APPENDIX A. SUPPLEMENTARY MATERIAL

Acute ecotoxicity assessment of a covalent organic framework

Marisa P. Sárria[a], Ana Vieira[a,b], Ângela Lima[a,b], Soraia P. S. Fernandes[a], Ivo Lopes[b], Anabela Gonçalves[b], Andreia C. Gomes[b], Laura M. Salonen*[a] and Begoña Espiña*[a]

a. INL - International Iberian Nanotechnology Laboratory, Avenida Mestre José Veiga, 4715-330 Braga, Portugal. E-mail: laura.salonen@inl.int; begona.espina@inl.int

b. CBMA - Center of Molecular and Environmental Biology, Department of Biology, University of Minho, Campus of Gualtar, 4710-057 Braga, Portugal.

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1. Materials and methods

1.1. Reagents and chemicals

o-Tolidine was purchased from TCI Chemicals. Triformylphloroglucinol (Tp) was synthesized following a literature-known procedure.[1] TpBD-Me_2[2] was prepared as described in the literature (details below). All other reagents and solvents were purchased from Sigma-Aldrich and Fisher Scientific. Commercial reagents and solvents were used as received. A dispersion of TpBD-Me_2 at 1 mg mL^{-1} in ultrapure water was used in the experiments (produced by Milli-Q Advantage A10 system; resistivity = 18.2 MΩ cm^{-1}). Cell culture media and supplements were acquired from Sigma-Aldrich Co. (Madrid, Spain). Resazurin Cell Viability Assays were purchased from GIBCO™ Invitrogen (Barcelona, Spain). Fetal bovine serum (FBS) was obtained from HyClone GE Healthcare Europe GmbH (Munich, Germany). Other chemicals and reagents not listed were of highest purity grade commercially available.

1.2. Characterization

Small-angle X-ray scattering (SAXS) measurements were performed in an Anton Paar SAXSess mc2 instrument operating at 40 kV and 50 mA. Data were collected with an image plate detector. Samples were placed in a holder with Mylar windows for the measurement. Data are background corrected.

Nitrogen sorption measurements were carried out using a Quantachrome Autosorb IQ2 automated analyzer. Prior to the measurements, samples were outgassed. Powder samples were outgassed by heating to 120 ºC (heating rate: 5 ºC min^{-1}, dwelling time: 720 min). Surface areas were estimated by the multipoint Brunauer–Emmett–Teller (BET) method using ASiQwin(TM) software. Pore size distributions were estimated using quenched-solid density functional theory (QSDFT) adsorption branch for cylindrical pores (N_2 at 77 K on carbon).

Scanning electron microscopy (SEM) studies were performed using a field emission (FEG) Quanta 650 (FEI) electron microscope, operated at 5 kV and employing Everhart-Thornley detector for secondary electrons for imaging, with a working distance (WD) of ca. 10 mm. The samples for SEM were prepared by adhesion of the sample powder directly on a conductive double-sided copper tape attached to SEM pin stub.

Infrared (IR) spectra were recorded on a Bruker VERTEX 80v FT-IR spectrometer in transmission mode. IR data is reported in frequency of absorption (cm^{-1}).

To gather advanced insights on a putative size-dependent impact of chorionic pore channels on TpBD-Me_2 COF embryotoxic effects (as these can act as selective barrier to their inlet[3]), vacant eggs shells of hatched larvae exposed to 0, 0.001 and 1 μg mL^{-1} of TpBD-Me_2 COF (FET, OECD test guideline 236), were imaged using a FEI Nova 200 NanoSEM ultra-high resolution field-emission scanning electron microscope (FEG–SEM), integrated to an energy dispersive spectrometer (EDS) and

electron backscatter diffraction (EBSD) detector, EDX-Pegasus X4M. With respect to the X-ray lines, an accelerating voltage of 15 kV was used.

In brief, samples were pre-treated 1 h at room temperature, and 1 h on ice to the EM fixative: 2% (*v*/*v*) paraformaldehyde, 1% (*v*/*v*) glutaraldehyde, 2 mM $CaCl_2$; solvent: 0.15 M cacodylate buffer, pH 7.40. A series of five washes (3 min at a time) using ice-cold EM fixative solvent was next conducted. At first post-fixation step, samples were incubated 1 h on ice to the RedOs mixture: 3% (*v*/*v*) $K_4Fe(CN)_6$, ice-cold 0.3 M cacodylate buffer pH 7.40, and 4 mM $CaCl_2$, 1% (*v*/*v*) aqueous OsO_4. Afterwards, samples were washed (series of five times, 3 min at a time) using ultrapure water, and exposed to 1% (*v*/*v*) thiocarbohydrazide (TCH; solvent: ultrapure water) for 20 min, at room temperature. Samples were post-fixed a second time, incubating to 1% (*v*/*v*) Osmium tetroxide (solvent: ultrapure water), for 30 min at room temperature. Another series of five washes (3 min at a time) using ultrapure water was subsequently conducted. Prior exposure to Walton's lead aspartate for 20 min at 60 °C, samples were incubated to ultrapure water for 30 min, at the same temperature. At last, samples were washed (series of five times, 3 min at a time) using ultrapure water, and dehydrated (5 min at a time) in a crescent (*v*/*v*) grade (20%, 50%, 70%, 90%, 100%, 100%) of ethanol. Mixtures of ethanol and hexamethyldisilazane (HMDS) at *v*/*v* ratios (%) of 75:25, 50:50 and 25:75 were used to gradually replace ethanol. To ensure residual ethanol removal, samples were washed several times using 100% (*v*/*v*) HMDS, and left overnight at this. Samples were mounted on SEM-stubs using adhesive foil, and dried at room temperature. To increase conductivity, an Au/Pd coating was inductively sputtered onto samples, using a Cressington 208HR High Resolution Sputter Coater, coupled to a MTM-20 Cressington High Resolution Thickness Controller.

