Supporting Information

The preparation of bifunctional hybrid nano-flowers and their application in the enzyme-linked immunosorbent assay for *Helicobacter pylori* detection

Tiantian Wang, a Xiangguang Li, a Lili Chen, a Youhuan Zhang, a Yujun Zheng, a Linjin Yu, a
Yingying Zhong, a Zhiyu Ye, b Huaqian Wang, a Xiping Cui, a Suqing Zhao. *a

a. Department of Pharmaceutical Engineering, School of Biomedical and Pharmaceutical Sciences, Guangdong University of Technology, Guangzhou 510006, People’s Republic of China.
b. College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, People’s Republic of China.

*Correspondence: E-mail: xgli@gdut.edu.cn and sqzhao@gdut.edu.cn.
Tel: 137 5189 7799 and 158 2025 8676
Preparation and purification of R-HP-Ab

HP suspension (1×10^8 CFU/mL) was mixed with adjuvant (in particular, freund’s complete adjuvant was used for primary immunization, followed by freund’s incomplete adjuvant) in a 1:1 ratio, and immunogens was made after being fully emulsified. Then, a New Zealand white rabbit was immunized twice a week, and its blood was collected from the ear vein after the third time of immunization. The blood was stored at 4°C overnight, then was centrifuged at 7 000 g for 10 min to obtain serum.

Serum titer was detected by iELISA regularly (n=3) and PBS served as the negative control, until the rabbit serum titer reached the highest. Then the rabbit was euthanized. The blood was taken and centrifuged and the obtained serum was stored at - 20°C.1-2

Then, appropriate amount of serum was taken out and purified by saturated ammonium sulfate two-step method (50%, 33%).3 Briefly, equal volume saturated (NH_4)_2SO_4 was added into the serum from the New Zealand white rabbit and mixed dropwise thoroughly. Then, it was precipitated overnight at 4°C. The next day, the supernatant was centrifuged and discarded, and the precipitation fully dissolved with appropriate PBS, and the saturated (NH_4)_2SO_4 was added slowly to make sure the final concentration was 33%. After full mixing, the suspension was settled at 4°C for 1 h. Next, the precipitation was centrifuged and dissolved with appropriate PBS. Then, it was dialyzed for 3 days against PBS buffer using an activated dialysis bag (MW = 14 000) to remove ammonium sulfate. Finally, the R-HP-Ab was stored and cryopreserved at - 20°C.

Preparation of HRP-labeled R-HP-Ab

The R-HP-Ab was labeled with HRP with the sodium periodate method. Briefly, HRP (5 mg) was dissolved in 0.5 mL ultra-pure water (at the moment, the solution was reddish brown). Then, 0.1 mL 2, 4-dinitrofluorobenzene anhydrous ethanol solution (1%) was added and stirred and the residues of α- and ε- amino groups in HRP were sealed and isolated from light for 1h at room temperature. Then, 1 mL NaIO_4 (0.06 M) was added and settled for 30 min (the solution was grass green), and the glycosyl o-hydroxyl groups independent of enzyme activity were oxidized to aldehyde groups. Then, 1 mL ethylene glycol (0.16 mol/L) was added to stop the oxidation reaction and the mixed solution obtained was dialyzed 3 times against carbonate buffer (0.05 mol/L, pH 9.5)
using an activated dialysis bag (MW = 14 000). Meanwhile, the dialysate was changed every 8 h. After that, the mixed solution was collected, and 1 mL purified rabbit polyclonal antibody (5 mg/mL) was added and stirred at room temperature in the dark for 3 h (at which time the color of the solution became light brown), so that the aldehyde group in the HRP molecule reacted with the amino group of the antibody protein to form schiff base structure. Then, 0.2 mL NaBH₄ (5 mg/mL) was added and settled at 4°C for 2 h, to reduce the excess aldehyde group in HRP enzyme and to form a stable compound. Then, the equal volume saturated (NH₄)₂SO₄ (pH 7.2) was added and stirred dropwise and the supernatant was discarded by centrifugation (7 000 g, 10 min) after being settled at 4°C for 30 min. After that, the precipitate was suspended with an appropriate amount of PBS and dialyzed 3 times against carbonate buffer (0.05 M, pH 9.5) using an activated dialysis bag (MW = 14 000) and then the supernatant was centrifuged (7 000 g, 10 min) and volume was fixed to 5 mL. The supernatant was separated and frozen for later use.

