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Ultrasensitive scanometric strategy for detection of matrix metalloproteinases using a histidine tagged peptide–Au nanoparticle probe†

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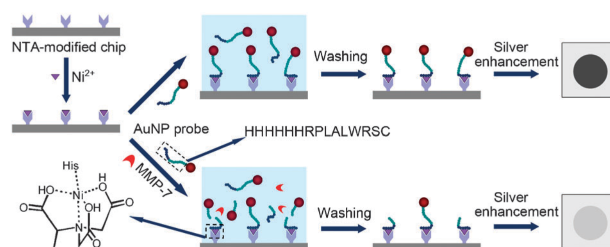
A simple scanometric strategy was developed for ultrasensitive assay of matrix metalloproteinases based on their discriminatory and proteolytic activity by integrating a newly designed peptide–gold nanoparticle probe, a nitrilotriacetic acid modified chip and silver signal amplification.

Matrix metalloproteinases (MMPs) constitute a family of extracellular and zinc-dependent proteinases that are capable of degrading all components of the extracellular matrix.[1] Abnormal expression and activities of MMPs are correlated with various diseases, such as arthritis, neurodegenerative diseases, atherosclerosis, multiple sclerosis and tumors.[2] Especially, MMPs play a crucial role in regulating tumor microenvironment, tumor cell invasion and metastasis.[2d] Therefore, the sensitive analysis of MMPs has become an important subject for understanding their role in disease development and providing a powerful tool for diagnosis, prognosis, and therapy monitoring.

Current technologies to detect MMPs mainly involve immunoassays, zymography and activity assays using labeled probes.[3] Immunoassays are sensitive, specific and quantitative techniques. But they require the availability of anti-MMPs antibodies and cannot evaluate the activity of MMPs. Zymography is an accurate and sensitive electrophoretic method to analyze the activities of MMPs. But this technique is tedious, difficult to quantitation, and limited to a few MMPs.[4] A wide variety of activity assays for MMPs have been developed using labeled probes, such as fluorescent, radio and electroactive reporter labeled substrate proteins or peptides.[5] However, the measurements are often complicated by the requirement of expensive devices for data readout.[5c] Thus, it remains a challenge to develop a simple, pragmatic and sensitive methodology for detection of MMPs in both basic research and clinical diagnostics.

Recently great progress has been made in the development of bioassay strategies owing to the immense application of biofunctionalized nanoparticles.[6] Among them, biofunctionalized gold nanoparticles (AuNPs) combining with electroless silver deposition have produced many bioanalytical strategies for convenient detection of nucleic acid, protein, carbohydrate and metal ion.[7] These called scanometric assays perform the information-recording and acquirement procedure with a simple flatbed scanner. However, a biomacromolecule modified chip is needed as a capture substrate in most scanometric assays. This work designed a convenient and sensitive scanometric approach for quantitative detection of MMPs by combining the especially proteolytic activity of target MMP with a novel peptide–AuNP probe and nitrilotriacetic acid (NTA) modified chip (ESI†).

MMP-7, an important biomarker for a number of tumors,[8] is present in serum of a healthy adult at several ng mL^{-1} level and significantly increases in that of a cancer patient,[8b,c] which was used as a model to verify the proposed strategy. Here, a 15 amino acid peptide was used as the sensing element, which contained a six-histidine (His) tag and a MMP-7 specific substrate sequence (ESI†), to construct a peptide–AuNP probe.[8a] As shown in Scheme 1, the designed peptide–AuNP probe could be trapped on the NTA modified chip using Ni^{2+}



Scheme 1 Schematic representation of the designed scanometric strategy for MMP-7 detection.

