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Detection of four phenolic oestrogens by a novel electrochemical immunosensor based on a hexestrol monoclonal antibody

Guo-zheng Zhao,[a] Meng Wei,[b] Ya-juan Wang,[b] Xiu-wen Wang,[b] Hu Zhao,[c] Jian Shen*[b] and Bo Zhao*[b]

[a] *Key Laboratory of Magnetic Molecules & Magnetic Information Materials Ministry of Education, The School of Chemistry and Material Science, Shanxi Normal University, Linfen 041004, China*

[b] *National and Local Joint Engineering Research Center of Biomedical Functional Materials, Jiangsu Key Laboratory of Biofunctional Materials, School of Chemistry and Materials Science, Nanjing Normal University, Nanjing 210023, China*

[c] *School of Life Science and Technology, China Pharmaceutical University, Nanjing 210009, China*

Experimental section

1. Two-step screening procedure

1.1. Indirect ELISA (iELISA)

Production of conjugated HEX-specific antibodies by hybridoma cells was shown using iELISA as follows. The ELISA plates (JET Biochemicals Int'l. Inc. Guangdong, China) were coated with 50 μL per well of HEX-BSA at a concentration of 1 μg· mL^{-1} in sodium bicarbonate buffer (0.05 M, pH 9.5) for 2 h at 37℃. After washing three times with a washing buffer (PBS containing 0.05% Tween-20, PBST), the supernatants were added to the plates (50 μL per well) and incubated for 1 h at 37℃. After another washing procedure, the plates were incubated for another 1 h at 37℃ with HRP-conjugated goat anti-mouse IgG and IgM antibodies diluted 1/10000 in PBST (50 μL per well). The final washing procedure was followed by 50 μL of TMB substrate added to each well for visualization at 37℃ for 20 min, and then 50 μL per well of 2 M sulfuric acid was added to stop the reaction. The absorbance was read at 450 nm in a micro plate reader (Thermo Electron Corporation, USA).

1.2. Indirect competitive ELISA (icELISA)

The icELISA procedure was used to accomplish the iELISA analysis. The procedure was identical to the iELISA described above except that after coating with 100 μL per well of HEX-BSA complex, a competition step was introduced by confusing the same volume (50 μL) of

*Corresponding authors. E-mail address: jshen@njnu.edu.cn (Jian Shen); zhaobo@njnu.edu.cn (Bo Zhao).

culture supernatants and HEX standard solution to each well. The subsequent steps were performed as above. In addition, icELISAs were performed using serial dilutions of the culture supernatant from those wells that afforded saturated signals in the first screening experiment. Relative absorbance, which used as the criterion for selecting the best antibody-secreting clones, was calculated using the formula B/B_0, where B_0 was the absorbance of the well without HEX and B was the absorbance of the well with HEX. With the icELISA format, the antibody that does not react with HEX would produce absorbance near 100%; conversely, the antibody that reacts with HEX would decrease in percentage of absorbance. Hence, these expanded hybridomas secreting desired antibodies will be screened.

2. Characterization of HEX monoclonal antibody

The immunoglobulin fraction was prepared from the ascitic fluids by precipitation with saturated ammonium sulfate and subsequently subjected to gel filtration chromatography on Sephacryl S200 (Healthcare Bio-Science Corp., UK). The protein content was determined by BCA kit (Pierce, USA). The HEX monoclonal antibody was isotyped with a mouse HEX monoclonal antibody isotyping test kit (Sigma-Aldrich, USA) according to the manufacturer's recommendations. The icELISA was used to evaluate the affinity (K_a) and sensitivity of each HEX monoclonal antibody. The dilution of purified HEX monoclonal antibody was systematically optimized. The median inhibitory concentration (IC_{50}) values were calculated by using Curve Expert 1.3 software to determine the sensitivity. Specificity was defined as the ability of structurally related analogs, including DES and DE, to bind to the specific antibody and cross-reactivity (CR, %) was calculated as: (IC_{50} of HEX)/(IC_{50} of analogs) ×100.

3. Direct competitive enzyme-linked immunosorbent assay (dcELISA) for HEX

Following characterization, the HEX monoclonal antibody 3F2 selected as the detecting antibody was coupled to HRP. The ELISA plates were coated with 100 μL per well of HEX-BSA in 0.05 M sodium bicarbonate buffer, pH 9.5, for 2 h at 37℃. After washing three times with PBST, 150 μL per well of 1% BSA in PBST was added to plates and incubated for 1 h at 37℃. After another washing procedure, the plates were incubated for another 1 h at 37℃ with confusing the same volume (50 μL) of HEX standard solution and HRP conjugated HEX monoclonal antibody 3F2 diluted in PBST. The subsequent steps were performed as above.

After conditions (e.g. dilutions/concentrations) for the dcELISA optimized, ELISA validation

experiments were carried out with HEX at lower (1 ng·mL^{-1}), medium (5 ng·mL^{-1}) and upper (20 ng·mL^{-1}). Validation experiments were performed by measuring each concentration level three times in separate assays.

