Electronic Supplementary Information

Ultrasensitive amplification-free detection of protein kinase based on catalyzed assembly and enumeration of gold nanoparticles

Tongtong Tian,[a] Yuanyuan Yao,[a] Beibei Yang,[a] Kun Zhang*[b] and Baohong Liu*[a]

[a] Department of Chemistry, Shanghai Stomatological Hospital, and State Key Lab of Molecular Engineering of Polymers, Fudan University, Shanghai, 200433, China

[b] Department of Neurosurgery, Shanghai Children's Hospital, Shanghai Jiao Tong University, Shanghai 200062, China

* Corresponding Author: E-mail: zhangkun@shchildren.com.cn

E-mail: bhliu@fudan.edu.cn

Contents

Experimental Section

1. Reagents and materials

PKA (catalytic subunit from bovine heart), N-(3-chlorophenyl)-6,7-dimethoxy-4-quinazolinamine (Tyrphostin AG1478), trizma hydrochloride, glycerine, DL-dithiothreitol (DTT), Forskolin (Fsk) and 3-isobutyl-1-methylxantine (IBMX) were purchased from Sigma-Aldrich (Shanghai, China). The γ-[6-aminohexyl]-ATP-biotin (γ-biotin-ATP) was purchased from Jena Bioscience (Jena, Germany). Cysteine-terminated kemptide (Cys-Leu-Arg-Arg-Ala-Ser-Leu-Gly, CLRRASLG) was obtained from Chinese Peptides Company (Nanjing, China). 6-Aminohexanoic acid, 4,4',5,5',6,6'-hexahydroxydiphenic acid 2,6,2',6'-dilactone (Ellagic acid), sodium chloride (NaCl), potassium hydroxide (KOH), magnesium chloride ($MgCl_2$) and 3-[(trimethoxysilyl)propyl]diethylenetriamine (DETA) were obtained from J&K Chemical Ltd (Beijing, China). Gold nanoparticles-streptavidin (avidin-GNPs) was purchased from RuiXi Biological Technology Co., Ltd (Xi'an, China). Glutaraldehyde (25 wt%) and ethylenediaminetetraacetic acid (EDTA) were obtained from Aladdin Bio-Chem Technology Co., Ltd (Shanghai, China). Coverslips (#12-548-C) made of optical borosilicate glass were purchased from Fisherfinest Premium (Pittsburgh, PA, USA). Other chemicals were of analytical grade or better and used as received without further purification. The ultrapure Milli-Q water (18.2 MΩ·cm) was used to prepare all the aqueous solutions.

2. Instruments

UV-vis extinction spectra were measured with an Aglient HP8453 spectrophotometer. The shape and size of avidin-GNPs were characterized using a JEOL JEM-2011 transmission electron microscope (TEM) at an acceleration voltage of 200 kV. Scanning electron microscope (SEM) images were taken on a Hitachi S-4800 microscope at an acceleration voltage of 1.0 kV.

3. Characterization of avidin-GNPs

The purchased 52 nm avidin-GNPs with a localized surface plasmon resonance

(LSPR) peak at 535 nm were characterized by TEM, SEM, dynamic light scattering, and UV-vis extinction spectroscopy (Figure S1).

4. Modification of glass microscope coverslips

The coverslips (2.5 cm × 2.5 cm) were first washed sequentially in acetone, ethanol, KOH (1 M) and ultrapure water for 15 min, respectively, followed by incubation in piranha solution (7:3 H_2SO_4-H_2O_2) for 30 min at 80 °C. The slides were rinsed thoroughly in water and absolute ethanol, then dried in a N_2 stream and heated at 120 °C for 1 h. Afterwards, the clean slides were immersed in 1 wt% DETA/methanol solution for 50 min. After washing thrice with absolute ethanol and drying in N_2 atmosphere, the slides were baked at 120 °C for 3 h to complete the aminosilylation process.

