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Miniaturization of a transthyretin binding assay using a fluorescent probe for high throughput screening of thyroid hormone disruption in environmental samples

Xiyu Ouyang [a, *, 1], Jean Froment [b, c, 1], Pim E.G. Leonards [a], Guttorm Christensen [d], Knut-Erik Tollefsen [b], Jacob de Boer [a], Kevin V. Thomas [b], Marja H. Lamoree [a]

[a] Department of Environment and Health, VU University Amsterdam, De Boelelaan 1108, 1081 HZ, Amsterdam, The Netherlands
[b] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349, Oslo, Norway
[c] Department of Chemistry, University of Oslo (UiO), PO Box 1033, Blindern, N-0316, Oslo, Norway
[d] Akvaplan NIVA, FramCentre, N-9296, Tromsø, Norway

HIGHLIGHTS

- A high throughput T4-TTR binding assay was developed in a 96-well microplate format.
- The dose response relationship of eight chemicals was determined in the assay.
- The thyroid hormone disrupting potency of 22 herring gull eggs was determined.

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ABSTRACT

Thyroid hormone (TH) disrupting compounds are potentially important environmental contaminants due to their possible adverse neurological and developmental effects on both humans and wildlife. Currently, the most successful bio-analytical method to detect and evaluate TH disruptors, which target the plasma transport of TH in environmental samples, is the radio-ligand thyroxine-transthyretin (T_4-TTR) binding assay. Yet, costly materials and tedious handling procedures prevent the use of this assay in high throughput analysis that is nowadays urgently demanded in environmental quality assessment. For the first time a miniaturized fluorescence T_4-TTR binding assay was developed in a 96 well microplate and tested with eight TH disrupting compounds. For most of the compounds, the sensitivity of the newly developed assay was slightly lower than the radio-ligand binding assay, however, throughput was enhanced at least 100-fold, while using much cheaper materials. The TH disrupting potency of 22 herring gull (*Larus argentatus*) egg extracts, collected from two different locations (Musvær and Reiaren) in Norway, was evaluated to demonstrate the applicability of the assay for environmental samples.

1. Introduction

Over the past decade, research in the field of endocrine disruption has greatly increased with regard to the occurrence of endocrine disrupting compounds in the environment and their effects in the ecosystem and humans (Crisp et al., 1998). Over the years, various end points such as estrogenic, androgenic, progestogenic, glucocorticoid and thyroidogenic activities (Crisp et al., 1998) have been included in endocrine disruption studies. The thyroid system comprises the hypothalamus, the pituitary and the thyroid gland. It is vulnerable to endocrine-disrupting effects through different mechanisms, such as binding of xenobiotics to thyroid hormone (TH) transport proteins and interference with the plasma hormone transport of THs (Boas et al., 2006). Together with thyroxine-binding globulin (TBG), transthyretin (TTR) is one of the most important plasma proteins associated with the transport of THs such as thyroxine (3, 3′, 5, 5′-tetraiodo-L-thyronine, T_4).

* Corresponding author.
E-mail address: xiyu.ouyang@vu.nl (X. Ouyang).
[1] The authors contributed equally to this work.

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Disruption of the binding of T_4 with TTR has already been observed in the presence of various environmental contaminants, such as hydroxylated polychlorinated biphenyls (OH-PCBs), perfluoroalkyl and polyfluoroalkyl substances (PFASs), hydroxylated polybrominated diphenyl ethers (OH-PBDEs) and other brominated flame retardants (BFRs); caused by their competition with the T_4 binding to TTR (Gutleb et al., 2010; Weiss et al., 2009; Cao et al., 2010; Hamers et al., 2006). Interference with the plasma transport of THs may lead to disruption of the targeted transport and metabolism of T_4 that may ultimately lead to perturbations of natural functions of THs in adults as well as maturation and development in juvenile or fetal life stages of vertebrates (Miller et al., 2009). Advanced bio-analytical methods have therefore been developed to evaluate disruption of T_4 transport. A classical method to assess such an effect uses ^{125}I labelled T_4 as the radioactive ligand in a competitive binding assay (Lans et al., 1993). The method was successfully applied in many studies and showed good sensitivity (Weiss et al., 2009; Meerts et al., 2000; Simon et al., 2013). However, due to the costly radioactive ligand, relatively complicated assay steps and safety issues related to the handling of ^{125}I as a radioactive tracer, the throughput of this approach is still rather limited. In addition to this classical radio-ligand binding assay, other approaches such as the TR-CALUX (thyroid hormone responsive chemically activated luciferase gene expression) assay and the ANSA (8-anilino-1-naphthalenesulfonic acid ammonium)-TTR competitive fluorescence displacement assay have been developed to assess the TTR binding capacity of TH disruptors in the environment (Montaño et al., 2012; Huang et al., 2011). Similar to the ANSA-TTR assay, another bioassay, based on a previous study (Smith, 1977), has also been developed to investigate the binding of OH-PBDEs to TH transport proteins (TTR and TBG) using the fluorescence probe fluorescein isothiocyanate (FITC) associated to T_4 (Ren and Guo, 2012). This assay was performed in cuvettes and the reported sensitivity for OH-PBDEs was comparable to the radioligand assay.

