Disclosing Environmental Ligands of L-FABP and PPARγ: Should We Re-evaluate the Chemical Safety of Hydrocarbon Surfactants?

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Abstract

Chemical contaminants can cause adverse effects by binding to the liver - fatty acid binding protein (L-FABP) and peroxisome proliferator-activated nuclear receptor γ (PPARγ), which are vital in lipid metabolism. However, the presence of numerous compounds in the environment has hindered the identification of their ligands, and thus only a small portion have been discovered to date. In the present study, protein **A**ffinity **P**urification with **N**ontargeted **A**nalysis (APNA) was employed to identify the ligands of L-FABP and PPARγ in indoor dust and sewage sludge. A total of 83 nonredundant features were pulled-out by His-tagged L-FABP as putative ligands, among which 13 were assigned as fatty acids and hydrocarbon surfactants. In contrast, only six features were isolated when His-tagged PPARγ LBD was used as the protein bait. The binding of hydrocarbon surfactants to L-FABP and PPARγ was confirmed using both recombinant proteins and reporter cells. These hydrocarbon surfactants, along with >50 homologues and isomers, were detected in dust and sludge at high concentrations. Fatty acids and hydrocarbon surfactants explained the majority of L-FABP (57.7 ± 32.9%) and PPARγ (66.0 ± 27.1%) activities in the sludge. This study revealed hydrocarbon surfactants as the predominant synthetic ligands of L-FABP and PPARγ, highlighting the importance of re-evaluating their chemical safety.

Keywords: L-FABP; PPARγ; Environmental obesogens; Surfactants; Nontargeted analyses

Synopsis: Hydrocarbon surfactants are the predominant synthetic ligands of L-FABP and PPARγ in the environment.

Introduction

Obesity is a worldwide public health epidemic,[1, 2] wherein nearly three-fourths of adults in the United States meet the criteria for being overweight or obese.[3] The increasing incidence of obesity has been partly attributed to increased exposure to environmental obesogens,[4-6] and the fatty acid binding protein (FABP) – peroxisome proliferator-activated nuclear receptor γ (PPARγ) pathway has been suggested as a major toxicity pathway for these obesogens.[7] As members of the lipid-binding protein superfamily, FABPs are vital in fatty acid metabolism and are involved in the proliferation of cancer.[8] FABPs are also key transporters to deliver lipophilic ligands to PPARs, and act as a cytosolic gateway for PPAR agonists.[9, 10] As a transcription factor, PPARγ plays a crucial role in lipid metabolism and adipocyte differentiation,[11] and thus inappropriate activation of PPARγ by environmental contaminants may promote adipogenesis and obesity. To date, many environmental contaminants (*e.g.,* phthalate monoesters) have been found to promote adipocyte formation via PPARγ activation.[12-14] This highlights the need to systematically identify environmental contaminants binding to PPARγ.

Extensive efforts have been made in the past several decades to identify the ligands of PPARγ[15] and other nuclear receptors (NRs)[16] using *in vitro* high-throughput screening (HTS) bioassays. Over 10,000 chemicals have been tested for PPARγ activities through the Tox21 program, among which many previously unrecognized PPARγ ligands were discovered.[17] However, due to the immense number (>350,000) of synthetic chemicals[18] as well as even more unknown transformation products, it is impractical to completely test the PPARγ activity of each individual compound. Indeed, only up to 4.2% and 9.3% of the PPARγ activity of river water[19] and

54 wastewater samples[20] can be explained by known chemicals. Effect-directed analysis (EDA) is an
55 alternative technology for the screening of harmful chemicals from environmental mixtures.[21] By
56 use of an EDA approach, Fang et al. made significant progress by identifying fatty acids as the
57 major (30-50%) natural PPARγ agonists in indoor dust, but suggested additional unknown
58 compounds are likely present in indoor dust.[22] Due to the large (~1440 Å^3) ligand binding pocket
59 of PPARγ[23, 24], a large number of PPARγ ligands may be present in the environment. Such an
60 abundance of compounds would exceed the separation capacity of liquid chromatography (LC)
61 and preclude EDA from accomplishing an in-depth identification of co-eluting ligands. As a result,
62 the majority of synthetic PPARγ ligands in the environment remain unknown to date.

63 A bioanalytical method "protein **A**ffinity **P**urification with **N**ontargeted **A**nalysis (APNA)"
64 has been proposed in our previous studies for the screening of protein ligands in the environment,
65 on the exposome-wide level.[25-29] Distinct from the conventional EDA method relying on LC
66 fractionation, APNA uses a tagged protein (*e.g.*, NR) as a "bait" to directly isolate ligands from
67 environmental mixtures consisting of thousands of chemicals.[29] This overcomes the major
68 challenge of co-elution in EDA, and largely reduces false discovery rates. This method has been
69 employed in our recent studies to identify chemical contaminants binding to multiple human[25-27]
70 and bacterial proteins[28]. A major advantage of APNA is the capacity to simultaneously isolate
71 multiple ligands from complex environmental mixtures, which is ideal for the identification of
72 unknown PPARγ ligands.

73 In this study, we aimed to employ APNA to uncover previously unknown chemical
74 contaminants in indoor dust and sewage sludge binding to human liver FABP (L-FABP) and

PPARγ. Fatty acids and four classes of hydrocarbon surfactants were identified as primary ligands in indoor dust and sewage sludge samples. Surprisingly, the identified hydrocarbon surfactants contributed comparably or even higher to PPARγ activities than fatty acids, which are the natural ligands of the receptor. This highlighted that hydrocarbon surfactants are putative environmental obesogens, even if they are considered safe from an acute toxicity perspective.

Materials and Methods

Protein Affinity Pulldown. To identify environmental contaminants binding to L-FABP and PPARγ in indoor dust and sewage sludge, APNA was employed.[25-29] In this study, indoor dust and sewage sludge were selected for testing because they represent important reservoirs for commercial chemicals, and have long been used as promising sources to measure the collective consumption or chemical exposure of humans.[30, 31] To prepare the indoor dust or sewage sludge extracts, approximately 0.5 g of indoor dust or freeze-dried sewage sludge was extracted twice with 5 mL methanol, and the extracts were blown down and reconstituted to a final volume of 1 mL before use. His-tagged full length L-FABP or His-tagged PPARγ ligand binding domain (LBD) were overexpressed in *E. coli* cells. See supporting information (SI) for more details.

To perform APNA experiments, crude lysates (400 µL) of *E. coli* cells overexpressing His-tagged L-FABP or His-tagged PPARγ LBD were incubated with 4 µL of indoor dust or sewage sludge extract. Lysates of wild-type *E. coli* cells were used as negative controls. 5 µL of His-select nickel magnetic agarose beads were added and transferred to a 96-well plate and incubated at 4 ºC for 60 min in a rotator at 20 rpm. The experiments were performed in triplicate (N = 3). After incubation, the 96-well plate was placed on a magnetic field plate to separate the beads. The

supernatant was removed, and the magnetic beads were washed 3 times using 100 μL of wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, and 10 mM imidazole, pH 8.0). 100 μL of elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, and 250 mM imidazole, pH 8.0) was added and mixed in a shaker at 170 rpm for 30 minutes. The solution was then transferred to a Zeba^{TM} spin 7k MWCO desalting plate (Thermo Fisher, Catalog# 89807). Following centrifugation at 1000 *g* for 2 minutes, the eluted solution was collected and transferred to 1.7 mL tubes. Proteins were denatured by addition of 400 μL methanol. The solution was vortexed for 1 minute, and centrifuged for 10 minutes at 10,000 *g*. The supernatant was transferred to sample vials for nontargeted analysis.