1.3. Cytotoxicity assessment

1.3.1. Cell culture. Human lung alveolar basal carcinoma (A549, ATCC®CCL-185™) and colorectal adenocarcinoma (Caco-2, ATCC®HTB-37™) epithelial cell lines, and Abelson murine leukemia virus-induced tumor (Raw 264.7, ATCC®TIB-71™) cells were procured from American Type Culture Collection (LGC Standards S.L.U., Spain). All assays with cells were performed within 10 consecutive passages. Cells lines were cultured every 3 to 4 days, and kept on a humidified 5% CO_2 incubator at 37 °C. Human epithelial cells and Raw 264.7 cell line were cultured using high glucose Dulbelco's Modified Eagle's Medium (DMEM), containing 0.584 g L^{-1} of l-glutamine and 1.500 g L^{-1} of $NaHCO_3$, supplemented to 10% (*v*/*v*) (A549, Raw 264.7) or 20% (*v*/*v*) (Caco-2) of heat-inactivated fetal bovine serum (FBS), and a mixture of 1% (*v*/*v*) penicillin streptomycin. Caco-2 cells culture medium was further supplemented to 1% (*v*/*v*) of Minimum Essential Medium (MEM) non-essential amino acids, and Raw 264.7 cells to 0.110 g L^{-1} of sodium pyruvate. As adherent cell lines, these were cultured in T75 flasks (TPP, Switzerland) as a sub-confluent monolayer.

1.3.2. Cell metabolic activity assay. Cells were seeded at an appropriate density according to the treated sterile culture plates used (Caco-2, $1x10^5$ cells mL^{-1} on 96-well plate; A549, $5x10^5$ cells mL^{-1} on 24-wells plate; Raw 264.7, $5x10^5$ cells mL^{-1} on 24-wells plate), and allowed to adhere overnight at 37 °C, under a humidified 5% CO_2 atmosphere. After this period, TpBD-Me_2 was diluted in respective cells culture media at the final nominal concentrations of 0.001, 0.01, 0.05, 0.1, 1, 10 and 100 μg L^{-1}. Adequate test controls were set: viability control (cells exposed to renewed culture media); and death control (cells exposed to 30% *v*/*v* of dimethyl sulfoxide, DMSO). Cells were incubated to the referred test conditions in quadruplicate per plate, for 24 h. Three independent experiments were conducted.

Two equivalent quantitative principle cell viability assays were considered to measure TpBD-Me_2 cytotoxic effects: PrestoBlue (PB), a resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) composed reagent; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), a tetrazolium salt. At the reducing microenvironment of metabolically active cells, these specific substrates are enzymatically converted to colored products, and detected thereafter using spectrophotometric methods6. Such reagents yield low background fluorescence (PB) or absorbance (MTT) in the absence of cells. Albeit, "cell free" controls of these were considered to certify for non-specific interactions and eventual interference of the dark orange suspension of TpBD-Me_2 at the wavelengths correspondent to the reagents readings. A blank control of specific "cell culture media" to detect "cell free" background was also tested, since these contained pH indicator phenol red.

At the end of the exposure period, PB was added to Caco-2 cells and allowed to react for 1 h at 37 °C, under a humidified 5% CO_2 incubator. Fluorescence intensity was measured at λ_{ex} 560 nm and λ_{em} 590 nm, on a microplate reader Synergy H1, Biotek. Cellular metabolic activity is detected as nonfluorescent blue resazurin is reduced to the fluorescent pink resorufin: the higher the toxic effect, the lower the fluorescence intensity mesured.[3] In the case of A549 and Raw 264.7 cells, these were incubated to 0.5 mg mL^{-1} of MTT for 2 h at 37 °C, under a humidified 5% CO_2 incubator. Purple formazan crystals generated were next solubilized using a mixture of ethanol and DMSO (1:1, *v*/*v*). Absorbance was measured at λ 570 nm, on a microplate reader SpectraMax Plus 384, Molecular Devices. Total aqueous-soluble formazan product generated correlates to cellular metabolic activity, and therefore is directly proportional to the number of living cells.[4]

Methodological rationale note: taking into account that the referred cell viability assays present equivalent quantitative principles, as MTT requires cell lysis using organic solvents,[4] and given that Caco-2 cells are less tolerant (in general) to these, as compared to A549 and Raw 264.7 cell lines,[5] besides tend to agglomerate, forming cells clumps of difficult detachment that can lead to data variability and affect the spectrophotometric readings, PB was specific selected for cytotoxicity assessment of these cells.

