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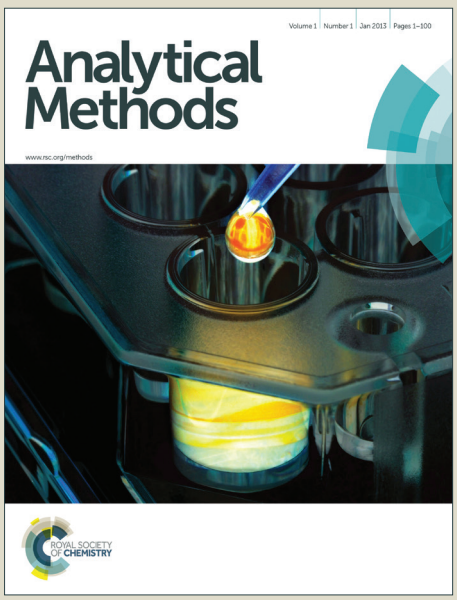





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ARTICLE TYPE

# Fluorescence Probe Techniques to Study the Interaction between Hydroxylated Polybrominated Diphenyl Ethers (OH-PBDEs) and Protein Disulfide Isomerase (PDI)

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**A selective and sensitive method to study the interaction between hydroxylated polybrominated diphenyl ethers (OH-PBDEs) and protein disulfide isomerase (PDI) was established in the assay. In this report, 3, 3', 5-triiodo-thyronine (T3) was conjugated with fluorescein (FITC) as fluorescence probe (F-T3) to study the competitive binding interaction of OH-PBDEs to PDI, which was said to have T3 binding activity. The findings suggest that some OH-PBDEs have the potential of binding to PDI and they share the same binding site with T3 to PDI and OH-PBDEs was able to act as a competitive inhibitor in PDI binding to T3.**

Polybrominated diphenyl ethers (PBDEs) have been widely employed in plastic products, sofa, carpet, condenser, computer etc as fire retardant over the past few decades. Because of their widespread use and high stability, PBDEs have become ubiquitous environmental contaminants and have attracted considerable attention recently.[1] Hydroxylated polybrominated diphenyl ethers (OH-PBDEs), which are analogous to PBDEs in structure, have been found in biota matrices, [2] and some researches have indicated that exposing hepatocytes to certain PBDEs will result in the formation of OH-PBDEs.[3] It has been reported that high bromine compounds can be metabolized to low bromine compounds, and the latter can be transformed to mono-hydroxy or di-hydroxy PBDEs by oxidizing enzymes such as cytochrome P450. [4] Hydroxylated PBDEs can combine with serum, accumulate in living organisms and exhibit a variety of potential toxicity. [5] Compared with their parent congeners, OH-PBDEs have stronger toxicity, and greater influence on the nervous system, such as estradiol toxicity, [6] anti-estrogen toxicity,[7] cytotoxicity, [8] interference on the oxidative phosphorylation.[6] In particular, OH-PBDEs have structural similarity to thyroxine (T4) and triiodo-thyronine (T3), and therefore exhibit similar characteristics with thyroid hormones (THs), disrupt the endocrine homeostasis, and compete with THs in binding to thyroid hormone transport proteins, transthyretin (TTR) as well as thyroxine-binding globulin (TBG). [9]