Nontargeted Analysis. Nontargeted analysis was conducted by use of a Q Exactive mass spectrometer equipped online with a Vanquish ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, Waltham, MA, USA). An Accucore Vanquish C18 column (50 × 2.1 mm, 1.5 μm, Thermo Scientific) was used for chemical separation. The injection volume was 2 μL, with ultrapure water (A) and methanol (B) as the mobile phases. The elution gradient was performed as follows: 0–7 min, 10–100% B; 7–11.5 min, 100% B; then, decreased to 10% B in 0.5 min and held for 1 min to allow for equilibration. The flow rate was 0.3 mL/min. The column and sample compartment temperatures were maintained at 40 °C and 10 °C, respectively. Data were acquired in negative mode by data-independent acquisition (DIA). Here, only negative mode was employed because ligands of L-FABP or PPARγ should be detectable in negative mode, as the binding is largely mediated by the negatively charged head group of the ligands.[32, 33] Parameters were one full MS^1 scan (100–1000 m/z) recorded at resolution R = 70 000

(at m/z 200) with a maximum of 3×10^6 ions collected within 100 ms, followed by DIA MS/MS scan (150–750 m/z) recorded at resolution R = 35 000 (at m/z 200) with a maximum of 1×10^5 ions collected within 50 ms. DIA data were collected by use of 15 m/z isolation windows per MS/MS scan. In total, there were 40 15-m/z wide windows between 150–750 m/z. The MS instrumental parameters were set as follows: spray voltage, 2.8 kV; sheath gas flow rate, 30 L/h; auxiliary gas flow rate, 6 L/h; capillary temperature, 300 °C.

Nontargeted Screening Algorithm. Nontargeted analysis was accomplished with an in-house R program as described in our previous studies.[34] Raw mass spectrometry files were first converted to mzXML format. A putative lock mass algorithm (PLMA) was applied for post-acquisition calibration of mass spectra before peak picking.[34] The 'XCMS' R package[35] was used for peak detection with a mass tolerance of 2.5 ppm. The peak features were aligned across samples with a mass tolerance of 2.5 ppm and retention time window of 20 seconds after retention time adjustment. The ratio between the peak abundance from *E. coli* overexpressing His-tagged L-FABP or His-tagged PPARγ LBD to that from wild type *E. coli* was calculated for each peak feature. The p value of the difference between the two groups was also determined by student's t-test. Only the features exhibiting greater peak intensities (fold change > 5, p value < 0.05) in extracts from the overexpressed L-FABP or overexpressed PPARγ LBD groups were considered as potential ligands. *E. coli* overexpressing His-tagged L-FABP or His-tagged PPARγ LBD without incubation with dust/sludge extracts was also employed as another negative control. The pulled-out LC-MS features were further filtered by using the second negative control with the same cutoffs. Isotopic peaks and adducts were excluded by matching chromatographic peaks and theoretical mass

differences. The final differentiated peak list from the output of the R program was manually checked by use of Qual Browser in Xcalibur software. Then, the differentiated peaks were searched against the LIPID MAPS database,[36] the United States Environmental Protection Agency (U.S. EPA) Toxic Substances Control Act Chemical Substance Inventory (TSCA Inventory),[37] and the Network of Reference Laboratories, Research Centers, and Related Organizations for Monitoring of Emerging Environmental Substances (NORMAN) Suspect List Exchange database[38] by an in-house R program.[34] A mass tolerance of 3 ppm was used. Confidence levels were assigned to all identities according to the Schymanski et al. scale.[39] In brief, level 1 confidence was assigned to chemicals for which authentic standards are available for confirmation by matching their retention time, MS^1, and MS^2 spectra. For chemicals without standards, the confidence levels fall to levels 2 or 3, as molecular ions and fragmentation patterns were both matched. For example, m/z 79.9559 (SO_3^-) and m/z 96.9587 (HSO_4^-) were characteristic fragments of sulfonate and sulfate, respectively.

In this study, four fatty acids (*i.e.,* palmitic acid, stearic acid, oleic acid, and pentadecanoic acid,) and two hydrocarbon surfactants (*i.e.,* dodecyl sulfate and octadecyl sulfate) were not identified by the APNA method due to the interfering background in the LC-MS system. However, their presence in the sample extracts and binding to L-FABP and PPARγ LBD were directly confirmed by using corresponding authentic standards and the fluorescence displacement assay.

Calculation of BEQ. In this study, the bioanalytical equivalent concentration (BEQ) concept[40] was used to determine the contributions of hydrocarbon surfactants to the L-FABP or PPARγ LBD binding activities of indoor dust or sewage sludge samples. The BEQ values from bioanalysis

159 (BEQ_{bio}) of extracts of indoor dust or sewage sludge samples were calculated based on the
160 inhibitory concentrations at 50% (IC_{50}) using equation 1:

161 $$BEQ_{bio} = \frac{IC_{50}(ref)}{IC_{50}(dust\ or\ sludge\ samples)} \quad (1)$$

162 where *ref* represents the reference compound. Perfluorooctane sulfonate (PFOS) was used as the
163 reference compound for L-FABP binding activity, whereas rosiglitazone was used for PPARγ
164 binding activity, since they are well-documented ligands for these respective proteins.[41, 42] The
165 IC_{50} value for each indoor dust and sewage sludge sample was derived from the fluorescence
166 displacement assay by dose-response curve fitting (see SI for more details).

167 To calculate the BEQ value from chemical analysis (BEQ_{chem}), the relative effect potency
168 (REP_i) values for hydrocarbon surfactants or fatty acids (i) detected in indoor dust or sludge
169 samples were first estimated by equation 2:

170 $$REP_i = \frac{IC_{50}(ref)}{IC_{50}(i)} \quad (2)$$

171 Then, BEQ_{chem} was calculated by multiplying the concentrations of hydrocarbon surfactants
172 or fatty acids (C_i) measured in samples by their respective REP values (equation 3):

173 $$BEQ_{chem} = \sum_{i=1}^{n} REP_i \times C_i \quad (3)$$

174 Finally, the contributions of hydrocarbon surfactants or fatty acids to the observed effects of
175 indoor dust or sewage sludge extracts were determined by equation 4:

176 $$Contribution\ (\%) = \frac{BEQ_{chem}}{BEQ_{bio}} \times 100\% \quad (4)$$

177

178 Detailed method descriptions of chemicals and reagents, protein overexpression and

purification, collection and extraction of indoor dust and sewage sludge samples, fluorescence displacement assay, calculation of dissociation constant (K_d) values, molecular docking, homologue series analysis, quantification of hydrocarbon surfactants and PFOS, PPARγ activity testing, and statistical analysis is available in the SI. This paper has also been deposited in the ChemRxiv server.[43]

Results and Discussion

Identification of L-FABP and PPARγ Ligands in Indoor Dust and Sewage Sludge. To identify the environmental ligands binding to L-FABP and PPARγ, the APNA method was employed. A proof-of-concept study was first conducted to benchmark the method by incubating a mixture of seven compounds including rosiglitazone, thyroxine (T_4), 3,3′,5-triiodo-L-thyronine (T_3), PFOS, and three unsaturated fatty acids (*i.e.,* oleic acid, linoleic acid, and linolenic acid) with three His-tagged human proteins including L-FABP, PPARγ LBD, and thyroid hormone α (TRα) LBD, separately. As expected, all potent ligands (K_d < 1 μM) including rosiglitazone, PFOS, and thyroid hormones (*i.e.,* T_3 and T_4) were significantly pulled-out by their corresponding proteins, *i.e.,* PPARγ LBD (rosiglitazone), L-FABP (PFOS), and TRα LBD (T_3 and T_4), respectively, demonstrating the high selectivity of the APNA platform (Figure S1). Unsaturated fatty acids, *i.e.,* oleic acid, linolenic acid, and linoleic acid were significantly pulled-out by both TRα LBD and L-FABP, which was in accordance with reports of them as potent ligands of both TRα[44] and L-FABP[45]. Interestingly, although PFOS and unsaturated fatty acids have been reported to bind to both L-FABP and PPARγ,[24, 46, 47] they were selectively pulled-out by L-FABP, but not by PPARγ. This

was likely due to the weaker binding affinity of PFOS and unsaturated fatty acids to PPARγ LBD (K_d=8.55 μM) than L-FABP (K_d=1.26 μM), as discussed below. Together this information confirmed that the APNA method can be used to selectively identify potent ligands binding to L-FABP or PPARγ.

