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Aligning AI Toxicity Predictions with Wet-Lab Biology for PFOA Toxicity in SH-SY5Y Cells

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Abstract

Perfluorooctanoic acid (PFOA) is a highly persistent per- and polyfluoroalkyl substance (PFAS) widely detected in the environment and biological systems. Its resistance to degradation and bioaccumulative behavior make it a critical toxicological and public health concern. The present study investigates whether probability based artificial intelligence (AI) toxicity predictions align with experimental in vitro findings in human SH-SY5Y neuroblastoma cells. Cells were exposed to PFOA at concentrations ranging from 0 to 2000 µM for 24, 48, and 72 hours, and cell viability was determined using the MTT assay. The resulting IC_{50} values419.52 µM, 174.97 µM, and 104.64 µM, respectively demonstrated a clear time-dependent increase in apparent cytotoxic potency (~4.01-fold from 24 to 72 h). These empirical data were compared against AI-derived toxicity probabilities from two external platforms: ProTox and CompTox/invitrodb. Calibration between predicted probabilities and observed biological outcomes was assessed using the Brier score. ProTox showed good calibration (Brier = 0.102), whereas CompTox/invitrodb yielded poor alignment (Brier = 0.537), highlighting the importance of endpoint- and time-matched probabilities. The results emphasize that AI models lacking temporal or biological context may underestimate toxicity, particularly when effects manifest gradually over prolonged exposures. This study presents a reproducible, curve-free workflow for integrating AI predictions with time-resolved in vitro toxicity data, providing a framework to enhance biological realism in computational toxicology and guide future PFAS risk assessments.

Keywords: PFOA; SH-SY5Y; artificial intelligence; cytotoxicity.

1. Introduction

Perfluorooctanoic acid (PFOA; CAS 335-67-1) is a synthetic per- and polyfluoroalkyl substance (PFAS) characterised by a fully fluorinated eight-carbon chain ending in a carboxylic acid moiety. It has been used industrially as a surfactant in the manufacture of fluoropolymers, such as polytetrafluoroethylene (PTFE), and in various consumer- and industrial-products (e.g., non-stick cookware coatings, firefighting foams) .PFOA is highly persistent in the environment due to the strength of the C–F bonds and its resistance to biotic/abiotic degradation, which has led to its designation as a “forever chemical”. Moreover, PFOA is bio-accumulative in humans and wildlife. According to the U.S. Environmental Protection Agency (EPA), PFOA and related salts were the subject of a human health toxicity assessment [1]. PFOA has become pervasive in both the environment and human populations. Because of its persistence and bioaccumulative nature, exposure to PFOA is continuous throughout life, beginning as early as the prenatal stage and extending into postnatal development [2], [3]. Due to its widespread environmental and human exposure, PFOA affects multiple organ systems in terms of toxicology. Experimental data from animal studies demonstrate that PFOA is readily absorbed via oral and inhalation routes, while dermal uptake appears to be relatively limited [4]. In humans, the compound exhibits prolonged biological persistence, with an estimated elimination half-life of approximately 4.4 years in occupationally exposed individuals [6]. PFOA preferentially accumulates in metabolically active tissues—including the liver and kidneys—and is frequently identified in human serum samples [7]. Comprehensive meta-analyses have demonstrated a clear correlation between PFOA exposure and hepatic injury, evidenced by significant elevations in serum alanine aminotransferase (ALT) levels—up to 117% higher among exposed groups [8]. Experimental studies have revealed that exposure to PFOA leads to increased neonatal mortality, delayed eye opening, impaired growth, and disruptions in pubertal maturation in rodents [9]. Furthermore, it has been demonstrated that PFOA exposure is associated with both structural and functional disturbances in major immune organs, including the spleen, thymus, and bone marrow [10]..Additionally, in vivo studies have shown that PFOA possesses tumor-promoting potential, whereas limited human epidemiological findings suggest possible links with malignancies of the liver, kidney, and testes [5]

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Due to its environmental persistence and occurrence at extremely low concentrations—often at the parts-per-trillion level in water and parts-per-billion in serum—PFOA detection demands the use of highly sensitive analytical platforms such as LC-MS/MS or high-resolution mass spectrometry. Conventional monitoring programs, however, remain labor-intensive, costly, and technically demanding. Furthermore, many of the adverse health outcomes linked to PFOA arise only after prolonged, low-level exposure spanning months or even years. This latency renders traditional toxicological and epidemiological investigations time-consuming and resource-heavy, requiring extensive follow-up and large study populations. In environmental surveillance, the need to repeatedly analyze diverse matrices such as water, soil, air particulates, biota, and human serum across different locations further complicates assessment efforts. Consequently, there is an urgent demand for rapid, accurate, and cost-efficient testing strategies that can enhance hazard identification, exposure evaluation, and risk prioritization processes.

