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Screening and Analysis of Thyroid-Disrupting Chemicals Based on Selective Recognition for a Thyroxine-Binding Peptide

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Thyroxine (TH) is an essential hormone for animal growth and development. Environmental exposure to thyroid-disrupting chemicals (TDCs) can alter the synthesis, secretion and transport of TH. In this study, a peptide with strong affinity for L-thyroxine (T4) was screened and synthesized, followed by attached to a gold electrode surface and then evaluated for its selective recognition ability for 36 chemicals with known activities towards TTR. The peptide-Au electrode could distinguish TCDs and thyroid-nondisrupting chemicals (non-TCDs) by impedance. The sensitivity, specificit and correct assignment of TDC action for these test compounds were 61.5, 73.9 and 69.4%, respectively. This method can therefore be used as a rapid, specific and cost effective way to identify TCDs.

Keywords: Thyroxine (TH); Thyroid-Disrupting Chemicals(TDCs); Peptide; Gold electrode; Impedance

1. INTRODUCTION

Thyroxine (TH) plays important roles in human growth, development and metabolism and is required for early brain and nerve development [1-4]. Following its synthesis in the thyroid, TH is transported and metabolized in target tissues but these processes can be altered in the presence of synthetic thyroid-disrupting chemicals (TDCs) such as brominated flame retardants, polychlorinated biphenyls and phthalates [5-7]. The presence of TCDs can lead to cognitive and behavioral disorders, obesity, some cancers and other problems [8]. For example, exposure to pesticides such as dichlorodiphenylethane (DDT) and hexachlorocyclohexane (chlordane, lindane) can affect the

homeostasis of the thyroid system and interfere with the development of the nervous system and brain [9]. Embryonic exposure to TDCs is also associated with attention deficit-hyperactivity and autism spectrum disorders [2]. Therefore, laboratory methods for the identification of potential TCDs are necessary for both animal and human health and safety.

There are currently 18 thyrotoxicological evaluation methods proposed by the Organization for Economic Cooperation and Development. The most utilized are assays involve transthyretin (TTR), a homotetrameric serum protein that transports L-thyroxine (T4) [10, 11]. The evaluation of T4 binding to TTR has been used as an assay for TDCs identification [12-14]. However, this method has disadvantages such that it requires large quantities of expensive pure protein, radioisotopes and is time- and labor-consuming [11, 12]. Therefore, an environmentally friendly, inexpensive and simple method to identify TCDs would enable testing on a larger scale.

Peptides have the advantages of simple structure, facile design and low cost [15]. In recent years, peptides were used to select some compounds [16, 17]. Thus, we are interested in the substitution of peptides for the *in vitro* TTR protein binding assay.

TH specifically binds TTR, thyroxine binding globulin (TBG) and thyroid hormone receptor (TR) proteins during its action [18]. Therefore, the amino acid sequence of structures near the TTR binding sites that are shared between these three proteins would be a starting point to form a TH-binding polypeptide. For economic reasons, we limited our analysis to 10 sequences near the binding site and this resulted in the identification of 7 peptides that were screened for T4 binding.

One key element for these types of assay screening procedures is the development of a simple method that has high sensitivity and low cost. Electrochemical detection possesses these advantages [19-21] and has been extensively used in environmental food science analyses and relies on the use of a gold electrode [22-24]. The gold electrode surface contains natural sulfhydryl groups that can be used to immobilize peptides and then serves as an electrochemical detection method for TDC evaluations.

Surface plasmon resonance technology (SPR) is a biomolecule detection method that utilizes a biosensor chip and is widely used and commercially available [25-27]. We used SPR to screen peptides that possessed T4 binding abilities that were then linked to the Au electrode surface. The potential of polypeptides in identifying TDCs was then evaluated by measuring Au-peptide impedance alterations using a cohort of 36 chemicals that have been identified by TTR in the literature [12-14].

2. EXPERIMENTAL

2.1. Chemicals and regents

Thyroxine and test chemicals were purchased from Aladin (Shanghai, China), peptide was synthesizeby Shanghai Keptide Biotechnology (Shanghai, China). Phosphate-buffered saline (PBS), 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (ECD), N-hydroxysuccinimide (NHS) and potassium ferricyanide were all analytical reagents purchased from Sinopill Chemical Reagent

(Shanghai, China). Experimental water was prepared using the Milli-R04 purification system (Millipore, Germany).