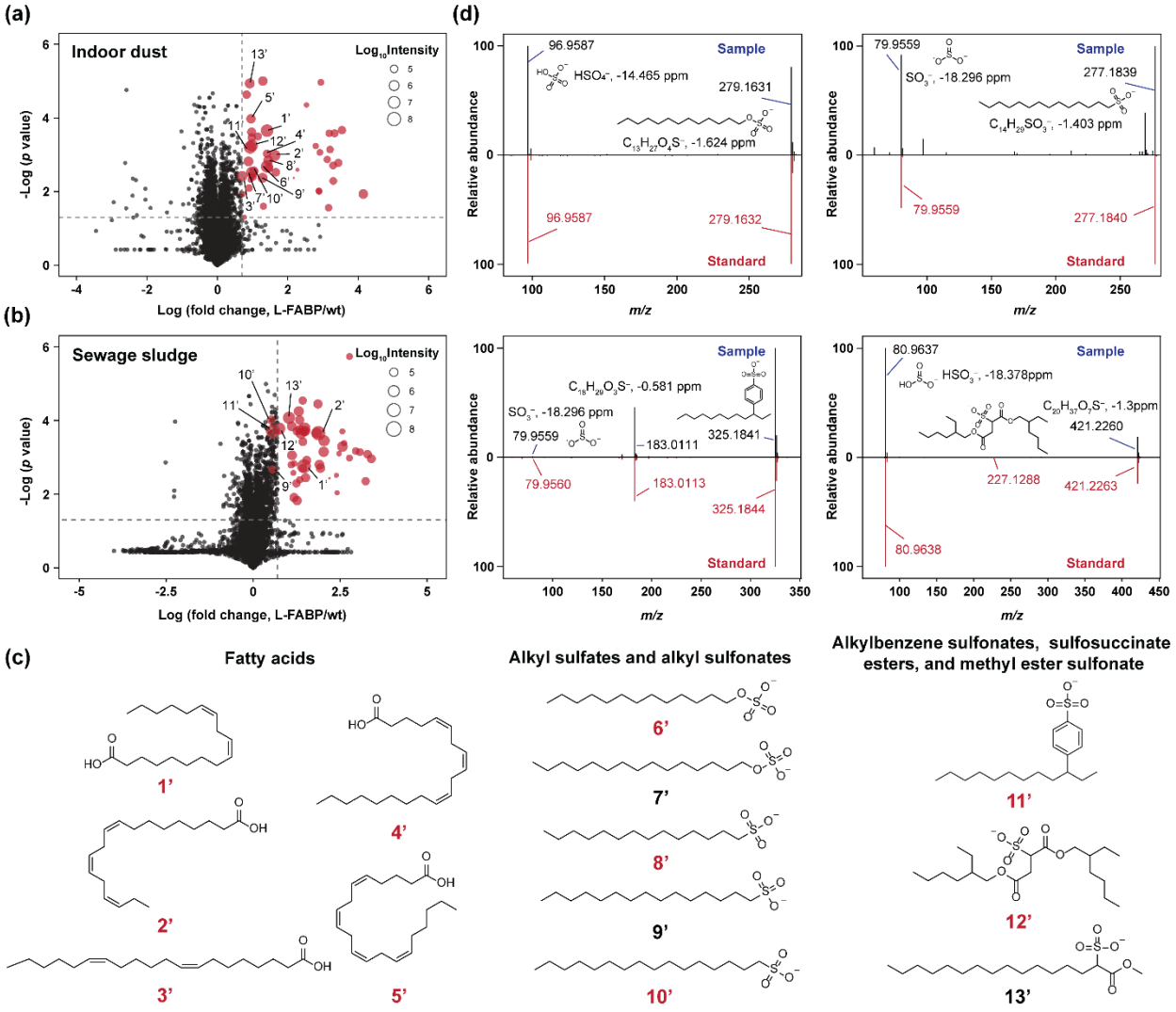

Figure 1. Identification of L-FABP ligands in indoor dust and sewage sludge by APNA. Volcano plots representing the log-transformed fold changes and corresponding *p* values of each feature detected in **(a)** indoor dust or **(b)** sewage sludge. Red dots indicate molecular features having significantly greater abundances (fold change > 5, $p < 0.05$) in *E. coli* lysates

overexpressing His-tagged L-FABP protein. Isotopic features and adducts were removed. Dot size represents the peak intensity for each pulled-out compound. **(c)** Structures of 13 tentatively identified environmental ligands of L-FABP. Compounds with commercially available standards were labeled in red. Pictured above is one potential isomer for each compound class, of which there are many possible isomers (*e.g.,* different isomeric alkyl groups). **(d)** Representative MS^2 spectra of four hydrocarbon surfactants and their corresponding authentic standards.

We then applied the APNA platform to identify unknown ligands of L-FABP and PPARγ. We first investigated L-FABP ligands by incubating His-tagged L-FABP with extracts of indoor dust or sewage sludge. Among the 12,249 and 12,412 features detected in pooled indoor dust and sewage sludge extracts, respectively, 46 and 45 LC-MS features were specifically pulled-out by His-tagged L-FABP as putative ligands after excluding isotopic peaks and adducts (Figure 1a and 1b). The *m/z* and retention time of all the pulled-out LC-MS features were manually inspected. During this process, four additional features (*i.e.,* 9', 10', 11' and 12' in Figure 1c) that had not been detected by the original nontargeted analysis algorithm due to background contaminations were found also significantly pulled-out by L-FABP, and then added into the pulled-out list. After excluding the repetitive features between dust and sludge, a total of 83 nonredundant features were detected (full list in Table S1). Among the 83 putative ligands, structures were successfully assigned to 13 features by matching to the LIPID MAPS, TSCA Inventory, and NORMAN databases with a mass tolerance of 3 ppm. Fortunately, these 13 features were the most abundant ligands (indicated by peak abundance) pulled-out by His-tagged L-FABP. All 13 features (Figure 1c) were assigned as fatty acids (1' to 5') or hydrocarbon surfactants, including alkyl sulfates (6'

and 7'), alkyl sulfonates (8' to 10'), alkylbenzene sulfonate (11'), sulfosuccinate ester (12'), and methyl ester sulfonate (13'). The identities of hydrocarbon surfactants were further supported by their characteristic MS^2 fragments (Figure 1d). For example, $[HSO_4]^-$ (m/z = 96.9587), $[SO_3]^-$ (m/z = 79.9559), and $[HSO_3]^-$ (m/z = 80.9637) were observed as characteristic fragments for sulfate, sulfonate, and sulfosuccinate ester, respectively (Figure 1d). In addition, the ethylene-substituted benzenesulfonate ion (m/z = 183.0111) has been reported as a predominant fragment ion for linear alkylbenzene sulfonate (LAS)[48], supporting their assigned structures. Standards were commercially available for 10 of the 13 ligands, which were purchased and successfully confirmed the identities by comparing MS^2 spectra (Figure 1d) and retention times. The identification of fatty acids (Figure 1c) including linoleic acid (1'), linolenic acid (2'), eicosadienoic acid (3'), eicosatrienoic acid (4'), and arachidonic acid (5') as a major ligand class is unsurprising, as they are well known natural ligands of L-FABP.[45] In addition to the fatty acids as the natural ligands, hydrocarbon surfactants (Figure 1c), *i.e.,* tridecyl sulfate (6'), tetradecane sulfonate (8'), hexadecane sulfonate (10'), dodecyl benzenesulfonate (11'), and dioctyl sulfosuccinate (12') were identified as the synthetic ligands of L-FABP. Consistent with this, our recent study identified alkyl sulfates as putative L-FABP ligands from Aqueous Film-Forming Foam (AFFF),[32] although authentic standards were not used for validation in that study.

In contrast to the large number of identified L-FABP ligands, only six LC-MS features were specifically pulled-out by His-tagged PPARγ LBD across the indoor dust and sewage sludge extracts (Figure S2 and Table S1). The peak intensities of the six features were about 1-3 orders of magnitude lower than those of fatty acids and hydrocarbon surfactants, and cannot be identified

by database searching. As mentioned above, some medium to low-affinity ligands (K_d>1 μM), *e.g.,* PFOS, could be missed by the APNA method when His-tagged PPARγ LBD was used as the protein bait. Fortunately, since L-FABP is a key transporter for delivering ligands to PPARγ, previous studies have thoroughly documented the similarities in ligand specificity between L-FABP and PPARγ.[9, 10] Supporting this, strong correlations were observed between the L-FABP and PPARγ activities across 74 per- and poly-fluoroalkyl substances (PFAS) (Figure S3), measured in our previous studies[32] and by Houck et al[49]. Therefore, we hypothesized that many of the 83 ligands of L-FABP isolated from indoor dust and sewage sludge would also bind to PPARγ (as shown below).

