

Supporting information

Ultrasensitive ELISA Using Enzyme-loaded

Nanospherical Brushes as Labels

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Experimental

1. Materials

Human chorionic gonadotrophin (hCG, freeze dried powder containing 2.5 μg hCG, the immuno-activity has been determined to be 22.8 IU) was purchased from China National Institutes for Food and Drug Control. Horseradish peroxidase (HRP, P8375) and ProClin[®] 300 were obtained from Sigma-Aldrich. Anti- α -hCG antibody and anti- β -hCG antibody were purchased from Hangzhou Clongene Biotech Co., Ltd. Biotinylation of anti- α -hCG antibody and HRP labeling of anti- β -hCG antibody (anti- β -hCG-Ab-HRP) were done by Abgent Biotechnology (Suzhou) Co., Ltd. Two HRPs were labeled on one anti- β -hCG antibody in average by measuring the UV-Visible spectrum and catalysis property of the anti- β -hCG-Ab-HRP conjugates. N-hydroxysuccinimide (NHS), BCA protein quantification kit and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was purchased from Pierce. N-(3-dimethyl-aminopropyl)-N'-ethylcarbodiimidehydro-chloride (EDC) was obtained from Aladdin Reagent (Shanghai) Co., Ltd. Streptavidin (SA) functionalized magnetic beads (Dynabeads M-270 Streptavidin) were purchased from life technologies. Fetal bovine serum (FBS) was obtained from Life Technologies-Gibco (USA).

SPAABs were synthesized via surface-initiated RAFT polymerization. The synthesis and characterization of SPAABs were reported elsewhere^{1,2}. Table 1 lists the key structural parameters of SPAABs used in this work. The PAA brush layer has an average polymerization degree of ca. 650. In aqueous solution, the SPAABs feature long-stretching PAA chains with high grafting density, abundant carboxyl groups, uniform distribution and excellent dispersity.

Table S1 structural parameters of SPAABs

Particles	D_c (nm)	L (nm)	N (mmol/g)	σ (nm ⁻²)	M_n (kD)	PDI
SPAABs	80	145	6.5	0.34	47.9	1.13

D_c , Diameter of the SiO₂ core; L , thickness of brush layer in aqueous solution (10 mM phosphate buffer, pH=7.2); N , content of carboxyl groups; σ , grafting density of PAA chains; M_n and PDI, number-average molecular weight and polydispersity of surface PAA (see Ref. 1,2 for measurement methods).

2. Preparation of SPAAB-HRP

HRP was covalently immobilized into SPAABs by the “chemical conjugation after electrostatic entrapment” (CCEE) process (Scheme 1a)¹. 0.3 mg of SPAABs were suspended in 10 mM 2-(N-morpholino) ethanesulfonic acid buffer (MES, pH=5.0, containing 0.05wt% Tween-20) and mixed with excess HRP at ambient temperature for 15 min. After removing the excess HRP by centrifugation and washing with MES, the electrostatically absorption of HRP was converted into covalent bond by adding 1

ml of 0.5 mM EDC. The conjugation was allowed to proceed at ambient temperature for 2 h. After centrifugation and discarding the supernatant, the obtained SPAAB-HRP complexes were washed with copious phosphate buffer saline (PBS, pH=7.4, containing 0.05wt% Tween-20) and stored in PBS (containing 0.05% ProClin[®] 300) at 4 °C with a concentration of 0.5 mg ml⁻¹. The SPAAB-HRP complex was characterized by a nanodrop 1000 (Thermo) (optical length: 0.1 cm) and a Tecnai G² Spirit Biotwin transmission electron microscopy (TEM) (FEI, USA).

The activity of immobilized HRP was measured and compared with free HRP using TMB/H₂O₂ as substrates. For kinetic measurements, SPAAB-HRP was mixed with TMB substrate in a square cuvette (optical length: 1 cm) to reach the final HRP concentration of 0.2 ng ml⁻¹. Absorbance at 652 nm was recorded at different time intervals (0~600s) after the mixing of HRP and TMB substrate. For end-point measurements, samples with known concentration of SPAAB-HRP were mixed with TMB substrate (with a total volume of 50 µL) in a 96-well microplate and incubated at 25 °C for 10 min. The reaction was stopped with the addition of 50 µL of 4 M H₂SO₄. The optical density was read out at 450 nm by a microplate reader (PerkinElmer 1420).

