4 °C for downstream assays. The final concentration of peptide-modified AuNPs was ~10.8 nM, assuming that there was no significant loss of AuNPs during the preparation process.

2.3. Activity assay of pancreatic elastase

The proteolysis reaction was started by adding a 30 μL aliquot of the peptide-modified AuNPs (~3.6 nM in AuNP concentration) in 27 μL phosphate buffer (10 mM, pH 8.0) containing a given concentration of pancreatic elastase. After 1 h incubation at 25 °C in a water bath, 3 μL streptavidin (15.8 μM) was added to the reaction mixture and incubated at 37 °C for 15 min on a shaker (Eppendorf, Hamburg, Germany).

2.4. Absorption spectrum, dynamic light scattering and transmission electron microscopy measurements

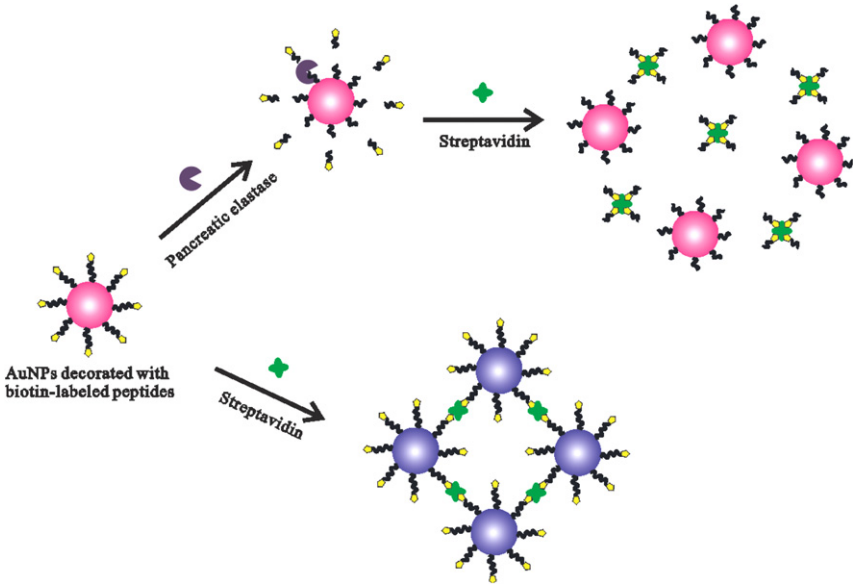
The surface plasmon absorption spectra of AuNPs were measured in the wavelength range from 400 nm to 800 nm at room temperature in a 60 μL quartz cuvette on a UV-2450 UV–vis absorption spectrophotometer (Shimadzu, Japan). To ensure homogeneity of the AuNP suspension, all solutions were agitated vigorously before the absorption measurement.

The hydrodynamic sizes of the AuNPs were determined by dynamic light scattering analysis using a Zetasizer 3000 HS particle size analyzer (Malvern Instruments, UK). The transmission electron microscope (TEM) images were obtained with a field-emission high-resolution 2100F TEM (JEOL, Japan) opened at an accelerating voltage of 200 kV. The sample films for TEM analysis were formed by dropping the solution of AuNPs on a carbon-coated copper mesh grid and left to dry in air condition at room temperature.

3. Results and discussion

3.1. Analytical principle of the biosensing strategy for visual screening of peptidase activity

This developed biosensing strategy relies on the proteolysis-mediated protection of substrate peptide-decorated AuNPs, as



Scheme 1. Illustration of the biosensing strategy for peptidase activity assay on the basis of proteolysis-mediated protection of gold nanoparticles.

illustrated in Scheme 1. The AuNPs are decorated with an artificial substrate peptide via self-assembly. For the substrate peptide, at its N′ terminal it is designed to have a biotinylated substrate sequence with a specific reacting site for peptide hydrolysis enzyme. At the C′ terminal of the substrate peptide, there is a thiolated, negatively charged spacer sequence which can minimize the steric hindrance in enzymatic reactions and enhance the stability of the peptide-decorated AuNPs.

