Metal-linked immunosorbent assay (MeLISA): the enzyme-free alternative to ELISA for biomarker detection in serum

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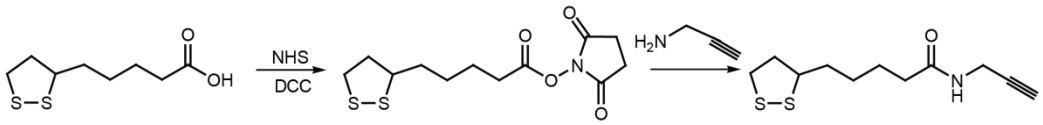
1. Reagents and Instrumentation

Lipoic acid, propargylamin, DCC, NHS and other chemicals were analytic reagents and used as received without any purification. Ultrapure water (18 MΩ) from a Millipore system was used to prepare solutions throughout the assay. BSA (bovine serum albumin) was purchased from Sigma-Aldrich. 96-well polystyrene plates were purchased from Corning Incorporated. Human AFP, CRP ELISA kits were obtained from Shanghai MLBIO Biotechnology Co. Ltd. The AFP antigen and antibodies, CRP antigen and antibodies were provided by prof. H.X.H. and the real samples for AFP clinical diagnosis were provided by prof. H.Y.W..

UV-Vis spectra were obtained on an Ocean optical USB 2000+ UV-Vis spectrometer.

2. Preparation of functionalized AuNPs



compound 2 compound 1

Synthesis of compound 1[1]**.** A solution of DCC (0.5 g, 2.5 mmol) in 3 mL dichloromethane was added to an ice-cooled solution of lipoic acid (0.5 g, 2 mmol) and N-hydroxysuccinimde (0.23 g, 2 mmol) in 4 mL dichloromethane. To fully precipitate dicyclohexylurea (DCU), the solution was placed in refrigerator overnight after stirring for 1 h. The filtrate was obtained after filtration of the precipitate and then was evaporated in vacuo. Yellowish solid was formed after using diethyl ether to treat with the residue and then was washed with ether. Thus the pure product of compound 2 was obtained. To synthesize compound 1, compound 2 (0.5 g, 1.56 mmol) was dissolved in 10 mL dichloromethane, and then added drop-wise an ice cold solution of propargylamine (100 μL 1.56 mmol) in dichloromethane. The resulting solution was diluted with 30 mL dichloromethane and saturated sodium carbonate after stirring for 3 h. The organic layer was washed three times with saturated brine, dried with anhydrous sodium sulfate over then evaporated to dryness. Subsequently the obtained residue was purified by silica gel column chromatography. Figure S1-S3 are the characterization of the target compound.

Synthesis of AuNPs. AuNPs were prepared by the citrate-induced reduction of $HAuCl_4$. In a 100 mL round bottom flask, 50 mL of 0.01 wt% $HAuCl_4$ was heated to rolling boil to reflux with vigorous stirring. Addition of 5 mL of 38.8 mM sodium citrate resulted in a color change of the solution from pale yellow to red. Heating was continued for 15 min and then removed while the stirring was kept for another 15 min. The resulting solution was allowed to cool to room

temperature and then characterized by UV-Vis spectrometer. The synthesized AuNPs was stored at 4℃ in a centrifuge tube.

Preparation of terminal alkyne-functionalized AuNPs. 200 μL solution of compound 1(1 mM in ethanol) was added drop-wise to 10 mL Au colloid, using sodium hydroxide to adjust the basicity of the solution to pH 9. The resulting solution was stirred for 24 h under room temperature and then was centrifuged for 20 min (9000 rpm) to remove the excess compound 1. The obtained alkyne-functionalized AuNPs was washed with water for three times, redispersed in distilled water and then stored in a refrigerator at 4℃.

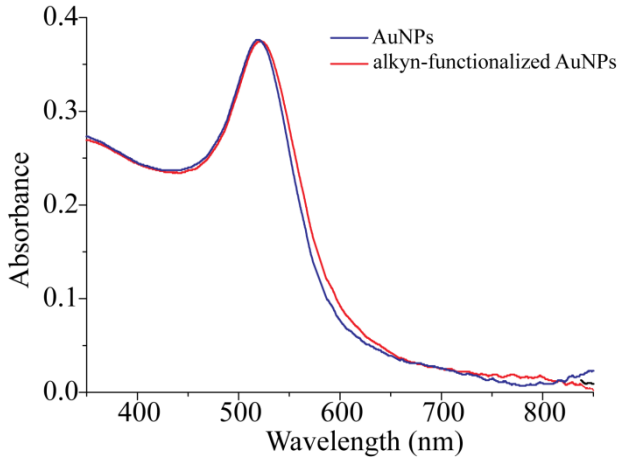


Figure S1. UV-Vis spectra of bare AuNPs and alkyne-functionalized AuNPs. As we can see, there is a slightly red shift in UV spectra after functionalization.

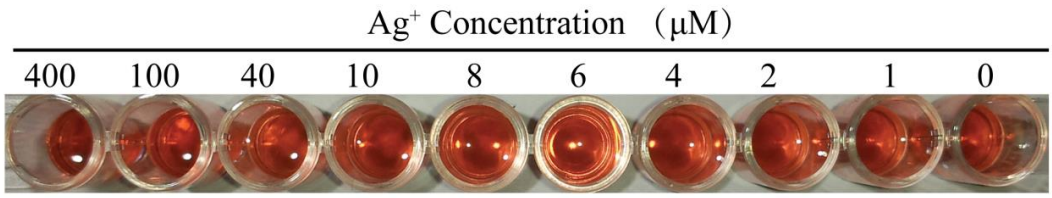


Figure S2. Mixed solution of bare AuNPs in the presence of different concentrations sliver ions.