PFOA represents just one compound within a vast group of thousands of PFAS chemicals, making it challenging to generalize toxicological properties across the entire class. Although regulatory agencies are increasingly adopting collective, class-based evaluation strategies, most analytical and monitoring methods remain concentrated on well-characterized compounds such as PFOA and PFOS. The immense diversity of PFAS structures, combined with the complexities of mixture interactions and long-term, low-dose exposures, continues to hinder comprehensive toxicological and risk assessments [11]. Thus, PFOA is of considerable concern in environmental toxicology and public health.

In this study SHSY-5Y treatment with PFOA in (0,100,250,500,750,1000,1500 and 2000 uM concentrations) at 24, 48, and 72 hours. The dateset offers valuable temporal resolution that enables.This time-dependent approach strengthens the analysis, as modeling toxicity over multiple exposure durations can reveal mechanistic insights such as delayed cytotoxic effects or cellular adaptive responses. The SH-SY5Y cell culture method used for evaluating PFOA toxicity presents several advantages, disadvantages, and practical challenges. Among its strengths, this approach offers a controlled experimental environment, human-relevant neurotoxicological data, cost-effectiveness, and ethical benefits compared to animal testing. It also allows for quantitative assessment of dose- and time-dependent effects through LC_{50} determinations at multiple exposure durations.However, the method has limitations, including the absence of systemic physiological interactions, low metabolic capacity, and restricted representation of diverse neural cell types. These factors can lead to an incomplete understanding of in vivo toxicity mechanisms.In addition, the technique poses certain challenges such as maintaining culture consistency, ensuring PFOA solubility and stability, and translating in vitro results to real-world exposure scenarios. Moreover, integrating these data into computational or AI-based models requires meticulous standardization and comprehensive metadata documentation.

There has been a rapid growth recently in AI and in-silico toxicology tools such as QSAR models, fingerprint-based classifiers, and deep learning that generate screening-scale predictions. Platforms such as ProTox provide probability outputs, while CompTox/HTS aggregates high-throughput bioactivity summaries. Yet how these AI outputs should be aligned with laboratory biology remains underspecified. Most studies report accuracy or AUC but overlook two essentials: probability calibration (how close a model's predicted probability is to the true event rate) and the time dimension of exposure (e.g., 24/48/72 h). In practice, alignment is hard because details like the cell model, when the assay is read, which form of the chemical is tested (APFO vs. acid), and serum protein binding can nudge the numbers in different directions [12].

Here we aim to test, in a transparent and curve-free manner, whether AI/in-silico probabilities agree with the biological activity of PFOA measured by SH-SY5Y cell viability at 24, 48, and 72 hours. We convert the viability data into simple threshold based labels (e.g., Active50 = 1 when the 50% effect threshold is met at a given timepoint) and compare these labels to model probabilities p (0–1) using the Brier score to quantify calibration. When time-resolved probabilities are available, we additionally check monotonicity ($p72 \geq p48 \geq p24$) to assess time-consistency; if only a single global probability is available, we report this as a limitation and interpret results in light of known context factors. The workflow requires no curve fitting, is reproducible with companion spreadsheets, and is designed to provide an interpretable, time-aware bridge between AI predictions and wet-lab biology.

2. Materials and Methods

2.1. Chemicals and Materials

Perfluorooctanoic acid (PFOA; CAS No. 335-67-1) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and

penicillin-streptomycin were also purchased from Sigma-Aldrich. The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) used for the cell viability assay was obtained from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals and reagents were of analytical grade and used without further purification.