Ligand Validation by the Fluorescence Displacement Assay. To verify the binding of these pulled-out ligands to L-FABP, a well-established fluorescence displacement assay was employed in which 1-anilinonaphtha-lene-8-sulfonic acid (1,8-ANS) was used as a fluorescent probe (see SI for more details).[32, 47] In this assay, if the tested compound competed with 1,8-ANS for the same protein binding site, the probe would be displaced, resulting in a decrease in the fluorescence signal (Figure S4 and S5). Supporting the APNA results, all 5 fatty acids (*i.e.,* linoleic acid, linolenic acid, eicosadienoic acid, eicosatrienoic acid, and arachidonic acid) pulled-out by the APNA method showed binding to L-FABP (Figure S6a and Table S2), with K_d values of 0.32 – 3.65 μM. Furthermore, four other common fatty acids (*i.e.,* palmitic acid, stearic acid, oleic acid, and pentadecanoic acid) that showed binding activities to L-FABP in previous studies[45, 50] were also tested. These four fatty acids were not identified by the APNA method mainly due to the interfering background in LC-MS (see Materials and Methods for more details). Among all the 9 tested fatty

acids, oleic acid was the most potent in binding to the L-FABP protein (K_d = 0.15 μM), whereas stearic acid had relatively weaker binding affinity (K_d = 2.70 μM). This is consistent with a previous isothermal titration calorimetry (ITC) study that oleic acid is a stronger human L-FABP ligand than stearic acid.[50]

We then moved forward to testing the binding of hydrocarbon surfactants to L-FABP. All 5 hydrocarbon surfactants identified by the APNA method were confirmed to bind to L-FABP with K_d values of 0.162 – 13.8 μM (Figure 2a and Table S2). For instance, dodecyl benzenesulfonate (K_d = 0.946 μM) and dioctyl sulfosuccinate (K_d = 0.22 μM) showed strong binding to L-FABP in sub μM range. Seven additional hydrocarbon surfactants with different carbon chain lengths (C_{2-18}; see complete list in Table S3) were also tested to establish the structure-related activity relationship. As shown in Figure 2a, shorter-chain (C_{2-8}) alkyl sulfates and alkyl sulfonates showed no binding to L-FABP (IC_{50} > 200 μM). The increasing binding affinities to L-FABP with stronger hydrophobicity was well-reflected by the negative relationship observed between K_d and their retention times on a C18 column (Figure 2c). Note that hydrocarbon surfactants with a carbon chain length >13 showed greater binding affinity to L-FABP than PFOS (K_d = 1.26 μM), a well-studied L-FABP ligand with a similar structure.[32, 47]

Then, we performed molecular docking to better understand the structure-related interactions between hydrocarbon surfactants and L-FABP. Taking dodecyl benzenesulfonate as an example, three hydrogen bonds were formed between the negatively charged sulfonate group and residues of Arg-122, Ser-39, and Ser-124 (Figure S7), which was similar to results previously reported for PFAS.[32, 47, 51] In addition, the alkyl chain formed hydrophobic contacts with the interior cavity of

L-FABP. It has been demonstrated that both the hydrogen bond and the hydrophobic interactions are crucial in stabilizing the ligand-protein complex of L-FABP.[47, 52] Since these hydrocarbon surfactants have similar acidic head groups (SO_3^- and SO_4^-), the stronger hydrophobic effects of longer-chain hydrocarbon surfactants may explain their stronger binding affinities to L-FABP.[53]

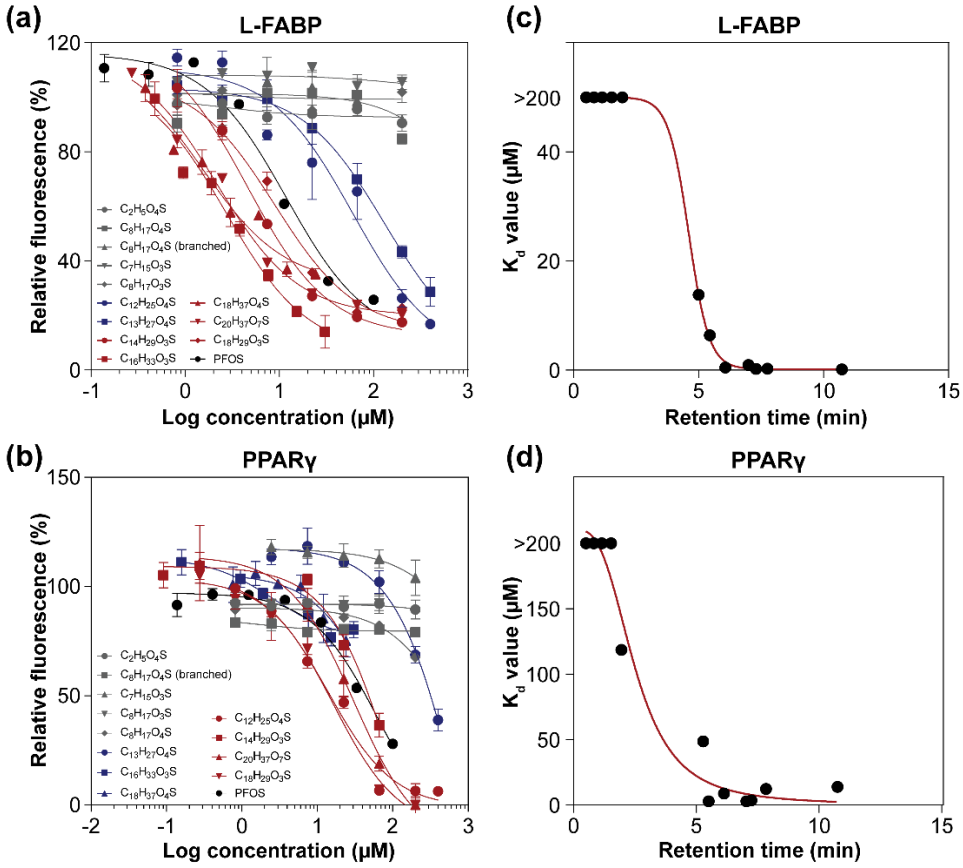

Figure 2. Binding of hydrocarbon surfactants to **(a)** L-FABP or **(b)** PPARγ LBD. The binding activity was determined by a fluorescence displacement assay. $N = 3$. Grey indicates inactive compounds while blue and red indicate medium- and high-affinity compounds. All data was normalized to solvent control. The relationships between K_d values of hydrocarbon surfactants binding to **(c)** L-FABP or **(d)** PPARγ LBD and their retention times on a C18 column were shown. A sigmoidal model was used to correlate K_d values and RTs. The K_d values for inactive surfactants were set at 200 μM for curve fitting.

