ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

Magnetic mesoporous $CoFe_2O_4$ labels reacted with TMB for use in a sandwiched photothermal immunoassay for thyroglobulin

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EXPERIMENTAL SECTION

Material and Chemical. Rabbit monoclonal [EPR9730] to thyroglobulin capture antibody (Ab_1; Application: Flow Cy, ICC/IF, IHC-P, WB; Reactivity: mouse, rat, human; Conjugate: unconjugated; Cat #: ab156008), rabbit monoclonal [CPT-R35.3-31] to thyroglobulin secondary antibody (Ab_2; Application: ELISA, IHC-P; Reactivity: human; Conjugate: unconjugated; Cat #: ab243093) and human thyroglobulin ELISA kit including different-concentration thyroglobulin (TG) (Description: human thyroglobulin ELISA kit; Reactivity: human; Sample type: cell culture supernatant, serum, plasma; Cat #: ab15544) were purchased from Abcam (Shanghai, China). All high-binding polystyrene 96-well microtiter plates were obtained from Greiner (Ref. 655061, Greiner, Frickenhausen, Germany). Bovine serum albumin (BSA, 96-99%), 3-glycidyloxypropyl trimethoxysilane ($C_9H_{20}O_5Si$, GOPS) and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sinopharm Chem. Re. Co., Ltd. (Shanghai, China). All other reagents were of analytical grade and were used without further purification. Ultrapure water obtained from a Millipore water purification system (18.2 MΩ, Milli-Q, Millipore) was used in all runs.

A pH 9.6 coating buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ and 0.2 g NaN_3) and a pH 7.4 phosphate-buffered saline (PBS, 0.01 M) (2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.24 g KH_2PO_4, 0.2 g KCl and 8.0 g NaCl) were prepared by adding the corresponding chemicals into 1000 mL distilled water, respectively. The blocking buffer and washing buffer (PBST) were obtained by adding 1.0% BSA (w/v) and 0.05% Tween 20 (v/v) in PBS, respectively.

Apparatus. Transmission electron microscopy (TEM) was carried out on FEI Talos F200S G2 at 200 KV accelerating voltage (Thermo Fisher Scientific Co., Ltd). N_2 adsorption–desorption analysis was measured on a Micromeritics ASAP 2000 instrument (Micromeritics, Norcross, GA, USA). Pore volumes were determined using the adsorbed volume at a relative pressure of 0.99. Multipoint Brunauer–Emmet–Teller (BET) surface area was estimated from the relative pressure range from 0.06 to 0.3. The pore size distributions of the as-prepared samples were analyzed using the Barrett–Joyner–Halenda (BJH) method. Magnetic measurements were made using Nanjing University Instruments on vibrating sample magnetometer (VSM) at room temperature that produces fields of up to 6 T on the sample. UV-vis absorption spectra was recorded on an

Infinite M200 Pro of TECAN GENIOS with QS-grade quartz cuvettes at room temperature. The temperature curve was measured by VICTOR 86 digital thermometer (Xi'an Beicheng Electronic Co., Ltd, China).

Synthesis of $CoFe_2O_4$ nanoparticles. $CoFe_2O_4$ nanoparticles were synthesized according to this literature[S1] as follows: $Fe(NO_3)_3 \cdot 9H_2O$, $Co(NO_3)_3 \cdot 6H_2O$ and glycine (Gly) were dissolved in distilled water (Fe^{3+} : Co^{2+} = 2 : 1, Gly : nitrate = 4 : 1, in molar ratio). After filtration, the red precursor solution obtained was concentrated by heating, forming mixtures of amorphous melted salts. Then combustion reaction fragmentarily appeared and rapidly diffused. Finally, the black loose powders (*i.e.*, $CoFe_2O_4$ nanoparticles) were obtained after combustion for several seconds.

Synthesis of Fe_3O_4 nanoparticles. Fe_3O_4 nanoparticles (~30 nm in diameter) were synthesized through the chemical co-precipitation method similar to the works.[S2,S3] To fabricate the magnetic nanoparticles, 44.44 g of $FeCl_3$ salt and 1.732 g of $FeCl_2$ salt were dissolved in 80-mL deionized water. Under reflux conditions in the presence of nitrogen gas and mechanical rotation at 1000 rpm, the temperature of the mixture could reach 70 °C with a gentle slope. After stirring the solution for 30 min, 20 mL of the ammonia was slowly added to the solution and stirred at the same temperature for 30 min. Eventually, a black precipitate was formed, which was then separated from the mixture with the help of a magnet and rinsed with a solution of deionized water and ethanol.

