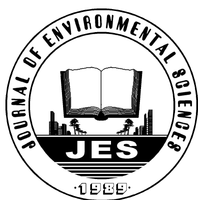

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In vitro fluorescence displacement investigation of thyroxine transport disruption by bisphenol A

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Abstract

Bisphenol A (BPA) is a chemical with high production volume and wide applications in many industries. Although BPA is known as an endocrine disruptor, its toxic mechanisms have not been fully characterized. Due to its structural similarity to thyroid hormones thyroxine (T4) and triiodothyronine (T3), one possible mechanism of BPA toxicity is disruption of hormone transport by competitive binding with the transport proteins. In this study, the binding interactions of BPA, T4, and T3 with three thyroid hormone transport proteins, human serum albumin (HSA), transthyretin (TTR), and thyroxine-binding globulin (TBG) were investigated by fluorescence measurement. Using two site-specific fluorescence probes dansylamide and dansyl-L-proline, the binding constants of BPA with HSA at drug site I and site II were determined as 2.90×10^4 and 3.14×10^4 L/mol, respectively. By monitoring the intrinsic fluorescence of tryptophan, a binding constant of 4.70×10^3 L/mol was obtained. Similarly, by employing 8-anilino-1-naphthalenesulfonic acid as fluorescence probe, the binding affinity of BPA with TTR and TBG was measured to be 3.10×10^5 and 5.90×10^5 L/mol, respectively. In general, BPA showed lower binding affinity with the proteins than T3 did, and even lower affinity than T4. Using these binding constants, the amount of BPA which would bind to the transport proteins in human plasma was estimated. These results suggest that the concentrations of BPA commonly found in human plasma are probably not high enough to interfere with T4 transport.

Key words: bisphenol A; human serum albumin; human transthyretin; human thyroxine-binding globulin; fluorescence displacement method

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Introduction

Many environmental pollutants have been found to have endocrine disruption activities that can lead to hormone imbalances, abnormal growth and reproductive functions. These chemicals interfere with physiological functions of endogenous hormones by affecting either their signaling pathways or metabolisms (Diamanti-Kandarakis et al., 2009; Ma, 2009; Foster, 2001). Bisphenol A (BPA) is used as the monomer for manufacturing polycarbonate plastic and epoxy resins, which is widely used in dental sealants and linings for food and beverage cans. The global production of BPA is estimated to be in excess of 2.9×10^9 kg/yr. Hydrolysis of ester bonds in BPA-based polymers has led to the release of BPA into environment. BPA has been detected in ambient air (0.1–4.41 ng/m^3, USA), water (about 0.14 μg/L in stream waters, USA) and soil (about 10.5 μg/g in river sediments, Germany), and has been reported to cause the adverse effects on organic systems in a variety of organism, including snail, fish, frog and human (Staples et al., 1998). BPA is a well-known environmental endocrine disruptor. It has shown such endocrine disruptive activities as uterotrophic effects, decreasing sperm production, simulation of prolactin release, promotion of cell proliferation in breast cancer cell lines, alteration in the onset of sexual maturity in females and change in the development of male reproductive organs, and influence on preimplantaton development (Tsai, 2006; Vom Saal and Hughes, 2005; Richter et al., 2007; Keri et al., 2007). Therefore, concerns are mounting regarding the effects of BPA pollution on environment and human health. However, the mechanisms of BPA-induced endocrine disruption in the human body have not been fully characterized.

Due to its structural similarity to thyroid hormones thyroxine (T4) and triiodothyronine (T3), one possible mechanism of BPA toxicity is the disruption of hormone transport by competitive binding with transport proteins. Endogenous thyroid hormones mainly bind to three transport proteins in human serum, namely thyroxine-binding globulin (TBG), transthyretin (TTR) and human serum albumin (HSA). Lines of evidences show that once BPA enters the blood stream, portion of BPA is metabolized to