PFOA stock solutions (100 mM) were prepared in dimethyl sulfoxide (DMSO). To ensure complete solubilization, the stock solution was vortexed for 30 seconds and gently warmed to 37°C, followed by visual confirmation that no precipitate remained. Working solutions were prepared by serial dilution into culture medium, and all final mixtures were inspected to verify clarity and absence of undissolved material.

2.2. Cell Culture

Human neuroblastoma SH-SY5Y cells (ATCC® CRL-2266™) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were routinely sub-cultured every 2–3 days to maintain exponential growth.

2.3. Experimental Design

For cytotoxicity testing, cells were seeded in 96-well plates at a density of 1×10^4 cells/well and allowed to adhere overnight. Subsequently, cells were exposed to PFOA at concentrations of 0, 100, 250, 500, 750, 1000, 1500, and 2000 μM for 24, 48, and 72 hours. A 0.1% DMSO vehicle control was included in each experiment.Following exposure, cell viability was assessed using the MTT assay. After adding 10 μL of MTT reagent (5 mg/mL) to each well, the plates were incubated for 4 hours at 37 °C. The resulting formazan crystals were dissolved in 100 μL of DMSO, and absorbance was measured at 570 nm using a microplate reader (BioTek Epoch, USA). All experiments were performed in triplicate and repeated independently three times.

The percentage of cell viability was calculated relative to the vehicle control. IC_{50} values were determined using nonlinear regression analysis (log[inhibitor] vs. response – variable slope) in GraphPad Prism v9 (GraphPad Software, San Diego, CA, USA). Data were expressed as mean ± standard deviation (SD). Statistical significance between groups was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, with $p < 0.05$ considered statistically significant.

2.4. Data Analysis

The percentage of cell viability was calculated relative to the vehicle control. IC_{50} values were determined using nonlinear regression analysis (log[inhibitor] vs. response – variable slope) in GraphPad Prism v9 (GraphPad Software, San Diego, CA, USA). Data were expressed as mean ± standard deviation (SD). Statistical significance between groups was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, with $p < 0.05$ considered statistically significant.

2.5. Curve-Free AI–Biology Alignment

For each time point $t \in \{24, 48, 72\}$, we defined a binary label;

$$y_t = \begin{cases} if\ the\ 50\%\ effect\ threshold\ was\ reached\ in\ the\ tested\ range \\ otherwise \end{cases}$$

In this study, IC50 values were determined at all three timepoints as provided above. Thus, $y_{24}=y_{48}=y_{72}=1$. Two externally available sources were used to obtain probability-style AI/in-silico signals for PFOA:

1. ProTox: a single global toxicity probability $p \in [0,1]$.

2. CompTox/invitrodb (ToxCast cytotoxicity summary): a global HTS activity ratio computed as

$$p = \frac{nhit}{ntested},$$

using the cytotoxicity summary row for PFOA. If time resolved probabilities $(p_{24}, p_{48}, p_{72})$are available from any AI source, they are used directly; otherwise, a single global $p$is evaluated against all timepoints.

Calibration between AI probabilities and biological labels was quantified with the Brier score if there is a time resolved case with

$$Brier = \frac{1}{3} \sum_{t \in \{24,48,72\}} (pt - yt)^2$$

And if it is single probability case (no $p_t$available) with $y_{24} = y_{48} = y_{72} = 1$,

$$\text{Brier} = (1 - p)^2.$$

We also report a simple calibration gap:

$$| \bar{y} - \bar{p} |, \quad \text{where } \bar{y} = \frac{1}{3}\sum y_t, \bar{p} = \begin{cases} \frac{1}{3}\sum p_t, \text{ if time-resolved} \\ p, \text{ if single } p \end{cases}$$

Lower Brier scores indicate better probability calibration; as a practical heuristic in this study, scores ≤ 0.20 were classified as good, 0.20–0.30 as moderate, and > 0.30 as poor.

When AI outputs provide time resolved probabilities at 24, 48, and 72 h, we assess time consistency by requiring a non-decreasing sequence (p72 ≥ p48 ≥ p24). Minor numerical jitter (absolute change ≤ 0.02) is tolerated and not counted as a violation to account for expected measurement noise. If the sequence is non-decreasing, the model is labeled *time-consistent*; otherwise, *non-monotonic*. When only a single global probability $p$is available, this check cannot be performed and is reported as a limitation. All calculations were implemented with simple spreadsheet formulas. No curve fitting across time is required; the procedure is fully replicable by pasting timepoint labels and AI probabilities.