In the absence of target peptidase, because the free terminal of the intact substrate peptide decorated on AuNPs is biotinylated, it can bind with streptavidin through the biotin–streptavidin interaction. Each streptavidin has more than two binding sites for biotin, thus, it can trigger a network-like assembly of the peptide-decorated AuNPs, subsequently inducing a significant variation in the plasmon resonance absorption peak with a visualized color change. In contrast, in the presence of target peptidase, the peptidase catalyzes the hydrolysis of the specific reacting site in the substrate peptide. The enzyme-catalyzed hydrolysis, thus, releases a biotinylated peptide fragment from the substrate peptide-decorated AuNP. As the added streptavidin can only bind with the released biotinylated fragment, AuNPs are well protected from the streptavidin-triggered network-like assembly. Note that the proteolysis-mediated protection of AuNPs is achieved only in the presence of the active target peptidase. Therefore, the resulting absorption spectral response of AuNPs can be immediately used for quantifying the activity of the target peptidase.

3.2. Typical characteristics of peptidase activity assay

For a biosensor developed using the substrate peptide of pancreatic elastase based on the strategy described in the present study, Fig. 1 depicts its typical absorption spectral response curves observed in the assay of pancreatic elastase. By dispersing the substrate peptide-decorated AuNPs (~1.8 nM) in aqueous solution, a homogeneous solution with a red color and a single surface plasmon absorption peak centered at 520 nm with the maximum intensity of 0.44 as obtained (curve a). With the addition of streptavidin (0.8 μM) and incubation for 15 min, the solution showed a rapid color change from red to purple and the absorption peak decreased by ~72% (~0.32, curve b) centered at 550 nm, exhibiting an obvious assembly of substrate peptide-decorated AuNPs. By contrast, incubating the substrate peptide-decorated AuNPs with 0.1 U/mL pancreatic elastase followed by the addition of streptavidin, compared with the substrate peptide-decorated AuNP solution, no significant color change appeared in the reaction solution and a surface plasmon absorption peak centered at 520 nm with the peak intensity slightly decreased to 0.43 was observed (curve c). It gave a direct evidence for no observable network-like assembly of substrate peptide-decorated AuNPs. Another control experiment using a peptide without any reacting site for pancreatic elastase instead of the substrate peptide was also performed. As anticipated, a rapid color change and peak intensity decreasing were observed (curve d) after treating such peptide decorated AuNPs (~1.8 nM) with pancreatic elastase (0.1 U/mL) followed by the addition of streptavidin (0.8 μM). The behavior of the solution was very similar to that of the solution of the substrate peptide-decorated AuNPs. It was suggested that pancreatic elastase was only active at the specific reacting site of peptide, and the protection of the AuNPs was highly selective for the pancreatic elastase-catalyzed hydrolysis. Taken together, the network-like assembly of the substrate peptide-decorated AuNPs in the assays was highly specific to the hydrolysis catalyzed by active pancreatic elastase, implying that the developed colorimetric

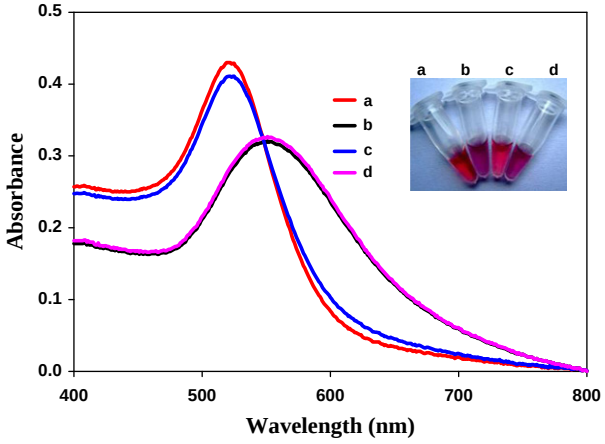


Fig. 1. Typical absorption spectra obtained in assays of pancreatic elastase: (a) substrate peptide-decorated AuNPs (~1.8 nM), (b) substrate peptide-decorated AuNPs reacting with 0.8 μM streptavidin, (c) substrate peptide-decorated AuNPs treated with 0.1 U/mL pancreatic elastase plus 0.8 μM streptavidin, and (d) peptide-decorated AuNPs without any reacting site for pancreatic elastase treated with 0.1 U/mL pancreatic elastase plus 0.8 μM streptavidin. The inset is the photograph for the corresponding systems.