We then tested if the L-FABP ligands could bind to PPARγ by using the same fluorescence displacement assay. As expected, hydrocarbon surfactants also showed binding to the PPARγ protein, albeit with binding affinities 5-20 times weaker than those to L-FABP (Figure 2b and Table S2). This confirmed our aforementioned hypothesis that medium to low-affinity ligands were not detected by the APNA method when PPARγ LBD was used as the protein bait but were captured when L-FABP was used. Similar to L-FABP, the retention times of hydrocarbon surfactants could roughly predict their binding affinities to PPARγ LBD (Figure 2d). To further investigate if hydrocarbon surfactants could modulate the transcriptional activities of PPARγ, a luciferase reporter cell assay which has been validated by using rosiglitazone as the positive control (Figure S8) was employed. We selected four representative hydrocarbon surfactants with stronger binding affinity from each surfactant class for reporter cell testing, including dodecyl benzenesulfonate, dioctyl sulfosuccinate, tetradecane sulfonate, and tridecyl sulfate. As shown in Figure 3, the dodecyl benzenesulfonate was the most potent PPARγ agonist (active at $\geq$ 12.5 μM), followed by dioctyl sulfosuccinate (active at $\geq$ 50 μM), tetradecane sulfonate (active at $\geq$ 58.6 μM), and tridecyl sulfate (active at $\geq$200 μM). The ranked order of PPARγ activity was consistent between the recombinant protein and reporter cells: dodecyl benzenesulfonate > dioctyl sulfosuccinate $\geq$ tetradecane sulfonate > tridecyl sulfate. Together, the results from the protein binding and reporter cell assays confirmed the bioactivities of hydrocarbon surfactants towards PPARγ. These results verified our hypothesis and further supported the use of His-tagged L-FABP as a "two birds with one stone" strategy for the isolation of ligands for both L-FABP and PPARγ at the same time. However, one limitation of the strategy could be that it may overlook certain ligands for PPARγ

that do not bind to L-FABP. Moreover, the ligands identified by L-FABP need to undergo an independent verification of their binding activities to PPARγ using another bioassay. In this study, the binding affinities and agonist activities of the identified hydrocarbon surfactants towards PPARγ were confirmed by two well-established bioassays, *i.e.,* the fluorescence displacement assay and the cell reporter assay, which strengthened our results. The PPARγ activity has been reported for a limited number of hydrocarbon surfactants,[54, 55] further supporting the APNA results in the current study. Although the PPARγ activity of hydrocarbon surfactants was not surprising given their structural similarity to fatty acids, this was the first time that they have been reported as the predominant synthetic ligands of PPARγ in the environment (as shown below).

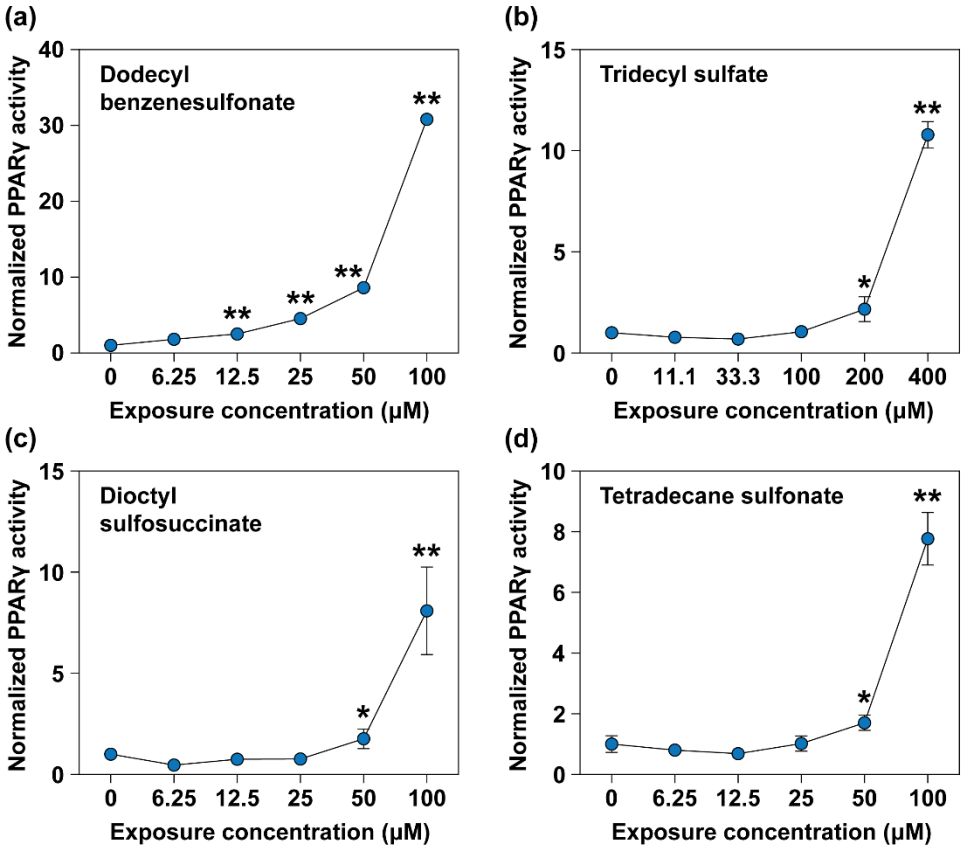

Figure 3. Activation of PPARγ transcriptional activity by hydrocarbon surfactants. The dose-

response relationship of PPARγ activation by four hydrocarbon surfactants including **(a)** dodecyl benzenesulfonate, **(b)** tridecyl sulfate, **(c)** dioctyl sulfosuccinate, and **(d)** tetradecane sulfonate were determined by the luciferase reporter cell. * $p < 0.05$; ** $p < 0.01$ ($N = 3$). Because of the cytotoxicity observed for dodecyl benzenesulfonate and dioctyl sulfosuccinate at high concentrations (>100 μM) and poor solubility of tetradecane sulfonate, the tested concentrations were different for the four hydrocarbon surfactants. Specifically, dodecyl benzenesulfonate was tested at 100, 50, 25, 12.5, and 6.25 μM; tridecyl sulfate was tested at 400, 200, 100, 33.3, and 11.1 μM; dioctyl sulfosuccinate was tested at 100, 50, 16.7, 5.6, and 1.9 μM; tetradecane sulfonate was tested at 117.2, 58.6, 16.7, 5.6, and 1.9 μM.

Quantification of Hydrocarbon Surfactants in Indoor Dust and Sewage Sludge. Despite the high production volume of hydrocarbon surfactants, limited information is available on their environmental occurrence. This is likely due to the fact that they are generally considered 'safe' chemicals. In order to quantify the concentrations of hydrocarbon surfactants in environmental samples, we collected additional 10 indoor dust samples and 9 sewage sludge samples in Canada, in addition to the previously mentioned indoor dust and sewage sludge samples. This resulted in a total of 11 indoor dust samples and 10 sewage sludge samples that underwent analysis. Interestingly, in addition to the aforementioned surfactants that we confirmed with commercially available standards, multiple homologues with a CH_2 unit difference were detected for each class of surfactants in both indoor dust (Figure 4) and sewage sludge (Figure S9) samples. The identities of these homologues were supported by their corresponding characteristic fragments, and their stepwise increasing retention times with carbon chain length, including 10 alkyl sulfates ($C_{10\text{-}18}$, and C_{20}, Figure 4a), 2 sulfosuccinate esters (C_{16} and C_{20}, Figure 4b), 4 alkyl sulfonates ($C_{14\text{-}17}$,

Figure 4c), and 6 alkylbenzene sulfonates ($C_{15\text{-}20}$, Figure 4d) in the indoor dust samples. In addition to the homologues, the chemical profile of hydrocarbon surfactants was further complicated by the presence of many isomers for each detected homologue, particularly for the sulfonates. Taken together, >50 hydrocarbon surfactants and isomers were detected in both indoor dust and sewage sludge. In this study, some ligands (*e.g.,* dodecyl sulfate) detected in indoor dust and sewage sludge were not isolated by the APNA method. This was because of their high instrumental background in the LC-MS, which further highlighted the difficulties in the identification of L-FABP/PPARγ ligands in complex environmental samples.