2. Cytotoxicity of TpBD-Me_2

In order to investigate the cytotoxic effects of TpBD-Me_2, considering the most probable routes of human environmental exposure, two *Homo sapiens* derived epithelial cell lines were specifically selected: Caco-2, colorectal adenocarcinoma used to resemble the human intestinal barrier and predict oral drug absorption, and A549, lung alveolar basal carcinoma used as model on inhalation toxicology testing. In addition, Raw 264.7, *Mus musculus* derived myeloid cell line, competent of replicating monocyte/macrophage-like endocytosis, was screened as a model to study a possible immune response elicited by exposure to COF.

One-factor ANOVA results on the metabolic activity of cells exposed to TpBD-Me_2 did not reveal any significant statistical effect in the different nominal concentrations tested up to 100 µg mL^{-1}, independently of the cellular model investigated (Figure S1, A: Caco-2 cell line; B: A549 cell line; C: Raw 264.7 cell line).

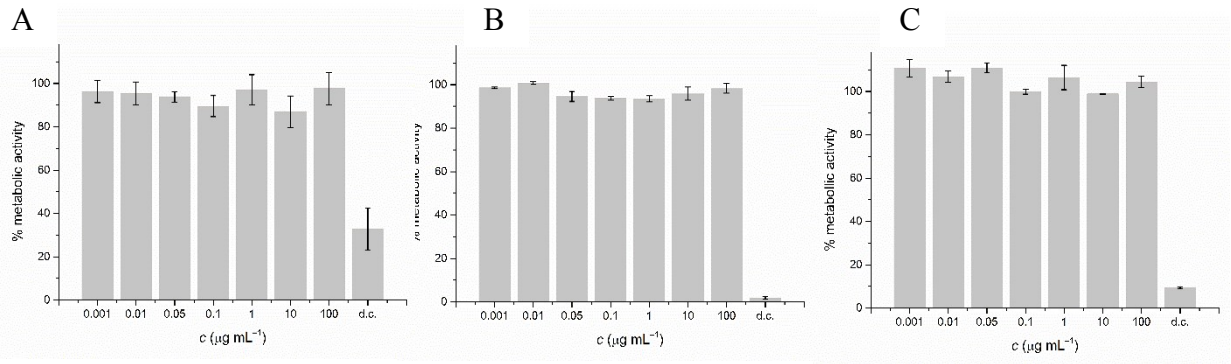


Figure S1: Effects on metabolic activity of a) Caco 2, b) A549, and c) Raw 264.7 cellular models representative of the most likely routes of human environmental exposure to TpBD-Me2. Results are mean ± S.E.M. of three independent experiments with four replicates each; d.c. = death control.

3. Synthetic procedures and characterization

3.1. Synthesis of TpBD-Me_2

TpBD-Me_2 was prepared as reported in the literature.[2] Briefly, Tp (32 mg, 0.15 mmol, 1 eq.) and *o*-tolidine (50 mg, 0.23 mmol, 1.5 eq) were placed in a 10 mL ampoule (Wheaton, pre-scored, borosilicate, 19 x 107 mm), and mesitylene (0.75 mL) and 1,4-dioxane (0.75 mL) were added. Then, aq. 3 M acetic acid was added (0.25 mL) and the mixture was sonicated to obtain a homogeneous suspension. The mixture was flash frozen at 77 K, the ampoule was sealed, and heated at 120 °C for 3 days. The precipitate was collected by centrifugation and washed with acetone, tetrahydrofuran, and dichloromethane to give TpBD-Me_2 as dark orange solid.

FT-IR (ATR; cm^{-1}): 1620 (s), 1578 (s), 1549 (m), 1497 (w), 1446 (s), 1384 (w), 1297 (s), 1279 (s), 1260 (s), 1237 (m), 1193 (w), 1119 (w), 1090 (w), 1065 (w), 1031 (w), 995 (w), 875 (w), 806 (w).