Synthesis of Co_3O_4 nanoparticles. Co_3O_4 nanoparticles (size: 25 – 40 nm in diameter) were synthesized according to this work.[S4] 1.0 mmol common Co_2O_3 powder and 15 mL ethanol-water (v/v = 1:1) mixed solvent was poured into Teflon-lined stainless-steel autoclave with 20 mL capacity in turn. After the mixture was agitated vigorously about 20 min, the autoclave was sealed and maintained at 160 °C for 6-24 h in a digital-controlled constant temperature oven. Then autoclave was cooled to room-temperature naturally. The obtained black precipitate was centrifuged and washed extensively with deionized water and absolute ethanol several times. Finally, the as-obtained precipitates were dried in a vacuum at 60 °C for storage.

Labeling of Ab_2 secondary antibody with $CoFe_2O_4$. Ab_2 secondary antibody was covalently conjugated onto the surface of $CoFe_2O_4$ by using the cross-linkage GOPS referring to this work.[S5] Briefly, 20 mg of $CoFe_2O_4$ was initially dried at 80 °C for 1 h, and then incubated with 5 mL GOPS (5%, v/v) in dry toluene for 12 h at room temperature under gentle stirring. During this

process, GOPS molecules were conjugated onto the magnetic beads through the reaction between –OH groups on the magnetic beads and $–OCH_3$ groups on the GOPS. The GOPS-functionalized magnetic beads were separated by an external magnet, and washed thoroughly with toluene and ethanol to remove the physically adsorbed GOPS. Following that, the obtained GOPS-functionalized $CoFe_2O_4$ was heated for 1 h at 80 °C under N_2 to achieve active epoxy groups on the surface. The GOPS-functionalized $CoFe_2O_4$ was dispersed into 2 mL PBS, pH 7.4,. Afterwards, 100 μL of Ab_2 secondary antibodies (1.0 mg mL^{-1}) were simultaneously added to the 2 mL of GOPS-functionalized $CoFe_2O_4$ suspension, and incubated for 12 h at 4 °C under gentle stirring. The excess biomolecules was removed by magnetic separation. Finally, the as-prepared probe (designed as Ab_2-$CoFe_2O_4$) was stored in 2 mL PBS, pH 7.4, at 4 °C when not in use (Conc. ≈ 10 mg mL^{-1}). Similarly, Ab_2-Fe_3O_4 and Ab_2-Co_3O_4 were prepared by the same method.

Preparation of Ab_1 capture antibody-coated microplate. A high-binding polystyrene 96-well microtiter plates (Ref. 655061, Greiner, Frickenhausen, Germany) were coated overnight at 4 °C with 50 μL per well of Ab_1 antibody at a concentration of 10 μg mL^{-1} in 0.05 M sodium carbonate buffer (pH 9.6). The microplates were covered with adhesive plastics plate sealing film to prevent evaporation. On the following day, the plates were washed three time with PBST, and then incubated with 300 μL per well of blocking buffer for 1 h at 37 °C with shaking. The plates were then washed as before for use.

Immunoreaction protocol and photothermal measurement. Initially, 50 μL of thyroglobulin (TG) standards or samples with various concentrations in PBS were added into the microtiter plates, and incubated for 1 h at 37 °C under shaking. After washing, 50 μL of the prepared-above Ab_2-$CoFe_2O_4$ suspension (Conc. ≈ 10 mg mL^{-1}) was added into the well and incubated for 1 h at 37 °C with shaking. The plates were washed again, and 100 μL of substrate solution containing 7.63 M H_2O_2 and 0.921 mM TMB in PBS (10 mM, pH 7.4) was added to each well. The plates were shaken for 15 min on a plate shaker for photothermal measurement. The temperature sensor was inserted into the microplate (close to the bottom of the well), and the temperature of the detection solution was determined coupling with the photo-heat conversion system on a portable VICTOR 86 digital thermometer under an 808-nm adjustable laser irradiation (2.5 W cm^{-2}). To avoid possible error resulting from different-batch introduction of digital thermometer, the signal for each well was recorded as the irradiation from 30 s (after

introduction of thermometer) until the equilibrium was reached at 10 min (optimized). The control tests with normal (negative) samples and the evaluations for human serum specimens were performed accordingly. The collected temperature relative to target TG concentration was recorded as the signal of the photothermal immunoassay. All the determinations were made at least in duplicate. All the measurements were carried out at room temperature (25 ± 1.0 °C), unless special statement. The sigmoidal curves were calculated by mathematically fitting experimental points using the Rodbard's four parameter function with Origin 6.0 software. Graphs were plotted in the form of absorbance against the logarithm of TG concentration.