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as a chelating bridge to bind NTA center and His.[9] In the presence of MMP-7, the proteolysis of the MMP-7 specific substrate sequence led to the cleavage of His tag from AuNPs. After silver enhancement, the bound AuNPs, whose amount depended on the quantity of target MMP, could be converted into a readily quantitative greyscale signal. Thus, the simple scanometric readout could finally be performed with a scanner for ultrasensitive detection of target MMP. In a similar way, the proposed approach was applicable to other MMPs and proteases by employing their specific peptide sequences to construct peptide–AuNP probes.[5a,d]

The AuNPs were protected with the bis(*p*-sulfonatophenyl) phenylphosphine dihydrate dipotassium salt (BSPP) to minimize the salt effect on the particle stability, and then conjugated with the peptide by the cystine residue. The TEM image showed the uniform size and monodispersity of peptide–AuNP probes and approximately 5 nm diameter of AuNPs (inset in Fig. 1a). UV-vis spectra of BSPP-protected AuNPs and peptide–AuNP probe exhibited a similar peak centered at 512 nm, proved the nanoparticle stability in the preparation process (Fig. 1a). The peptide–AuNP probe was also characterized by 3% agarose gel electrophoresis (Fig. 1b). BSPP-protected AuNPs showed well-defined dark-red bands. Meanwhile, the prepared peptide–AuNP probe showed a dispersed band with relative less migration due to increased molecular weight and modified surface charge, indicating successful conjugation of the peptide to AuNPs.

The peptide–AuNP probe bound on the NTA modified chip was characterized by AFM observation and the collected height and phase data (Fig. S1 in ESI†). After introducing the peptide–AuNP probe on the chip surface, the cross-sectional view of the AFM image showed a complete, homogeneous, and well ordered AuNP monolayer, which led to the height fluctuation of about 5 nm, visibly larger than that of the NTA modified substrate and similar with the diameter of AuNPs determined by the TEM. The formation of the AuNP monolayer proved that a large number of peptide–AuNP probes could be bound on the chip due to the strong interaction between the His tag of the peptide–AuNP probe and chelated Ni^{2+} on the NTA modified surface, inducing a sensitive methodology for scanometric detection of MMP-7 by AuNP catalyzed silver enhancement.

A five-spot NTA-modified chip was used to examine the designed scanometric strategy (Fig. S2 in ESI†). After the

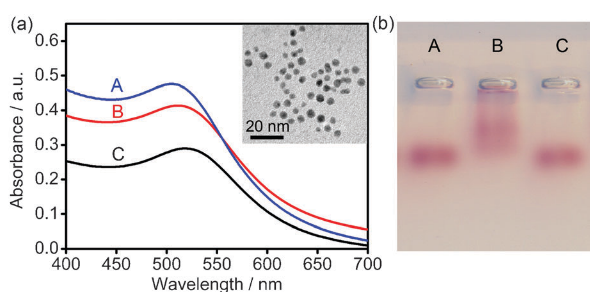

Fig. 1 (a) UV-vis spectra of (A) AuNPs, (B) BSPP-protected AuNPs and (C) peptide–AuNP probe. Inset: TEM image of peptide–AuNP probes. (b) 3% agarose gel electrophoresis photos of (A, C) BSPP-protected AuNPs and (B) peptide–AuNP probe.

peptide–AuNP probe binding and sliver enhancement, the spot showed significantly deep black due to the strong interaction between His tag and chelated Ni^{2+} (spot A). In the presence of MMP-7, the cleavage and release of the His tags from AuNPs counteracted the binding process, resulting in a very weak grey spot (spot E). It was proved that the peptide–AuNP probe and the designed method could be applied for convenient detection of MMP-7. Meanwhile, the control spots showed nearly invisible grey (spots B–D), indicating little nonspecific adsorption of AuNPs on these spots, which was crucial to obtain high sensitivity and good precision of the designed method. The little nonspecific adsorption of AuNPs attributed to the more immaculate surface of the NTA-modified chip than the previous biomacromolecule modified chip. The relative intensity herein was defined as the greyscale value of the chip background subtracted from that of a given spot. The affinity binding capabilities of a series of divalent metal ions were examined (Fig. S3 in ESI†). The Ni^{2+} ion produced the highest signal. So Ni^{2+} was used for the affinity binding.

