Electronic supplementary information

Ultrasensitive colorimetric assay based on a multi-amplification strategy employing Pt/IrO_2@SA@HRP nanoflowers for detection of progesterone in saliva samples

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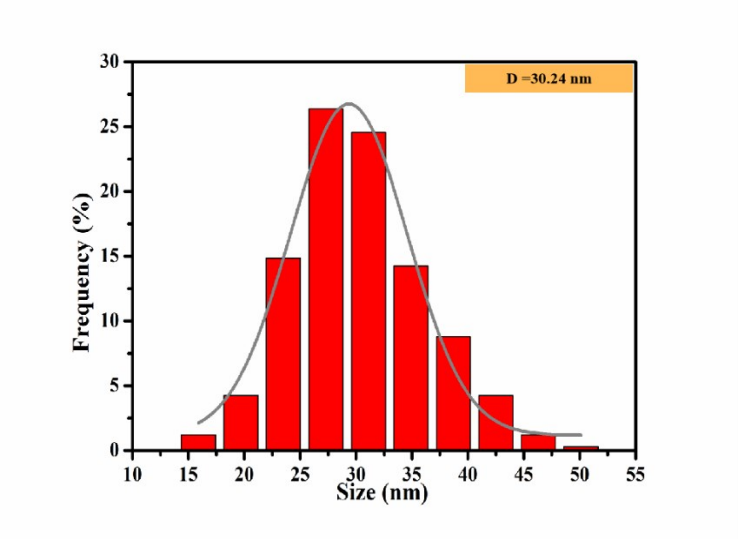

Figure S1. Distribution of diameter of the as-prepared Pt/IrO_2 NPs based on counting more than 300 particles by using "NANO MEASURER software".

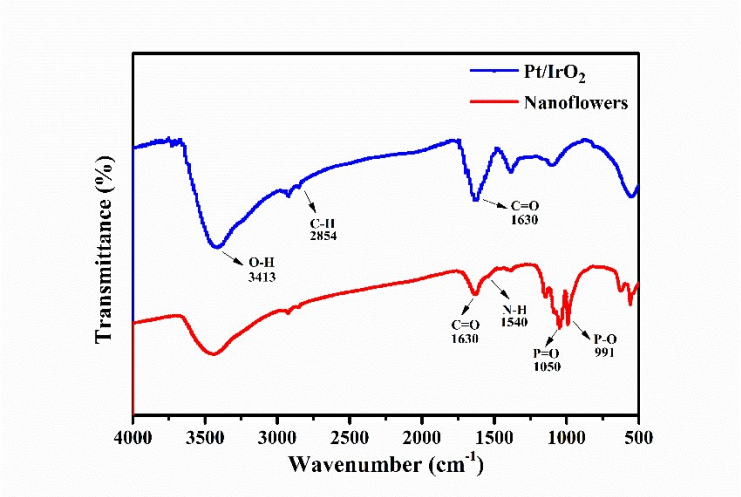

Figure S2. Fourier transform infrared (FTIR) spectra of Pt/IrO_2 NPs (blue line) and Pt/IrO_2@SA@HRP nanoflowers (red line).