Calculation of photothermal conversion efficiency. Photothermal conversion efficiency of nanoparticles (NPs) was calculated according to the previous reports.[S6,S7] Detailed calculation was given as follows. The total energy balance for the whole system is

$$\sum_i m_i C_{p,i} \frac{dT}{dt} = Q_{NP} + Q_{Dis} - Q_{Surr} \quad (1)$$

Where: m and C_p are the mass and heat capacity, respectively. T refers to the solution temperature. Q_{NP} is the photothermal energy input of nanoparticles. Q_{Dis} is the photothermal energy input of solvent and water and container. Q_{Surr} is the heat energy conducted away from the system to the surrounding.

Q_{NP} expresses heat dissipated by electron-phonon relaxation of the plasmon on the surface of nanoparticles under the 808 nm (λ) laser irradiation.

$$Q_{NP} = I(1-10^{-A_\lambda})\eta \quad (2)$$

Where: I is the incident power of the NIR laser (mW), A_λ is the absorbance of the nanoparticles at the NIR laser wavelength (λ) of 808 nm in aqueous solution, and η is the photothermal conversion efficiency of nanoparticles from the incident NIR laser energy to thermal energy. Q_{Surr} represents a temperature-dependent parameter, which is linear with thermal energy lost

$$Q_{Surr} = hS(T - T_{Surr}) \quad (3)$$

Where: h is the heat transfer coefficient, S is the surface area of the container, T is temperature of system surface, and T_{Surr} is the surrounding temperature, respectively.

Q_{Dis} is the heat associated with the light absorbed by solvent water and quartz cuvette sample cell. Once the NIR laser power is defined, the heat input ($Q_{NP} + Q_{Dis}$) will be finite, the heat input is equal to the heat output at the maximum steady-statue temperature, so the equation could be:

$$Q_{NP} + Q_{Dis} = Q_{Surr\text{-}Max} = hS(T_{Max} - T_{Surr}) \quad (4)$$

T_{Max} is the equilibrium temperature, standing for no heat conduction away from the system surface by air. Besides, Q_{Dis} represents the heat dissipated from the photo absorption of the quartz cuvette sample cell itself, and it was measured independently to be using a sample cell containing pure water without nanoparticles.

In order to obtain photothermal conversion efficiency (η), substituting eq 3 for Q_{NP} into eq 5 and rearranging, η can be expressed as following:

$$\eta = \frac{hS(T_{Max} - T_{Surr}) - Q_{Dis}}{I(1-10^{-A_\lambda})} \quad (5)$$

Therefore, in this equation, only the hS is unknown for the calculation of η. In order to obtain hS, we introduce a Θ defined as dimensionless driving force temperature, and a τ_s representing a time constant of sample system,

$$\theta = \frac{T - T_{Surr}}{T_{Max} - T_{Surr}} \quad (6)$$

$$\tau_s = \frac{\sum_i m_i C_{p,i}}{hS} \quad (7)$$

which substituted into eq (2) and rearranged to yield

$$\frac{d\theta}{dt} = \frac{1}{\tau_s}\left[\frac{Q_{NP} + Q_{Dis}}{hS(T_{Max} - T_{Surr})} - \theta\right] \quad (8)$$

When the nanoparticles were cooling, the laser radiation ceases and $Q_{NP} + Q_{Dis} = 0$ eq (8) could be expressed to:

$$dt = -\tau_s \frac{d\theta}{\theta} \quad (9)$$

and the final expression after integrating

$$t = -\tau_s In\theta \quad (10)$$

All the parameters using in the equation are as follows. For the measurement of nanoparticles, the T_{Max} was 37.7 °C and the T_{Surr} was 25.1 °C. Through linear fitting, τ_s was about 285.74 s. The temperature change (T_{Max} - T_{Surr}) was 12.6 °C. Compared with m_{H_2O}, the m_{NP} (2.0 × 10^{-9} kg) was too little so it could be neglected. Therefore, the $m_iC_{p,i}$ was calculated by m_{H_2O} (1.0 × 10^{-3} kg） and $C_{p,i}$ (4.2 J/g·°C). According to the results mentioned before, the *hS* was deduced to be 14.69 mW/°C. In addition, the laser power *I* was 1000 mW where the area of light spot was 1.0 cm^2, and the absorbance of the nanoparticles at 808 nm (A_{808}) was 0.2057. Q_{Dis} was measured

independently to be 29.38 mW. Thus, the photothermal conversion efficiency (η') of nanoparticles could be calculated by substituting according values of each parameters to eq (6).

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