3. The stability of the alkyne-functionalized AuNPs in the presence of different metal ions

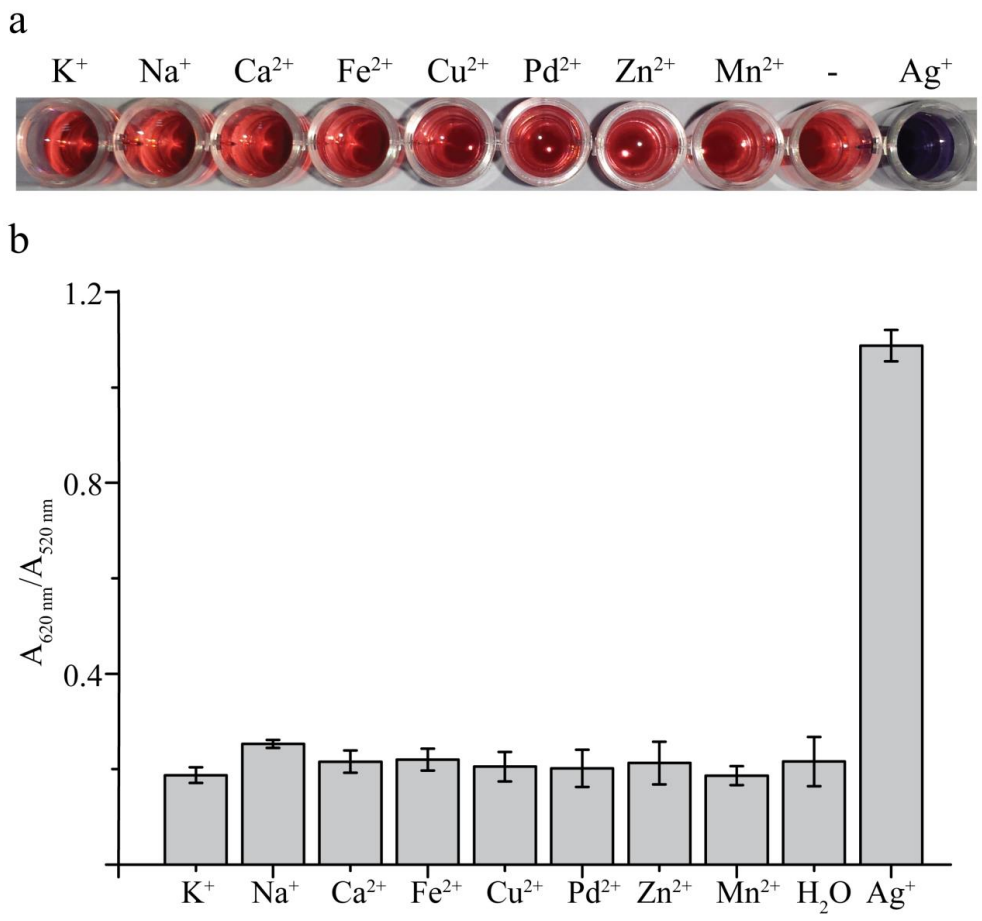


Figure S3. a) Mixed solution of alkyne-functionalize AuNPs and different metal ions. The concentration of Ag^{+} was 4 μM, while other metal ions were in a concentration of 40 μM, ten times more than the Ag^{+}. Well 9 was blank control, adding distilled water instead. b) Responses of A_{620nm}/A_{520nm} value with different metal ions. Actually, Au^{3+} could also induce the aggregation of alkyne-functionalized AuNPs and the reason remained unclear.

Target analyte	Time	Limit of detection	Reference
Hydrogen peroxide	15 min	Color change in a narrow range from 100 μM to 120 μM	14
Cu^{2+}	10 min	1 μM	21
Glucose	5 min	0.5 mM	26
Hg^{2+}	20 min	0.1 μM	24
Hydrogen peroxide	20 min	10 μM	22
Ag^{+}	1 sec	1 μM	This work

Table S1. Comparison of reaction time and limit of detection typically reported in literatures. Conventional AuNPs-based colorimetric assay generally based on either growth (ref. 22) or aggregation (ref. 14, 21, 26, 24) of AuNPs. Current AuNPs aggregation induced by interparticle crosslinking of biomolecules often requires approximately 10 minutes, and the growth of AuNPS would require more time. Therefore, the significant advantage of this work is to reduce the colorimetric time to second level.

4. Preparation of antibody labeled AgNPs

Synthesis of 70-nm AgNPs. Silver nanoparticles were prepared according to a classic method. 18 mg $AgNO_3$ were dissolved in 100 mL distilled water, heated to boil with vigorous stirring. 2 mL 1% sodium citrate was added in the boiling solution and continued heating for 30 min. The Ag sols were stored in dark bottles after cooling to room temperature.

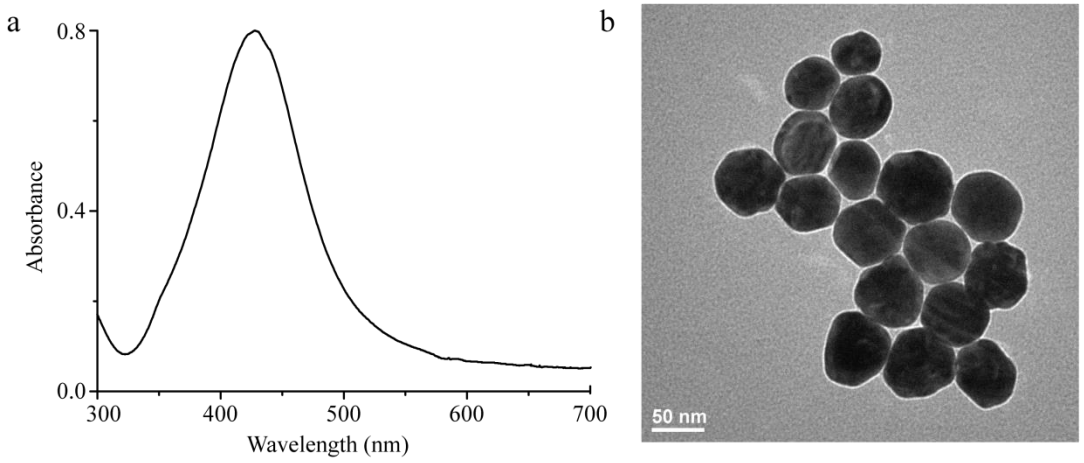


Figure S4. UV-Vis spectrum (a) and TEM image (b) of the synthesized silver nanoparticles with a diameter about 70 nm.