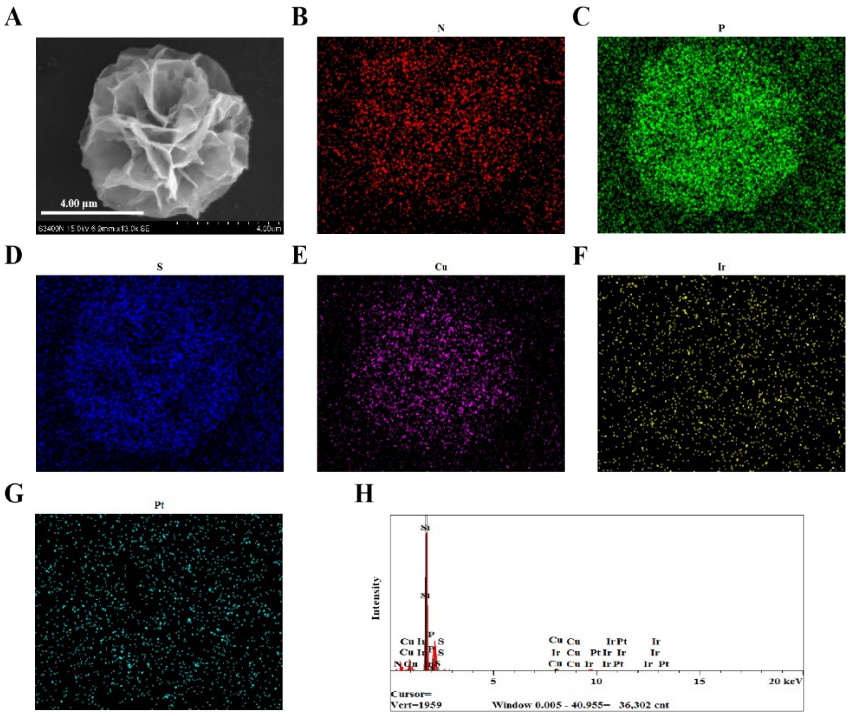

Figure S3. (A) The SEM image of Pt/IrO_2@SA@HRP nanoflowers. (B)-(G) Corresponding SEM elemental mappings of the N, P, S, Cu, Ir, and Pt signals; (C) Energy Dispersive Spectrometer (EDS) result of Pt/IrO_2@SA@HRP nanoflowers.

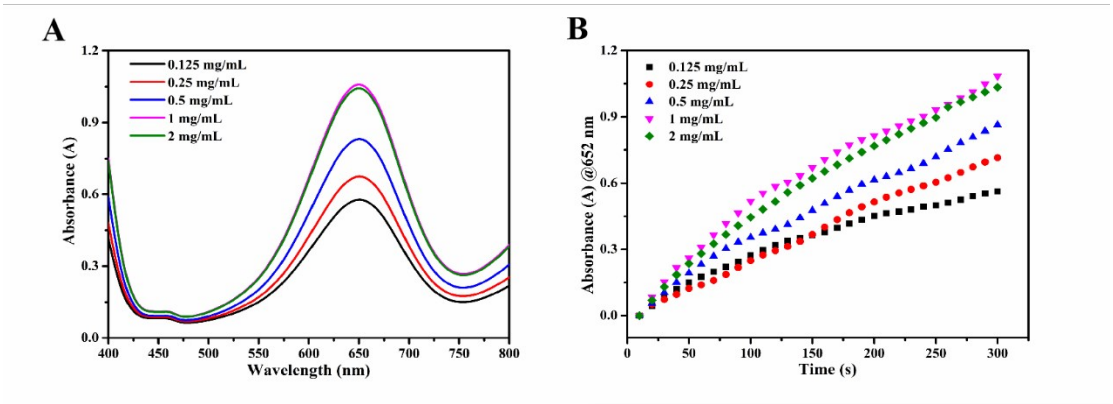

Figure S4. (A) UV-vis absorption spectra of the various ratio of Pt/IrO_2 catalyst. (B) The time-dependent absorption for the peroxidase-like activity of various ratio of Pt/IrO_2 catalyst after mixing for 5 min.