5. Phosphorylation of kemptide and assembly of GNPs-kemptide on silanized slices

To immobilize kemptide on the slide surface, we first dipped the amino group-functionalized slide in 5 wt% glutaraldehyde solutions for 1 h at 37 °C, and then immersed it in a PBS (10 mM, pH 7.4) solution containing a desired amount kemptide. Kemptide was immobilized on the slide through aldimine condensation between glutaraldehyde and kemptide. Next, the slide was treated with 5 mM 6-aminohexanoic acid solution for 1 h to minimize the nonspecific binding. After that, the slide was bathed in ultrapure water, dried under N_2 stream, and then sealed to a silicone culture well gasket (3.0 mm diameter, 1.0 mm depth, 50 holes, Grace Bio-labs) for a PKA-catalyzed phosphorylation reaction (Figure S2). 10 μL of PKA assay buffer (50 mM Tris-HCl, 20 mM $MgCl_2$, 25 μM γ-biotin-ATP, pH 7.4) containing a certain amount of PKA was added into certain milliwells and incubated with kemptide-modified coverslips. After a period of reaction, the redundant buffer in wells were removed and every well was cleaned thoroughly with ultrapure water. Finally, 10 μL of avidin-GNPs (0.01 mg/mL) was transfered into milliwells and incubated at 37 °C for several minutes to allow avidin-GNPs binding to the biotinylated substrate kemptides. After the reaction, all the wells were washed completely with ultrapure water and dried gently under a jet of N_2.

6. Optimization of the detection conditions

To achieve the best sensing performance, we optimized the detection conditions. Three replicate tests for each sample were performed according to the above procedures with the presence of 0.001 U/μL of PKA.

7. Dark-field microscopic imaging and data analysis

All dark-field images were collected by a 50× Olympus dark field condenser (0.5 NA) that coupled to a HORIBA Xplore microscope system with a true color CCD. The imaging conditions of each reaction system were kept consistent during the experimental process. Images of ultrapure water were regarded as the background images of the reaction systems. Eight images derived from different locations near the center of the reaction well were obtained for each sample.

The image J software (version 1.45) was used for data analysis. Generally, a central region of 750 × 560 pixels in each image was used for counting. The number of GNP counts was acquired by using "analyze particles" function with the particle size at 2-12 pixels and calculated according to eq 1:

$$N_{net} = N_{PKA} - N_{negative} \quad (1)$$

where N_{net} is the net number of GNPs, $N_{negative}$ is the corrected number of GNPs without PKA, and N_{PKA} is the corrected number of GNPs under the participation of PKA.

8. Kinase activity inhibition evaluation

For PKA inhibition assay, a series of concentrations (0-14 μM) of ellagic acid or Tyrphostin AG 1478 were added into the reaction mixture with a fixed concentration of PKA (5×10^{-4} U/μL). The relative activity of PKA was calculated according to eq 2:

$$\text{Relative Activity} = \frac{N_i - N_{negative}}{N_t - N_{negative}} \times 100\% \quad (2)$$

where N_t and N_i represent the corrected number of GNPs in the presence of PKA, and in the coexistence of PKA and inhibitor. The relative activity was plotted against the concentrations of ellagic acid or Tyrphostin AG 1478, and the IC_{50} value of ellagic acid was calculated from the fitting curve.

9. Preparation of MCF-7 breast cancer cell lysates

MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum under a humidified atmosphere containing 5% CO_2 at 37 °C. The cells were treated with 1 mL of serum-free medium for additional 4 h before stimulation. Subsequently, 25 μM of Fsk/IBMX dimethyl sulfoxide (DMSO) or ellagic acid DMSO solution was added to the cells (10^6 cells) for 0.5 h to activate or inhibit the intracellular PKA activity, respectively. Cells incubated with the equal volume of DMSO were used as the unstimulated control. After that, the cells were rinsed thrice with PBS buffer (10 mM, pH 7.4) and then treated with a commercial protein extraction kit (BSP022, Sangon Biotech, Shanghai, China) on ice for 10 min. The cell lysates were centrifuged at 4000 rpm for 10 min at 4 °C and stored at -80 °C before use.