3. Preparation of SPAAB-HRP-Ab

The anti-β-hCG antibody was conjugated to the periphery of the brushes via NHS/EDC process (Scheme 1b). The SPAAB-HRP complex was transferred into MES by centrifugation and resuspension. The complex was activated by 0.1 M NHS and EDC at ambient temperature for 20 min and then washed with MES three times with the help of centrifugation to remove excess reagents. Then, anti-β-hCG antibody was added and the conjugation was done in MES for 2h. After conjugation, the remaining NHS ester was quenched by 0.1 M Tris-HCl (pH=7.4). The obtained SPAAB-HRP-Ab was then blocked by 0.5% BSA at 4 °C overnight and stored in PBS (containing 0.05% ProClin[®] 300) at 4 °C with a concentration of 0.5 mg ml⁻¹. The binding capacity of antibody was measured by depletion method using BCA protein quantification kit.

4. Sandwich ELISA of hCG

The brush-amplified ELISA of hCG was done using SPAABs-HRP-Ab as labels and magnetic beads as solid-phase substrate (Scheme 1c). Firstly, the SA functionalized magnetic beads were decorated with biotinylated anti-α-hCG antibody (Mag-Ab) via SA-biotin affinity interaction. 3 mg of magnetic beads were mixed with 30 µg biotinylated anti-α-hCG antibody and rotated at ambient temperature for 0.5 h in 300 µL PBS. The obtained Mag-Ab was washed 4 times with PBS and stored in PBS at a concentration of 5 mg ml⁻¹. Then, 15 µg aliquot of Mag-Ab, 1 µg of SPAAB-HRP-Ab and 100 µL of analyte (hCG, dissolve and diluted with PBS) was added and mixed in a 96-well microplate. The mixture was incubated at 37 °C for 1h to allow the formation of immunocomplex, which can be easily separated with the help of external magnetic field. The immunocomplex was washed six times with Tris-HCl buffer saline (TBS, pH=7.4, containing 0.05% wt Tween-20) before the

addition of 100 μL TMB substrates. After incubation at 25°C for 10 min, the immunocomplex was magnetically separated and 50 μl of the supernatant was transferred to another well containing 50 μl of 4 M H_2SO_4 to stop the reaction. The optical density was read out at 450 nm by a PerkinElmer 1420 microplate reader. Detection of hCG in FBS was done by a two-step protocol: (1) In a 96-well microplate, 100 μL of hCG in FBS was firstly mixed with 15 μg aliquot of Mag-Ab and incubated at 37°C for 1h. (2) The magnetic beads was washed five times with TBS. Then, 1 μg of SPAAB-HRP-Ab and TBS was added to reach the final volume of 100 μL . The mixture was incubated for another 1 h and washed with TBS for six times before the addition of TMB substrates. The detection procedure was the same as that used for the detection of hCG in PBS. The conventional magnetic beads-based ELISA system, as comparison, underwent the same procedure except that anti- β -hCG-Ab-HRP conjugates were used as label. For each well, 20 μL of 1 $\mu\text{g ml}^{-1}$ anti- β -hCG-Ab-HRP was added.

Optimization of binding conditions in CCEE process

The optimal pH is 4.5~5.0 in step 1 where the largest binding capacity of ca. 1.6 mg HRP per mg SPAABs can be obtained (Figure S1a). Aggregation of SPAAB-HRP is observed when the pH of buffer is lower than 4.0. The optimal EDC concentration in step 2 is 0.5 mM. The final covalent binding capacity of HRP is 0.75 mg mg⁻¹ measured by depletion method utilizing the 403-nm characteristic absorption of HRP. (Figure S1b). This result generally agrees with the binding capacity estimated from the UV-Visible spectrum of the obtained SPAAB-HRP in Figure 1c. The binding capacity of HRP on both SPAABs and SiO₂-COOH is lower than that of bovine serum albumin (BSA) in our previous report¹. This can be attributed to the fact that there are only two lysine residues available for EDC conjugation in one HRP molecule³, whose content is much lower than BSA and most other proteins.