**Selection and optimization of the concentration of copper ions**

**Methods for selecting metal ions:** Different kinds of divalent metal ions (120mM) were added to HRP solution (0.01 μg/mL), 100 μL per well was added to the 96-well plate (n=3). Then 100 μL color rendering liquid containing TMB and H₂O₂ was added to each well, and the solution reacted at 37°C for 5 min. Next, 50 μL termination fluid was added to each well to terminate the reaction. And the OD₄₅₀nm was measured within 3-5 min. The choice of metal ions depends on whether their addition will adversely affect the enzyme activity of HRP or make the experiment inconvenient.

**Optimization of the concentration of copper ions:** Copper ion solution 120 mM was diluted and 100 μL per well was added to the 96-well plate (n=3). Then 100 μL color rendering liquid containing TMB and H₂O₂ was added to each well, and the solution reacted at 37°C for 15 min. Next, 50 μL termination fluid was added to each well to terminate the reaction. And the OD₄₅₀nm was measured within 3-5 min. OD₄₅₀nm < 0.1 was considered to have no effect on the system. That is, the reaction system would not cause false positive due to copper ions.

**Synthesis of R-HP-Ab-HRP@Cu²⁺ NFs, HRP@Cu²⁺ NFs and R-HP-Ab@Cu²⁺ NFs**

**The synthesis of R-HP-Ab-HRP@Cu²⁺ NFs:** 15 μL R-HP-Ab-HRP (HRP: R-HP-Ab = 1:1,
1 mg/mL) and 20 μL copper ion solution (120 mM) was added to 965 μL PBS buffer liquid system and mixed thoroughly. Then, it was settled at 4°C for 16 h. R-HP-Ab-HRP@Cu^{2+} NFs were naturally formed. Next, the sediment was collected by centrifugation (7 000 g, 5 min) and washed three times with PBS to remove the excess R-HP-Ab-HRP. Finally, R-HP-Ab-HRP@Cu^{2+} NFs solution was obtained and stored at 4°C.

**The synthesis of HRP@Cu^{2+} NFs:** 13 μL HRP (1 mg/mL) and 20 μL copper ion solution (120 mM) was added to 967 μL PBS buffer liquid system and mixed thoroughly. Then, it was settled at 4°C for 16 h. HRP@Cu^{2+} NFs were naturally formed. Next, the sediment was collected by centrifugation (7 000 g, 5 min) and washed three times with PBS to remove the excess HRP. Finally, HRP@Cu^{2+} NFs solution was obtained and stored at 4°C.

**The synthesis of R-HP-Ab@Cu^{2+} NFs:** 13 μL R-HP-Ab (1 mg/mL) and 20 μL copper ion solution (120 mM) was added to 967 μL PBS buffer liquid system and mixed thoroughly. Then, it was settled at 4°C for 16 h. R-HP-Ab@Cu^{2+} NFs were naturally formed. Next, the sediment was collected by centrifugation (7 000 g, 5 min) and washed three times with PBS to remove the excess R-HP-Ab. Finally, R-HP-Ab@Cu^{2+} NFs solution was obtained and stored at 4°C.

**Preparation of NFs containing different proportions of HRP and R-HP-Ab**

Five kinds of R-HP-Ab-HRP with different proportions of HRP and R-HP-Ab 20 μL and 20 μL copper ion solution (120 mM) were added to 960 μL PBS buffer liquid system and mixed thoroughly and settled at 4°C overnight. The bifunctional hybrid NFs were naturally formed. Finally, R-HP-Ab-HRP@Cu^{2+} NFs solution was obtained and stored at 4°C for standby.