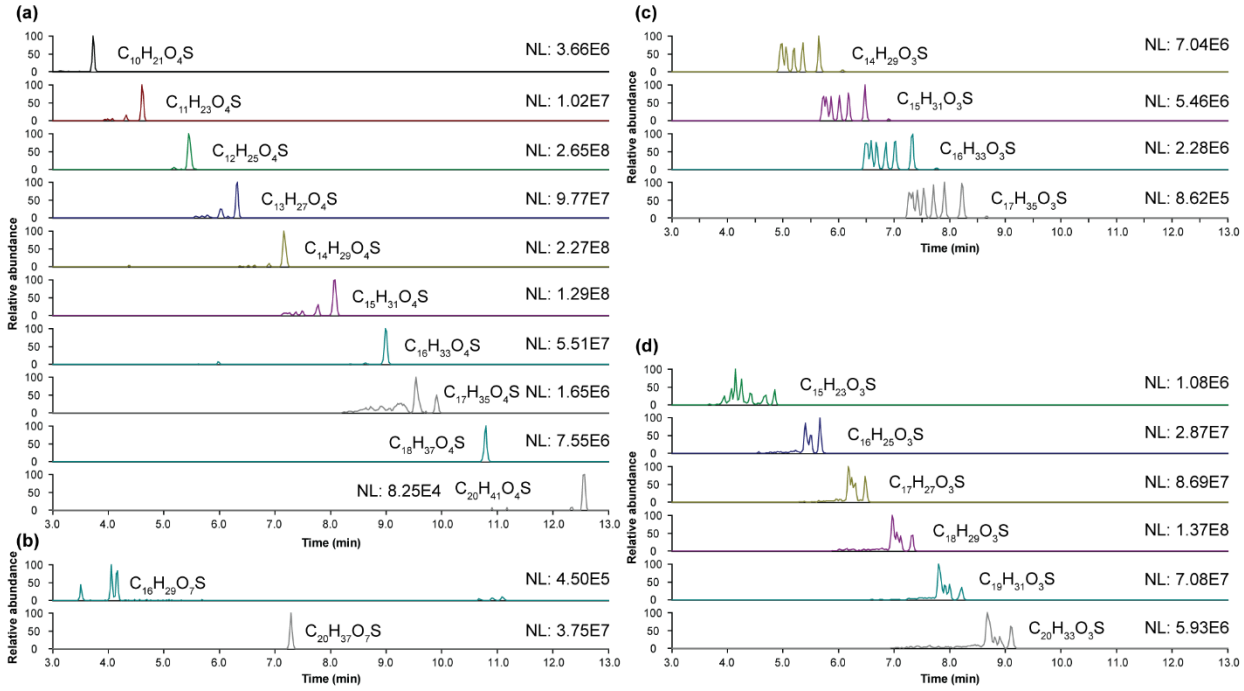

Figure 4. Representative chromatograms of homologues and isomers of hydrocarbon surfactants in an indoor dust sample. **(a)** alkyl sulfates; **(b)** sulfosuccinate esters; **(c)** alkyl sulfonates; **(d)** alkylbenzene sulfonates.

Due to the lack of authentic standards for most homologues and isomers, their concentrations were estimated by standards from the same class with similar retention times, and all isomer peaks were summed to quantify each homologue by assuming their similar instrumental responses. As shown in Figure 5, extremely high concentrations of alkylbenzene sulfonates (mean ± SD, 4660 ± 4459 μg/g) and alkyl sulfates (1454 ± 1414 μg/g) were detected in dust, followed by sulfosuccinate esters (49.9 ± 72.3 μg/g) and alkyl sulfonates (38.8 ± 76.5 μg/g). While high concentrations in sludge were also detected for alkylbenzene sulfonates (29238 ± 33134 μg/g dry weight, dw), the concentrations of alkyl sulfates (93.2 ± 146 μg/g dw) were comparable to that of alkyl sulfonates (32.3 ± 22.7 μg/g dw) and sulfosuccinate esters (25.5 ± 44.0 μg/g dw). The lower predominance of alkyl sulfates in sludge compared to dust may be attributed to the hydrolysis of sulfates during wastewater treatment.[56] Accordant with our results, previous studies have reported high concentrations of alkylbenzene sulfonates in floor dust (34 to 1500 μg/g dust)[57] and in treated sludge (up to 30200 μg/g dw)[58], but information was not available for other hydrocarbon surfactants. It should be noted that the measured environmental concentrations of hydrocarbon surfactants were greater than their concentrations required to activate PPARγ. Further studies are warranted to test their bioactivities using *in vivo* models. Moreover, epidemiological investigation and monitoring of these hydrocarbon surfactants in human blood are also urgently needed.

In addition to hydrocarbon surfactants, we quantified the concentrations of PFOS in the 11 indoor dust and 10 sewage sludge samples. As shown in Table S4, PFOS was detected in only 2 indoor dust samples at 0.214 and 1.65 ng/g, respectively. For sewage sludge, PFOS was detected in 8 out of 10 samples at 0.117 – 2.24 ng/g. In comparison to hydrocarbon surfactants, its

environmental concentration was about 6 orders of magnitude lower, further emphasizing the importance of hydrocarbon surfactants as primary synthetic L-FABP/PPARγ ligands.

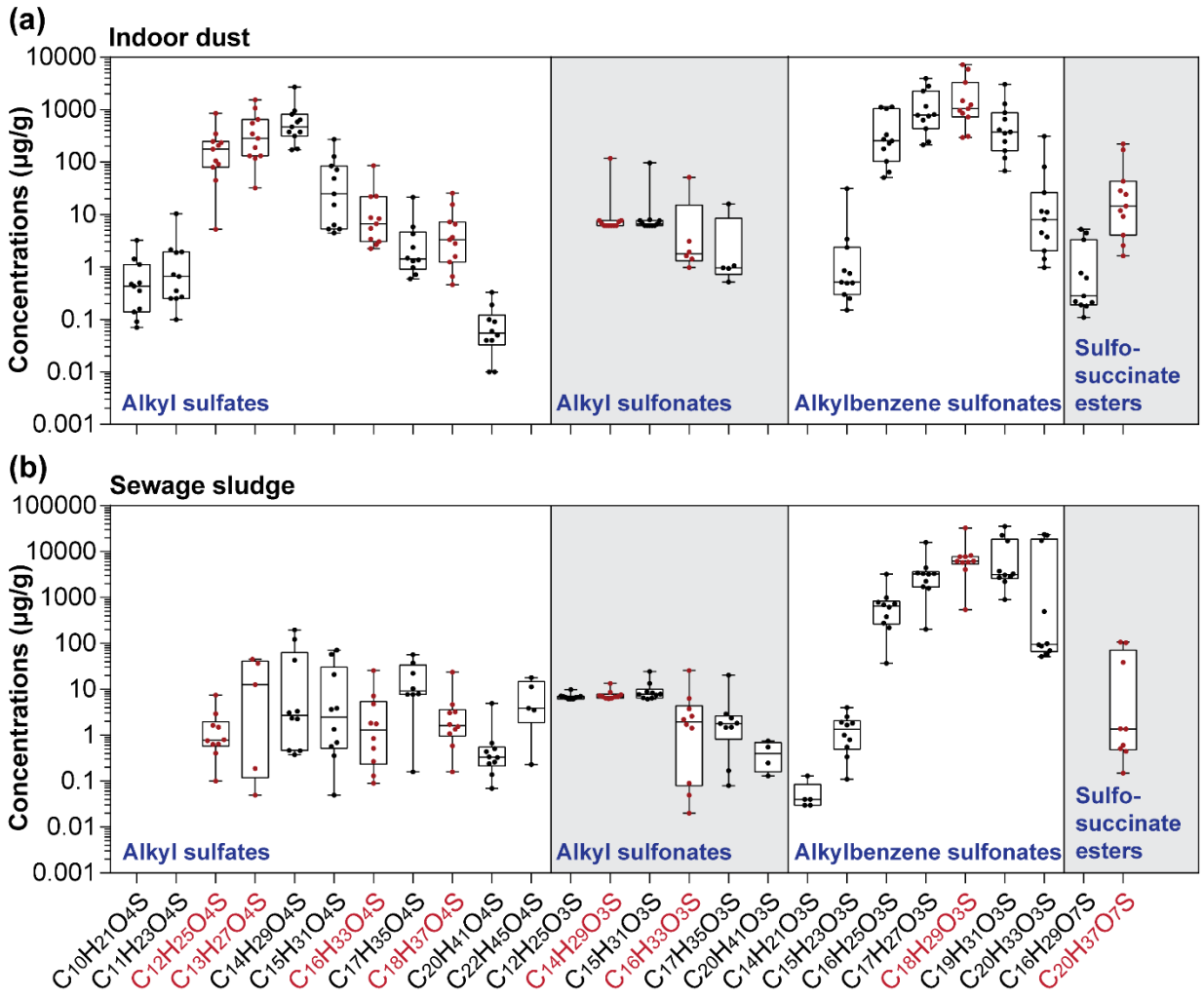

Figure 5. Estimated concentrations of hydrocarbon surfactants in **(a)** indoor dust and **(b)** sewage sludge samples. Hydrocarbon surfactants with available authentic standards were labeled in red.