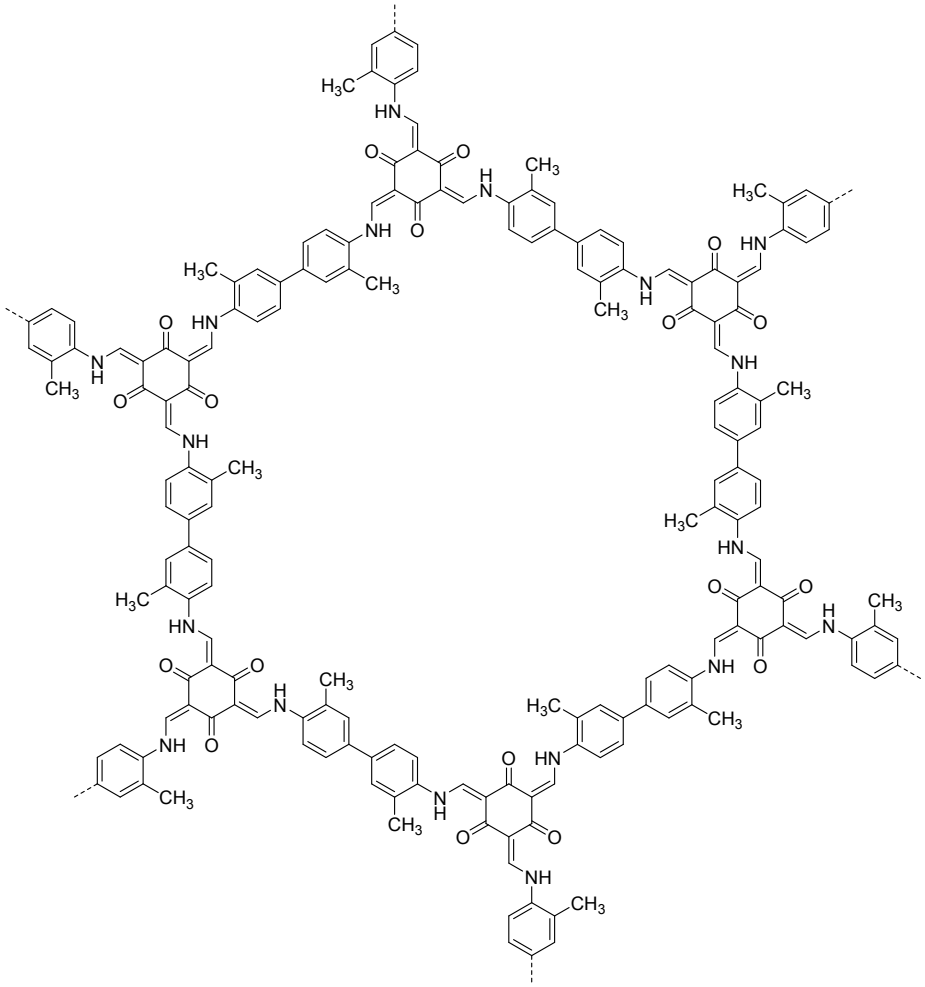


Figure S2. Chemical structure of TpBD-Me_2.

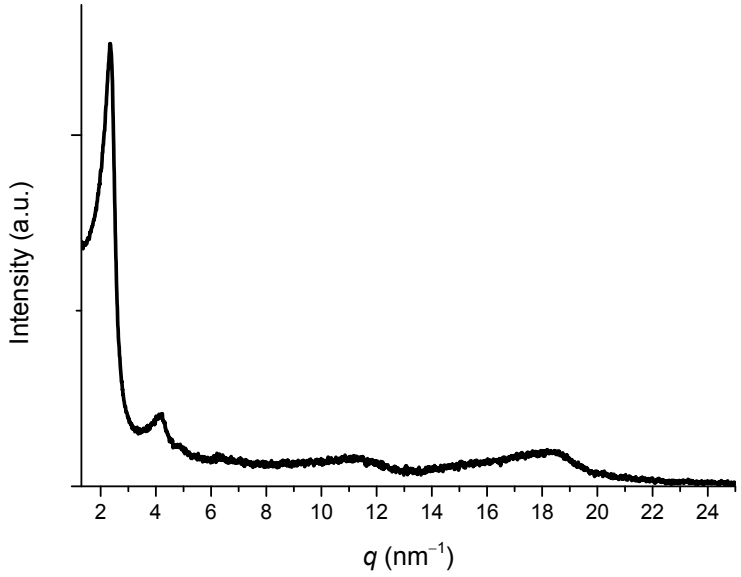


Figure S3. SAXS pattern of TpBD-Me_2.

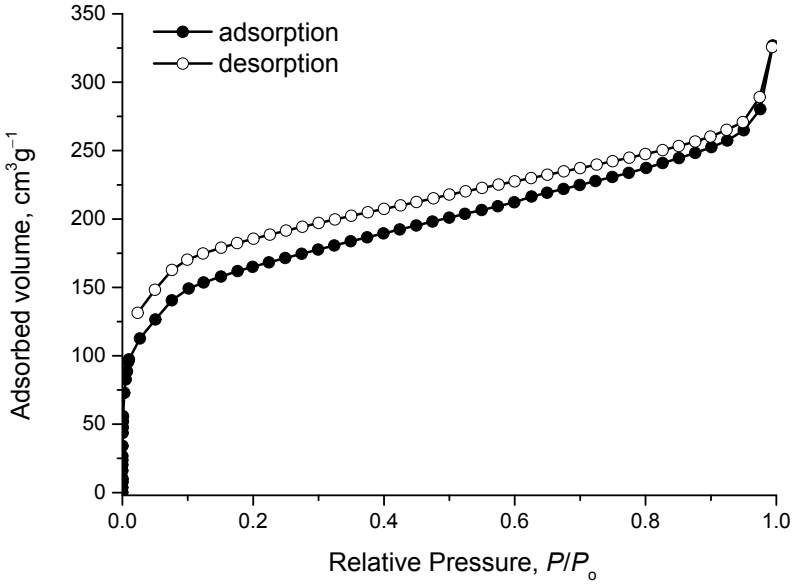


Figure S4. Nitrogen adsorption (full spheres) and desorption (hollow spheres) isotherm profiles of TpBD-Me_2 measured at 77 K.