**Preparation of R-HP-Ab-HRP@Cu^{2+} NFs after different formation time**

A total of 20 μL R-HP-Ab-HRP and 20 μL copper ion solution (120 mM) were added to 960 μL PBS buffer liquid system, and mixed thoroughly. And, it was settled at 4°C for 4 h, 8 h, 12 h, 16 h, 24 h and 32 h. The R-HP-Ab-HRP@Cu^{2+} NFs were naturally formed. Then, the sediment was collected by centrifugation (7 000 g, 5 min) and washed three times with PBS to remove the excess R-HP-Ab-HRP. Finally, NFs solution was obtained and stored at 4°C for standby.
The encapsulation efficiency about R-HP-Ab-HRP in R-HP-Ab-HRP@Cu$^{2+}$ NFs

Different amounts of R-HP-Ab-HRP (10 μL, 15 μL, 20 μL or 25 μL) and 20 μL copper ion solution (120 mM) were added to 1 mL PBS system, mixed thoroughly and settled at 4°C for 16 h. Then, the sediment was collected by centrifugation (7 000 g, 5 min) and washed three times with PBS to remove the excess R-HP-Ab-HRP. Finally, R-HP-Ab-HRP@Cu$^{2+}$ NFs solution was obtained and the encapsulation efficiency was calculated.

HRP performance test

A total of 50 μL R-HP-Ab-HRP@Cu$^{2+}$ NFs, HRP@Cu$^{2+}$ NFs or R-HP-Ab@Cu$^{2+}$ NFs were added to a 96-well plate (n=3), and then 100 μL color rendering liquid containing TMB and H$_2$O$_2$ was added. The solution reacted at 37°C for 15 min. Next, 50 μL termination fluid was added to terminate the reaction. And the OD$_{450}$ nm was measured immediately.

R-HP-Ab performance test

A total of 100 μL $10^8$ CFU/mL HP was added to a 96-well plate for each well and settled at 37°C for 1 h and then 4°C overnight. The plate was washed with PBST 3 times, pat dry with absorbent paper, and then, 270 μL skim milk powder (3%) was added and incubated at 37°C for 1 h. After the plate was washed with PBST 3 times and pat dry with absorbent paper, 100 μL R-HP-Ab-HRP@Cu$^{2+}$ NFs, HRP@Cu$^{2+}$ NFs or R-HP-Ab@Cu$^{2+}$ NFs was added and settled at 37 °C for 1 h. Next, the plate was washed with PBST 3 times, pat dry with absorbent paper, and then 100 μL color rendering liquid containing TMB and H$_2$O$_2$ was added and reacted at 37°C for 15 min. Next, 50 μL termination fluid was added to terminate the reaction. Finally, the OD$_{450}$ nm was measured within 3-5 min (n=3).

Storage stability of R-HP-Ab-HRP@Cu$^{2+}$ NFs and R-HP-Ab-HRP

R-HP-Ab-HRP@Cu$^{2+}$ NFs or R-HP-Ab-HRP were stored at 4°C and the activity was measured every day for the first ten days and every five days after ten days by iELISA. Finally, the test value of the first day was taken as 100% for statistical analysis of the remaining test results (n=3).
Temperature stability of R-HP-Ab-HRP@Cu²⁺ NFs and R-HP-Ab-HRP

A total of 100 μL R-HP-Ab-HRP@Cu²⁺ NFs or R-HP-Ab-HRP were added to a 96-well plate and settled at 4 °C, 20 °C, 37 °C, 50 °C or 70 °C for 2 h. Then it was taken out and restored at room temperature and then TMB catalytic color development analysis was conducted. The 4 °C value observed during each test was set as 100%, and the remaining values were statistically analyzed (n=3).

pH stability of R-HP-Ab-HRP@Cu²⁺ NFs and R-HP-Ab-HRP

The R-HP-Ab-HRP@Cu²⁺ NFs and R-HP-Ab-HRP solutions were respectively adjusted to different pH (pH=1-14) and 100 μL was added to a 96-well plate. Then the catalytic experiment of TMB substrate was carried out. The biggest value observed during each test was set as 100%, and the rest values were statistically analyzed (n=3).