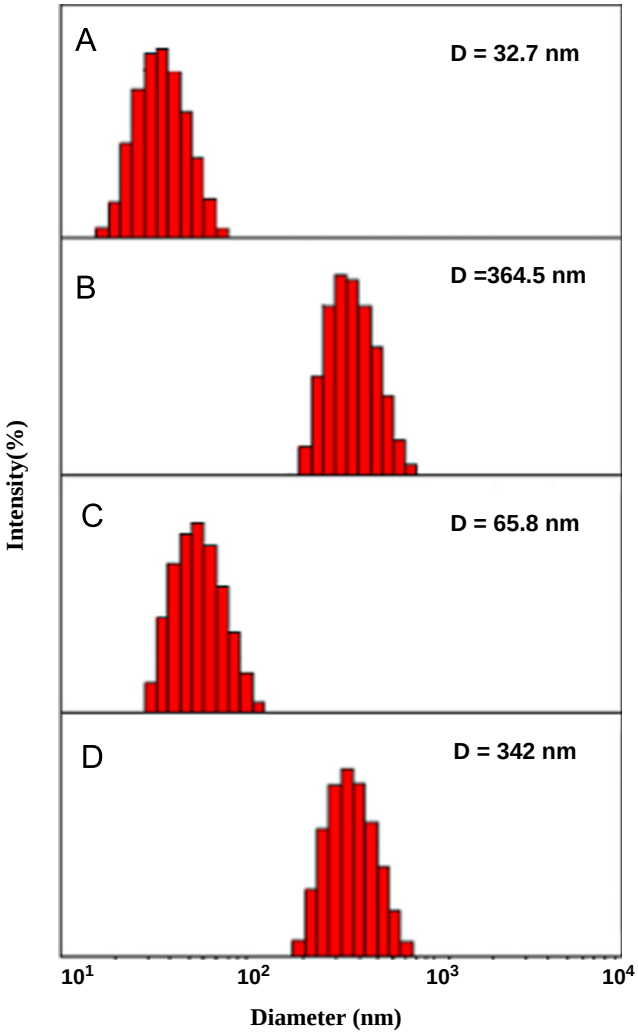


Fig. 2. Hydrodynamic sizes of AuNPs determined by DLS analysis: (A) substrate peptide-decorated AuNPs; (B) substrate peptide-decorated AuNPs reacting with 0.8 μM streptavidin; (C) substrate peptide-decorated AuNPs treated with 0.1 U/mL pancreatic elastase plus 0.8 μM streptavidin; and (D) peptide-decorated AuNPs without any reacting site for pancreatic elastase treated with 0.1 U/mL pancreatic elastase plus 0.8 μM streptavidin.

biosensing strategy may provide a selective and visualized platform for activity screening of peptide hydrolysis enzymes.

3.3. Dynamic light scattering and transmission electron microscopy characterization of the biosensing strategy for peptidase activity assay

Because the AuNP aggregate usually displayed a large hydrodynamic diameter, dynamic light scattering and transmission electron microscopy analysis could provide straightforward evidences for the assembly of AuNPs. Hence, DLS and TEM were performed to inspect the assembly of AuNPs in the assays in order to further verify the mechanism of the developed biosensing strategy. As shown in Figs. 2A and 3A for the peptide-decorated AuNPs, an average hydrodynamic diameter of ~32.7 nm was observed. This diameter value was much larger than the core size (~13 nm) of AuNPs because of the additional peptide and hydration layers. After treating the substrate peptide-decorated AuNPs with streptavidin, a substantial increase in the average hydrodynamic diameter (~346.5 nm) was observed for the AuNPs, giving an immediate evidence for the assembly of the peptide-decorated AuNPs into large aggregates (Figs. 2B and 3B). For the reaction system that consecutively treats the peptide-decorated AuNPs with pancreatic elastase and streptavidin, an average hydrodynamic diameter of ~65.8 nm was obtained for the AuNPs (Figs. 2C and 3C). The value of the diameter was little larger than that observed in the system only containing the peptide-decorated AuNPs, but much smaller than that observed in the system containing the peptide-decorated AuNPs and streptavidin. On the other hand, in the control experiment where the peptide had no active site for pancreatic elastase, we obtained a hydrodynamic diameter of an average of ~342 nm for AuNPs after treating with pancreatic elastase and streptavidin (Figs. 2D and 3D). These results were very consistent with those obtained with the absorption spectral measurements, which further confirmed that the pancreatic elastase catalyzed hydrolysis allowed well protection of the substrate peptide-decorated AuNPs from network-like assembly by streptavidin, and such protection was highly specific for the active pancreatic elastase.