Combination of AgNPs to antibody. We prepared the antibody labeled Ag nanoparticle by using electrostatic adhesion method, which is similar with the commercialized product (gold nanoparticle labeled with antibody). Thus, the robustness and universal of our functionalization is unquestionably good. In detail, the synthesized silver nanoparticles were condensed using centrifugation and finally dispersed in 10 mM (or lower) PBS (pH 7.4, avoiding chloridion), to which 1 mL antibody was added in quickly. The mixed solution was shaked for 12 h and then blocked for another 12 h with 500 μL 1% BSA. The solution was repeatedly centrifuged under optimized conditions to get rid of the excess antibody and silver nanoparticles. AgNPs-AB (AgNPs conjugated with antibody) was dispersed in 0.1% BSA (20 mM PBS with 150 mM Cl^{-1}) and then stored at 4℃. Note that, the concentration of silver nanoparticles should be higher than that of the detected antigen.

The reason for choosing 70 nm Ag nanoparticles is a compromise of sensitivity and practicability. Although larger diameter of Ag nanoparticles would produce higher sensitivity, lager Ag nanoparticles with uniform shape and size is difficult to synthesis and is hardly adapted into immunosorbent assay because of the less stable suspension. The diameter of 70 nm is employed since it is enough for the sensitivity and it could easily suspend in the solution.

5. The dissolution of AgNPs in the presence of hydrogen peroxide

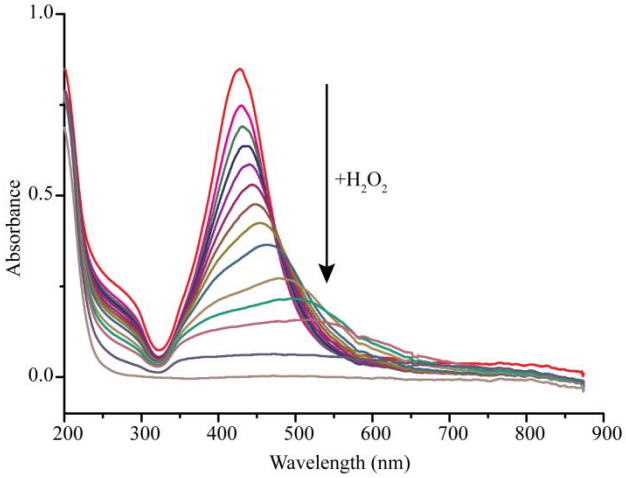


Figure S5. UV-Vis spectra of silver nanoparticles in the presence of 5 mM hydrogen peroxide. The process could complete in approximately five minutes. While it is worth mentioning that the process could be accomplished in a shorter time with a higher concentration of hydrogen peroxide.

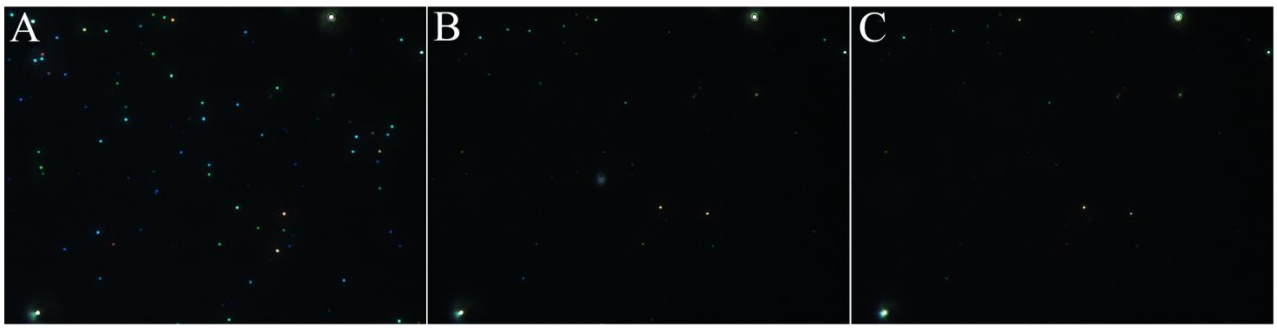


Figure S6. Dark-field images of the silver nanoparticles before (A) and after incubation with hydrogen peroxide for 2 min (B) and 5 min (C).

6. Enhanced MeLISA for AFP detection

Sandwich assay for AFP. 100 μL of capture antibody diluted 500 times in sodium bicarbonate (50 mM, pH 9.6) was added into each well of 96-well polystyrene plate, and then the plate was incubated at 4℃ overnight. After removing the coating solution, the plate was washed three times by filling the wells with 200 μL PBST. The plate was loaded with 200 μL 1% BSA for 2 h in room temperature to block the remaining protein binding site. After three times washing, 100 μL of appropriately diluted AFP solution from 10^{-6} g mL^{-1} to10^{-12} g mL^{-1} were added into each well. The blank was set as negative control by adding buffer only. After a 1 hour's incubation at 37℃, the plate was washed three times with 200 μL PBST. A solution of AgNPs-AB dissolved in blocking

buffer was added and the plate was incubated for 40 min at 37℃. The plate was washed three times and 50 μL hydrogen peroxide with a concentration of 0.1 M was added into each well. The plate was incubated at room temperature for 5 min to fully dissolve the AgNPs and 50 μL functionalized AuNPs was added. The addition of functionalized AuNPs induced a color change of the solution within one second, at this time, results were recorded as photographs.