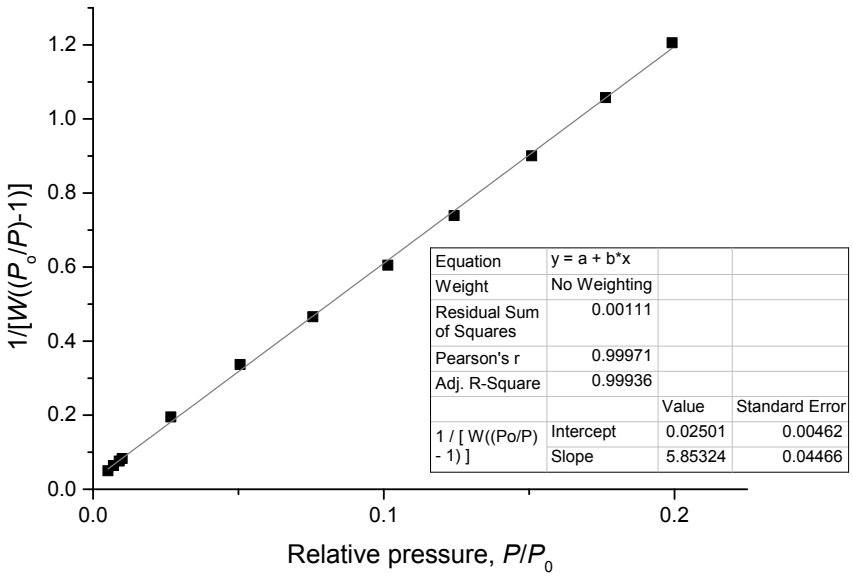


Figure S5. Multi-point BET plot and linear fit.

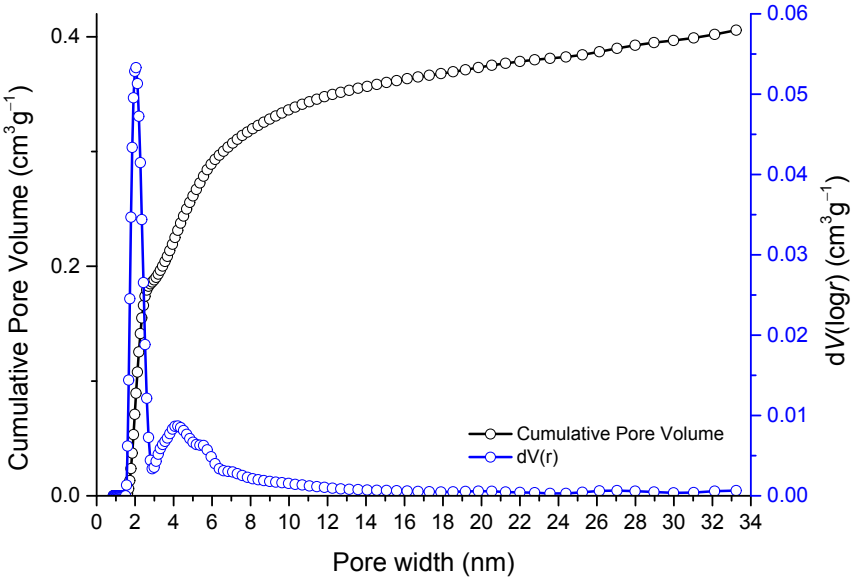


Figure S6. Pore size distribution (blue) and cumulative pore volume (black) profiles.

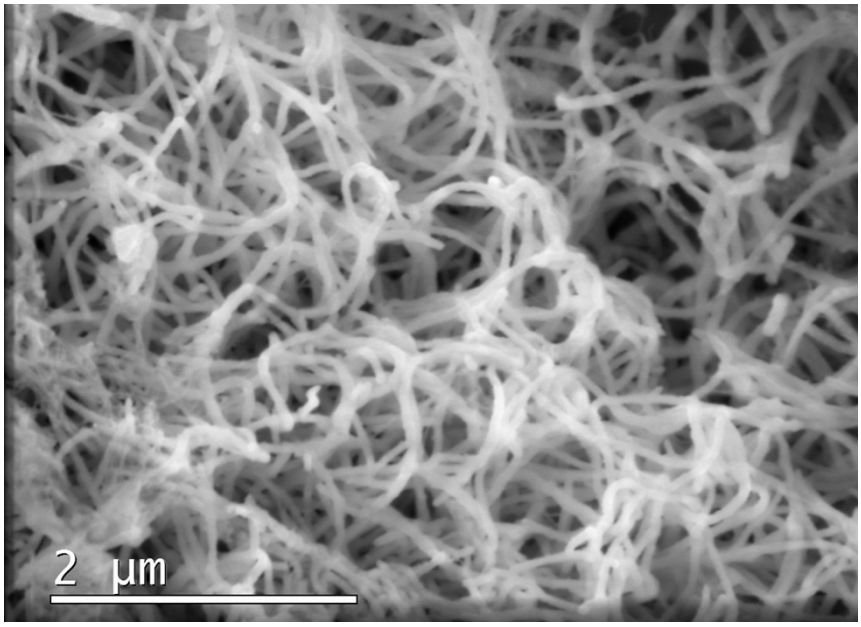


Figure S7. SEM image of TpBD-Me_2.

3.2. Stability studies of TpBD-Me_2 on the zebrafish embryo toxicity assays media

In order to evaluate the stability of COF material on the zebrafish embryo toxicity assay media, TpBD-Me_2 (10 mg) was suspended in culture media (10 mL) and incubated at 27 ºC for 7 days. Then, the sample was filtered (0.2 µm PES syringe filter, Fisher Scientific) and the aqueous medium was concentrated using a modular centrifugal evaporator SPEEDVAC SPD121P. The concentrated sample was resuspended in acetonitrile (1 mL) and its content was analyzed measuring the fluorescence intensity (excitation wavelength 320 nm, emission wavelength 500 nm for Tp, and 450 nm for *o*-tolidine) in a BioTek Synergy H1 microplate reader.