Protein Disulfide Isomerase (PDI) is a multifunctional protein, whose main role is to assist the folding process of proteins containing disulfide bonds,[10,11] it catalyzes thiol/disulfide exchange reactions of protein, including disulfide formation, reduction and isomerization.[12] Recent studies indicate that PDI catalyzes the reduction of the active site in vitamin K epoxide redox enzyme (VKOR)[13] and the reduction of dehydroascorbic acid in vitro.[14] And PDI involves several brain impairments, such as Parkinson's disease,[15] Alzheimer's disease.[16] In addition to that, PDI has other biological functions, for example, it is reported that PDI is an intracellular 3, 3', 5-triiodo-thyronine (T3)-binding protein,[17] and that PDI behaves as a chaperone,[18] inhibiting the aggregation of misfolded proteins, such as rhodanese,[19] and alcohol dehydrogenase.[20] PDI exists ubiquitously in endoplasmic reticulum, nuclear membranes, cytosol and so on.[21] The isomerization of protein disulfide bridges mediated by PDI plays a key role in the formation of three-dimensional structure of protein molecules, and also in protein renaturation. As an important multifunctional protein, PDI's expression has been said to be regulated by T3 and 17 β -estradiol(E2).[22,23] Primm et al. had indicated that PDI has only one estradiol binding site with modest affinity and two binding sites with comparable affinity for T3. T3 and E2 share the same binding site, and the other binds to the hydrophobic probe, bis-ANS.[24] Since T3 plays a crucial role in neural development, protein synthesis, endocrine homeostasis, the significance of both binding to PDI requires more study and needs further explanation. However, some research teams have reported that bisphenol A (BPA), hydroxylated polychlorinated biphenyls (PCB), PBDEs and their derivatives as well as a series of environmental phenolic compounds specifically inhibit T3 binding to PDI competitively and suppress the oxidative refolding of reduced RNase A by PDI.[25,26,27] The competitive replacement of T3 by the targets from PDI may result in the enzyme activity decreasing, intracellular protein folding in error and the physiological functions of PDI destroyed. Thus the reaction caused widespread concern, and there are some research teams paying a lot of attention to it.[25,26] However, the method they used was the competitive radioligand binding assay. By using $^{125}$I labeled T3 and $^{4}$H labeled E2 as the radioactive tracers in the assay, they revealed that BPA, hydroxylated Polychlorinated Biphenyls (OH-PCBs), T3 and E2 share the same binding site on PDI. However, the radiolabeling method has some deficiencies. Firstly, it

requires physical separation between protein-bound and free $^{125}$I-T3, which is a practical inconvenience and a potential source of deviation from equilibrium.[28] Radiolabelled materials are very costly and need expensive counting equipment. More importantly, there exists a great potential hazard to human. Therefore, a more simple and harmless method must be explored as a powerful means to study the interaction between PDI and the targets which can bind to PDI.

In our present study, a convenient fluorescence assay for the competitive binding reaction to PDI between OH-PBDEs and T3were proposed. A fluorescein-3, 3', 5-triiodo-thyronine (F-T3) conjugate was designed, synthesized, and characterized as a site specific fluorescence probe for the competitive binding assay.

The principle of the fluorescence assay for the competitive binding reaction is shown in Fig.1. Firstly, the amino group in T3 reacts with the isothiocyanate bond in FITC, thus forming the required fluorescence probe F-T3. Similar to the report and experimental data,[19] FITC itself was a very efficient fluorophore while FITC-labelled T3 represented an exception. We suggest that there exists a process of intramolecular fluorescence quenching in the F-T3 conjugate. This may be due to the iodine atoms in T3 close to the FITC, and resulted in a reduction in fluorescence emission intensity. Molecules containing an iodine atom are often considered to be fluorescent quenchers by collisional or heavy atom mechanisms. When F-T3 was bound to PDI, the iodine atoms were enclosed inside the PDI binding pocket and could not contact the fluorophore. As a result, the fluorescence restored. OH-PBDEs have a similar chemical structure with T3, therefore they can competitively replace F-T3 from the same binding site of PDI. Iodine atoms are exposed, and the fluorescence intensity decreases again. This provides the theoretical basis for us to detect OH-PBDEs.

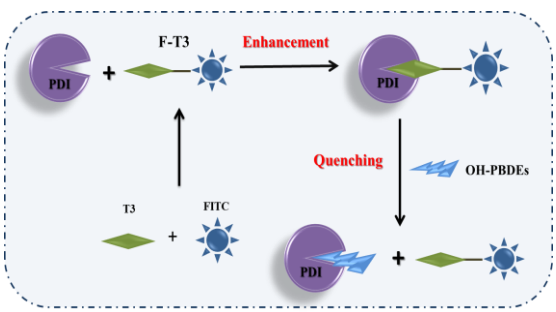


Fig. 1 Mechanism for OH-PBDEs detection based on the fluorescence competitive binding assay.

A simple experiment was performed to verify our mechanism. As shown in Fig. 2, when excited with light of 490 nm, the free F-T3 shows a weak fluorescence at 516 nm (curve a). After the addition of 5nM PDI, the fluorescence intensity increased significantly (curve d). Further addition of the target, 4-OH-BDE-42 (curve b) and BPA (curve c), cause the quenching of fluorescence. These results are consistent with our hypothesis.