To obtain high analytical performance, some important detection parameters were optimized (Fig. S4 in ESI†). With the increasing concentration of the peptide–AuNP probe, the relative intensity of the greyscale sharply increased and tended to a steady value after 62.5 nM. Considering the sensitivity and dynamic range, 125 nM was chosen as the optimal concentration. The relative intensity increased with the increasing incubation time of the peptide–AuNP probe, and reached a constant intensity at 90 min due to a saturated binding. In the presence of MMP-7, the relative intensity decreased with the increasing incubation time due to more cleavage of AuNPs. For sufficient cleavage of AuNPs by MMP-7, 90 min was chosen as the optimal incubation time. In order to simplify the protocol, the peptide–AuNP probe and target MMP were mixed for simultaneous binding and proteolytic reactions both in solution and on the chip surface, which led to more sufficient cleavage than that for activity assay by two sequential binding and proteolytic reactions on the chip surface.[5b]

Fig. 2a shows the scanometric image of spots responding to various concentrations of MMP-7. With the increasing target MMP-7 level, the greyscale gradually changed from deep black to slight grey. Under the optimal conditions, the relative intensity of the greyscale was proportional to the logarithmic value of MMP-7 concentration over a 3-decade range from 0.1 to 100 ng mL^{-1} with a linear correlation coefficient of 0.996 (Fig. 2b). The detection limit for MMP-7 concentration was calculated to be 0.097 ng mL^{-1} (4.8 pM) in a 3σ rule, which was lower than that of 2 ng mL^{-1} MMP-2 for the fluorescence resonance energy transfer method based on quantum dots and bioluminescence,[5a] and comparable with those of 0.5 pM MMP-2 for immunoassay based on the optical biosensor,[10] 3.4 pM MMP-7 for electrochemical detection using the ferrocene labeled proteolytic beacon,[5b] and 0.1 ng mL^{-1} MMP-2 for colorimetric detection on nanoporous silicon photonic films.[11] Considering the fact that the volume of analyte was only 2.5 μL, much smaller than those used in these mentioned methods, the designed method could detect MMP-7 down to 1.2×10^{-17} moles. The ultra-high sensitivity of the designed method was attributed to the strong

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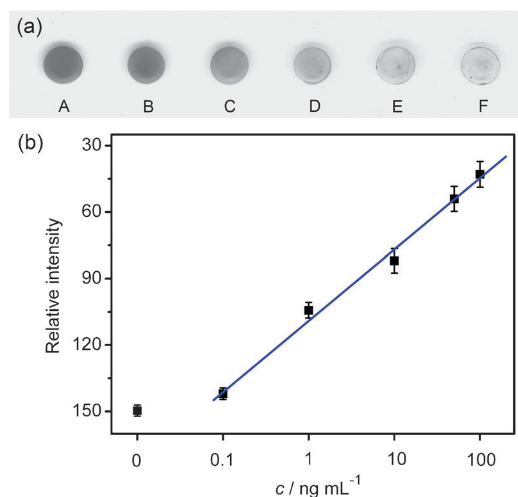

Fig. 2 (a) Scanometric image of the spots responding to 0, 0.1, 1, 10, 50, and 100 ng mL^{-1} MMP-7 (A–F), and (b) quantitative dynamic range of the designed strategy. The error bars represent the standard deviations calculated from three different spots.

interaction between His tag and chelated Ni^{2+}, little non-specific adsorption of AuNPs on the NTA-modified chip and silver signal amplification. More importantly, such a performance was obtained only using a flatbed scanner rather than any sophisticated analytical instrument.

The intra-assay precision of the scanometric strategy was evaluated by five replicative measurements of MMP-7 at two levels in one assay. The intra-assay variation coefficients were 2.3% and 4.4% at MMP-7 concentrations of 1 and 10 ng mL^{-1}, respectively, showing a good repeatability. While the inter-assay variation coefficients for five independent assays at these concentrations were 7.8% and 8.4%, respectively, indicating acceptable fabrication reproducibility. Moreover, the peptide–AuNP probe stored for one week and three weeks at 4 °C could induce 98.1% and 91.3% of the initial relativity intensity, respectively, indicating acceptable stability of the peptide–AuNP probe.