10. Supplementary figures and table

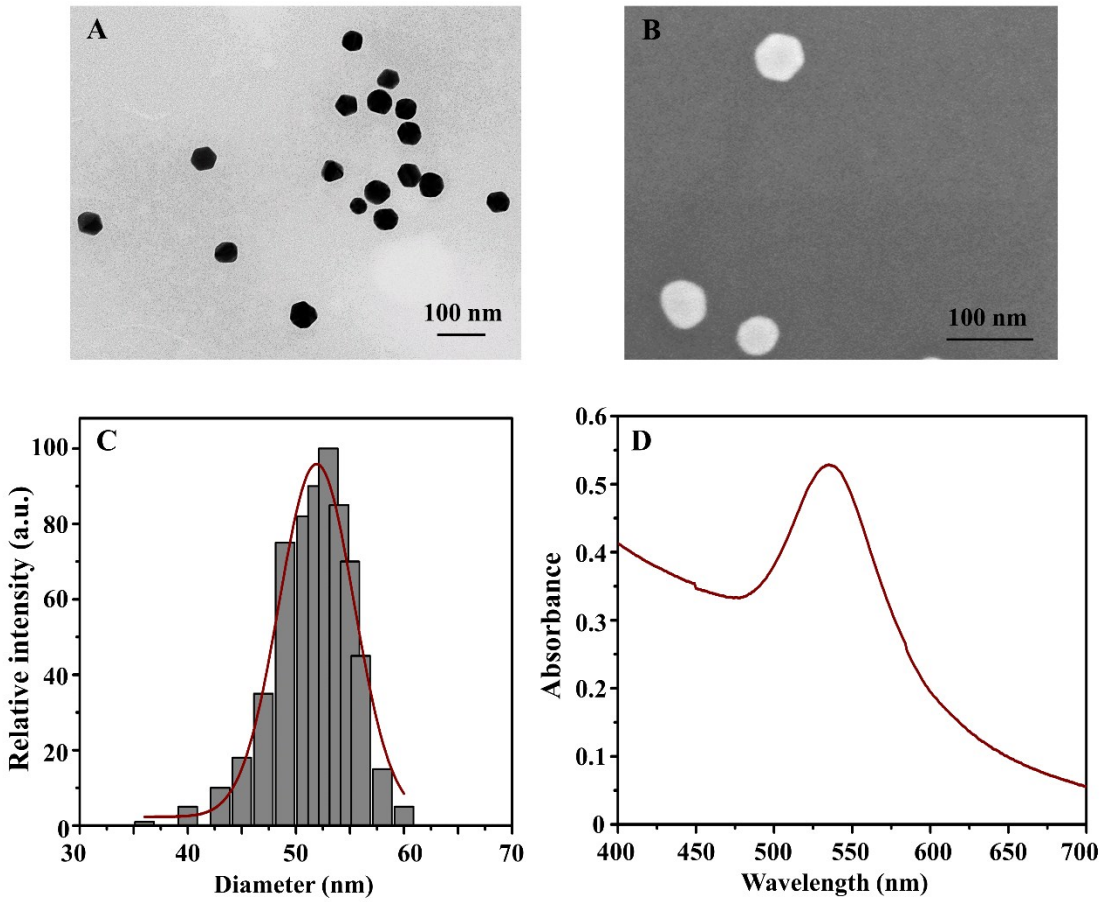

Figure S1. (A, B) TEM and SEM images of avidin-GNPs, (C) Size distribution obtained from TEM measurement (150 particles). (D) UV-vis absorption spectra of avidin-GNPs.

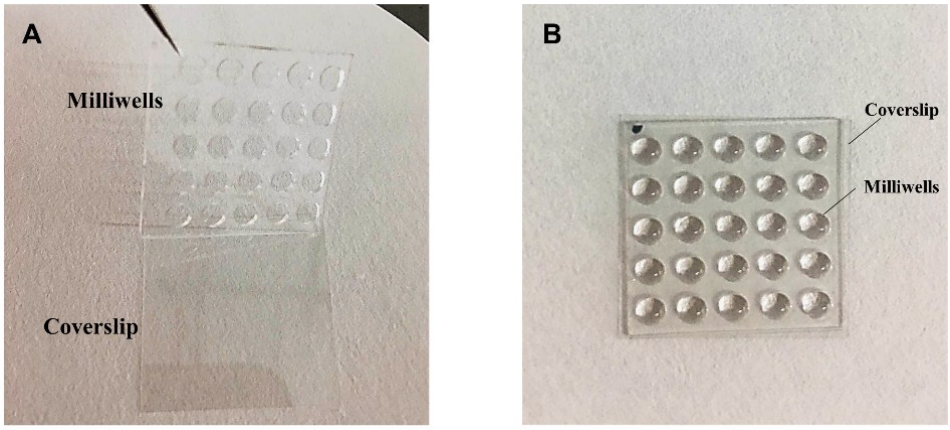

Figure S2. (A) Photograph showing a silicon gasket with 25 milliwells (A) before and (B) after affixing a coverslip. 10 μL of PKA assay buffer (50 mM Tris-HCl, 20 mM $MgCl_2$, 25 μM γ-biotin-ATP, pH 7.4) containing 0.001 U/μL of PKA was added into milliwells in B.