Calibration curves were prepared using acetonitrile as solvent in final concentrations of 0.5, 0.35, 0.25, 0.1, 0.075, 0.05, 0.025, and 0.01 mg mL^{-1}, and analyzed as described above. Then, using the software Origin9®, the known concentrations were plotted against their corresponding fluorescence, and a non-linear pharmacology dose-response fitting was applied. No significant amount of the building blocks (<0.1% from initial amount in the material) was found in the freshwater, indicating that there is no significant degradation or release of the COF building blocks into the water.

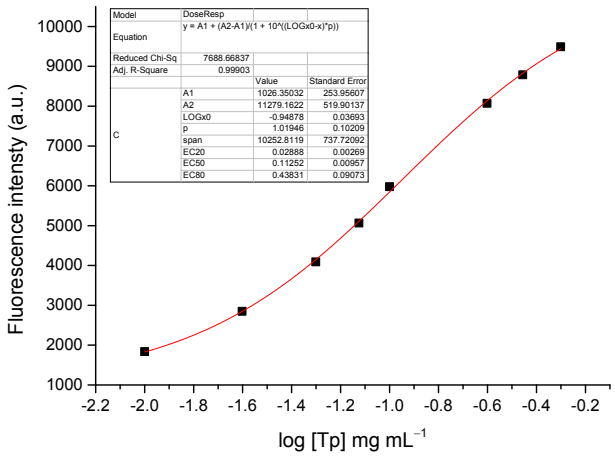


Figure S8. Tp calibration curve in acetonitrile.

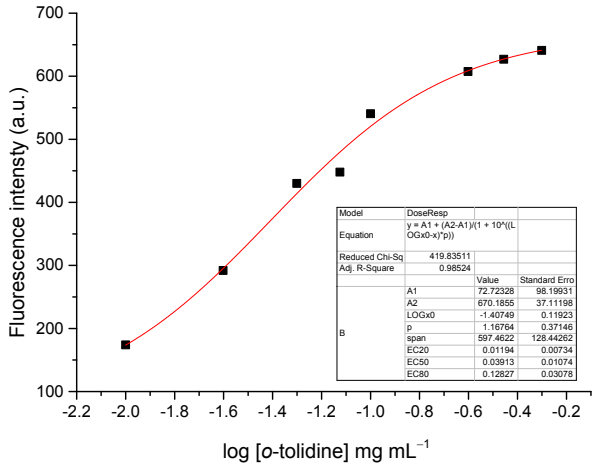
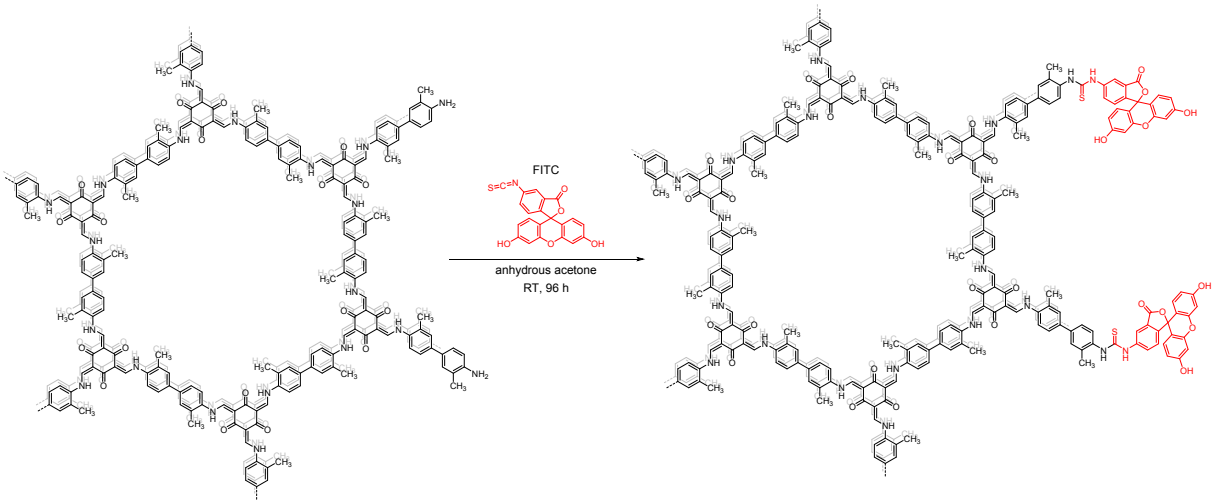


Figure S9. *o*-Tolidine calibration curve in acetonitrile.