The incubation time for AgNP-AB. AgNP-AB was used to replace the enzyme-linked antibody in ELISA. Considering this difference, we optimized the incubation time for AgNP-AB. For this purpose, identical protocol was performed as "sandwich assay for AFP" (the concentration of AFP was 10^{-8} g mL^{-1}). As described in Figure S6, within 40 min the specific antigen-antibody reaction was already reached maximum degree. The incubation time is similar to that in classic immunoassay.

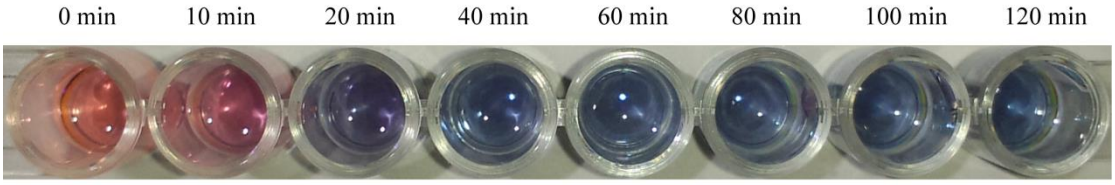


Figure S7. The incubation time for AgNP-AB had been optimized. Finally we employed 40 minutes as the adjusted incubation time.

The amount of antibody adsorbed to silver nanoparticles. The concentration of antibody was optimized to better combine with silver nanoparticles in AFP detection. An AFP concentration of 10^{-8} g mL^{-1} was used to he experiment. As depicted in Figure S7, the concentration of antibody should be five times higher than that of AgNPs. In the following experiment, a concentration of antibody that of ten times AgNPs was used to do the combination.

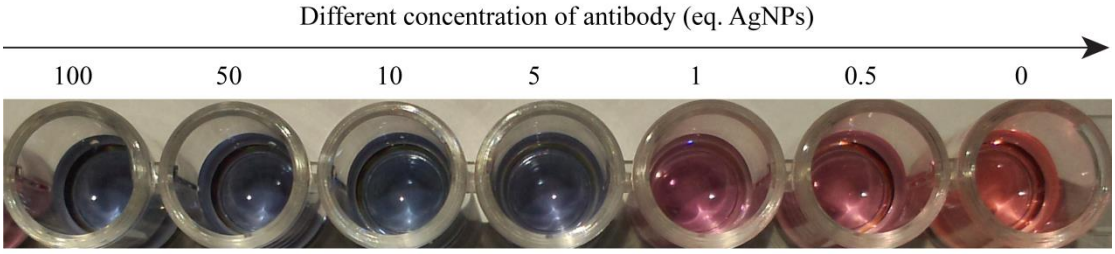


Figure S8. Different concentrations of antibody were used to conjugate with AgNPs. The immunoassay procedure was identical to the sandwich assay for AFP.

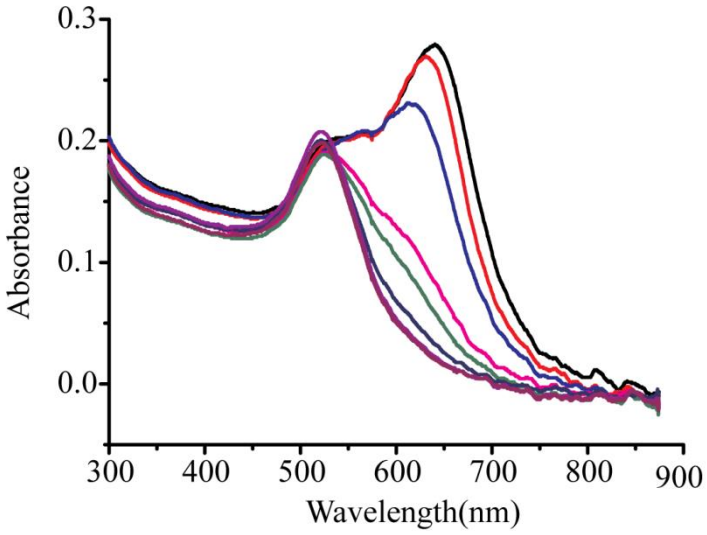


Figure S9. UV-Vis spectra of the solution in each well in the detection of AFP using this enhanced MeLISA.

7. Enhanced MeLISA for CRP detection.

AgNPs with a diameter of 15 nm were prepared via sodium borohydride reduction. The combination process to antibody was similar to 70-nm AgNPs. The detection procedure for CRP was identical to the detection of AFP. For the diameter of 15 nm and 70 nm, the generated silver ions differ by two orders of magnitude. The assay performance for CRP detection differs approximately two orders of magnitude likewise.