To further evaluate the application potential, the proposed approach was used to detect MMP-7 with a wide range of concentrations in the cell culture supernatants, respectively (Fig. 3). The MMP-7 concentration in the culture supernatant of the K562 cell was detected to be 1.38 ng mL^{-1}, which was in good agreement with 1.20 ng mL^{-1} obtained with a conventional ELISA method. The relative intensity decreased proportionally with the increasing concentration of spiked MMP-7. According to the calibration curve, the recovery for 1, 10, and 50 ng mL^{-1} MMP-7 spiked in culture supernatant was 100.6%, 104.8% and 107.5%, respectively. Thus, the designed method allowed accurate quantification of MMP-7 in complex biological matrices without the matrix effect.

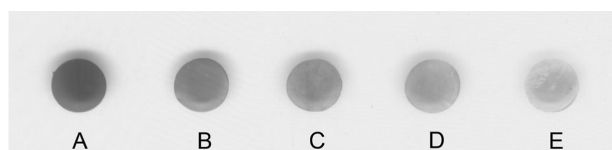

Fig. 3 Scanometric image of the spots responding to fresh culture fluid (A), initial cell culture supernatant of the K562 cell (B), and (B) spiked with 1, 10, and 50 ng mL^{-1} of purified MMP-7 (C–E).

In summary, a facile strategy is developed for ultrasensitive detection of MMPs by combining their discriminatory and proteolytic activities with the peptide functionalized AuNP probe, NTA modified chip, silver signal amplification and convenient scanometric readout. This method shows a broad dynamic range, high sensitivity, low matrix effect, and good reproducibility, stability and accuracy. By using multiplex peptide–AuNP probes, the strategy could be developed for multiple detection of MMPs. We anticipate that this method could be readily expanded for detection of MMPs in clinical diagnosis, prognosis, and therapy monitoring, and would become a powerful tool for underlying the roles of MMPs in pathophysiological processes.

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Ultrasensitive scanometric strategy for detection of matrix metalloproteinases using histidine tagged peptide-Au nanoparticle probe

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Experimental details

Materials and reagents. Human MMP-7, nitrilotriacetic acid (NTA), 3-glycidoxypropyltrimethoxysilane (GPTMS), bis(p-sulfonatophenyl)phenylphosphine dihydrate dipotassium salt (BSPP), nickel(II) chloride and silver enhancer solutions A and B were purchased from Sigma-Aldrich (USA). Chloroauric acid ($HAuCl_4$ $4H_2O$) and trisodium citrate were obtained from Shanghai Reagent Company (Shanghai, China). Sodium borohydride ($NaBH_4$) was obtained from Sinopharm Chemical Reagent Co. Ltd (China). The enzyme-immunoassay kit for human MMP-7 was purchased from Boster Biological Technology Co., Ltd. (China). 15 amino acid peptide (HHHHHHRPLALWRSC) was synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (China). All other reagents were of analytical grade. All aqueous solutions were prepared using ultra-pure water ($\geqslant$ 18 MΩ, Milli-Q, Millipore).

Apparatus. The UV-vis absorption spectrum was recorded with an UV-3600 UV-vis-NIR spectrophotometer (Shimadzu, Japan). The transmission electron microscopic (TEM) image was observed under a JEM-2100 transmission electron microscope (JEOL Ltd, Japan). Agarose gel electrophoresis was performed at PowerPac electrophoresis apparatus (Bio-Rad, U.S.A.). The AFM images were examined with an Agilent 5500 atomic force microscopy (AFM, U.S.A.) Scanometric images were obtained with a flatbed scanner (HP scanjet 2400, Hewlett-Packard).

Preparation of peptide-AuNP probe. 5-nm Au nanoparticles (AuNPs) were synthesized according to the method reported previously with some modification.[1] All glassware used in the synthesis procedures were immersed in freshly prepared aqua regia (HNO_3: HCl = 1:3) for 24 h, then washed with water and dried before use. 0.6 mL of ice-cold 0.1 M NaBH4 was added into 50 mL aqueous solution containing 0.25 mM $HAuCl_4$ and 0.25 mM trisodium citrate with stirring. The orange-red solution was stirred for 1 h to obtain AuNPs solution, which was stored at 4 °C.