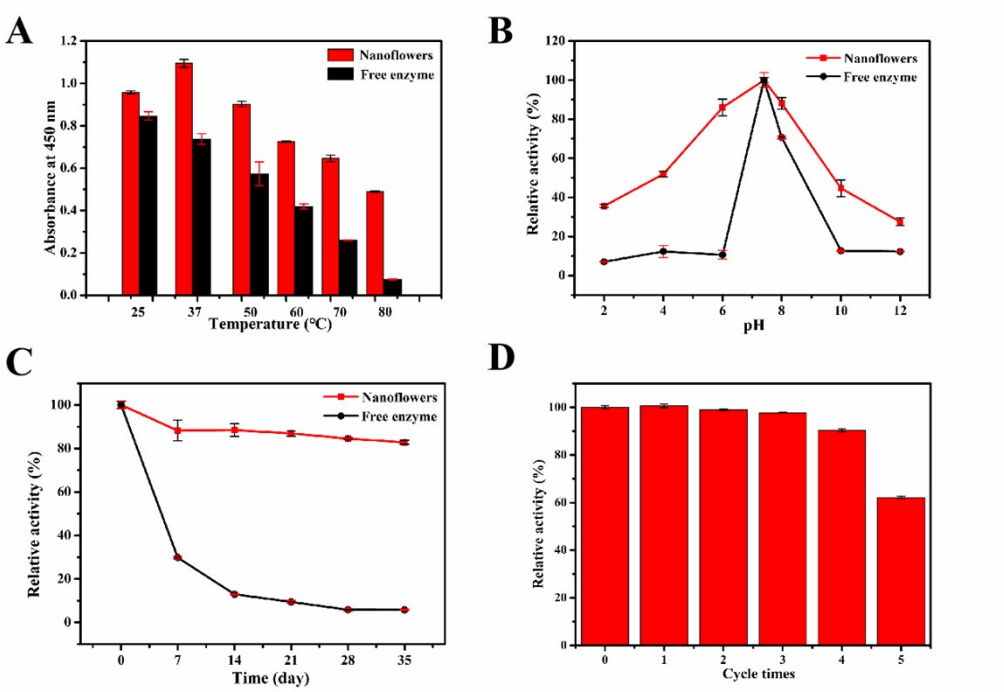

Figure S5. Comparison of (A) thermal stability, (B) pH stability and (C) storage stability at 25 °C of Pt/IrO_2@SA@HRP nanoflowers and free enzyme. (D) Reusability of Pt/IrO_2@SA@HRP nanoflowers.

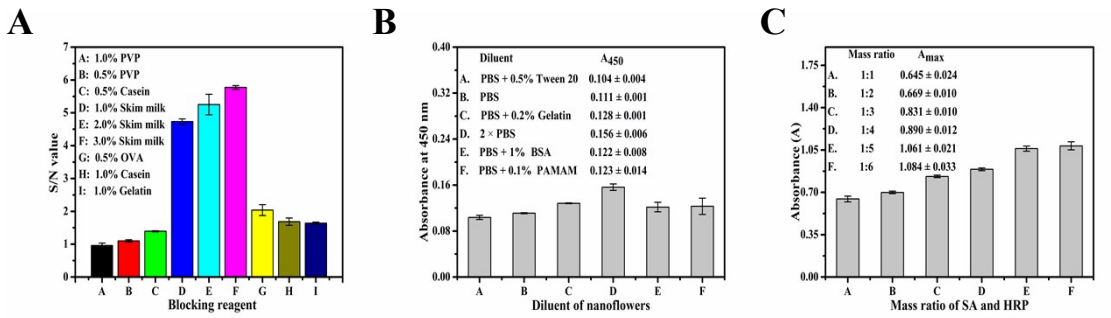

Figure S6. Suitable working conditions of the multiple-catalysis ELISA: (A) the blocking reagent, (B) diluent of Pt/IrO_2@SA@HRP nanoflowers and (C) mass ratio of SA and HRP.

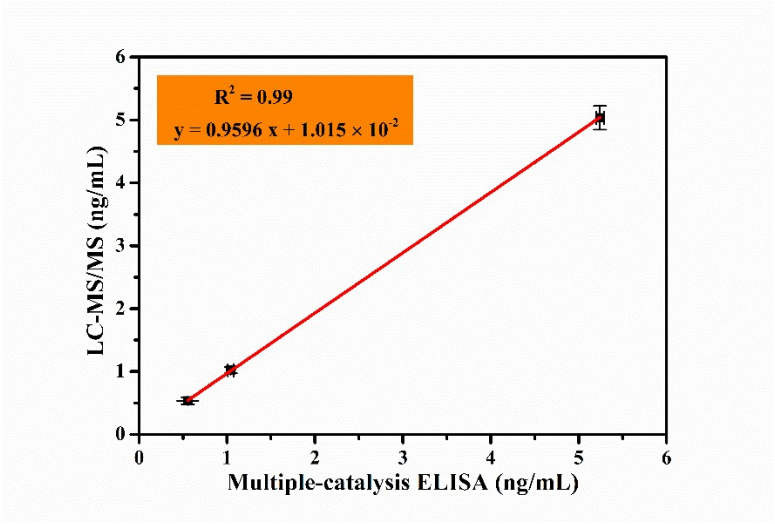

Figure S7. Correlation analysis between multiple-catalysis ELISA and LC-MS/MS results for the spiked saliva samples. The error bars were from triplicate samples (*n*=3).