2.2. Apparatus

The instruments in the study were a CHI830D electrochemical workstation (Chenhua,Shanghai,China), a vacuum drying oven (Yiheng, Shanghai, China), a THZ-22 table temperature oscillator (Taicang Pei Ying THZ-22, Suzhou, China) and a SPR (Affinite, Canada), a circular dichroism (JASCO J-1500, Japan). A 3-electrode system was used for electrochemical tests and the working electrode was the Au-peptide, the reference electrode was a calomel electrode and the auxiliary electrode was a platinum wire electrode.

2.3. Peptide screening

The specific TH binding sites in the proteins TTR, TBG and TR were identified from the Protein Data Bank (https://www.rcsb.org/). We selected 10 amino acid sequences near the TH binding sites and 7 were selected for screening. The kinetic constants (Ka, Kd) and affinity constant (KD) of the interaction between these peptides and T4 were determined using SPR method.

2.4. Au electrode-peptide construction and evaluation

Both polypeptide and gold electrode have natural sulfhydryl groups on the surface, they can combine each other well. The specific steps are as follows: first polishing the gold electrode, then dissolving 1mg peptide in 1mL of aqueous solution, placing the gold electrode in the polypeptide aqueous solution and leaving it at rest for one night, and washing it with deionized water before being used to remove physical adsorption. The change in impedance of the peptide-modified Au electrode was examined in the presence of T4 and a collection of 36 compounds that are known TDCs and non-TDCs. The specific parameters were as follows: starting voltage: 0.197 V, high frequency: $e10^5$ HZ, low frequency: 0.1 HZ, amplitude: 0.005 V.

We define an ef value as the impedance of each compound divided by the T4 impedance detected by Au-peptide. For ef > 0, the chemicals were classified as TTR binders; for ef = 0, the chemicals were classified as TTR non-binders.Thus, a screening analysis was performed between this work with TTR Method to determine whether the peptides have the potential to recognize TCDs.

3. RESULTS AND DISCUSSION

3.1. Peptide screening

The Sequence and source of 7 peptides were listed in Table 1. For instance, the sequence of peptide 1 is FTKIITPAITRVVD, and TR (218-231) indicated that the sequence of peptide 1 was the

truncated segment 218-231 of TR protein. We analyzed 7 peptides using for their ability to bind TH using SPR. Peptide 4 possessed the highest affinity for T4 (4.21e-6) and was one order of magnitude greater than for peptides 2 and 5. In contrast, peptides 1, 3 and 7 displayed only weak binding (Table 1). We therefore selected peptide 4 MIGACHASRFL for the remainder of the experiments. SPR screening of peptide 4 was also evaluated using tetrachlorobisphenol A (TCBPA), tetrabromobisphenol A (TBBPA) and perfluorooctanoic acid (PFOA). All of these known TDC chemicals were able to bind peptide 4 and the affinity of TBBPA surpassed that of the control T4 (Figure 1).

Table 1. Peptides information, kinetics and affinity constants determined using SPR for experimental peptides and T4.

No	Sequence	Source (location)	MW(bp）	Ka（1/（M*S)	Kd (1/s)	KD (M)
1	FTKIITPAITRVVD	TR (218-231)	1573.87	†-	-	-
2	MEIMSLRAAVR	TR (256-266)	1276.57	1.15e2	6.57e-3	5.72e-5
3	SETLTLNGEM	TR (325-334)	1094.19	-	-	-
4	MIGACHASRFL	TR (430-440)	1205.45	2.59e2	1.09e-3	4.21e-6
5	SSKTLKKWNRL	TBG (285-295)	1360.6	4.51e2	2.02e-2	4.49e-5
6	ILERSTRSILF	TBG (395-405)	1334.56	-	-	-
7	IAALLSPYSYSTTA	TTR (127-140)	1457.62	7.09e1	1.09e-2	1.54e-4

† indicates that the affinity was so low that it can be ignored.