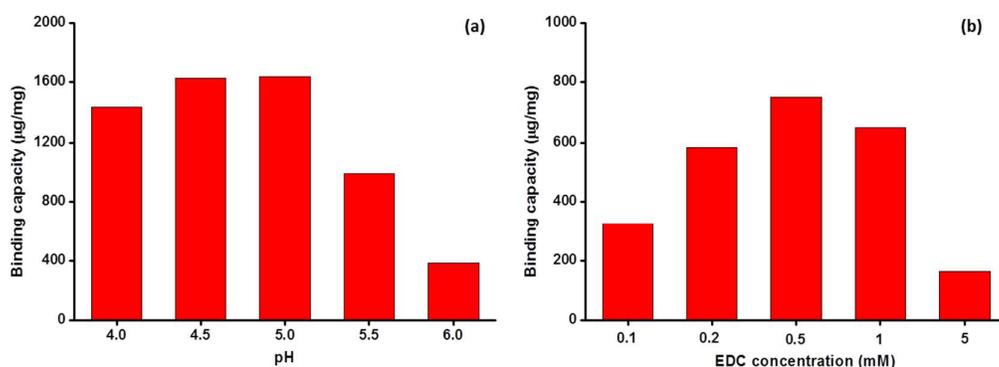


Figure S1 Influence of (a) buffer pH and (b) EDC concentration on the HRP binding capacity in electrostatic entrapment (step 1) and chemical conjugation (step 2) in CCEE process. The binding capacity of HRP was measured by depletion method utilizing the 403-nm characteristic absorption of HRP.

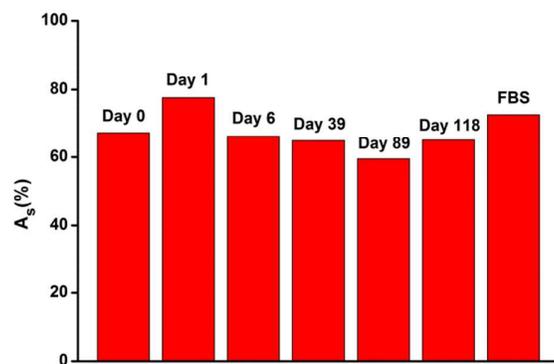


Figure S2 Stability of catalytic activity of HRP immobilized in SPAABs over time and measured in biological medium (FBS). The SPAAB-HRP was stored in PBS at 4°C with a concentration of 0.5 mg ml⁻¹ and measured at different time. The last column showed the data that SPAAB-HRP dispersed in FBS was measured. Within the experimental error, the activity of HRP immobilized in SPAABs remained constant for at least 4 months or measured in biological sample.

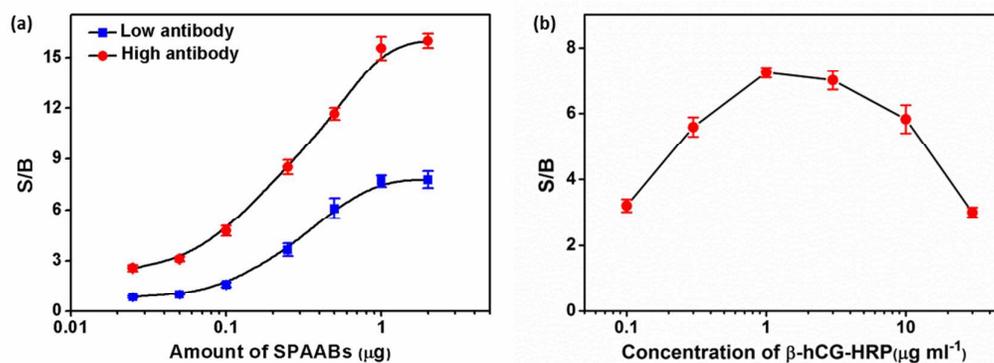


Figure S3. Influence of (a) antibody binding capacity and dosage of SPAAB-HRP-Ab in SPAAB-amplified system (b) concentration of anti- $\beta\text{-hCG-Ab-HRP}$ in conventional system on the detection signal. The signal-to-background ratio (S/B) was plotted as a function of these elements. The binding capacities of SPAAB-HRP-Ab with high (SPAAB-HRP-Ab_H) and low (SPAAB-HRP-Ab_L) antibody densities were 134 and 67 $\mu\text{g mg}^{-1}$ measured by depletion method using BCA protein quantification kit. The signal and background were obtained at the hCG concentration of 1 mIU ml^{-1} and 0 mIU ml^{-1} for SPAAB-amplified system, and 100 mIU ml^{-1} and 0 mIU ml^{-1} for conventional system, respectively. The rapid decrease of S/Bs in conventional system for anti- $\beta\text{-hCG-Ab-HRP}$ higher than 10 $\mu\text{g ml}^{-1}$ resulted from an increased detection background.

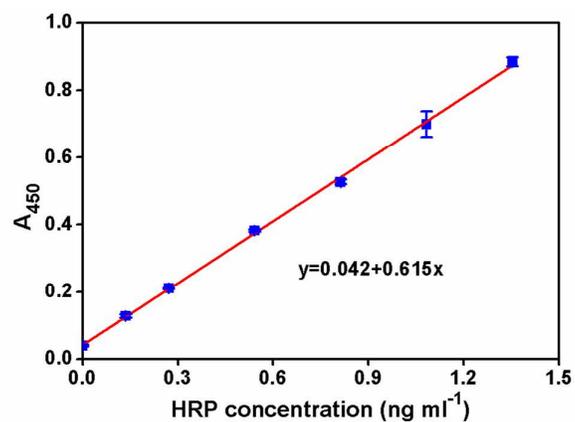


Figure S4 Catalytic efficiency of SPAAB-HRP-Ab_H using TMB/H₂O₂ as substrates. After immobilization of antibody via NHS/EDC process, the immobilized HRP in SPAABs retained 59% of their catalysis activity as judged from the slopes of linear regression fitting (compared with SPAAB-HRP in Figure 2b). Reduction in HRP number during antibody modification was found negligible (<2%).