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BPA-glucuronide by the liver and excreted via the kidneys and urine (Tsai, 2006), whereas the remaining population may compete for blood transport protein with endogenous hormones, leading to toxic effects on endocrine system. Several studies have examined the capability of BPA to bind TTR and TBG. For example, Meerts et al. (2000) used ^{125}I-labeled T4 to investigate competitive binding of BPA and T4 with TTR *in vitro* and found a weaker binding affinity of BPA with TTR than that of T4. Although BPA has a lower affinity for TTR and TBG than some other chemicals such as hydroxylated PBDEs, long-term low dose exposure to BPA still can produce serious toxicological effects (Goodman et al., 2009; Vandenberg et al., 2009). Yamauchi et al. (2003) revealed that BPA inhibited T3 binding to TTR more strongly than it did on thyroid receptor (Ishihara et al., 2003). In addition, a recent study using surface plasmon resonance (SPR) biosensor-based screening method also indicated that BPA could bind with TTR and TBG (Marchesini et al., 2008). Despite these earlier studies, the binding interactions between BPA and transport proteins have not been fully characterized. In particular, in most of the previous studies, the binding affinity of BPA with TTR and TBG was quantified with IC_{50}, which makes it difficult to assess the possibility of BPA disruption on hormone transport in any quantitative way.

In this study, the binding reactions of BPA, T4 and T3 with the three thyroid hormone transport proteins were investigated by fluorescence. Binding constants were obtained from either fluorescence displacement or intrinsic fluorescence measurement. The information obtained from the experiments allowed us to estimate the amount of BPA that would be able to bind to the transport proteins in human plasma and compare it with thyroid hormones.

1 Materials and methods

HSA (fraction V, essentially fatty acid free, ⩾ 96% purity), dansylamide (DNSA), dansyl-L-proline (DP) and 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANSA) were purchased from Sigma-Aldrich (St Louis, USA). Stock solutions (100 μmol/L) of DNSA and DP were prepared in acetonitrile. HSA used for fluorescence displacement assay was rehydrated with phosphate buffer (100 mmol/L NaH_2PO_4, 100 mmol/L Na_2HPO_4, pH 7.4). Its concentration was determined spectrophotometrically using extinction coefficient ($E^{1\%}_{1\,cm}$) of 5.3 at 280 nm wavelength and a molecular weight of 66,400 Da (Zia and Price, 1976). BPA was purchased from Alfa Aesar (Ward Hill, USA) and dissolved in methanol to a concentration of 20 mmol/L. T4 and T3 were purchased from Fitzgerald Industries International, Inc. (Concord, USA) and dissolved in methanol to a concentration of 5 mmol/L. Human TTR (98%) and human TBG (99.9%) were purchased from Calbiochem (San Diego, USA). TTR, TBG and ANSA were all dissolved in Tris buffer (50 mmol/L Tris-HCl, 100 mmol/L NaCl, pH 7.4). Molecular weight of 55 and 58 kD was used to calculate the molar concentration of TTR and TBG, respectively (Somack et al., 1982; Hocman, 1981). All chemicals and organic solvents were of analytical grade.

Fluorescence measurements were performed on a Perkin Elmer LS 55 fluorescence spectrometer (Waltham, USA) equipped with a right angle configuration and a 3 mm-light path quartz cuvette. Fluorescence of ANSA was excited at 375 nm, and the emission spectrum was recorded from 400 to 600 nm, using a 390 nm cutoff filter. DNSA and DP were excited at wavelengths 350 and 375 nm, respectively, and emission spectra (slit width = 10 nm) were recorded from 440 to 600 nm. HSA tryptophan fluorescence was measured by using an excitation wavelength of 295 nm, and the emission spectrum was recorded from 315 to 400 nm. The excitation wavelength of 295 nm was used to minimize the interference from tyrosine residues (Petersen et al., 1996).