3.4. Optimization of peptidase activity assay conditions

The developed strategy relied on the proteolysis-mediated protection of AuNPs from streptavidin-triggered network-like assembly, so the performance of the biosensor highly depended on the concentration of streptavidin as well as the reaction time of the peptidase-catalyzed hydrolysis. Fig. 4 depicts the dependency of absorbance responses at 520 nm for the biosensing strategy on the concentrations of streptavidin. It was observed that with the increasing concentration of streptavidin, absorption intensities were decreased substantially at first, and then increased when the concentration was larger than 0.8 μM. Therefore, this concentration of streptavidin was taken throughout the subsequent experiments. Fig. 5 depicts the dependency of the

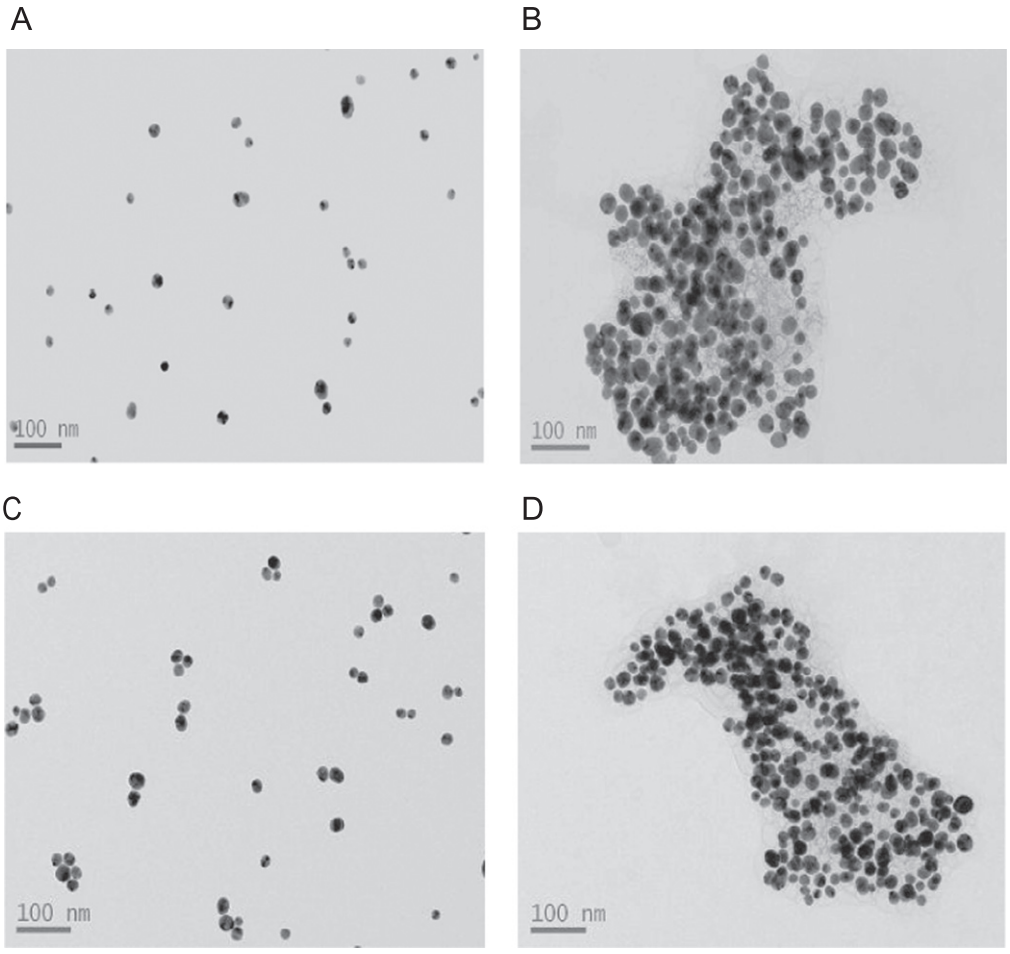


Fig. 3. TEM images of AuNPs in different situation: (A) substrate peptide-decorated AuNPs; (B) substrate peptide-decorated AuNPs reacting with 0.8 μM streptavidin; (C) substrate peptide-decorated AuNPs treated with 0.1 U/mL pancreatic elastase plus 0.8 μM streptavidin; and (D) peptide-decorated AuNPs without any reacting site for pancreatic elastase treated with 0.1 U/mL pancreatic elastase plus 0.8 μM streptavidin.