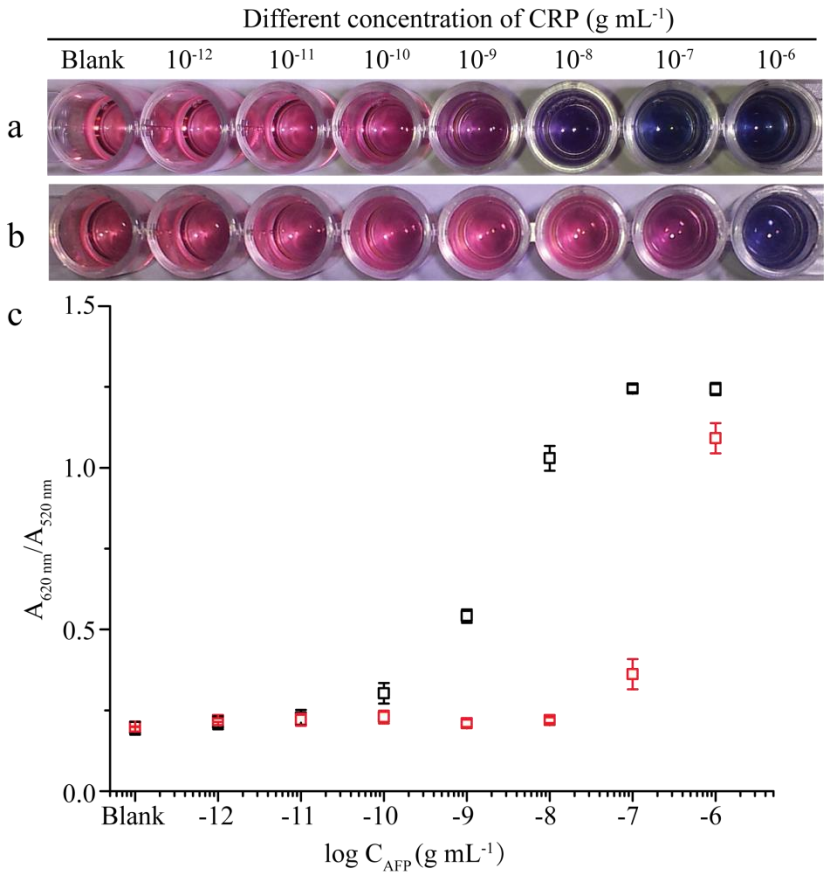


Figure S10. Photograph for the detection of different concentrations of CRP using silver nanoparticles with a diameter of 70 nm (a) and 15 nm (b). c) Plots of the $A_{620\ nm}/A_{520\ nm}$ values against different concentrations of CRP.

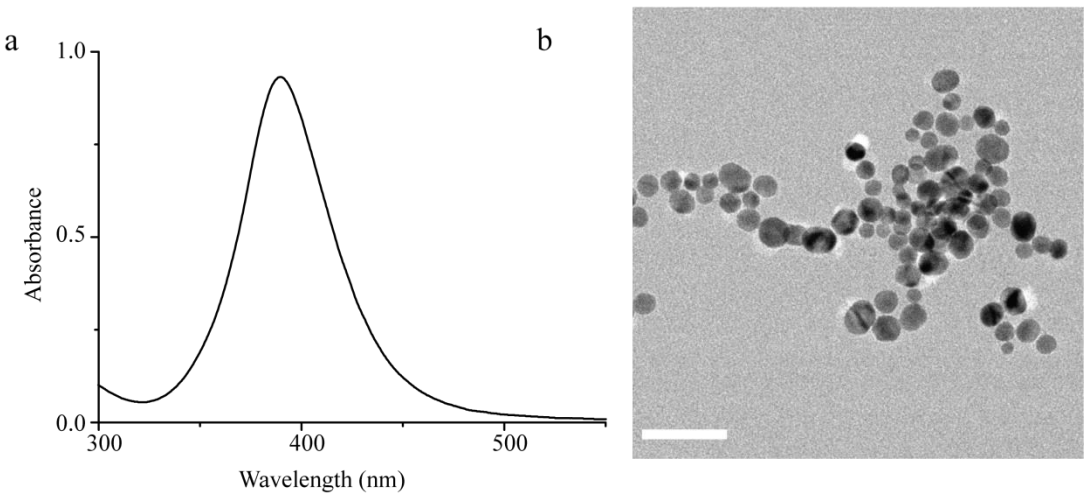


Figure S11. UV-Vis spectrum (a) and TEM image (b) of the synthesized silver nanoparticles with a diameter about 15 nm. Scale bar: 50 nm.

8. Procedure for HRP-based ELISA

Human ELISA kit was taken out of the fridge 20 minutes before each use. 50 μL target concentrations of analyte (different antigens) were added to each microwell. The plate was shaked and then placed in 37 ℃ for 1 hour. After discarding the solution, the plate was washed 5 times with washing buffer to remove the remaining antigen. The plate was incubated with 100 μL HRP-linked antibody for 1 hour in 37 ℃. Following the same washing steps, 50 μL chromogenic A and 50 μL chromogenic B were added to the plate successively and then the plate was incubate in 37 ℃ for 30 minutes. At last, 50 μL stop solution was added to stop the catalytic process and the value of $OD_{450\ nm}$ was obtained through a microplate reader.

9. The detection protocol for clinical samples

The serum samples were collected from patients with hepatocellular carcinoma and normal subjects, regarding as positive and negative samples respectively. The detection procedure was identical to sandwich assay for AFP detection. Using diluted sera instead of AFP antigen during the whole process, and the results were obtained in a microplate reader for convenience. Compared with other types of assays (electrochemical, fluorescent, magnetic etc.), in MeLISA, the results could be obtained using the same equipment with ELISA. The great advantage of MeLISA lies in that it could be directly adapted to conventional ELISA without employing extra equipment.

The assay performance performed by the novice. The novice, here, is a second-year master student in our laboratory, who is skilled in nanoparticles synthesis and functionalization, but unfamiliar with any immunosorbent assay. A detailed protocol but no further training was provided prior to performing the experiments. Actually, success was obtained in the second attempt. And the first attempt failed because of inadequate washing. Note that the last washing should be more severe and thorough to avoid false-positive results.