2 Results and discussion

2.1 Fluorescence probe binding with thyroid hormone transport proteins

In this study, a fluorescence displacement method was employed to investigate the binding interaction of BPA with thyroid hormone transport proteins. In the method, a fluorescent protein probe is employed. The probe does not emit fluorescence when free in solution, but becomes highly fluorescent after it binds to a protein. If a chemical of interest competes with the probe for the same site of the protein, it will displace the probe from the protein and reduce its fluorescence intensity. From the titration curve, an IC_{50} value (the concentration of the chemical required to reduce fluorescence by 50%) is obtained, and the binding constant of the chemical with the protein can be calculated. Before the fluorescence displacement measurement was performed, binding of the fluorescent probes with HSA, TTR and TBG was investigated first to identify the best experimental conditions.

HSA is the most abundant protein in blood plasma, and is typically present at approximately 0.6 mmol/L. X-ray crystallography has shown that HSA is a heart-shaped protein with three homologous domains (designated as I, II and III), each having two subdomains (A and B) (He and Carter, 1992; Ghuman et al., 2005). HSA has seven fatty acid binding sites, five thyroxine binding sites and two drug binding sites. Drug site I (located in subdomain IIA) overlaps with fatty acid site 7 and thyroxine site 1, while drug site II (located in subdomain IIIA) overlaps with fatty acid sites 3 and 4 and thyroxine site 4 (Sudlow et al., 1975). The fluorescent probes, DNSA and DP, can specifically bind to drug site I and II, respectively (Ghuman et al., 2005). Upon binding, the fluorescence intensity of the probes greatly increases due to the hydrophobic interactions within HSA. Therefore, the intensity change of probe fluorescence reflects the interaction between the probe and HSA.

As shown in Fig. 1, in 2 μmol/L HSA the fluorescence intensity of DNSA and DP first increased with increasing probe concentration (0–2 μmol/L) and then leveled off

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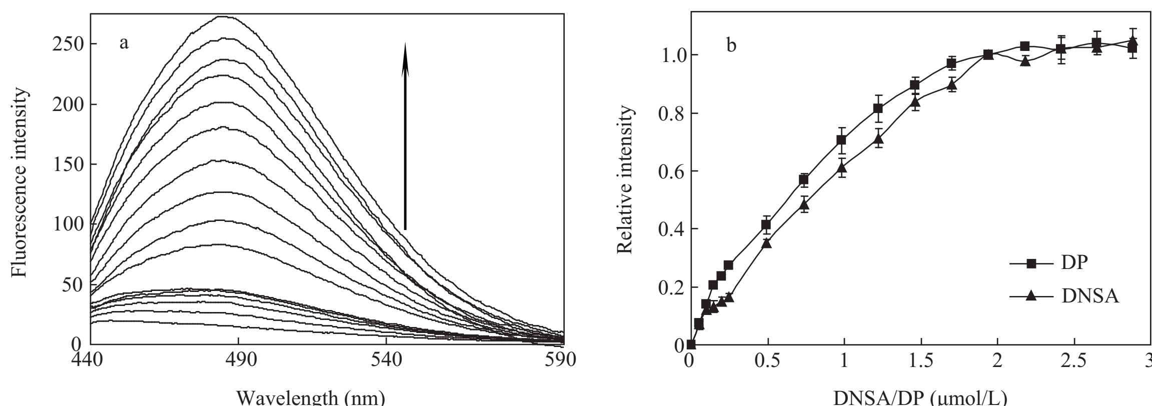

Fig. 1 Fluorescence intensity of DNSA (a) and fluorescence titration curve of DNSA and DP (b) titrated into 2 μmol/L HSA in 100 mmol/L PB buffer, pH 7.4. Arrow in (a) indicates increasing DNSA concentration. Each titration was repeated three times.