Timepoint labels were defined operationally: for each $t \in \{24,48,72\}$, we set $y_t = 1$ if the 50% effect threshold was reached within the tested range at time t(IC50 present by the in-vitro procedure), and $y_t = 0$ otherwise. Numerical IC50 values and their time trends are reported in the Results. AI probabilities were obtained from two sources for PFOA only (CAS 335-67-1):

i. ProTox, providing a single global probability $p \in [0,1]$
ii. The CompTox/invitrodb cytotoxicity summary for PFOA, from which we computed a global HTS ratio $p = \text{nhit}/\text{ntested}$. When time-resolved probabilities (p_{24}, p_{48}, p_{72}) were available, they were used directly; otherwise, the single $p$was evaluated against all timepoints.

3. Results

3.1. Cytotoxicity Results

SH-SY5Y viability assays yielded IC_{50} values of 419.52 μM (24 h), 174.97 μM (48 h), and 104.64 μM (72 h), indicating a ~4.01-fold increase in apparent potency from 24 to 72 h. These experimentally derived concentration–response metrics were subsequently integrated into the AI probability framework for calibration and comparative validation against computational toxicity predictors (Figure 1).

3.2. AI Probability Inputs

Two external sources were queried for probability-style signals. ProTox returned a single global toxicity probability of p = 0.68. The CompTox/invitrodb cytotoxicity summary for PFOA reported nhit = 24 and ntested = 90, yielding a global high-throughput activity ratio of p = 0.267.

Calibration Against Active50 Labels

Because Active50 = 1 at all three timepoints, the single-probability Brier score reduces to $(1-p)^2$. ProTox achieved Brier = 0.102 with a calibration gap $|\ 1-p\ | = 0.320$, which falls in the *good* range per our predefined heuristic. In contrast, CompTox/invitrodb yielded Brier = 0.537 with a gap of 0.733, indicating *poor* calibration and systematic underestimation of the observed biological activity.

Time Consistency

Time-resolved probabilities (p_{24}, p_{48}, p_{72}) were not available from either source; therefore, the monotonicity criterion (non-decreasing $p$from 24→72 h) could not be tested and is noted as a limitation. The pronounced ~4-fold potency shift nevertheless underscores the value of time-aware probability outputs for future alignment analyses.

4. Discussion

This study evaluates whether probability-based AI predictions align with time-resolved biological responses to PFOA in SH-SY5Y cells. Using a curve free alignment, we converted viability readouts into threshold labels (Active50) and assessed probability calibration with the Brier score. The biology showed a clear time-dependent ≈4.01-fold potency shift from 24 to 72 h. Against these labels, ProTox yielded good calibration (p=0.68; Brier=0.102), whereas the CompTox/invitrodb cytotoxicity summary ratio provided poor calibration (p=0.267; Brier=0.538). Taken together, the results indicate that alignment depends strongly on how the AI signal is defined and whether it preserves the biological context.

Our interpretation is that the two sources differ in both semantics and context. ProTox returns a calibrated probability intended to approximate risk, while the invitrodb "global hit ratio" is an assay aggregated activity frequency that is endpoint- and time-agnostic. On the other hand, our in vitro findings provide a clear time-dependent toxicity profile: SH-SY5Y viability assays yielded IC_{50} values of 419.52 μM (24 h), 174.97 μM (48 h), and 104.64 μM (72 h), reflecting an approximately four-fold increase in apparent potency over the exposure period. This progressive decrease in IC_{50} suggests cumulative cytotoxic stress or delayed cellular responses to PFOA exposure, consistent with previous reports describing time-dependent reductions in neuronal cell viability following PFAS treatment [13], [14]. Such temporal sensitivity supports the interpretation that short-term assays may underestimate PFOA's true neurotoxic potential, whereas extended exposures better capture its progressive mitochondrial and oxidative effects. Therefore, incorporating time-resolved experimental data into AI calibration improves the biological realism of toxicity prediction models and enables a more accurate alignment between computational probability outputs and actual cellular outcomes. Because potency increases at longer exposures, context-free ratios are likely to underestimate activity when effects develop over time.