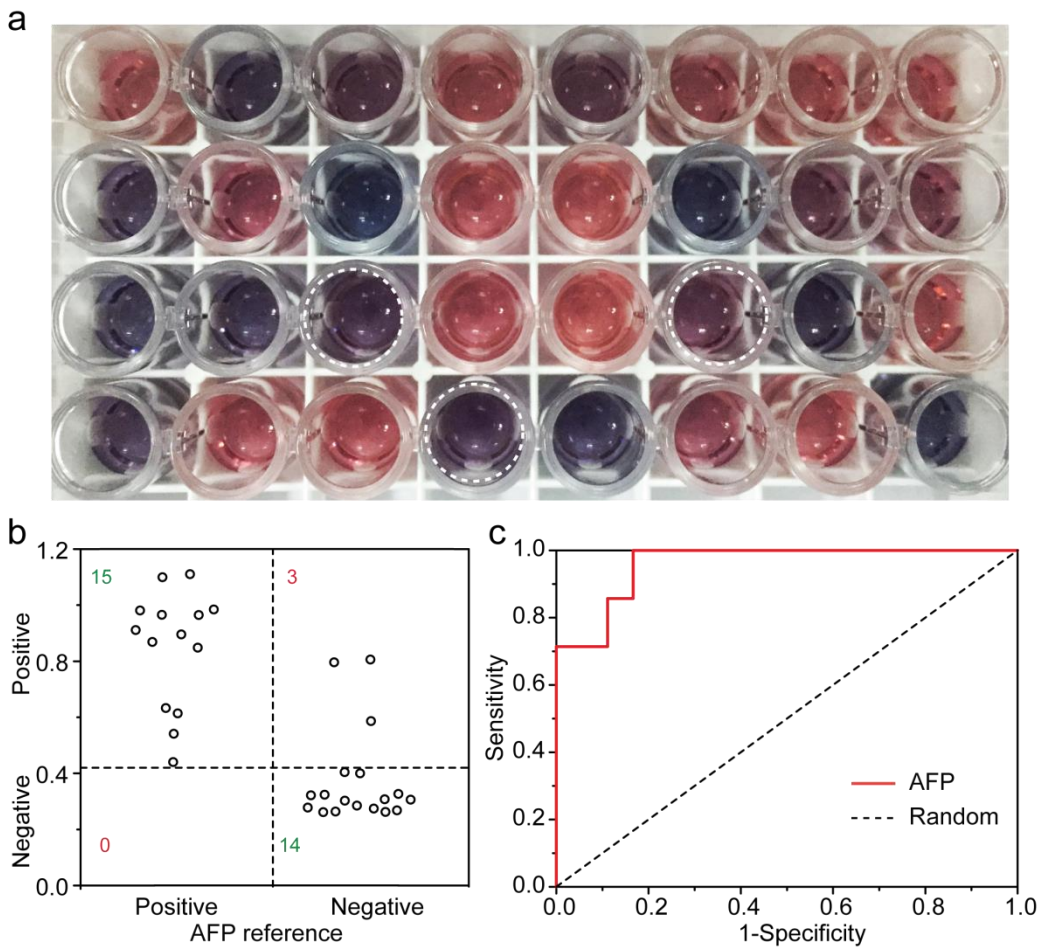


Figure S12. Results of double-blind experiment performed by a student who is not familiar with this system. a) Colorimetric double-blind results obtained from the enhanced MeLISA consisted of 32 serum samples. b) $A_{620\ nm}/A_{520\ nm}$ values of serum specimens that are positive and negative for HCC. c) ROC curves for HCC.

Distribution in microwell	Concentration of AFP by ECLIA (ng mL^{-1})	positive and negative distribution by ECLIA	$A_{620\ nm}/A_{520\ nm}$	positive and negative distribution by MeLISA
A1	2.4	negative	0.247	negative
A2	177.5	positive	0.337	positive
A3	0.7	negative	0.251	negative
A4	1.9	negative	0.248	negative
A5	1.9	negative	0.248	negative
A6	>1210	positive	1.318	positive
A7	2.6	negative	0.261	negative
A8	>1210	positive	1.232	positive
A9	3.3	negative	0.261	negative
A10	146.9	positive	0.324	positive
A11	2.9	negative	0.268	negative
A12	5.4	negative	0.272	negative
B1	228	positive	1.263	negative
B2	100.3	positive	0.266	negative
B3	>1210	positive	1.311	positive

B4	27	positive	0.269	negative
B5	2.1	negative	0.262	negative
B6	>1210	positive	1.304	positive
B7	>1210	positive	1.436	positive
B8	1.5	negative	0.273	negative
B9	0.9	negative	0.281	negative
B10	>1210	positive	1.254	positive
B11	348.5	positive	0.947	positive
B12	2.2	negative	0.29	negative
C1	1.1	negative	0.249	negative
C2	>1210	positive	1.346	positive
C3	>1210	positive	1.346	positive
C4	18.9	negative	0.268	negative
C5	>1210	positive	1.391	positive
C6	3.6	negative	0.291	negative
C7	4.5	negative	0.265	negative
C8	>1210	positive	1.213	positive
C9	>1210	positive	1.359	positive
C10	>1210	positive	1.354	positive
C11	>1210	positive	1.375	positive
C12	>1210	positive	1.382	positive
D1	>1210	positive	1.347	positive
D2	2.1	negative	0.252	negative
D3	>1210	positive	1.358	positive
D4	1.1	negative	0.256	negative
D5	282.9	positive	0.46	positive
D6	985.5	positive	1.301	positive
D7	9.5	negative	0.286	negative
D8	5.5	negative	0.263	negative
D9	>1210	positive	1.458	positive
D10	>1210	positive	1.376	positive
D11	>1210	positive	1.21	positive
D12	2.1	negative	0.285	negative
E1	2.4	negative	0.269	negative
E2	18.9	negative	0.268	negative
E3	>1210	positive	1.288	positive
E4	699.2	positive	1.211	positive
E5	5.1	negative	0.284	negative
E6	406	positive	0.621	positive
E7	>1210	positive	1.299	positive
E8	1027	positive	1.211	positive
E9	1.8	negative	0.281	negative
E10	2.5	negative	0.28	negative
E11	>1210	positive	1.283	positive
E12	54.9	positive	0.282	negative
F1	>1210	positive	1.284	positive
F2	3.7	negative	0.259	negative
F3	>1210	positive	1.383	positive
F4	>1210	positive	1.393	positive