at higher concentrations. From the experimental data, the probe-protein dissociation constant can be calculated by the Scatchard equation (Eq. (1)):

$$r/C_{\text{probe}} = n/K_{\text{d}} - r/K_{\text{d}} \quad (1)$$

where, K_d (mol/L) is the dissociation constant, C_{probe} (mol/L) is the concentration of unbound probe at equilibrium, n is the number of equal and independent sites in the protein, and r is the average number of bound probe per protein under a given condition (Möller and Denicola, 2002). The plot of r/C_{probe} against r is linear with slope $-1/K_d$ and X-axis intercept of n. With known concentrations of bound or free probe at equilibrium and total protein, the protein-probe dissociation constant can be determined. Based on Eq. (1), the dissociation constant of DNSA and DP with HSA was calculated to be 0.79 and 0.5 μmol/L, respectively. These results are in accordance with our previously published data (1.07 and 0.31 μmol/L for DNSA and DP, respectively), which were obtained in 100 μmol/L HSA (Chen and Guo, 2009). The lowered protein concentration in our current measurement is necessary because the three chemicals under investigation, BPA, T4 and T3, have relatively low solubility in aqueous solutions. In 100 μmol/L HSA, the chemicals did not show displacement effect even at the highest concentration tested.

TTR is the major T4 transport protein in plasma of most mammalian species. It is a homotetrameric plasma protein with two equivalent T4 binding sites (Morais-de-Sa et al., 2004). TBG is a typical serpin protease inhibitor and has a binding area, which transports most of the T4 in blood of higher mammals (Lans et al., 1994; Zhou et al., 2006). ANSA is a well established fluorescence probe for proteins, and is also known as a competitive inhibitor with thyroxine for the binding sites on TTR and TBG (Cheng et al., 1977; Cody and Hazel, 1977). In a separate study, binding of ANSA with TTR and TBG was investigated by fluorescence titration, and a dissociation constant of 280 and 45.5 nmol/L was obtained for TTR and TBG, respectively (Cao et al., 2010).

2.2 Binding of BPA, T4 and T3 with HSA

Binding of BPA, T4 and T3 with HSA was then investigated separately by the fluorescence displacement measurement. To examine the binding interaction of the chemicals at the drug site I of HSA, the chemical was titrated into a mixture of 2 μmol/L DNSA and 2 μmol/L HSA based on the results in Fig. 1. Since the stock solutions of all the chemicals were prepared in organic solvents, the final content of the solvent in the protein solution was kept below 5% to minimize any interference. Figure 2 shows that, as BPA was added into the solution mixture, DNSA fluorescence was reduced gradually until it reached the background. From the plot of maximum fluorescence intensity versus BPA concentration, an IC_{50} value of 122 μmol/L was obtained. The binding constant of BPA/HSA is then calculated according to Eq. (2):

$$K_{\text{b}} = (1 + K_{\text{a}}/C_{\text{DNSA}})/\text{IC}_{50} \quad (2)$$

where, K_b (L/mol) is the binding constant, C_{DNSA} (mol/L) is DNSA concentration, and K_a (mol/L) is DNSA-HSA dissociation constant obtained from Eq. (1) (Cheng and Prusoff, 1973). The calculated binding constant of BPA/HSA at drug site I is 2.9×10^4 L/mol. Using the same approach, the binding constant of T4 and T3 with HSA at drug site I was found to be 4.41×10^5 and 1.76×10^4 L/mol, respectively. Therefore, BPA seems to bind to HSA at drug site I with similar affinity as T3, but 10 times weaker than T4.

DP is a fluorescent probe specific for HSA drug site II. To examine the binding interaction of the chemicals at drug site II of HSA, the chemical was titrated into a mixture of 2 μmol/L DP and 2 μmol/L HSA. From the fluorescence displacement curve illustrated in Fig. 3, the binding constant at drug site II of HSA was calculated to be 3.1×10^4 L/mol for BPA, 2.2×10^5 L/mol for T4, and 5.9×10^6 L/mol for T3, respectively. At this site, BPA binds to HSA approximately 10–100 fold weaker than T3 and T4. Loun and Hage (1995) used warfarin and indole as site-specific probes to determine the binding affinity of thyroid hormones with HSA at drug site I and II, respectively. They obtained a binding constant of 1.7×10^4 L/mol for T3 and 1.4×10^5 L/mol for T4 at drug site I, and 3.3×10^5 L/mol for T3 and 5.7×10^5 L/mol for T4 at drug site II (Loun and Hage, 1995). Our results are slightly different, but fall in the same range of magnitude as the constants they obtained.