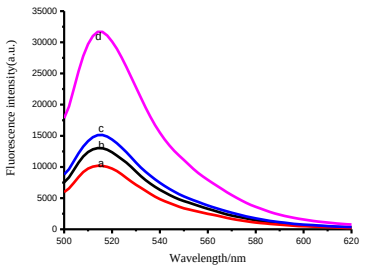


Fig. 2 Fluorescence emission spectra of (a) 200nM F-T3, (b) 200nM F-T3+5nM PDI+1μM 4-OH-BDE-42, (c) 200nM F-T3+5nM PDI+1μM BPA, (d) 200nM F-T3 +5nM PDI.

In order to obtain better assay results, several factors such as incubation time and the concentration of F-T3 were optimized. The effect of incubation time on the binding reaction between F-T3 and PDI was shown in Fig. S2(A). The fluorescence intensity increased progressively until 30 min and then reached a plateau. Hence, 30 min was chosen as the optimized condition in the direct binding assay. Furthermore, Fig. S2(B) showed the effect of incubation time on the competitive binding reaction between OH-PBDEs and PDI. After the addition of 1μM 4-OH-BDE-42 into the direct binding assay, the fluorescence intensity reduced gradually until 10 min and then reached a plateau. Hence, 10 min was chosen as the optimized condition in the competitive binding assay.

The concentrations of the PDI and F-T3 have been optimized too. Fix PDI's concentration to 5nM, and change the fluorescence probe F-T3 from 0nM to 500nM. As shown in Fig. S3, the fluorescence intensity ratio was largest when the concentration was 200nM. We then performed a similar study in a fixed F-T3 concentration of 200nM, only changing the PDI concentration from 0nM to 10nM. As shown in Fig. 3, the fluorescence intensity increased gradually and reached a plateau until 7nM. These results show a hyperbolic behavior and the saturation of the signal. From this experiment, we can calculate the Kd value for F-T3 and PDI interaction, that gives a value of 2.5nM. Considering the price and content of PDI, we finally chose 5nM as ous experimental dosage.

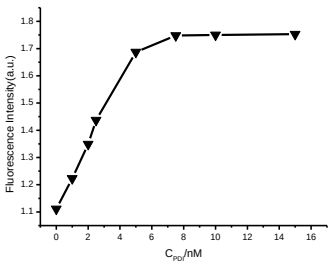


Fig.3 Fluorescence intensity ratio at different PDI concentrations, using a fix F-T3 200nM.

In the previous experiment, we synthesized F-T3 fluorescence probe by attaching FITC to the amino group of T3. We speculated that F-T3 will bind to PDI at the T4 binding site and the labeling of FITC will not modify the binding properties of the T3 molecule. In order to confirm our speculations,

determination of CD spectrum (See Fig.S4) and intrinsic fluorescence of PDI was carried out.

It is well known that among the amino acids that make up proteins, trptophan (Trp), tyrosine (Tyr), and phenylalanine (Phy) are associated with the intrinsic fluorescence of proteins. In addition, Trp accounts for the largest proportion of PDI fluorescence and is very sensitive to the tiny change of microenvironment induced by the interaction of small molecules and PDI. This sensitivity allows us to study the conformational changes and binding properties in PDI while binding to T3 or F-T3. When excited with 290nm light, the fluorescence emission intensities were obtained. As shown in Fig 4, upon addition of 1μM T3 into 200nM PDI, intrinsic fluorescence of PDI decreased by about 25%. This is due to T3 binding to PDI, which led to the conformational change of PDI. Similar results were obtained by adding F-T3 in place of T3, suggesting that F-T3 binds to PDI at the same binding site as T3.

The above fluorescence data suggested that F-T3 shares the same binding site with T3 in PDI, and F-T3 can be used as fluorescence probe in our experiment while not modifying the binding properties of T3 and PDI.

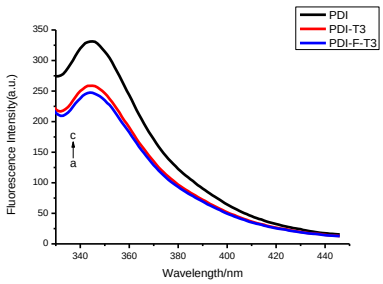


Fig. 4 Intrinsic fluorescence intensity of (a)PDI, (b)PDI+T3, (c)PDI+F-T3.