1 mg BSPP was added to 4 mL AuNPs solution, and the mixture was stirred overnight. The AuNPs were then subjected to ultrafiltration using Vivaspin concentrator (Sartorius, 10,000 MW) at 14,000 g for 10 min to remove excessive BSPP. The upper phase was washed twice with pH 7.4 10 mM HEPES, and dissolved in 500 μL HEPES to obtain a solution of BSPP-protected AuNPs. 20 μL of 100 μM peptide was then added to the solution and left overnight at 4 °C under shaking. The resulting mixture was ultrafiltrated using Vivaspin concentrator (10,000 MW) at 14,000 g for 10 min at 4 °C to remove the non-conjugated peptide. The upper phase was washed thrice with HEPES by ultrafiltration. The obtained peptide-AuNP probe was dissolved in 100 μL pH 7.4 10 mM HEPES and store at 4 °C. The concentration of prepared peptide-AuNP probe was estimated to be approximately 250 nM from their optical absorbance at 520 nm.[2] Prior to use, peptide-AuNP probe was diluted with reaction buffer of MMP-7, which is pH 7.4 10 mM HEPES containing 0.2 M NaCl, 10 mM $CaCl_2$ and 50 μM $ZnCl_2$.

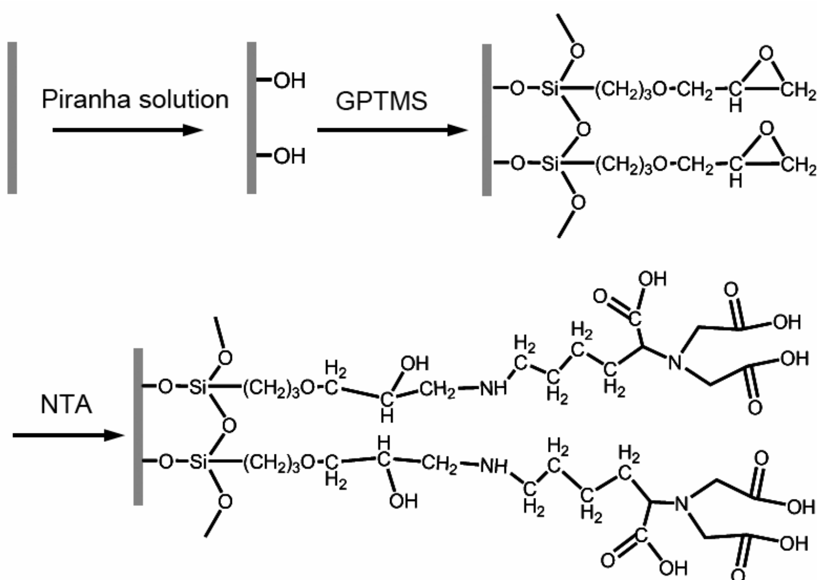
Preparation of NTA modified chip. A glass slide (25 mm × 75 mm × 1 mm) was firstly treated with piranha solution (30% hydrogen peroxide and 70% sulfuric acid) for 24 h, and silylanized by dipping it

in toluene solution of 1% GPTMS for 24 h at room temperature to obtain epoxy-coated slide.[3] 5 μL of 10 mM NTA solution was dropped on the epoxy-coated slide at a defined location, respectively, to form a droplet array of NTA, which was then incubated at room temperature for 3 h in a humidified chamber. The remaining active epoxy groups were reduced with sodium borohydride (1 mg in 4 mL 25% ethanol). After the slide was thrice washed with ultrapure water, the resulting NTA-modified chip was used for following operation.