Figure S3. Dark-field image of GNPs with the concentration of kemptide up to 50 μM. Conditions: 2 h reaction of kemptide, 30 min and 37 °C of phosphorylation, and 30 min reaction of avidin-GNPs

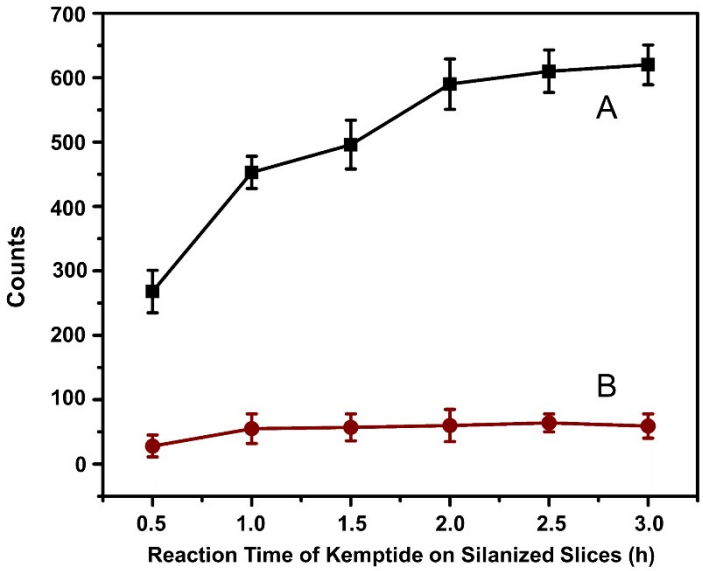

Figure S4. Optimization of the reaction time of kemptide. Conditions: 5 μM of kemptide, 30 min and 37 °C of phosphorylation, and 30 min reaction of avidin-GNPs, A) in the presence of 0.001 U/μL of PKA; B) in the absence of PKA.

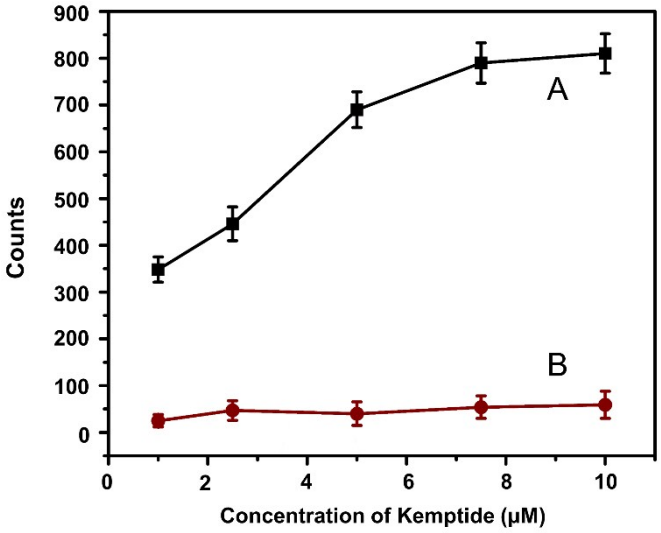

Figure S5. Optimization of the concentration of kemptide. Conditions: 2 h reaction of kemptide, 30 min and 37 °C of phosphorylation, and 30 min reaction of avidin-GNPs, A) in the presence of 0.001 U/μL of PKA; B) in the absence of PKA.