HSA contains a single tryptophan residue (Trp^{214}) that is located in the subdomain IIA (Kragh-Hansen et al.,

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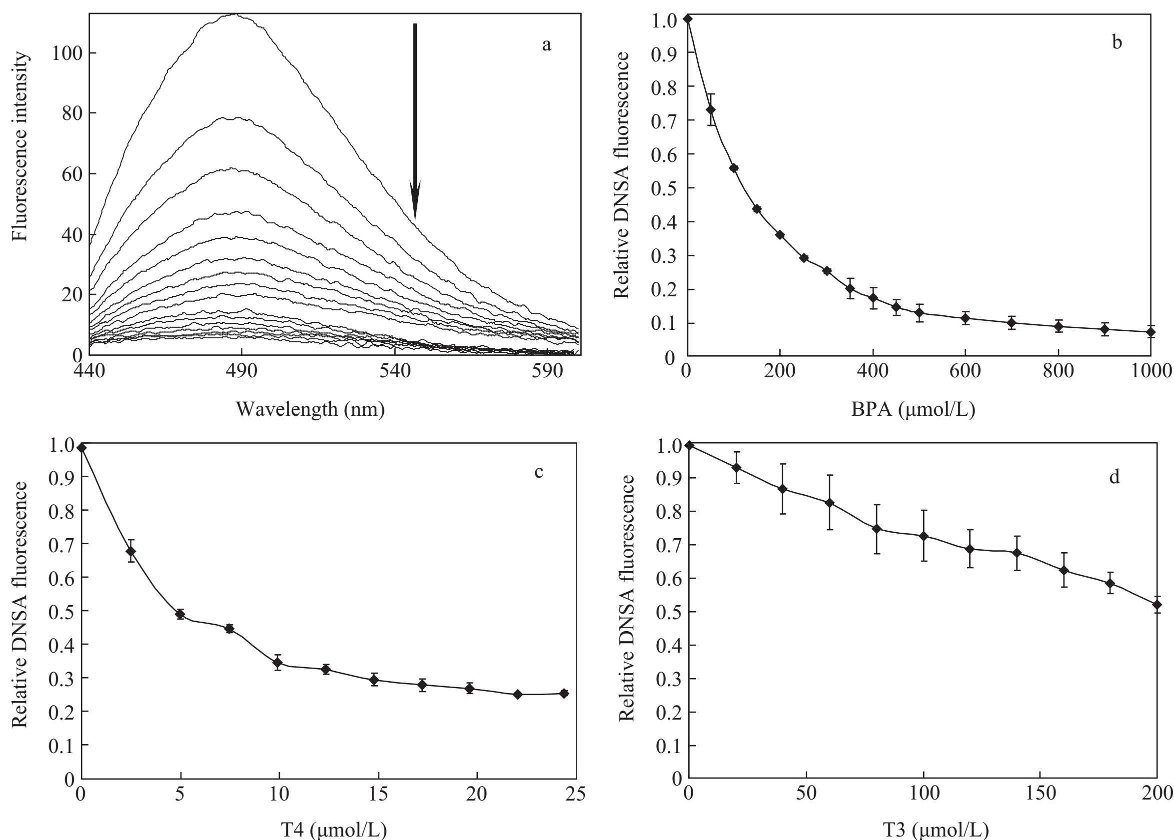

Fig. 2 Fluorescence spectra of 2 μmol/L DNSA in 2 μmol/L HSA with increasing BPA concentration. Arrow indicates increasing BPA concentration (a), fluorescence displacement curve of BPA (b), T4 (c) and T3 (d) titrated into 2 μmol/L DNSA + 2 μmol/L HSA. Each titration was repeated three times.