Contributions of Hydrocarbon Surfactants to Total L-FABP/PPARγ Activities. To determine the contributions of hydrocarbon surfactants to the observed bioactivities, the total L-FABP and PPARγ LBD binding activities of 11 dust and 10 sludge extracts were tested by the fluorescence replacement assay. Of the 21 indoor dust/sewage sludge extracts, twenty demonstrated dose-

dependent decreases in the fluorescence intensity with strong L-FABP and PPARγ LBD binding activities (Figure 6). This result demonstrated the wide presence of L-FABP and PPARγ ligands in indoor dust and sewage sludge. A positive correlation was found between L-FABP and PPARγ LBD binding activities across these samples ($r = 0.90$, $p < 0.0001$, Figure S10), further supporting that L-FABP and PPARγ have common ligands.

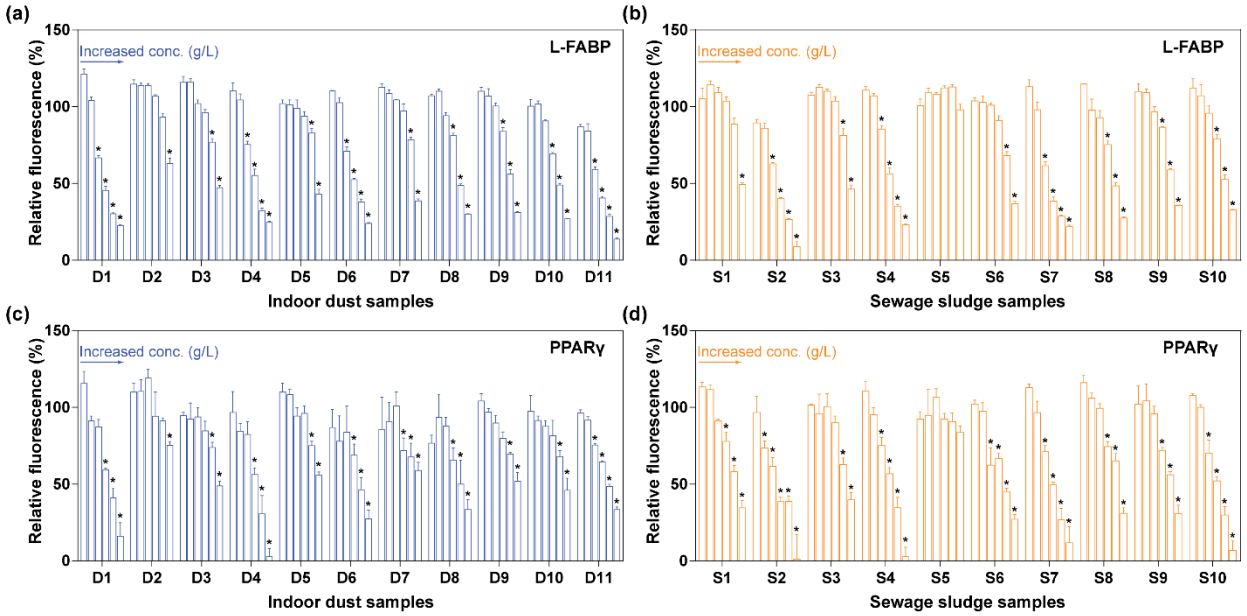

Figure 6. Fluorescence displacement assay to evaluate the binding activities of indoor dust and sewage sludge samples to L-FABP and PPARγ LBD proteins at different concentrations. For L-FABP binding activities **(a and b)**, hydrocarbon surfactants were tested at 0.002, 0.017, 0.063, 0.125, 0.25, and 1 g indoor dust or dry sewage sludge/L. For PPARγ binding activities **(c and d)**, hydrocarbon surfactants were tested at 0.017, 0.063, 0.125, 0.25, 0.5, and 1 g indoor dust or freeze-dried sewage sludge/L. * indicates $p < 0.05$ ($N = 3$). All data was normalized to solvent control. Moreover, an additional sample-only control was used to correct any potential signal caused by the color of the sample extracts. See SI for more details.

By using PFOS as the reference compound for L-FABP, the biological PFOS equivalents

423 (BEQ_{bio}) were calculated as 9.89 – 109 mg/g, and ND – 115 mg/g for indoor dust and sewage
424 sludge samples, respectively. The BEQ_{chem} values derived from chemical analysis were calculated
425 for the 9 fatty acids and 13 hydrocarbon surfactants for which L-FABP binding affinities were
426 determined by commercially available standards. This could lead to the underestimation of
427 BEQ_{chem} as other fatty acids and hydrocarbon surfactant homologues were not taken into
428 consideration due to their chemical standards being unavailable. Similarly, standards were not
429 available for most isomers of the same homologue (*e.g.,* $[C_{18}H_{29}O_3S]^-$), and thus we used isomer
430 mixtures or linear isomers with available standards for BEQ_{chem} calculation by assuming similar
431 bioactivities across isomers. This could also lead to the underestimation of BEQ_{chem}, as previous
432 studies reported the stronger L-FABP and PPARγ activities of branched fatty acids than linear
433 isomers.[59] Despite the two potential sources of underestimation, the 22 selected fatty acids and
434 surfactants could explain the majority of the L-FABP activities of the sewage sludge (57.7 ± 32.9%)
435 and a large portion of that of the indoor dust (35.4 ± 16.9%) (Figure 7a and Table S5). Fatty acids
436 were the predominant ligands of L-FABP, followed by alkylbenzene sulfonates, while the three
437 other classes of hydrocarbon surfactants contributed minorly (<5%) to the total activities (Figure
438 7c).

439 Similarly, by using rosiglitazone as the reference compound, the BEQ_{bio} equivalents of PPARγ
440 were calculated as 0.241 – 0.955 mg/g for the indoor dust samples and 0.338 – 1.35 mg/g for the
441 sewage sludge samples. By comparing the BEQ_{bio} and BEQ_{chem} values (Figure 7b and Table S6),
442 the 22 fatty acids and hydrocarbon surfactants with commercially available standards could
443 explain 36.5 ± 15.4% and 66.0 ± 27.1% of PPARγ activities in dust and sludge, respectively.

Different from L-FABP, for which fatty acids were the predominant ligands, alkylbenzene sulfates contributed the highest PPARγ activities among all the tested chemicals (Figure 7d). Notably, when only considering the PPARγ activities induced by synthetic chemicals (*i.e.,* by removing the contributions of fatty acids), the hydrocarbon surfactants alone can explain 61.2 ± 21.7% of PPARγ activities in sewage sludge samples, leaving only ~39% of effects unexplained, which further demonstrated that hydrocarbon surfactants are predominant synthetic PPARγ ligands in sewage sludge (Table S7). While the contribution of hydrocarbon surfactants to PPARγ in indoor dust was at 29.6 ± 16.7%, their contributions were underestimated as authentic standards are not available for most hydrocarbon surfactants. These results are highly intriguing, as only up to 4.2% and 9.3% of the activity could previously be explained by known synthetic chemicals in river water[19] and wastewater[20] samples, respectively. Because the actual contributions of hydrocarbon surfactants were underestimated due to the lack of authentic standards, we concluded that hydrocarbon surfactants should be the predominant synthetic ligands for both L-FABP and PPARγ in indoor dust and sewage sludge. With that being said, considering that only samples collected from several locations in Canada were tested in this study, a more diverse set of samples from other locations across the world are warranted to confirm this conclusion.