Detection protocol. 5 μL of 10 mM nickel chloride solutions were added on the defined NTA spots and incubated for 1 h in a humidified chamber to immobilize Ni^{2+} ions onto the chelating NTA centers. The slide was washed thrice with ultrapure water to remove the non-immobilized Ni^{2+}. Then 5 μL of 1:1 mixture of peptide-AuNP probe and analyte was added on each Ni^{2+}- immobilized spot, and incubated at 37 °C for 1.5 h in a humidified chamber. The slide was carefully washed thrice with pH 7.4 10 mM HEPES containing 0.05% Tween 20, rinsed twice with ultrapure water to remove the non-bound AuNP probe, and dried under a stream of nitrogen. Finally, the sliver enhancement was preformed on each spot by reaction with 5 μL 1:1 mixture of silver enhancer solutions A and B for 4 min. After rinsed with ultrapure water and dried under a stream of nitrogen, the resulting chip was scanned with a flatbed scanner. The greyscale values of the spots were obtained with an Adobe Photoshop software.

Detection of MMP-7 in cell culture supernatant. Human leukemic K562 cell line were cultured in a flask in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (FCS, Sigma), penicillin (100 μg mL^{-1}) and streptomycin (100 μg mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO2. After culture for 12 h, the cell culture fluid was collected and centrifuged at 1,000 rpm for 10 min to remove cell debris. The obtained supernatant and fresh culture fluid served as the real samples for detection of MMP-7 with the designed method. The MMP-7 in the supernatant was also quantified with conventional ELISA method according to the manufacturer’s instruction.

Reaction scheme for preparation of NTA modified chip



Scheme S1. *Schematic representation of preparation of NTA modified chip.*

Characterization of peptide-AuNP probe binding

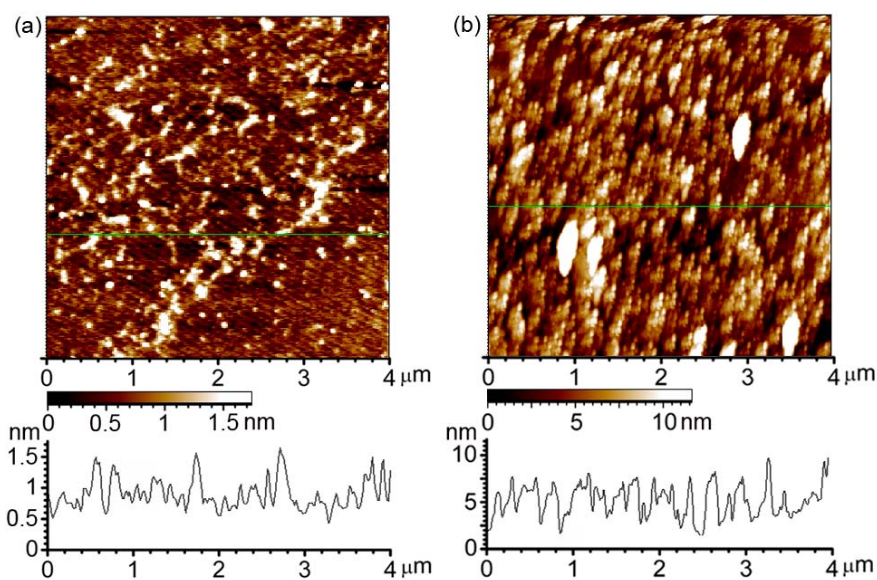


Fig. S1. *AFM image of (a) NTA modified mica chip and (b) peptide-AuNP probe bound on NTA modified mica chip by* N^{i2+} *chelation. The freshly cleaved mica chip was used to replace the glass chip.*

Examination the designed scanometric strategy

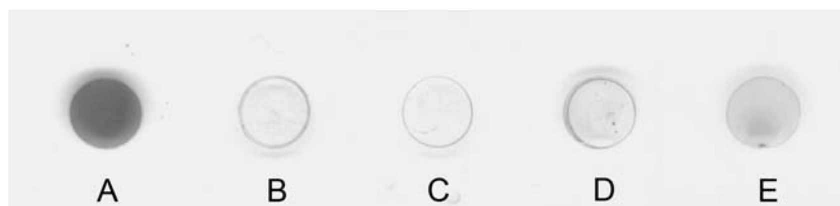


Fig. S2 *Scanometric image of spots on a NTA-modified chip with different treatments followed by identical sliver enhancing step. Incubation with (A) peptide-AuNP probe and (B) AuNPs after* N^{i2+} *chelation, (C) peptide-AuNP probe without preliminary* Ni^{2+} *chelating step, (D) peptide-AuNP probe after* Ni^{2+} *chelation and blocking with excess free peptide, and (E) the mixture of peptide-AuNP probe and 100 ng* mL^{-1} *MMP-7 after* Ni^{2+} *chelation. The concentrations of* Ni^{2+}*, AuNPs and peptide-AuNP probe were 10 mM, 125 and 125 nM, respectively.*