Results and discussion

1. Analysis of HEX-BSA complex

The method used in the present study utilized CDAP activation method for HEX to link to carrier proteins (BSA), which ensured stable crosslinking of haptens with proteins. Full scan experiments (scan range 220 to 400 nm) were performed to analyze the changes of the absorption spectra and the characteristic absorbance contribution of the new HEX-BSA conjugate. UV spectrum showed qualitative differences between the conjugate and the corresponding carrier protein. The characteristic absorbance for HEX-BSA showed a red-shift at 280 nm compared with the 278 nm for BSA, which could demonstrate that the coupling is successful.

In addition to the band corresponding to the BSA carrier protein, the conjugate sample showed two other bands: a major band with a median molecular weight (MW) of 55.5 kDa and a minor higher band of MW 142 kDa. It is probably due to the fact that the polypeptide chains of BSA consists of 30-35 being accessible for conjugation whilst per HEX contains two hydroxyphenyl groups with identical activity. When CDAP activates HEX, exchanging a cyano group for each hydroxyphenyl hydrogen, creating a highly reactive cyanoester, which can be reacted directly with the epsilon amines of lysine on BSA carrier protein to form stable O-alkyl-isourea linkages, including intra-polypeptide chain and inter-polypeptide chain cross-linking.

2. Evaluation of hybridomas

The culture supernatants from 865 hybridoma-positive wells were evaluated by iELISA on ten days after fusion. We found that the supernatants from 27 hybridomas contained antibodies that bound specifically to HEX-BSA complex. After expansion to 24-well plates, 15 primary clone hybridomas tested positive in the iELISA. The positive hybridomas were subsequently tested with icELISA, the capacity of HEX added in solution at a single concentration (1 μg·mL^{-1}) to inhibit the binding of antibodies to coated HEX-BSA complex. We found that antibodies from 6 hybridomas bound free HEX in solution (Table S1), and free HEX couldn't inhibit the antibodies from the rest 9 hybridomas bounding to coated antigen, probably due to immunoreactivity of the

clones to the HEX-BSA chemical linker in the ELISA antigen that resulted in false ELISA positives. In total, 6 hybridoma cell lines specific for free HEX were cloned and stabilized.

3. Characterisation of HEX monoclonal antibody

Table S1 Characteristics of HEX monoclonal antibody

Hybridoma	Idiotype	Titer of ascetic fluid	Affinity constant (per monal)	IC_{50} (ng·mL^{-1})
2D4	IgG1	1:640000	7.5×10^{9}	4.56
3F3	IgG1	1:1280000	2.4×10^{11}	1.28
3G11	IgG2a	1:320000	4.0×10^{9}	3.02
5E9	IgG1	1:640000	6.1×10^{10}	1.60
6E8	IgG1	1:640000	1.2×10^{10}	4.89
9E3	IgG1	1:1280000	5.8×10^{9}	2.74

Table S1 summarized the characterization of selected clones in terms of titer, affinity, IC_{50} values, class, and subclass, demonstrating that the antibodies are of G1 and G2a subclasses with κ light chain. These are high-affinity-type antibodies ranging from 10^{9} to 10^{11} per mol. The IC_{50} values ranged from 1.28 to 4.89 ng·mL^{-1} with the best for HEX monoclonal antibody 3F3. The specificity of the produced HEX monoclonal antibody was evaluated by cross-reactivity with other structurally related compounds, including DES and DE. The values of cross-reactivity for DES and DE were 82.5% and 61.4%, which may be due to a similar structure for the hydroxyphenyl group.

4. Detection of HEX by dcELISA

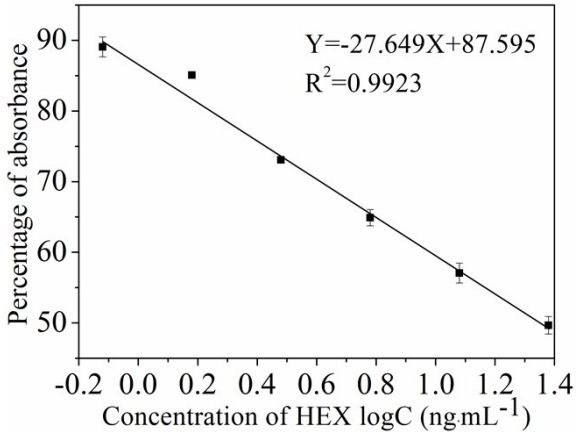


Fig. S1 Standard curve for HEX in PBS was detected by dcELISA. Vertical bars represent standard deviations for three independent assays.