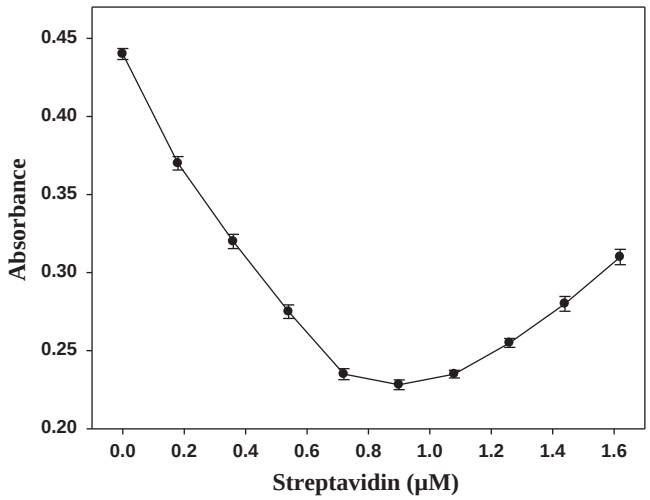


Fig. 4. Dependency of the absorbance responses at 520 nm for the biosensing strategy on concentrations of streptavidin. Concentration of substrate peptide-decorated AuNPs is ~1.8 nM.

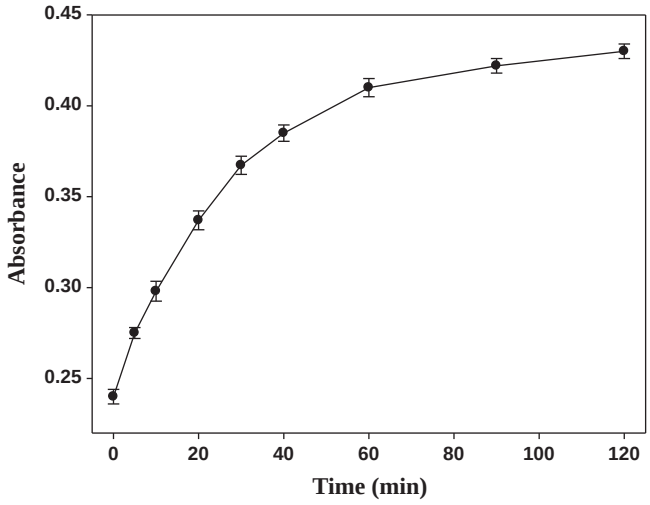


Fig. 5. Dependency of the absorbance responses at 520 nm for the biosensing strategy on the hydrolysis reaction time. Concentrations of substrate peptide-decorated AuNPs and pancreatic elastase are ~1.8 nM and 0.1 U/mL, respectively.

absorption spectral signal of the peptidase activity assay on hydrolysis reaction time. With prolonged reaction time, the observed absorption intensity at 520 nm gradually increased and became almost leveled off at 1 h. Thus, the time for peptidase-catalyzed hydrolysis reaction was set to 1 h throughout the subsequent experiments.

3.5. *Quantitative assay of peptidase activity using the biosensing strategy*

We also investigated the ability of the developed biosensor for quantitative assay of the peptidase activity. In the assays, a series of samples containing pancreatic elastase of different concentrations were incubated with the substrate peptide-decorated AuNPs followed by the addition of streptavidin, and then the absorption spectra were recorded. Fig. 6A shows the typical absorption spectral signals of the developed biosensing strategy obtained in response to varying concentrations of pancreatic elastase. With the increasing of pancreatic elastase concentration, the absorption peaks were also found to display gradual increase. A plot of the absorbance readings