Results and discussion of optimum conditions

Working conditions on assay performance were determined basing on the values of IC_{50}, the maximum absorbance value (A_{max}), and the ratio of A_{max} /IC_{50}.[1] A checkerboard titration was implemented to evaluate suitable concentrations of the immobilized coating antigen and the biotinylated antibody. Following the report of literature protocols, ELISA is more sensitive when the recorded maximum absorbance (A_{max}) is approximately 1.0.[2]

Heterologous coating antigens, including the length linking arms, the structure of spacer, the categories of carrier protein, or the junction site between the carrier protein and hapten, are different from the immunogen for preparation of antibody.[3] In this study, we have investigated the influence of heterologous and homologous coating antigens on the sensitivity of multiple-catalysis ELISA. It was obviously observed in Figure. 5A that Progesterone-3CMO-OVA (1.821 ng/mL) and Dehydroepiandrosterone-OVA (3.065 ng/mL) were used in the heterologous immunoassay to obtain lower IC_{50} value than homologous coating antigen Progesterone-OVA (30.19 ng/mL). To our astonishment, the sensitive of multiple-catalysis ELISA using heterologous coating antigen Progesterone-3CMO-OVA (1.821 ng/mL) was about 16-fold higher than that of using Progesterone-OVA (30.19 ng/mL). Hence, Progesterone-3CMO-OVA served as the best coating antigen in subsequent experiments.

Biotin and streptavidin are known to have high affinity and strong specificity, originating from their noncovalent biological interaction.[4] Immunoassay used biotinylated antibody is able to amplify the signal, which greatly improve the sensitivity of detection. Commercial activated biotin ramifications are often applied to chemical modification of antibodies. Covalent coupling process in biotins and antibodies is of importance in signal amplification. Accordingly, the different theoretical mole ratio (5:1, 10:1, 20:1, 40:1) of sulfo-NHS-LC-biotin to polyclonal antibody were used in multiple-catalysis ELISA, and the coupling effects were estimated by the A_{max}/IC_{50} value. As illustrated in the Figure. 5B, A_{max}/IC_{50} value was increased from 0.344 to 0.569 when theoretical mole ratio from 5:1 to 20:1. The results might be contributed to when a certain amount of biotin increased, the more likely SA could capture biotin which achieving signal amplification. Nevertheless, when the theoretical mole ratio reached 40:1, the ratios of A_{max} and IC_{50} was precipitously decreased. Excessive biotin modification on the antibody will take up the specific binding epitope of the antigen and antibody, which will result in a decrease sensitivity in immunoassay.[5] Therefore, we chose a mole ratio of 20:1 for the following experiment.

Nonspecific adsorption is a worrying problem in multiple-catalysis ELISA. Adopting suitable blocking buffer and diluent of nanoflowers are effective methods to reduce nonspecific adsorption. With the purpose of evaluating the effect of background interference on sensitivity at blocking procedure, the S/N values (the ratio between specific and nonspecific absorption) of block buffers were computed and background values (nonspecific absorption) were recorded. As shown in Figure. S6A, the S/N values of the other blocking buffers were as follows: 1.0% PVP (0.963), 0.5% PVP (1.099), 0.5% Casein (1.394), 1.0% Skim milk (4.733), 2.0% Skim milk (5.252), 3.0% Skim milk (5.773), 0.5% OVA (2.037), 1.0% Casein (1.685), 1.0% Gelatin (1.639). The results gave some information about that 3.0% Skim milk had maximum S/N value among these blocking buffers. Thus, 3.0% Skim milk was chosen as optimum blocking reagent.