Calculation of number of antibody and effective HRP

The molecular weight of silica core in SPAABs is calculated assuming that the silica core is uniform solid sphere with a density of 2 mg cm^{-3} . The content of PAA chains relative to the silica core is 114% determined by thermogravimetric analysis. Thus the molecular weight of SPAABs (M_{SPAABs}) can be calculated. In the same way we can calculate the molecular weight of $\text{SiO}_2\text{-COOH}$ used as comparison in this work.

As shown in Table 1, the number of effective HRP in SPAAB-HRP is 7100. After immobilization of antibody, the activity of immobilized HRP decreased to 59%. Thus, the number of effective HRP in SPAAB-HRP- Ab_H is about 4200.

The binding capacity (σ) of anti- β -hCG antibody on SPAAB-HRP is $134 \text{ }\mu\text{g per mg SPAABs}$. Assuming that anti- β -hCG antibody has a molecular weight of 150 kD (M_{ab}). The number of antibody (N) is calculated to be ca. 600 with the following equation:

$$N = \sigma \times \frac{M_{\text{SPAABs}}}{M_{\text{Ab}}}$$

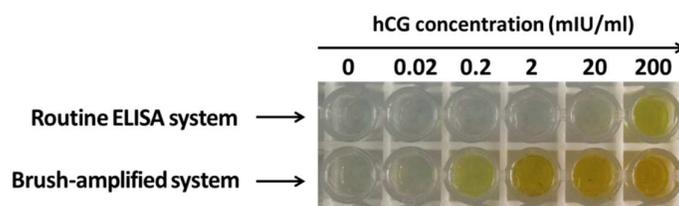


Figure S5 Colorimetric ELISA detection of hCG with routine system and brush-amplified system. It is evident that the signal is amplified by 2~3 order of magnitude even judged by naked eye. At the hCG concentration of 2, 20 and 200 mIU ml⁻¹, the color produced in the SPAAB-amplified system exceeds the linear range. A precise result can be obtained with a microplate reader (Figure 3).

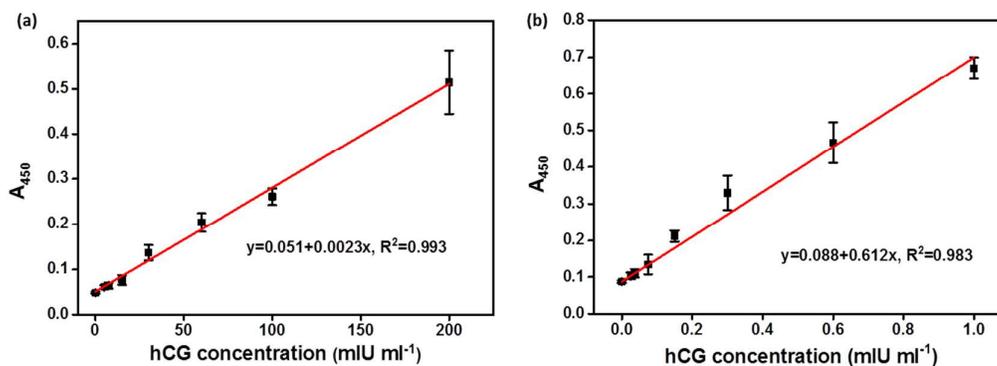


Figure S6 Detection of hCG in FBS by (a) conventional system. (b) SPAAB-amplified system. The SPAAB-amplified system has a LOD of 0.025 mIU ml⁻¹ for the detection of hCG in FBS, about 200-fold higher than conventional system (5 mIU ml⁻¹).

(1) Qu, Z.; Chen, K.; Gu, H.; Xu, H. *Bioconjugate Chem.* **2014**, *25*, 370-378.

(2) Qu, Z.; Hu, F.; Chen, K.; Duan, Z.; Gu, H.; Xu, H. *J. Colloid Interface Sci.* **2013**, *398*, 82-87.

(3) Hermanson, G. T. *Bioconjugation techniques*, 2nd ed.; Academic Press: San Diego, 2008; pp 963.