Optimization of detection conditions

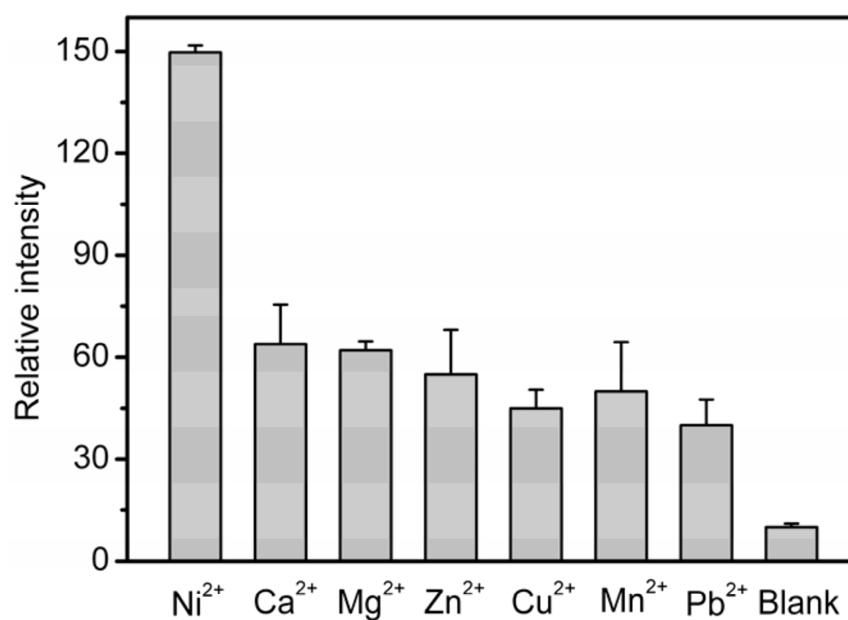


Fig. S3*. Dependence of relativity intensity of greyscale on chelated metal ions for binding peptide-AuNP probe. The concentration of each metal ion was 10 mM.*

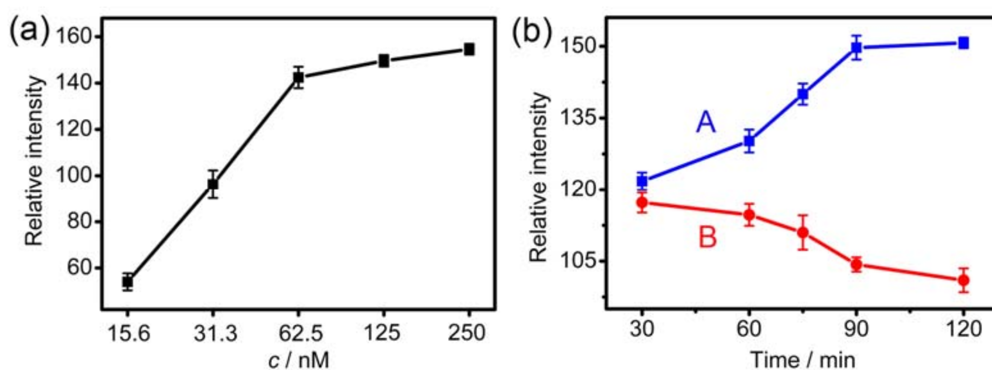


Fig. S4. *Dependence of relativity intensity of greyscale on (a) peptide-AuNP probe concentration and (b) incubation times of (A) 125 nM peptide-AuNP probe and (B) a mixture of 125 nM peptide-AuNP probe and 1 ng mL^{-1} MMP-7.*

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