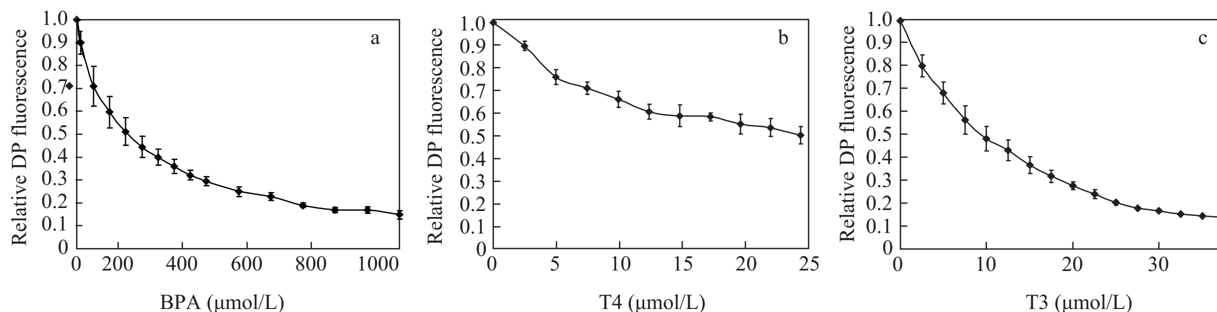

Fig. 3 Fluorescence displacement curve of BPA (a), T4 (b) and T3 (c) titrated into 2 μmol/L DP + 2 μmol/L HSA. Each titration was repeated three times.

2001). The binding of chemicals with HSA often introduces specific structural changes in subdomain IIA, and subsequently alters the microenvironment of tryptophan, resulting in change in its fluorescence. This intrinsic fluorescence property has been explored frequently in the studies of small molecule/HSA interactions (Wu et al., 2000). Tryptophan fluorescence of 2 μmol/L HSA was excited at 295 nm, and the emission spectrum was recorded from 315 to 400 nm. The fluorescence peak intensity decreased slowly with addition of BPA, and reached the background when BPA concentration exceeded 1 mmol/L (Fig. 4). Using Eq. (1), the binding constant of BPA with HSA was calculated to be 4.7×10^3 L/mol. Similarly, tryptophan fluorescence was also quenched with addition of either T4 or T3. From the titration curves, the binding constant for T4 and T3 with HSA was determined to be 9.28×10^5 and 3.72×10^5 L/mol, respectively. These results are in good agreement with those reported in the literature (4.4×10^5 and 1.0×10^5 L/mol for T4 and T3, respectively, Petersen et al., 1996). Obviously, BPA binds to HSA much weaker than T4 and T3 at the tryptophan site.

2.3 Binding of BPA with TTR and TBG

As mentioned in the introduction section, TBG, TTR and HSA are the three major T4 transport proteins in human plasma, each carrying 75%, 20% and 5% of total T4. Based on the results described in Section 2.1, ANSA was employed as the fluorescence probe in the displacement measurement. To investigate the binding interaction of BPA with TBG, BPA was titrated into a mixture solution of 0.5 μmol/L ANSA and 0.5 μmol/L TBG. Once again, reduction in ANSA fluorescence intensity was observed with addition of BPA into the mixture solution (Fig. 5). From

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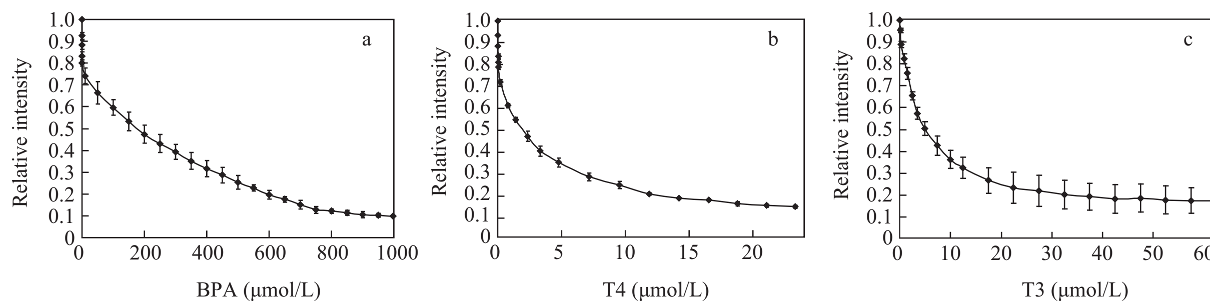

Fig. 4 Tryptophan fluorescence quenching curve of BPA (a), T4 (b) and T3 (c) titrated into 2 μmol/L HSA. Each titration was repeated three times.