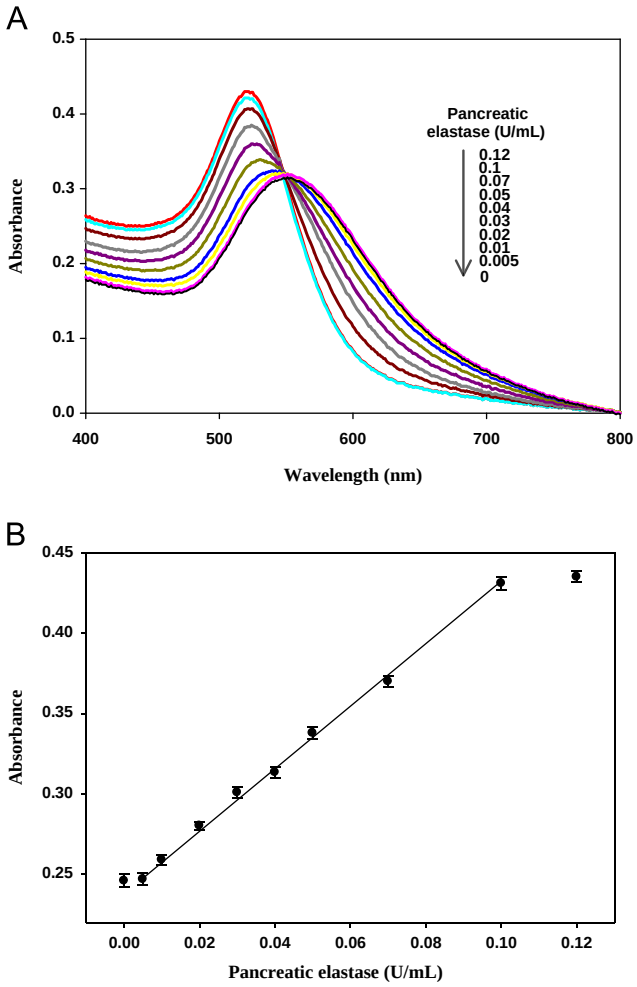


Fig. 6. (A) Typical absorption spectral responses of the biosensing strategy to pancreatic elastase of varying concentrations and (B) corresponding peak absorbance readings at 520 nM versus pancreatic elastase concentrations. Error bars are standard deviations across four repetitive experiments.

at 520 nm versus concentrations of pancreatic elastase revealed a dynamic correlation between the peak absorbance and pancreatic elastase concentrations in the range from 0.005 U/mL to 0.12 U/mL (Fig. 6B). A linear correlation was obtained for concentration range of 0.005–0.10 U/mL with a detection limit of 0.003 U/mL, a concentration readily available in pancreatic elastase analysis. It was also observed that the strategy exhibited excellent reproducibility due to its homogeneous assay format with simple operations. Relative standard deviations (RSDs) of luminescence peak intensities were 1.9%, 1.3%, 1.8% and 2.1% in four repetitive assays of 0.01, 0.03, 0.05, and 0.1 U/mL peptidase target respectively. Therefore, we might conclude that the developed colorimetric biosensor holds great potential for quantitative activity assay of pancreatic elastase with desirable sensitivity and reproducibility.

4. Conclusion

A novel homogeneous biosensing strategy for visual activity screening of peptidases was proposed based on the proteolysis-mediated protection of substrate peptide-decorated AuNPs against the network-like assembly which can be triggered by the biotin–streptavidin binding event. This strategy allowed a homogeneous assay of the peptidase activity in a very simple format as well as simplified instrumentation owing to the

visualized detection, which made the assays robust, easily automated, scalable for parallel assays of hundreds of samples. Using pancreatic elastase as a model case, the developed strategy was demonstrated to display desirable selectivity and sensitivity in peptidase assay. A wide linear response range from 0.005 to 0.10 U/mL with a detection limit of 0.003 U/mL was achieved. By designing proper substrate peptides and decorating them on AuNPs, this strategy also can be used for sensitive and specific activity screening of other peptidases. In view of these advantages, the developed peptidase biosensing strategy was expected to provide an intrinsically robust, convenient, and sensitive platform for visualized peptidase activity analysis and related biochemical studies.