Optimization of the concentration of R-HP-Ab-HRP@Cu²⁺ NFs and R-HP-Ab-HRP

In the iELISA process based on R-HP-Ab-HRP@Cu²⁺ NFs or R-HP-Ab-HRP, different concentrations of R-HP-Ab-HRP@Cu²⁺ NFs or R-HP-Ab-HRP were added, and the experiments were carried out according to the conventional scheme. Then, the optimal concentrations of R-HP-Ab-HRP@Cu²⁺ NFs and R-HP-Ab-HRP used in the following detection system were obtained through the above experiments.

Optimization of chromogenic time of R-HP-Ab-HRP@Cu²⁺ NFs and R-HP-Ab-HRP

In the iELISA process based on R-HP-Ab-HRP@Cu²⁺ NFs or R-HP-Ab-HRP, the positive and negative test holes were set in parallel, and different chromogenic time was set at the same time. The reaction was terminated by adding the termination solution at 37°C for 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 40 min, 50 min, 60 min, 70 min, 80 min, or 90 min. And, the OD_{450nm} was detected within 3-5 min.
Optimization of the sealing fluid

In the iELISA process based on R-HP-Ab-HRP@Cu^{2+} NFs or R-HP-Ab-HRP, different concentration of skim milk powder (0.1%, 0.3%, 0.6%, 1%, 3%, 6%, 8%), casein (0.1%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%), and bovine serum albumin (BSA) (0.1%, 0.3%, 0.6%, 1%, 3%, 6%, 8%) was chosen as the sealing fluid, and the iELISA process was set according to the conventional scheme.

Optimization of incubation time for R-HP-Ab-HRP@Cu^{2+} NFs and R-HIP-Ab-HRP

In the iELISA process based on R-HP-Ab-HRP@Cu^{2+} NFs or R-HP-Ab-HRP, the positive and negative test holes were set in parallel, and different incubation time was set at the same time. The 96-well plates were washed with PBST 3 times at 37\(^\circ\)C after 5 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 70 min, 80 min, 90 min, 100 min and 110 min respectively. The plates were then dried with blotting paper and the experiment continued according to the routine scheme.