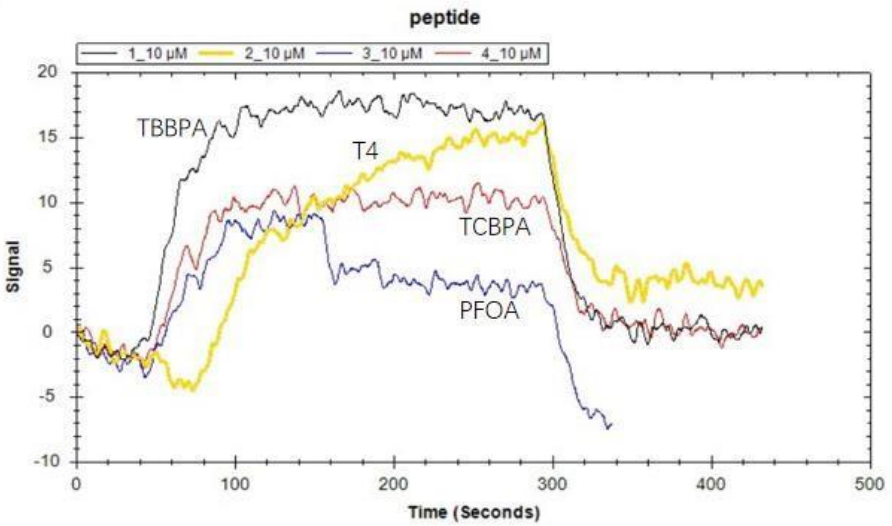

Figure 1. SPR analysis of immobilized peptide 4 (TR 430-440) binding to T4, tetrachlorobisphenol A (TCBPA), tetrabromobisphenol A (TBBPA) and perfluorooctanoic acid (PFOA).

3.2. Characterization and testing of the peptide-modified Au electrode

We utilized AC impedance spectroscopy (EIS) to examine the interface properties at the electrode surface before and after peptide 4 linkage [28, 29]. The electron transfer resistance of the

electrode surface is a measure of the electrochemical properties of the electrode. The Au electrode before peptide modification showed only traces of resistance. In contrast, following modification the resistance rose to ~ 13000 Ω (Figure 2a). This indicated that the electrode had been successfully modified after its linkage to peptide 4. We additionally examined peptide 4 using circular dichroism (CD) to characterize its secondary and tertiary structure [30, 31]. The presence of T4, TCBPA, TBBPA and PFOA did not significantly alter the structural characteristics of peptide 4 (Figure 2b).

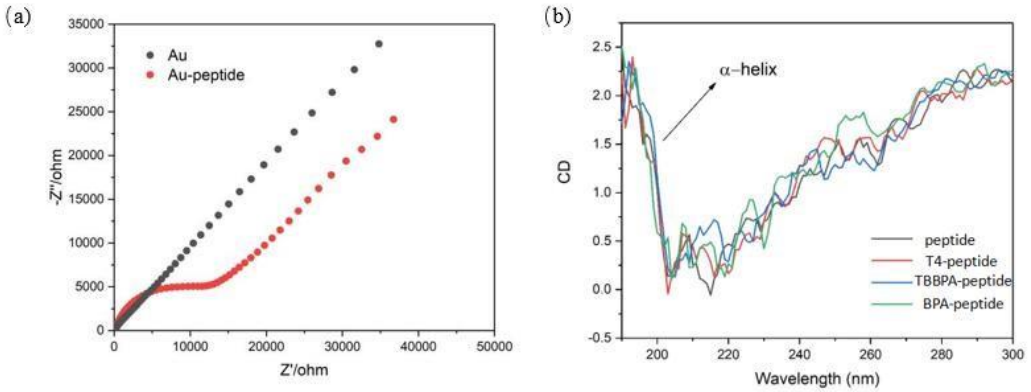

Figure 2. (a). Au electrode resistance measurements before and after peptide modification (b). CD analysis of peptide 4 in the presence and absence of the indicated compounds

3.3. Evaluating the Au-peptide 4 electrode for TDCs identification

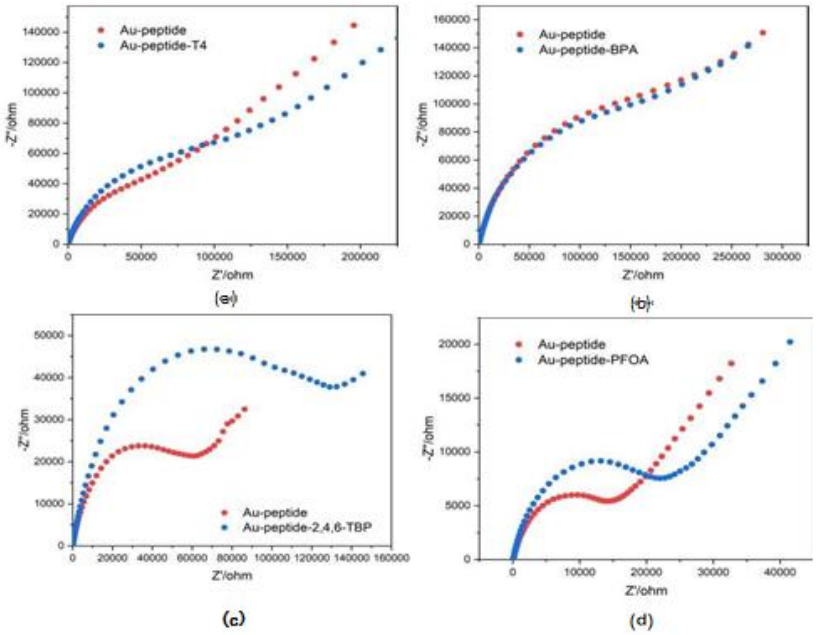

Figure 3. Impedance changes following absorption onto the Au-peptide 4 electrode for (a) T4 positive control (b) BPA negative control and the known TCDs (c) 2,4,6-TBP and (d) PFOA.