3.3. Post-Synthetic modification of TpBD-Me$_2$



Scheme S1. Post-synthetic modification of TpBD-Me$_2$

3.4. Characterization of FTpBD-Me$_2$

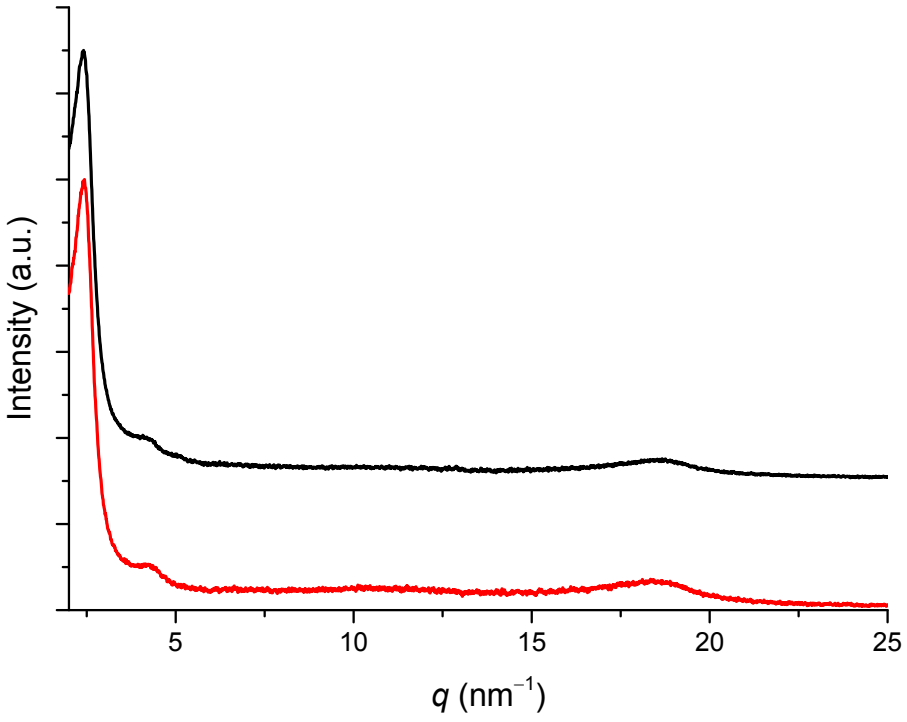


Figure S10. SAXS pattern of TpBD-Me$_2$ as synthesized (black) and after post-synthetic modification with FITC, FTpBD-Me$_2$ (red).

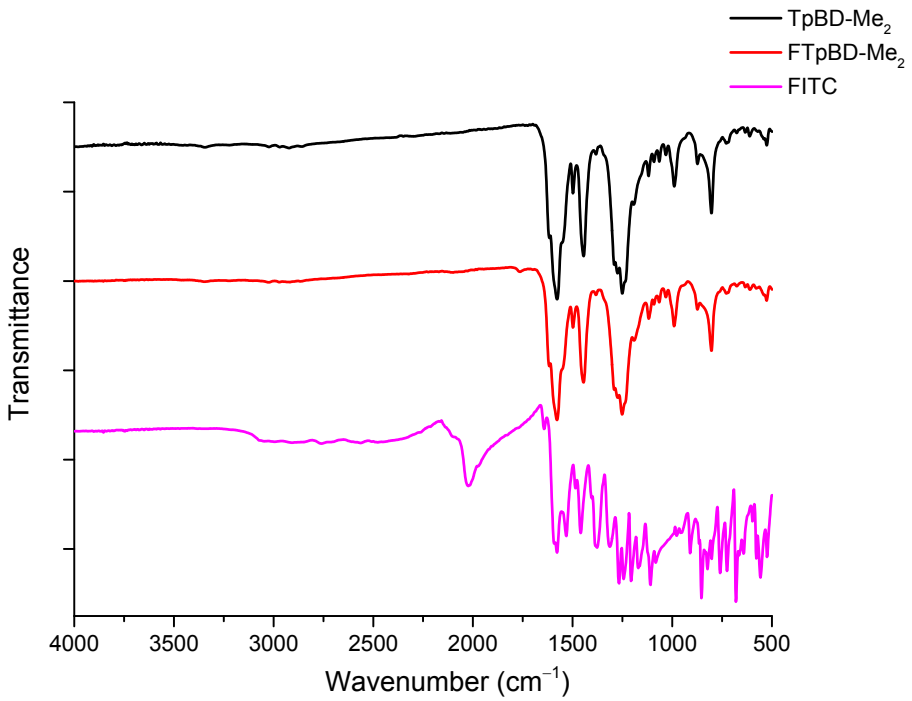


Figure S11. FT-IR spectra of TpBD-Me_2 as synthesized (black), after post-synthetic modification with FITC, FTpBD-Me_2 (red), and FITC (magenta).