Comparative analysis of the existing non-invasive detection methods of HP

Previously, Zambon et al. mentioned that the \(^{13}\)C/\(^{14}\)C isotope breath test can be used for HP detection, but we all know that \(^{14}\)C is radioactive with a long half-life.\(^4\) Not suitable for pregnant and lactating women. Wu et al. used \(^{15}\)NH\(_4\)\(^+\) excretion test to detect HP, but does not apply to the liver kidney function incomplete person.\(^5\) Monteiro et al. proved that there are inhibitors present in feces which prevent the use of PCR for the detection of HP, and it made the molecular biology techniques methods unstable.\(^6\) Fischbach et al. pointed out that a positive serotonin test might be due to a previous infection.\(^7\) Chen et al. established a method based on immunomagnetic beads and fluorescent quantum dots (IMBs-QDs) to detect HP, and the detection sensitivity reached \(10^2\) CFU/mL.\(^8\) In our study, the iELISA based on R-HP-Ab-HRP@Cu^{2+} NFs were firstly applied to detect HP, and the detection range is \(0-10^5\) CFU/mL (R=0.9952) with detection limit low as 50 CFU/mL. Furthermore, there is not restricted to the application of this method.
Table S1: A comparison of the performance between different non-invasive detection methods.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Methods</th>
<th>Applicability</th>
<th>Linear range</th>
<th>Sensitivity / LOD</th>
<th>Else</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zambon et al.⁴</td>
<td>¹³C/¹⁴C Isotope Breath Test</td>
<td>Most people</td>
<td>--</td>
<td>Positive or Negative</td>
<td>Radioactive</td>
</tr>
<tr>
<td>Wu et al.⁵</td>
<td>¹⁵N Urine Ammonia Discharge Test</td>
<td>Most people</td>
<td>--</td>
<td>Positive or Negative</td>
<td>--</td>
</tr>
<tr>
<td>Monteiro et al.⁶</td>
<td>Molecular Biology Techniques</td>
<td>All people</td>
<td>--</td>
<td>Positive or Negative</td>
<td>Instability and Professionals</td>
</tr>
<tr>
<td>Fischbach et al.⁷</td>
<td>Serotonin Test</td>
<td>All people</td>
<td>--</td>
<td>Positive or Negative</td>
<td>Hysteresis</td>
</tr>
<tr>
<td>Chen et al.⁸</td>
<td>IMBs-QDs Based Immunoassay</td>
<td>All people</td>
<td>10⁻⁶ CFU/mL</td>
<td>10² CFU/mL</td>
<td>Previous study in our lab</td>
</tr>
<tr>
<td>Our study</td>
<td>HP-Ab-HRP@Cu²⁺ NFs Based Immunoassay</td>
<td>All people</td>
<td>0-10⁵ CFU/mL</td>
<td>50 CFU/mL</td>
<td>--</td>
</tr>
</tbody>
</table>

Fig. S1. The titers of rabbit polyclonal antibodies before (A) and after (B) purification.
Fig. S2. The effect of different metal ions on the enzyme activity of HRP. (A): The relative activity of HRP after different metal ions added (The activity of HRP without metal ions was treated as 100%). (B): The results of the significant difference between the HRP activity response with and without metal ions (** **P<0.0001).

Fig. S3. SEM images of R-HP-Ab-HRP@Cu²⁺ NFs growing naturally for 16h (A, B) and 48h (C, D).
Fig. S4. TEM images of R-HP-Ab-HRP@Cu$^{2+}$ NFs growing naturally for 16h (A, B and C) and 48h (D, E and F).

Fig. S5. Element mapping of the R-HP-Ab-HRP@Cu$^{2+}$ NFs via EDS: images (A-F) exhibit the element sensitive maps of carbon, oxygen, nitrogen, phosphorus, sulfur and copper; (G) SEM of the sample; (H) EDS spectrum of complete element distribution.
Fig. S6. The results of the detection method established based on R-HP-Ab-HRP@Cu$^{2+}$ NFs

Fig. S7 The curves of the optimization of ELISA detection conditions. The concentration of R-HP-Ab-HRP@Cu$^{2+}$ NFs (A) and R-HP-Ab-HRP (B). The chromogenic time of R-HP-Ab-HRP@Cu$^{2+}$ NFs (C) and R-HP-Ab-HRP (D).
Fig. S8 Optimization of the sealing fluid. Type and concentration of the sealing fluid in the iELISA process based on R-HP-Ab-HRP@Cu^{2+} NFs (A) and R-HP-Ab-HRP (B). The influence of the sealing fluid in the iELISA process based on R-HP-Ab-HRP@Cu^{2+} NFs (C) and R-HP-Ab-HRP (D).

Fig. S9 The curves of incubation time of R-HP-Ab-HRP@Cu^{2+} NFs and R-HP-Ab-HRP. (A) incubation time of R-HP-Ab-HRP@Cu^{2+} NFs, (B) incubation time of R-HP-Ab-HRP.
Fig. S10. The results of the detection method established based on R-HP-Ab-HRP@Cu^{2+} NFs (A) or R-HP-Ab-HRP (C) for detecting HP in idea system. (B) The standard curves of Linear calibration curve for HP detection in idea system based on R-HP-Ab-HRP@Cu^{2+} NFs.

References