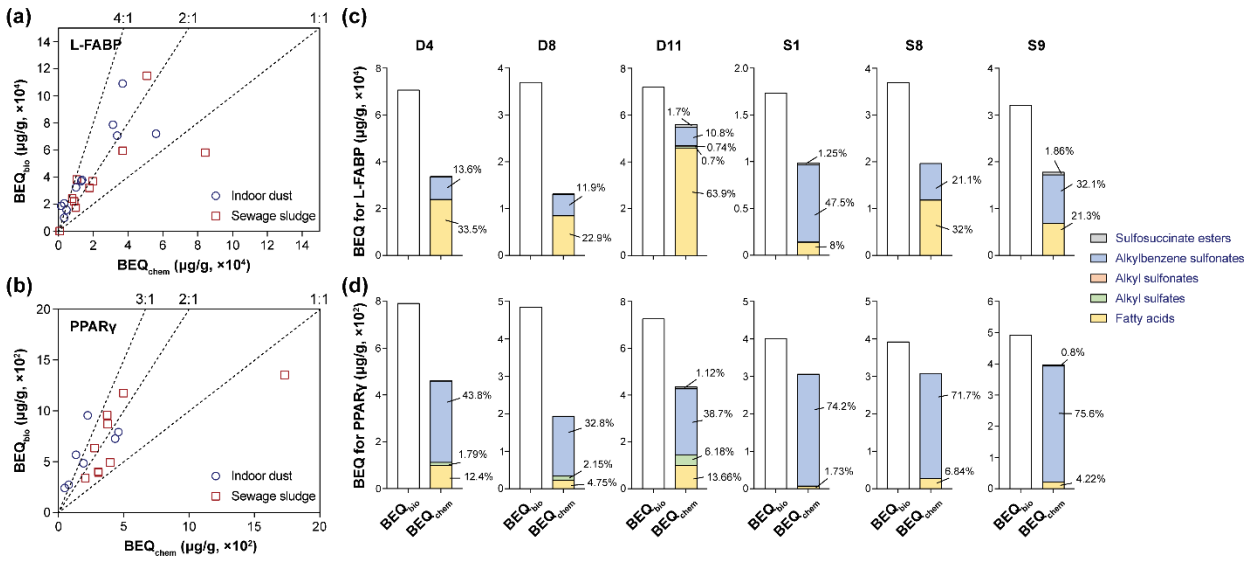

Figure 7. Comparison of biological equivalent concentration from bioanalysis (BEQ_{bio}) and chemical analysis (BEQ_{chem}) for **(a)** L-FABP **(b)** and PPARγ LBD binding activity. Blue circle and red square represent indoor dust and sewage sludge samples, respectively. Contributions of hydrocarbon surfactants to the total **(c)** L-FABP **(d)** or PPARγ LBD binding activities in representative indoor dust or sewage sludge samples were shown as bar plots. D represents indoor dust samples while S represents sewage sludge samples.

Development of a Free-Access Database for Unidentified Ligands. While a total of 83 and 6 ligand features were detected by the APNA method, only 13 L-FABP ligands (14.6%) were identified by nontargeted analysis, and none of the 6 PPARγ ligands could be identified. This demonstrated that the majority of L-FABP and PPARγ ligands were beyond the current chemical databases. For instance, transformation products of synthetic compounds (*e.g.,* phthalates[13]) may be potent PPARγ ligands, but they were outside the scope of the current chemical databases. The ligands that remain unidentified in this study can contribute to the unexplained L-FABP and PPARγ activities observed, making their identification crucial for comprehensively disclosing

environmental ligands of L-FABP and PPARγ. However, the identification of the other 70 L-FABP ligands and 6 PPARγ ligands would require *de novo* nontargeted analysis, which demands a significant amount of work that could not be achieved by any single research group alone. Fortunately, the APNA method could predict compound formulas, retention times, MS^1 spectra, and MS^2 spectra for all pulled-out ligands, which provides an excellent opportunity for the nontargeted analysis community to annotate the structures of these ligands. To achieve this, we have built an open-access "**e**nvironmental **C**hemical-**P**rotein **I**nteraction **N**etwork (eCPIN)" database and deposited information on all of the pulled-out ligands from this study (https://penggroup.shinyapps.io/ecpin/). Moreover, although the identities of most of these ligand features are currently unknown, they are physically linked to a protein target and are thus intrinsically interesting and functionally interpretable. The development of the eCPIN database could ultimately accelerate the distribution and utility of this ligand dataset within the nontargeted analysis and toxicology communities.

Implications. While extensive efforts have been invested over the past several decades to identify PPARγ ligands[19, 20, 60], our study represents the first identification of the major synthetic ligands of PPARγ in the environment. This was achieved by employing the APNA approach, which is fundamentally distinct from conventional EDA and high-throughput screening methods. The expansion of the APNA method to a broader set of human proteins is of great interest. One possible drawback of the current study is that xenobiotic metabolism and bioaccumulation in whole organisms was not taken into consideration. Future studies are warranted to apply the APNA method to human samples to identify physiologically relevant ligands. Another limitation is that

498 authentic standards were not available for most isomers and homologues, which could potentially
499 lead to inaccurate concentration estimates, and an underestimation of their contributions towards
500 L-FABP/PPARγ activities.

501 The identification of hydrocarbon surfactants as the predominant synthetic ligands of PPARγ
502 is significant. Compared to the widely studied PPARγ ligands including phthalate monoesters[13]
503 and PFAS,[61] which have been reported to have postive associations with obesity in human
504 populations[62, 63], hydrocarbon surfactants were detected in sludge at 2-6 orders of magnitude
505 higher concentrations and exhibited comparable or even more potent PPARγ activities. Although
506 hydrocarbon surfactants have been considered safe due to their weak acute toxicities, information
507 on their chronic toxicities is surprisingly limited. Two previous studies have reported the *in vivo*
508 obesity effects of dioctyl sulfosuccinate in mice,[55, 64] which is consistent with their potent PPARγ
509 activities that were detected in the current study. Given the strong persistence and bioaccumulation
510 of PFAS, finding 'safe' replacements is urgent, and nonfluorinated surfactants are possibly one of
511 the best options. However, recent studies have documented the stronger toxicity of nonfluorinated
512 surfactants than PFAS to zebrafish embryo[65] and terrestrial plant *Brassica rapa*[66]. Our study
513 further highlights the need for in-depth evaluation of the toxicity of nonfluorinated surfactants to
514 avoid making regrettable substitutions, as certain hydrocarbon surfactants exhibited stronger
515 PPARγ activity than PFOS. Notably, shorter-chain alkyl surfactants with a carbon chain length <
516 8 exhibited minor PPARγ activities and may be preferred as replacements for PFAS. Future studies
517 using *in vivo* animal models and epidemiological investigations are warranted to systematically
518 evaluate the chemical safety of hydrocarbon surfactants. Overall, our study revealed hydrocarbon

surfactants as the predominant synthetic ligands of L-FABP and PPARγ in the environment for the first time, highlighting the importance of re-evaluating their chemical safety.

Supporting Information Available

The supporting information provides text, tables, and figures addressing: (1) Supplementary materials and methods; (2) List of pulled-out LC-MS features; (3) The inhibitory concentrations ($IC_{50/10}$) and K_d values; (4) List of standards; (5) Detected concentrations of PFOS; (6) Contributions of hydrocarbon surfactants to L-FABP and PPARγ activities; (7) Benchmarking of the APNA method by known ligands; (8) APNA by using PPARγ as the protein bait; (9) Comparison of binding activities of 74 PFAS to L-FABP and PPARγ; (10) Benchmarking of the fluorescence displacement assay; (11) Binding activities of fatty acids to L-FABP and PPARγ LBD; (12) Binding mode of dodecyl benzenesulfonate to L-FABP; (13) Validation of the PPARγ reporter cell assay; (14) Homologous profiles of hydrocarbon surfactants in sewage sludge; (15) Correlations between L-FABP and PPARγ activities across 21 indoor dust and sewage sludge samples.

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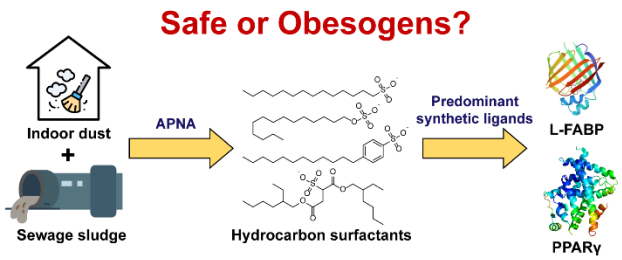
767 **TOC**



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