F5	3.8	negative	0.256	negative
F6	180.6	positive	1.238	positive
F7	21.3	positive	0.281	negative
F8	>1210	positive	1.258	positive
F9	>1210	positive	1.404	positive
F10	4.5	negative	0.271	negative
F11	>1210	positive	1.301	positive
F12	>1210	positive	1.342	positive
G1	1.7	negative	0.254	negative
G2	>1210	positive	1.383	positive
G3	78.4	positive	0.511	positive
G4	2.2	negative	0.263	negative
G5	2.1	negative	0.26	negative
G6	11.8	negative	0.269	negative
G7	1.8	negative	0.304	negative
G8	>1210	positive	1.335	positive
G9	3.9	negative	0.278	negative
G10	1.8	negative	0.269	negative
G11	>1210	positive	1.356	positive
G12	921.2	positive	0.658	positive
H1	>1210	positive	1.303	positive
H2	30	positive	0.256	negative
H3	3.1	negative	0.253	negative
H4	4.8	negative	0.254	negative
H5	1	negative	0.269	negative
H6	1.9	negative	0.281	negative
H7	>1210	positive	1.333	positive
H8	>1210	positive	1.277	positive
H9	3	negative	0.283	negative
H10	2.5	negative	0.271	negative
H11	1.5	negative	0.278	negative
H12	3.6	negative	0.296	negative

Table S2. Concentrations of AFP in these unrelated sera obtain from the hospital.

Distribution in microwell	Concentration of PSA by ECLIA (ng mL^{-1})	positive and negative distribution by ECLIA	$A_{620\ nm}/A_{520\ nm}$	positive and negative distribution by MeLISA
A1	5.66	positive	0.894	positive
A2	5.81	positive	1.106	positive
A3	31.25	positive	1.332	positive
A4	5.85	positive	1.207	positive
A5	6.45	positive	1.052	positive
A6	0.01	negative	0.261	negative
B1	0.01	negative	0.292	negative
B2	0.51	negative	0.303	negative
B3	7.4	positive	0.831	positive
B4	18.69	positive	1.292	positive
B5	0.09	negative	0.272	negative

B6	0.41	negative	0.319	negative
C1	4.56	positive	0.843	positive
C2	0.47	negative	0.315	negative
C3	0.32	negative	0.340	negative
C4	9.43	positive	1.145	positive
C5	0.41	negative	0.341	negative
C6	0.04	negative	0.250	negative
D1	0.44	negative	0.278	negative
D2	0.46	negative	0.288	negative
D3	0.86	negative	0.291	negative
D4	100	positive	1.369	positive
D5	0.3	negative	0.295	negative
D6	6.21	positive	1.011	positive
E1	0.76	negative	0.353	negative
E2	7.19	positive	1.168	positive
E3	0.01	negative	0.274	negative
E4	10.53	positive	1.291	positive
E5	5.18	positive	1.248	positive
E6	4.07	positive	0.995	positive

Table S3. Concentrations of PSA in these sera obtain from the hospital.

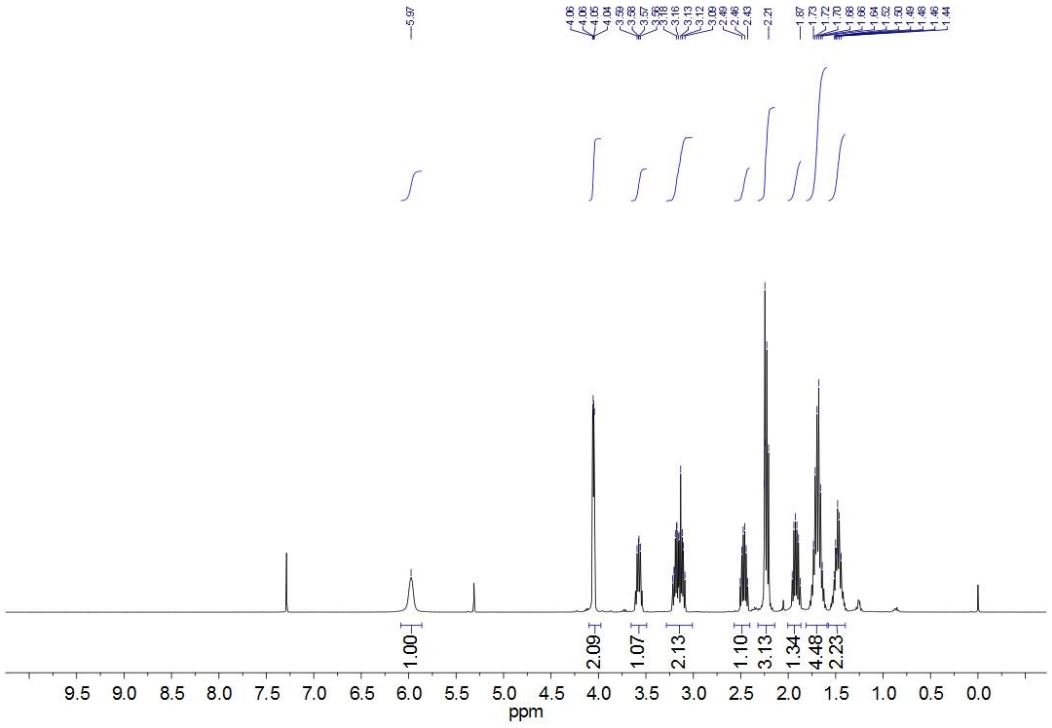


Figure S13. ^{1}H NMR spectrum of 5-(1,2-dithiolan-3-yl)-N-(prop-2-ynyl) pentanamide