Pt/IrO_2@SA@HRP nanoflowers solutions obtained by dissolving in diverse diluents, including PBS containing 0.5% Tween 20, PBS, PBS containing 0.2% Gelatin, 2×PBS, PBS containing 1% BSA and PBS containing 1% PAMAM, were determined. From Figure. S6B, an analogously nonspecific signal intensity of nanoflowers solutions were as followed: 0.104 (PBS containing 0.5% Tween 20), 0.111 (PBS), 0.128 (PBS containing 0.2% Gelatin), 0.156 (2×PBS), 0.122 (PBS containing 1% BSA) and 0.123 (PBS containing 1% PAMAM). The addition of Tween 20, Gelatin or BSA could not effectively reduce background interference. The highest nonspecific adsorption value, which was from nanoflowers diluted in 2×PBS, showed that higher ionic concentration possibly made Pt/IrO_2@SA@HRP nanoflowers tend to aggregate and led to a higher background signal. Compared with PBS, no remarkable improvement in background interference of PBS containing 0.5% Tween 20 was observed. Therefore, we selected PBS as a diluent in the further research.

The intensity and sensitivity of the proposed method was mainly affected by both the amount of recognition unit SA and one of the catalyst unit HRP immobilized on the Pt/IrO_2@SA@HRP nanoflowers. When the theoretical mass ratio of SA and HRP was 1:5, the maximal absorbance achieved, making it the optimized ratio (Figure. S6C).

Progesterone was weakly water-soluble and requires the help of organic solvent to improve its solubility. To investigate organic solvent tolerance of biotinylated antibody, the effect on dimethylformamide (DMF) volume fraction of 10%, 20%, 30% and 40% in assay solution were evaluated (Figure. 5C). As the volume fraction of DMF increased from 10% to 20%, the IC_{50} value of 20% DMF

(1.714 ng/mL) was basically the same as 10%DMF (1.737 ng/mL). The IC_{50} of 30% DMF and 40% DMF were 4.374 ng/mL and 3.746 ng/mL. However, the A_{max} markedly decrease when the volume fraction of DMF increased from 10% to 40%. According to the above data, we inferred that specific recognition of antigens and antibodies was vulnerable to the interference of superfluous organic solvents owing to the fact that the high concentration of DMF would destroy the spatial structure of the proteins. Considering the highest proportion of A_{max}/IC_{50} was appeared when the volume fraction of DMF reached 10%, the volume fraction of DMF was used for optimization test.

For eliminating possible interference that may be encountered, for instance, in serum, urine or saliva specimens, it was of great significance to further optimize ionic strength and pH of assay solution in reaction system. The influence of different degrees of ionic strength (5 mM, 10 mM, 20 mM and 40 mM PBS) on the assay performance were exhibited in Figure. 5D. With increasing concentration of assay solution from 5 mM to 40 mM, the sensitivity on assay gradually increased, whereas the highest value of A_{max}/IC_{50} (0.688) was obtained in 10 mM PBS, at which ionic strength was probably similar to the physiological environment. Another related indicator of assay, pH value, was examined for optimal experiment (Figure. 5E). The value of IC_{50} declined when the pH increased from 6.8 to 8.0. The most satisfying group of IC_{50} and A_{max} (IC_{50} = 1.620 ng/mL, A_{max} = 1.129) was achieved at pH 7.4. This reveals that a slightly alkaline pH is prefer to using in multiple-catalysis ELISA. The standard curve of a traditional ELISA and dual-catalysis ELISA are illustrated in Figure. 5F.

LC–MS/MS analysis

In order to verify results of multiple-catalysis ELISA, with a Hypersil GOLD C18 (10 × 2.1 mm, 1.9 µm, Thermo Scientific), 2 µL of sample solution volume was injected with running time of 8 min. The analysis procedures were performed at a flow-rate of 0.3 mL/min, the mobile phase constituted of solvent A (0.1% formic acid in water) and solvent B (Methanol). The gradient profile was as follows: started with 40% B (hold time 1.0 min); continued with linear change to 95% B up to 3.0 min; continued 95% B up to 6.0 min; returned to initial condition at 6.1 min, followed by equilibration until 8 min. Meanwhile, the temperature of column oven was maintained at 40 °C.

For quantitative and qualitative tracing of the progesterone, electrospray ionization (ESI) probe operated in positive ion mode was also performed in MS system. Ion source and MS parameters were as follows: Spray Voltage, 3500 V; Vaporizer Temperature, 350 °C; Capillary Temperature, 320 °C; Sheath Gas pressure, 35 Arb; AUX Gas Pressure, 10 Arb; Sweep Gas Pressure, 0 Arb. Xcalibur software was used for data acquisition and processing in SRM mode.

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