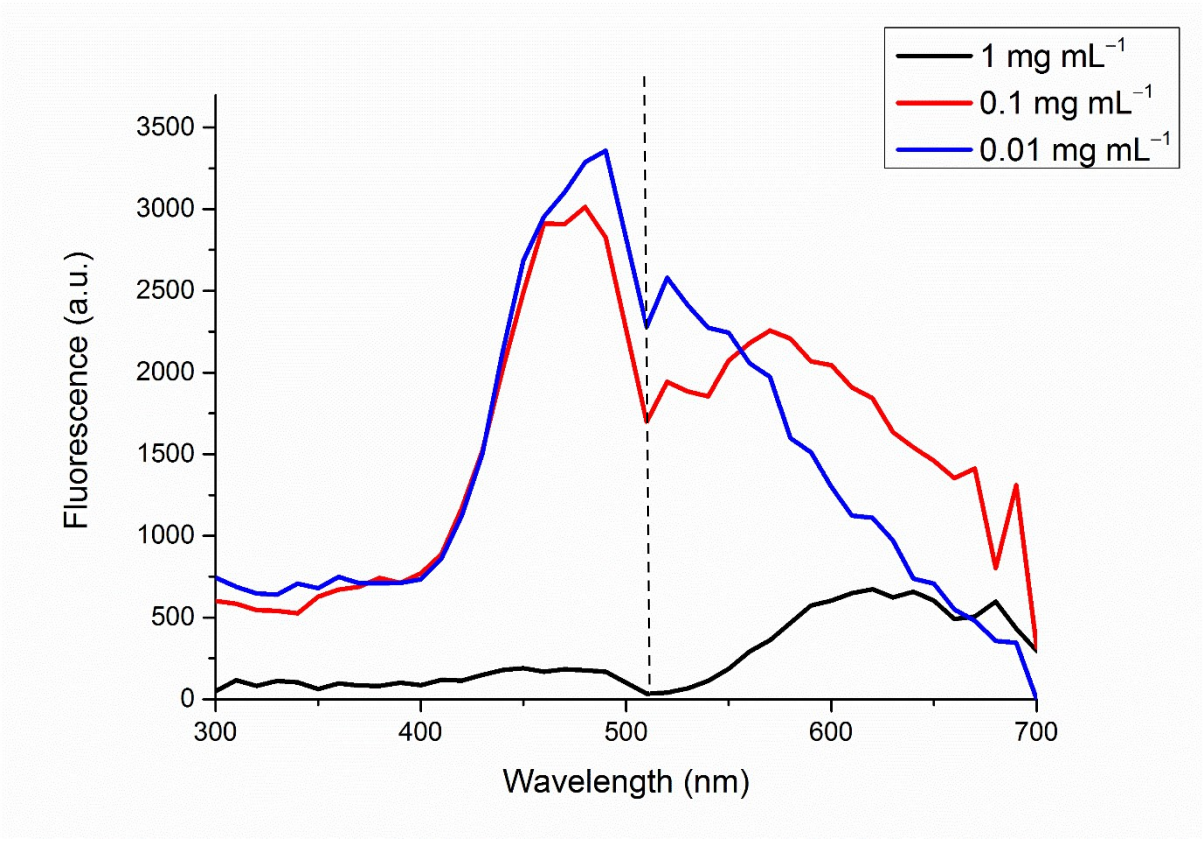


Figure S12. Excitation (wavelengths on the left of the dashed line) and emission (wavelengths on the right of the dashed line) spectra of FTpBD-Me_2 collected with an excitation wavelength of 490 nm for emission spectrum and at fixed emission of 525 nm for excitation spectrum, corresponding to the maximum peaks for FITC.

4. Statistical analysis

Table S1: Statistics of (standard) FET upon exposure to 0, 0.001, 0.01, 0.05, 0.1 and 1 μg mL^{-1} of TpBD-Me_2 COF, for 80 h_{pf}. Effects on characteristic embryonic developmental stage-correspondent events are listed, and referred to "non-significant" (-) or "significant" (+) statistical differences among groups on tested independent variables.

	h_{pf}	developmental age	independent variables	statistical analysis	TpBD-Me_2 COF	Effect
Morphometric Traits	8	phylotypic period	*epipolic arc*	One-Factor ANOVA	$F(5,102)=1.693$; $P=0.143$	-
	8	phylotypic period	*yolk volume*	ANCOVA	$F(5,102)=0.951$; $P=0.452$	-
	32	phylotypic period	*head-trunk angle*	One-Factor ANOVA	$F(5,87)=1.172$; $P=0.330$	-
	56	eleuthero period	*pupil surface*	ANCOVA	$F(5,69)=1.951$, $P=0.097$	-
Neuro-Mediated Traits	32	phylotypic period	*spontaneous movements*	Chi-Square test	$\chi^2=30.137$; DF=5; $P<0.05$	+
	32	phylotypic period	*heart rate*	One-Factor ANOVA	$F(5,52)=2.282$; $P=0.060$	-
	56	eleuthero period	*heart rate*	One-Factor ANOVA	$F(5,53)=3.941$; $P<0.05$	+
	56	eleuthero period	*hatching rate*	Chi-Square test	$\chi^2=0.000$; DF=5; $P=1.000$	-
	80	eleuthero period	*free-swimming*	One-Factor ANOVA	$F(5,18)=1.554$; $P=0.223$	-
	80	eleuthero period	*cumulative survival*	Chi-Square test	$\chi^2=5.764$; DF=5; $P=0.330$	-

5. References

1 J. H. Chong, M. Sauer, B. O. Patrick, M. J. MacLachlan, *Org. Lett.,* 2003, **5**, 3823–3826.

2 S. Chandra, S. Kandambeth, B. P. Biswal, B. Lukose, S. M. Kunjir, M. Chaudhary, R. Babarao, T. Heine and R. Banerjee, *J. Am. Chem. Soc.*, 2013, **135**, 17853–17861.

3 L. A. Fuiman, *Special Considerations of Fish Eggs and Larval. In: Fuiman L.A., Werner R.G. (Ed), Fishery Science: the Unique Contributions of Early Life Stage.*, Oxford: Blacwell Science, 2002.

4 H. H. K. Präbst, H. Engelhardt, S. Ringgeler, *Basic Colorimetric Proliferation Assays: MTT, WST, and Resazurin*, 2017, vol. 1601.

5 L. Jamalzadeh, H. Ghafoori, R. Sariri, H. Rabuti, J. Nasirzade, H. Hasani and M. R. Aghamaali, *Avicenna J Med Biochem.*, 2016, **4**, e33453.