To verify the selectivity and specificity of the fluorescence probe F-T3 previously synthesized, some other enzymes or proteins with relatively high concentrations were used in place of the PDI(the concentration is shown in Fig. S5) to test the fluorescence recovery. The concentrations are at least 50 times higher than that of the PDI. The results demonstrated that they were unable to react with F-T3 as shown in Fig. S5. And the vertical axis represents fluorescence intensity changes. So the fluorescence could be markedly recovered only when PDI is present. Therefore, the developed method shows noteworthy selectivity.

Fig. 5 depicts the typical fluorescence responses with different concentrations of target 4-OH-BDE-42 at the optimized conditions. With an increase in target 4-OH-BDE-42 concentration, the fluorescence intensity decreased gradually, implying that more F-T3 was replaced by 4-OH-BDE-42. Meanwhile, it is found that there is a linear relationship between the fluorescence intensities and the logarithm of 4-OH-BDE-42 concentrations in the range$10^{-10}$-$10^{-6}$mol $L^{-1}$. The regression equation is:

$$y=-4944-2343\log C,\ R^2=0.99715.$$

Where C is the target 4-OH-BDE-42 concentration, R is the regression coefficient.

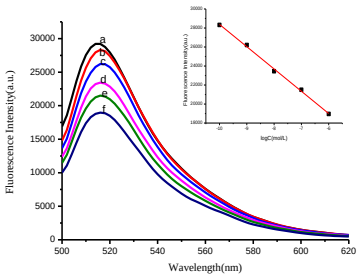


Fig. 5 (A):Fluorescence spectra after incubation with varying concentrations of 4-OH-BDE-42, 200nM F-T3 + 5nM PDI with 4-OH-BDE-42 (a) 0nM; (b) $1\times10^{-10}$nM; (c) $1\times10^{-9}$nM; (d) $1\times10^{-8}$nM; (e) $1\times10^{-7}$nM; (f) $1\times10^{-6}$nM. (B): The linear relationship between fluorescence intensity and logarithm of 4-OH-BDE-42 concentration.

In a large number of OH-PBDEs congeners, 4-OH-BDE-42, 6-OH-BDE-47, 2-OH-BDE-28, 6'-OH-BDE-99, 3-OH-BDE-7 as well as BPA, the structural analogues, were chosen as test chemicals to compare their binding activity towards PDI. The results were shown in Fig. S6, and the vertical axis represents fluorescence quenching ratio. As we expected, OH-PBDEs and BPA can make fluorescence quenching, although some of them had a very small quenching degree. Furthermore, the present findings and reported literatures demonstrated that the 2- and 4- hydroxyl groups in the phenyl ring of OH-PBDEs had a stronger inhibitory effect than the OH-substitutions at the 3-,6- position. We speculated that the position of bromine atoms substituted may also influence the binding affinity. Moreover, the current research suggests that OH-PBDEs, BPA, T3 share the same binding site on PDI because of the competitive binding reaction to PDI among them.

In summary, we have proved that the multifunctional protein PDI is a target of OH-PBDEs, BPA, and T3, and they share the same binding site. Moreover, we also successfully developed an approach for the study of competitive replacement by using a fluorescein-3, 3', 5-triiodo-thyronine (F-T3) conjugate as the probe. The application of the fluorescence probe F-T3, unlike radioactive tracers, has several attractive properties such as simple and secure preparation, simplified experimental process, and conventional fluorescence spectrometer. Our study will facilitate the deep understanding of significance of PDI and hazards of OH-PBDEs to living beings and the results suggest that OH-PBDEs are able to act as competitive inhibitors in PDI binding to T3.

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## Notes and references

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† Electronic Supplementary Information (ESI) available: experimental details. See DOI: 10.1039/b000000x/

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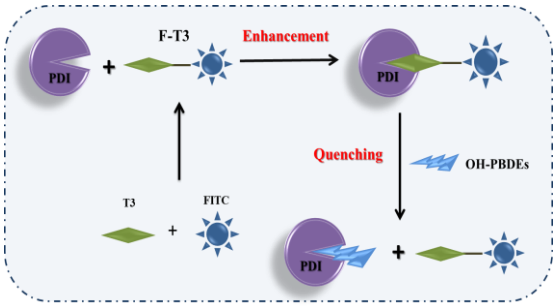


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