In the present study, the same principle of FITC-T_4 was applied, however in order to further enhance assay throughput, it was miniaturized in a 96 well microplate format. First, the new downscaled protocol was optimized to achieve similar performance as the assay performed in cuvettes. Then, the new protocol was tested with eight compounds from seven different groups (OH-PCBs, OH-PBDEs, BFRs, PFASs, bromophenols, phthalates and antibacterial agents) known to interfere with the T4-TTR binding. The observed activities were compared with those obtained in the classical radioligand binding assay. The T_4 displacing potency of herring gull extracts from two locations in Norway was evaluated using the new protocol to demonstrate the applicability of the assay for testing environmental samples. In addition, in order to further explain the activities determined using the bioassay, target analysis of OH-PCBs based on gas chromatography (GC) using electron capture detection (ECD) was performed for the most potent sample, as high levels of PCBs have previously been detected in these samples (Muusse et al., 2015).

2. Materials and methods

2.1. Chemicals

Fluorescein isothiocyanate (FITC, >90%) and L-thyroxine (T_4, >98%) were supplied by Sigma-Fluka (Zwijndrecht, The Netherlands). Anhydrous pyridine (99.8%) and triethylamine (>99%) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Water was obtained from a Milli-Q Reference A+ purification system (Millipore, Bedford, MA, USA). Perfluorooctanoic acid (PFOA, 96%) and triclosan were purchased from Sigma-Fluka. Perfluorooctanesulfonic acid (PFOS, 98%) was purchased from RTI laboratories (Livonia, MI, USA). 2, 4, 6-tribromophenol (2, 4, 6-TBP, 99%) were purchased from Riedel-de Haen (Seelze, Germany). Mono (2-ethylhexyl) phthalate (MEHP, 100 μg/mL in methyl tert-butyl ether) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). 2,2′,3,4′,5,5′-hexachloro-4-biphenylol (4-OH-CB-146, 50 μg/mL in nonane), 2,2′,4,4′,5,5′-hexachloro-3-biphenylol (3-OH-CB-153, 50 μg/mL in nonane) and 2,2′,3,3′,4′,5,5′-heptachloro-4-biphenylol (4-OH-CB-172, 50 μg/mL in nonane) were obtained from Wellington Laboratories (Guelph, ON, Canada). 2,3,3′,4′,5-pentachloro-4-biphenylol (4-OH-CB-107, 0.99 mg/g in 4-methyl-2-pentanol), 2,2′,3,4′,5,5′,6-heptachloro-4-biphenylol (4-OH-CB-187, 2 mg/g in 4-methyl-2-pentanol), 6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (6-OH-BDE-47, 1 mg/mL in dimethyl sulfoxide) and tetrabromobisphenol A (TBBPA, 1 mg/mL in dimethyl sulfoxide) were obtained from the group of Prof. Bergman, ACES, Stockholm University, Sweden.

2.2. Synthesis of the fluorescent probe

The fluorescent labelled thyroxine (FITC-T_4) has been synthesized and purified according to a previous study (Smith, 1977). In short, 51.4 mM of FITC reacted with 25.7 mM of L-thyroxine in a pyridine/water/triethylamine medium (9:1.5:0.1, v/v/v) for one hour at 37 °C. The reaction products were precipitated by adding 20 vol of 0.2 M ammonium acetate buffer and collected after centrifugation (10 min, 1000 × g, Biofuge Stratos, Heraeus Instruments, Hanau, Germany). After removal of the supernatant, the precipitate was washed with 20 vol of MilliQ water and centrifuged again at the same condition. The precipitate was then re-dissolved in 8 vol of 0.005 M of ammonium bicarbonate. A few drops of ammonia solution (10%, v/v) were added until the precipitate dissolved. The obtained solution was applied to a Sephadex G-50 fine column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) which was previously equilibrated with a solution of 5 mM ammonium bicarbonate. The fluorescent impurities were thereafter removed by washing the column with 10 column volumes of 5 mM ammonium bicarbonate solution. The purified product (FITC-T_4) was eluted from the gel using MilliQ water and then freeze dried for 48 h under 0.7 mbar, −20 °C. Before use, the obtained FITC-T_4 was dissolved in Tris-NaCl buffer (50 mM Tris−HCl/100 mM NaCl, pH 7.4) and its concentration was measured by absorbance at 490 nm using a Smartspec 3000 spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Herring gull egg collection and extraction

Sample collection and extraction was described in a previous study (Muusse et al., 2015). During spring 2012, 22 herring gull (*Larus argentatus*) eggs were collected from two locations in Norway: 1) the Musvær Island (69°52′N, 18°33′E), a remote island in the north of the country in the municipality of Tromsø and 2) the Reiaren Island (59°8′N, 10°27′E), a more populated area off the southeastern coast in the municipality of Tjøme. After collection all the eggs were frozen as fast as possible in order to prevent embryo development. Each egg was collected from a different nest without knowing the age. The egg yolk extraction was performed in an ultrasonic bath for 15 min with acetone and cyclohexane (3:2, v/v). After shaking for 1 h, the extracts were centrifuged for 10 min (1300×*g*) and the lipids were removed by gel permeation chromatography (GPC; Waters 2695 separations module coupled to a Waters 486 absorbance detector at 254 nm) fitted with Envirogel columns (19 mm × 150 mm + 19 mm × 300 mm; Waters). The extracts were collected between 14.40 and 21.00 min and the dichloromethane used as mobile phase was evaporated under a