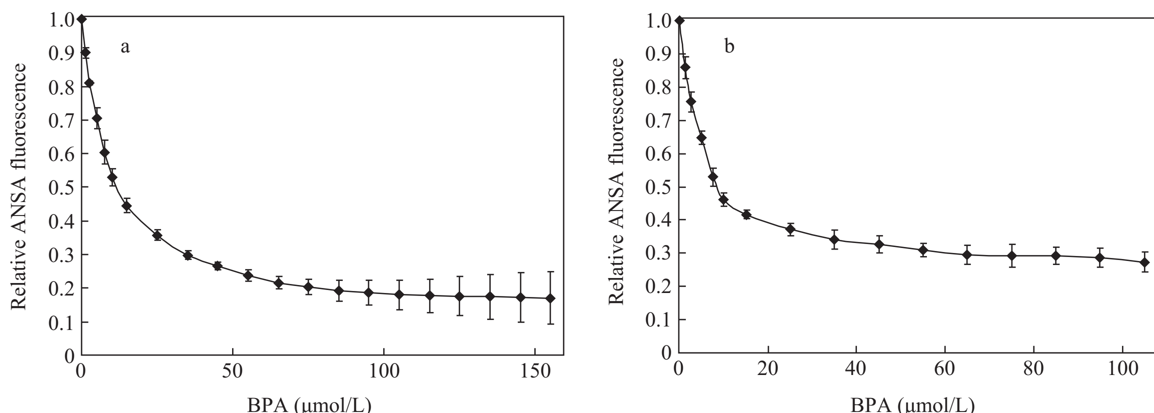

Fig. 5 Fluorescence displacement curve of BPA titrated into 0.5 μmol/L TBG + 0.25 μmol/L ANSA (a), and 0.5 μmol/L TTR + 0.5 μmol/L ANSA (b). Each titration was repeated three times.

the displacement curve, a binding constant of 5.9×10^5 L/mol for BPA with TBG was obtained. Using the same experimental condition, the binding constant between BPA and TTR was also measured (3.1×10^5 L/mol). These constants are smaller than T4/TBG affinity by a factor of 300 to 2666, demonstrating high affinity of TBG with endogenous thyroid hormones. All the binding constants are listed in Table 1.

2.4 Evaluation of thyroxine transport disruption by BPA in human serum

Based on the measured binding constants in Table 1, the amount of the protein-bound chemical in human serum can be estimated with Eqs. (3)–(5):

$$C_{\text{ligand-P}} + C_{\text{ligand}} = C_{\text{ligand-T}} \quad (3)$$

$$C_{\text{ligand-P}} + C_{\text{P}} = C_{\text{P-T}} \quad (4)$$

$$C_{\text{ligand-P}} = K_{\text{b}} \times C_{\text{ligand}} \times C_{\text{P}} \quad (5)$$

where, $C_{\text{ligand-P}}$ (mol/L) is the ligand concentration bound to one of the three transport proteins (HSA, TTR and TBG), C_{ligand} (mol/L) is the concentration of free ligand, $C_{\text{ligand-T}}$ (mol/L) is the total concentration of ligand, C_{P} (mol/L) is the concentration of the free protein, $C_{\text{P-T}}$ (mol/L) is the total concentration of protein, and K_{b} (L/mol) is the binding constant of the ligand with the protein. To simplify the calculation, it is assumed that BPA, T3 and T4 binds independently to each transport protein, and the three binding sites of HSA are equal and independent, and their concentrations are the same as that of HSA. If the binding sites are not equal and dependent, there would be more equations and more variables in the model, which would make the calculation very complex and difficult to solve. Nonetheless, since BPA binds stronger to TTR and TBG than HSA, and TTR and TBG carry the majority of the thyroid hormones, our general conclusion would still hold.