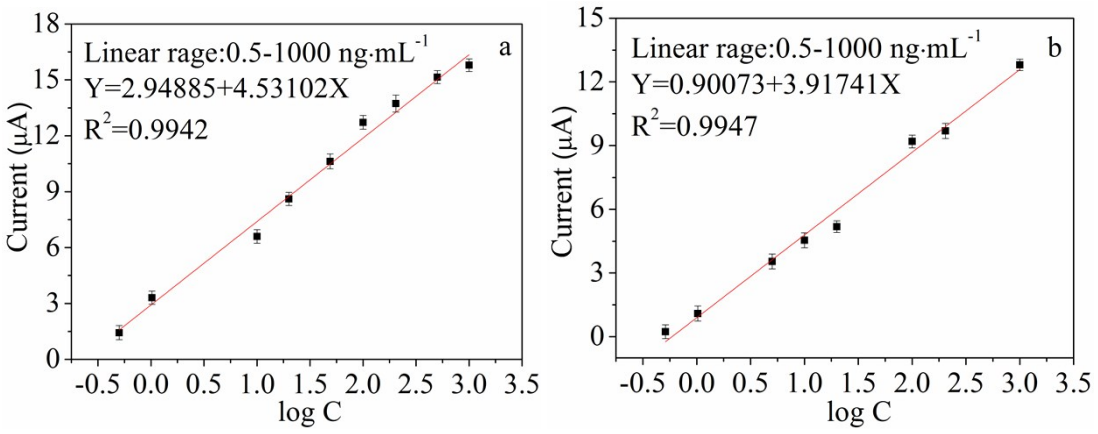
The purified HEX monoclonal antibody was tested for their performance in icELISA using serial dilutions of the free HEX, after checkerboard optimization of the coating antigen (HEX-BSA) and detecting antibody (HRP-3F2) concentration, a representative standard curve for the

dcELISA of HEX is depicted in Figure S1. As shown in Figure S1, a strong negative linear relationship between absorbance and log concentration of HEX was obtained in the range 25 to 0.78 ng·mL^{-1} HEX. The limit of detection (LOD) was 0.40 ng·mL^{-1} HEX. The limit of quantitation was 0.53 ng·mL^{-1} HEX.

Table S2 Intra-assay and inter-assay precision of HEX detection by dcELISA

Assay	Standard solutions (ng·mL^{-1})	Mean±SD	RSD (%)	Accuracy (%)
Intra-assay	1	0.96 ± 0.05	4.9	95.8
	5	4.82 ± 0.06	1.2	96.3
	20	20.43 ± 1.51	7.4	102.2
Inter-assay	1	0.98 ± 0.04	3.7	97.8
	5	5.06 ± 0.35	7.0	101.2
	20	21.54 ± 1.89	8.8	107.7

Intra-assay and inter-assay precision were studied continuously with three standard solutions of HEX at 1, 5 and 20 ng·mL^{-1}, which reflect concentrations at the lower, medium and higher ends of the standard curve. Intra-assay precision was evaluated by relative standard deviation (RSD) of detection of HEX from well to well (n = 3) in the same plate and inter-assay precision was obtained from different plates (n = 3). From the results of Table S2, the maximum RSD of intra-assay was not more than 7.4%, while that of inter-assay was 8.8%, accompanied by satisfied accuracy between 95.8% and 107.7%.



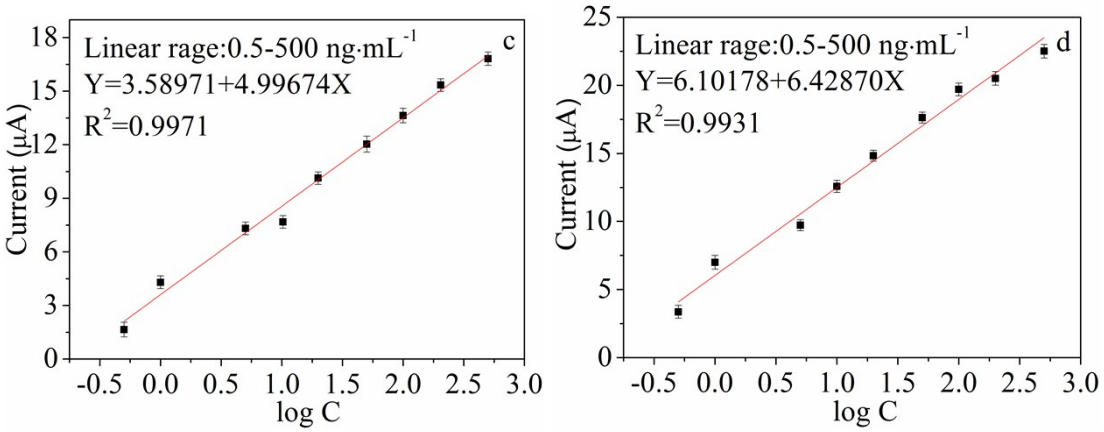


Fig. S2 Calibration curve based on the change of the DPV peak currents versus the standard solution concentrations of (a) HEX, (b) DES, (c) DE and (d) BPA detected by HEX monoclonal antibody/MACA/nanogold/GCE.