We examined the basic function of the peptide-Au electrode by measuring impedance changes in the presence of known TDCs and non-TDCs. The impedance measurements for the interaction of T4 and the activated electrode were only slightly different and were assigned as the positive control values (Figure 3a). The interaction with the non-TDC BPA indicated non-adsorption since the curves were superimposable (Figure 3b). In contrast, 2,4,6-TBP and PFOA were absorbed by the electrode surface and resulted in a large alterations in the impedance signals (Figure 3c and d). Therefore, an increase in impedance was associated with the binding of 2 known TCDs.

The Au-peptide 4 electrode was then used to evaluate 36 chemicals that are known TDCs and Non-TDCs. We found that 13 of these chemicals generated ef values >1 (Table 2). For example, we successfully identified the decabromodiphenyl ethers (BDE), TCS, TCBPA, TBBPA, PBP and TBBPS as known TDCs, and another 16 as known non-TDCs. In contrast, the results were equivocal for 11 of the compounds, which included 5 benzo (A) pyrene derivatives (Table 2). Overall, the sensitivity and specificity from this work were at 61.5, 73.9 and 69.4 %, respectively, indicating that peptide 4 could be used for TDCs identification. We also generated receiver operating characteristic curves (ROC). The AUC values were 0.677 (AUC>0.5), indicating that the interaction of peptide 4 was similar to that of TTR. Therefore, peptide 4 Au electrode can be used as a facile method for TDCs screening that is cost effective and specific for the identification of compounds for interacting with TTR.

Table 2. Comparisons of test chemical binding in this work with known TTR binding results in literature.

No	Compound	ef	Activity in this work	Activity in TTR
1	DEHP	0	Non-binder	Non-binder[32]
2	MeP	0	Non-binder	Non-binder[33]
3	EtP	0	Non-binder	Non-binder[33]
4	PrP	0	Non-binder	Non-binder[33]
5	BuP	0.009	Non-binder	Non-binder[33]
6	BPA	0	Non-binder	Non-binder[34-36]
7	BPS	0.032	Binder	Non-binder[37]
8	BP-2	0	Non- binder	Binder[33]
9	BP-6	0	Non-binder	Non-binder[11]
10	TCS	0.773	Binder	Binder[32]
11	TCC	0	Non-binder	Non-binder[32]
12	TBBPA	1.5	Binder	Binder[34]

13	TCBPA	0.477	Binder	Binder[34]
14	OP	1.09	Binder	Non-binder[34]
15	NP	0	Non-binder	Non-binder[38]
16	2,4,6-TCP	0	Non-binder	Binder[39]
17	2,4,5-TCP	0	Non-binder	Binder[39]
18	2,4,6-TBP	0	Non-binder	Binder[40]
19	PBP	0.068	Binder	Binder[41]
20	PCBs	0	Non-binder	Non-binder[32]
21	4-OH-BDE 17	0.773	Binder	Binder[42]
22	3-OH-BDE 47	1.636	Binder	Binder[34]
23	5-OH-BDE 47	1	Binder	Binder[36]
No	Compound	ef	Activity in this work	Activity in TTR
24	DEP	0	Non-binder	Non-binder[34]
25	BPE	0	Non-binder	Non-binder[34]
26	BPF	0	Non-binder	Non-binder[34]
27	BPZ	0.218	Binder	Non-binder[34]
28	BPAF	0	Non-binder	Non-binder[11]
29	BPAP	0.15	Binder	Non-binder[11]
30	NAP	0	Non-binder	Non-binder[40]
31	PYR	0	Non-binder	Non-binder[40]
32	BP-1	0	Non-binder	Binder[38]
33	BP-3	0.636	Binder	Non-binder[38]
34	BP-8	0.109	Binder	Non-binder[38]
35	BP-12	0	Non-binder	Non-binder[38]
36	TBBPS	0	Binder	Binder[36]

4. CONCLUSIONS

In this study, a peptide that specifically bound T4 was screened,we evaluate its selective recognition ability for TCDs by measuring its impedance using an Au-peptide electrode. We performed a preliminary screen using 36 compounds that indicated the electrode can rapidly and selectively recognize TCDs in aqueous solution. This method can replace more expensive TTR assays that rely on large quantities of purified TTR protein.

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