The plasma concentrations of the three transport proteins, HSA, TTR and TBG, are 600, 5 and 0.2 μmol/L, respectively (Marchesini et al., 2008). The concentration of BPA in human blood has been reported to be in the range of 0.5–22.3 μg/L, or 2.1–97.6 nmol/L (Padmanabhan et al., 2008). Total T4 and T3 concentrations in normal human serum are in the range of 58–161 nmol/L and 1.54–3.08 nmol/L, respectively (Thienpont et al., 2005). Using these numbers, the concentration of protein-bound BPA, T4 and T3 in human serum is calculated, and listed in Table 2.

With these numbers calculated, we can now evaluate

Table 1 Binding constants of BPA, T4 and T3 with the three major thyroid hormone transport proteins obtained from fluorescence measurement

Compound	HSA			K_{TTR} (L/mol)	K_{TBG} (L/mol)
	$K_{\text{drug site I}}$ (L/mol)	$K_{\text{drug site II}}$ (L/mol)	$K_{\text{tryptophan site}}$ (L/mol)		
T4	4.41×10^5	2.19×10^5	9.28×10^5	0.93×10^8 (ref)	1.62×10^9 (ref)
T3	1.76×10^4	5.92×10^6	3.72×10^5	0.99×10^7 (ref)	2.17×10^8 (ref)
BPA	2.90×10^4	3.14×10^4	4.70×10^3	3.10×10^5	5.90×10^5

ref: Cao et al., 2010.

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Table 2 Calculated concentration of BPA, T4 and T3 bound to thyroxine transport proteins

Chemical	Total concentration (nmol/L)	Concentration of drug site I of HSA-bound chemical (nmol/L)	Concentration of drug site II of HSA-bound chemical (nmol/L)	Concentration of tryptophan site of HSA-bound chemical (nmol/L)	Concentration of TTR-bound chemical (nmol/L)	Concentration of TBG-bound chemical (nmol/L)
T4	58.00–161.00	57.78–160.39	57.56–159.78	57.90–160.71	57.87–160.64	57.76–158.69
T3	1.54–3.08	1.41–2.81	1.54–3.08	1.53–3.07	1.51–3.02	1.47–2.93
BPA	2.10–97.60	1.98–92.29	1.99–92.68	1.55–72.05	1.28–59.05	0.21–9.84

quantitatively the potential of BPA disruption on T4 transport in human plasma. Because TBG carries the majority of T4, we will consider this transport protein first. As can be seen from Table 2, due to extremely high affinity of TBG with T4, almost all the hormone can bind to the protein. On the other hand, because BPA binding with TBG is very modest, the concentration of TBG-bound BPA amounts to only 1/6 of TBG-bound T4 even if total BPA reaches 97.6 nmol/L. This suggests that, at the current BPA level in human plasma, its disruption effect on T4 transport by TBG is insignificant. Turning to TTR, the concentration of TTR-bound BPA (59 nmol/L) is comparable to the low end of TTR-bound T4 (57.87 nmol/L) if total BPA reaches 97.6 nmol/L. However, since there is a large excess of TTR in human plasma (5 μmol/L) relative to T4 and BPA, the two chemicals do not compete with each other for TTR binding. This is also the case with HSA.

3 Conclusions

In this study, binding interaction of BPA, T4 and T3 with three major thyroid hormone transport proteins was investigated by fluorescence displacement and intrinsic fluorescence measurements. Binding affinity of the three chemicals with HSA was measured by using DNSA and DP as two site-specific fluorescence probes for HSA drug binding site I and site II, and by the intrinsic tryptophan fluorescence. The binding constant of BPA was found to be in the range of 10^3–10^4 L/mol, and 10–100 fold smaller than T4. BPA binding with TTR and TBG was assessed by using ANSA as the fluorescence probe. Its affinity was found to be weaker than T4 by 300 to 2666 folds. Based on the binding constants obtained in this work, possibility of competitive displacement of thyroid hormones from the transport proteins by BPA was evaluated. It was found that the current level of BPA in human plasma was not high enough to disrupt T4 transport.

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