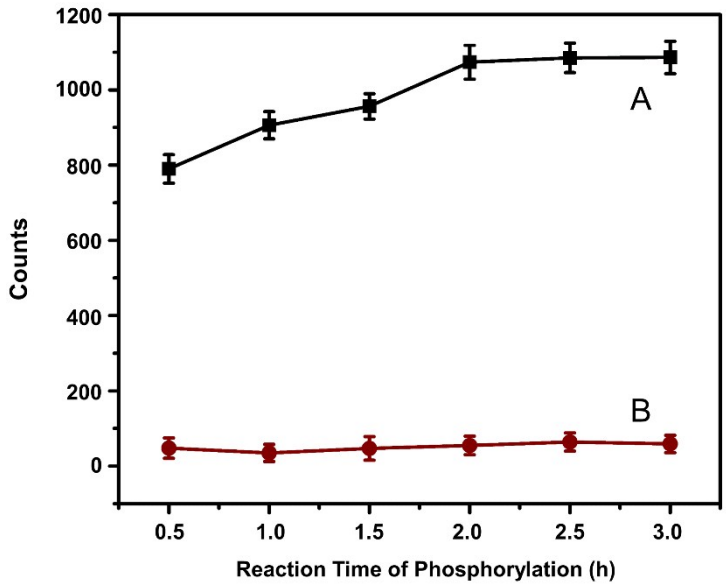

Figure S6. Optimization of the reaction time of phosphorylation. Conditions: 2 h and 7.5 μM of kemptide, 37 °C of phosphorylation, and 30 min reaction of avidin-GNPs, A) in the presence of 0.001 U/μL of PKA; B) in the absence of PKA.

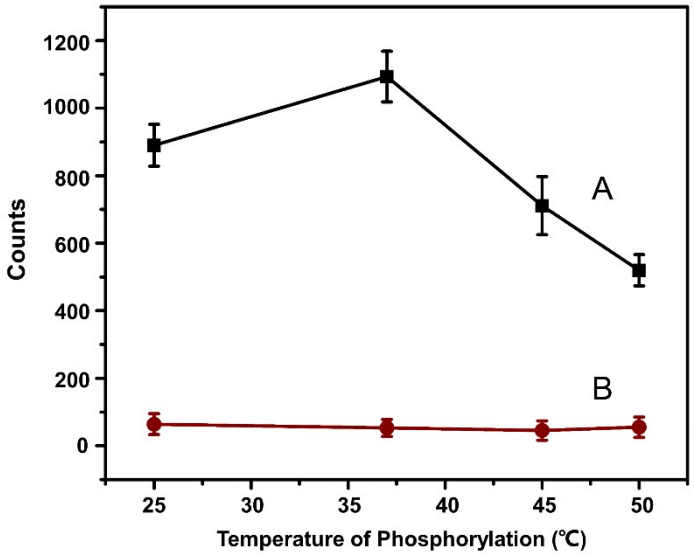

Figure S7. Optimization of the temperature of phosphorylation. Conditions: 2 h and 7.5 μM of kemptide, 2 h of phosphorylation, and 30 min reaction of avidin-GNPs, A) in the presence of 0.001 U/μL of PKA; B) in the absence of PKA.

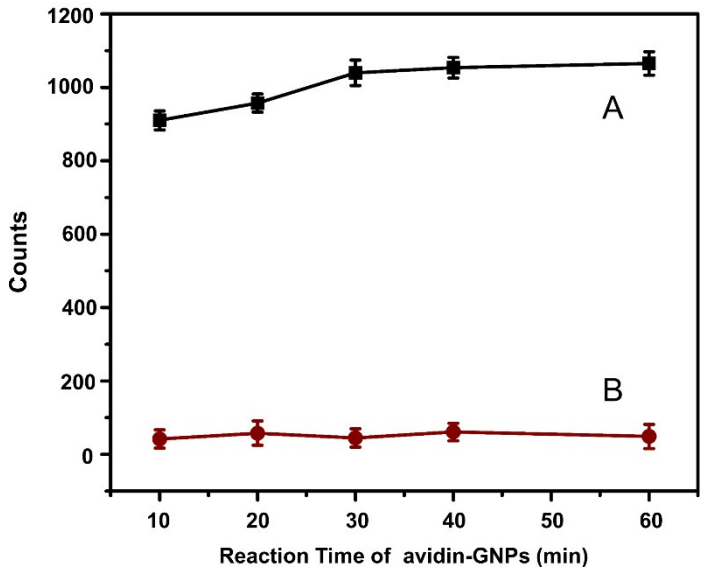

Figure S8. Optimization of the reaction time of avidin-GNPs. Conditions: 2 h reaction and 7.5 μM of kemptide, 2 h and 37 °C of phosphorylation, A) in the presence of 0.001 U/μL of PKA; B) in the absence of PKA.