Acknowledgments

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References

[1] C. Lopez-Otin, L.M. Matrisian, Nat. Rev. Cancer 7 (2007) 800–880.
[2] J. Müller, T. Schwann, Arch. Anat. Physiol. (1836) 66–89.
[3] J.P. Alao, Mol. Cancer 6 (2007) 24.
[4] B. Bowerman, T. Kurz, Development 133 (2006) 773–784.
[5] I. Rushton, Nurs. Stand. 21 (2007) 68–72.
[6] R.M. Siegel, Nat. Rev. Immunol. 6 (2006) 308–317.
[7] F. Molinari, Hum. Mol. Genet. 12 (2003) 195–200.
[8] R.B. Singh, Mol. Cell. Biochem. 263 (2004) 241–256.
[9] H.D. Klenk, W. Garten, Trends Microbiol. 2 (1994) 39–43.
[10] H. Schlüter, D. Hildebrand, C. Gallin, A. Schulz, J. Thiemann, M. Trusch, Anal. Bioanal. Chem. 392 (2008) 783–792.
[11] D. Wahler, J.L. Reymond, Curr. Opin. Chem. Biol. 5 (2001) 152–158.
[12] L.C.G. Oliveira, V.O. Silva, D.N. Okamoto, M.Y. Kondo, S.M.B. Santos, I.Y. Hirata, M.A. Vallim, R.C. Pascon, I.E. Gouvea, M.A. Juliano, L. Juliano, Anal. Biochem. 421 (2012) 299–307.
[13] Y.P. Kim, Y.H. Oh, E. Oh, S. Ko, M.K. Han, H.S. Kim, Anal. Chem. 80 (2008) 4634–4641.
[14] B.A. Williams, E.J. Toone, J. Org. Chem. 58 (1993) 3507–3510.
[15] R.E. Ionescu, Anal. Chem. 78 (2006) 6327–6331.
[16] R. Ludwig, R. Lucius, R. Mentlein, Biochimie 77 (1995) 739–743.
[17] J.A. Richard, L. Jean, A. Romieu, M. Massonneau, P.N. Fraissignes, P.Y. Renard, Org. Lett. 9 (2007) 4853–4855.
[18] W. Zhao, M.A. Brook, Y.F. Li, ChemBioChem 9 (2008) 2363–2371.
[19] Y. Du, B.L. Li, E. Wang, Bioanal. Rev. 1 (2010) 187–208.
[20] L.M. Zanoli, R. D'Agata, G. Spoto, Anal. Bioanal. Chem. 402 (2012) 1759–1771.
[21] J. Zhang, S.P. Song, L.H. Wang, D. Pan, C.H. Fan, Nat. Protoc. 2 (2007) 2888–2895.
[22] C.L. Schofield, R.A. Field, D.A. Russell, Anal. Chem. 79 (2007) 1356–1361.
[23] J. Zhang, L.H. Wang, D. Pan, S.P. Song, F.Y.C. Boey, H. Zhang, C.H. Fan, Small 4 (2008) 1196–1200.
[24] H. Wei, B.L. Li, J. Li, E. Wang, S.J. Dong, Chem. Commun. (2007) 3735–3737.
[25] M.Y. Liu, C.P. Jia, Y.Y. Huang, X.H. Lou, S.H. Yao, Q.H. Jin, J.L. Zhao, J.Q. Xiang, Analyst 135 (2010) 327–331.
[26] W.T. Lu, S.R. Arumugam, D. Senapati, A.K. Singh, T. Arbneshi, S.A. Khan, H.T. Yu, P.C. Ray, ACS Nano 4 (2010) 1739–1749.
[27] J.W. Harper, R.R. Cook, C.J. Roberts, B.J. McLaughlin, J.C. Powers, Biochemistry 23 (1984) 2995–3002.
[28] J.G. Bieth, S. Dirrig, M.L. Jung, C. Boudier, E. Papamichael, C. Sakarellos, J.L Dimicoli, Biochim. Biophys. Acta 994 (1989) 64–74.
[29] J. Liu, Y. Lu, Nat. Protoc. 1 (2006) 246–252.
[30] H.D. Hill, C.A. Mirkin, Nat. Protoc. 1 (2006) 324–336.
[31] R. Levy, N.T.K. Thanh, R.C. Doty, I. Hussain, R.J. Nichols, D.J. Schiffrin, M. Brust, D.G. Fernig, J. Am. Chem. Soc. 126 (2004) 10076–10084.