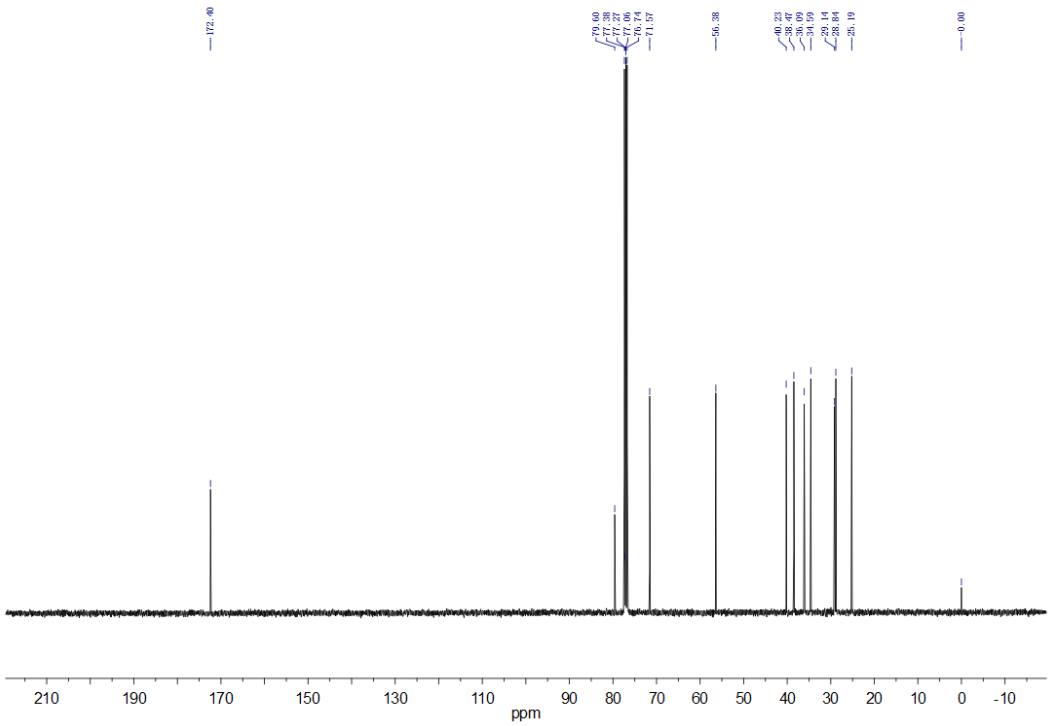


Figure S14. ^{13}C NMR spectrum of 5-(1, 2-dithiolan-3-yl)-N-(prop-2-ynyl) pentanamide

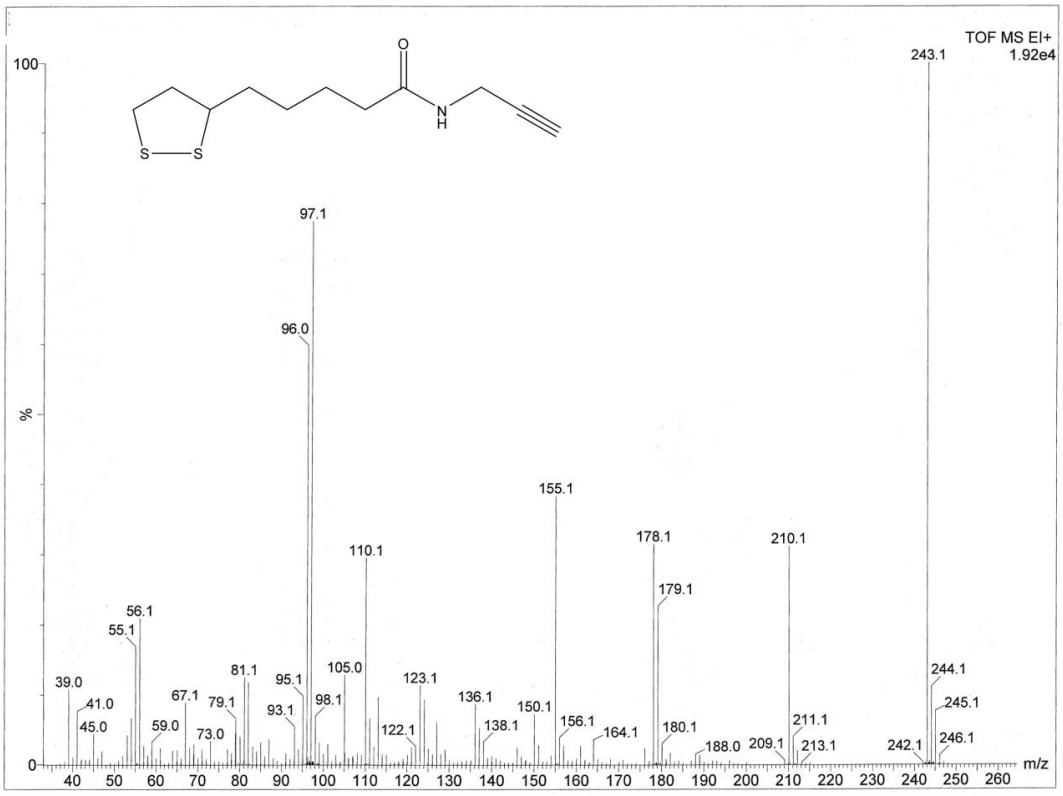


Figure S15. Mass spectrum (EI+) of 5-(1, 2-dithiolan-3-yl)-N-(prop-2-ynyl) pentanamide

Reference

1. Shi L, Jing C, Ma W, et al. Plasmon resonance scattering spectroscopy at the single-nanoparticle level: real-time monitoring of a click reaction. Angew Chem Int Ed. 2013; 52:6011–4.