Table S1. The comparison of different assays for PKA activity.

Sensing Strategy	Detection limit	Liner Range	Ref.
Fluorescent quenching of silver nanoclusters	0.5 U/mL	1-2000 U/mL	1
Enrichment on TiO_2 coated magnetic microbeads	0.1 U/mL	0.5-500 U/mL	2
Quenching effect of grapheme oxide	0.134 U/mL	-	3
Aggregation of gold nanoparticles	0.232 U/mL	-	4
FRET based on up conversion nanoparticles	0.05 U/mL	0.1-10 U/mL	5
Fluorescence off-on reaction of the polyU-peptide assembly	0.05 U/mL	0.1-3.2 U/mL	6
FRET based on quantum dots	0.0093U/mL	0.01-100 U/mL	7
Photoelectrochemical Biosensors	0.005 U/mL	0.008-1 U/mL	8
Surface-Enhanced Raman Spectroscopy	0.005 U/mL	-	9
Degradation of gold nanoclusters	0.004 U/mL	0.01-40 U/mL	10
Enrichment on single microbead	0.00012 U/mL	0.0005-0.004 U/mL	11
Gold nanoparticle enumeration with dark-field microscope	0.00015 U/mL	0.0005-10 U/mL	This work

As shown in Table S1, the presented work provides a very high sensitivity with a limit of detection (LOD) of 1.5×10^{-4} U/mL. The improved detection performance of our work may be explained by the following aspects: first, we directly measure the localized surface plasmon resonance of individual immobilized GNPs in this work where no other energy transferring processes such as FRET are involved, thus a higher optical measurement efficient is expected. Second, the optical scattering of GNPs under dark illumination is strong and stable, so that some side effects such as photobleaching and degradation of dyes in fluorescence analysis are not occurred here. Additionally, the rationale of the presented work is that one phosphorylated peptide molecule, in principle, should bind to one Au particle, especially when the PKA activity is very low, which is different to the design of the literature work where one QD is expected to bind several peptide molecules.[7]

11. References

1. C. Shen, X. Xia, S. Hu, M. Yang and J. Wang, *Anal. Chem.* 2014, **87**, 693-698.
2. J. Bai, Y. Zhao, Z. Wang, C. Liu, Y. Wang and Z. Li, *Anal. Chem.* 2013, **85**, 4813-4821.
3. J. Zhou, X. Xu, W. Liu, X. Liu, Z. Nie, M. Qing, L. Nie and S. Yao, *Anal. Chem.* 2013, **85**, 5746-5754.
4. J. Zhou, X. Xu, X. Liu, H. Li, Z. Nie, M. Qing, Y. Huang and S. Yao, *Biosens. Bioelectron*. 2014, **53**, 295-300.
5. C. Liu, L. Chang, H. Wang, J. Bai, W. Ren and Z. Li, *Anal. Chem.* 2014, **86**, 6095-6102.
6. Y. Xu, W. Shi, X. He, X. Wu, X. Li and H. Ma, *Anal. Chem.* 2017, **89***,* 10980-10984.
7. L. Wang, Y. Yang and C. Zhang, *Anal. Chem.* 2015, **87**, 4696-4703.
8. Z. Yan, Z. Wang, Z. Miao and Y. Liu, *Anal. Chem.* 2016, **88**, 922-929.
9. S. He, Y. M. E. Kyaw, E. K. M. Tan, L. Bekale, M. W. C. Kang, S. S. Y. Kim, I. Tan, K.P. Lam and J. C. Y. Kah, *Anal. Chem.* 2018, **90**, 6071-6080.
10. W. Song, R. Liang, Y. Wang, L. Zhang and J. Qiu, *Chem. Commun.* 2015, **51**, 10006-10009.

11. X. B. Zhang, C. H. Liu, H. H. Wang, H. Wang, and Z.P. Li, *Angew. Chem., Int. Ed.* 2015, **54**, 15186-15190.