Perfluorooctanoic acid (PFOA) is a persistent environmental contaminant that falls under the category of per- and polyfluoroalkyl substances (PFAS). Its biological effects are varied and depend significantly on the cell type and the duration of exposure. Recent studies have shown that the cellular response to PFOA is influenced by these two factors, pointing to the compound's diverse mechanisms of action and the unique metabolic and defensive characteristics of different cell types.For instance, in neuronal cells, the cellular response is strongly affected by both exposure duration and concentration. In human SH-SY5Y neuronal cells, exposure to concentrations ranging from 10 to 100 μM for 24–48 hours resulted in mitochondrial dysfunction and metabolic changes, while higher concentrations (above 250 μM) led to cell death and reduced ATP production [14]. In neuroblastoma S1 cells, even a brief one-minute exposure decreased GABA receptor-mediated currents, indicating a rapid but reversible effect on neurophysiology [19]. These results imply that neuronal systems are particularly sensitive to short-term exposure to PFOA, with effects varying over time.

In liver-derived cells, PFOA exposure results in dose-dependent changes in lipid metabolism and oxidative stress that are also specific to the cell type. For example, in HepaRG cells, even low concentrations (25 μM) disrupted lipid homeostasis [18], while in HepG2 cells, a 24-hour exposure led to cytotoxic effects and depletion of glutathione without significantly increasing reactive oxygen species (ROS) production [20]. These findings indicate that even brief PFOA exposure can cause metabolic disturbances in liver cells through different pathways.

Endocrine and reproductive cells also show responses dependent on cell type and hormonal context. In porcine theca and granulosa cells, PFOA was found to inhibit steroid hormone secretion, both alone and in conjunction with gonadotropin stimulation, underscoring its potential as an endocrine disruptor [16]. In Japanese medaka fish, a 21-day exposure resulted in time- and sex-dependent changes in the transcription of reproductive genes such as

vitellogenin and choriogenin [17].When examining breast epithelial cells, PFOA's effects on cell proliferation and migration varied significantly by cell type. In non-cancerous MCF-10A cells, concentrations of 50–100 μM enhanced proliferation by accelerating the transition from G_0/G_1 to S phase [21], while in ER-negative MDA-MB-231 cells, it promoted migration through activation of the PPARα–FA2H signaling pathway [22].

In summary, the evidence indicates that responses to PFOA are both cell type-specific and time-dependent, with initial metabolic disruptions often occurring before noticeable cytotoxic effects. This underscores the need to consider temporal dynamics and cellular context when assessing the toxicological impacts of PFOA, especially regarding human health risk evaluations.

The results coming from ProTox and CompTox/invitrodb suggest that alignment depends on how the AI signal is defined and whether it preserves the biological frame (endpoint and exposure window). A calibrated, endpoint-relevant probability (ProTox) aligns well with Active50, while a global HTS hit ratio (invitrodb) can misrepresent activity because it collapses heterogeneous assays and lacks time resolution [12]. This interpretation is consistent with the literature reporting that PFAS responses vary by cell type, endpoint, and exposure duration, and that shorter, context-mixed HTS readouts may miss effects that emerge over time. Filtering HTS to viability/mitochondrial panels and where possible stratifying by exposure window would likely improve agreement.

The contribution of this work is methodological as well as practical. However, limitations include a single compound and cell line, and the absence of time-resolved probabilities from external sources (precluding a formal monotonicity test). Even so, the framework is readily extensible by requesting from different AI models, applying endpoint-specific filtering to HTS, and expanding to a PFAS panel to examine generalizability.In conclusion, a simple, curve-free calibration framework showed that probability style AI predictions can align with time resolved SH-SY5Y responses to PFOA when the AI signal is defined in the same endpoint and exposure windows. A calibrated, endpoint relevant probability (ProTox) exhibited good agreement with Active50 labels, whereas a context agnostic global HTS hit ratio (CompTox) was poorly calibrated, highlighting the risk of underestimating effects that emerge with longer exposure. These results support adopting time-aware, endpoint-matched probabilities for benchmarking AI against wet-lab biology. Despite limitations such as single compound/cell line and no time-resolved external probabilities, the workflow is reproducible and readily generalizable; extending it to additional PFAS, adding mechanism weighted composites, and incorporating time resolved probabilities are clear next steps.

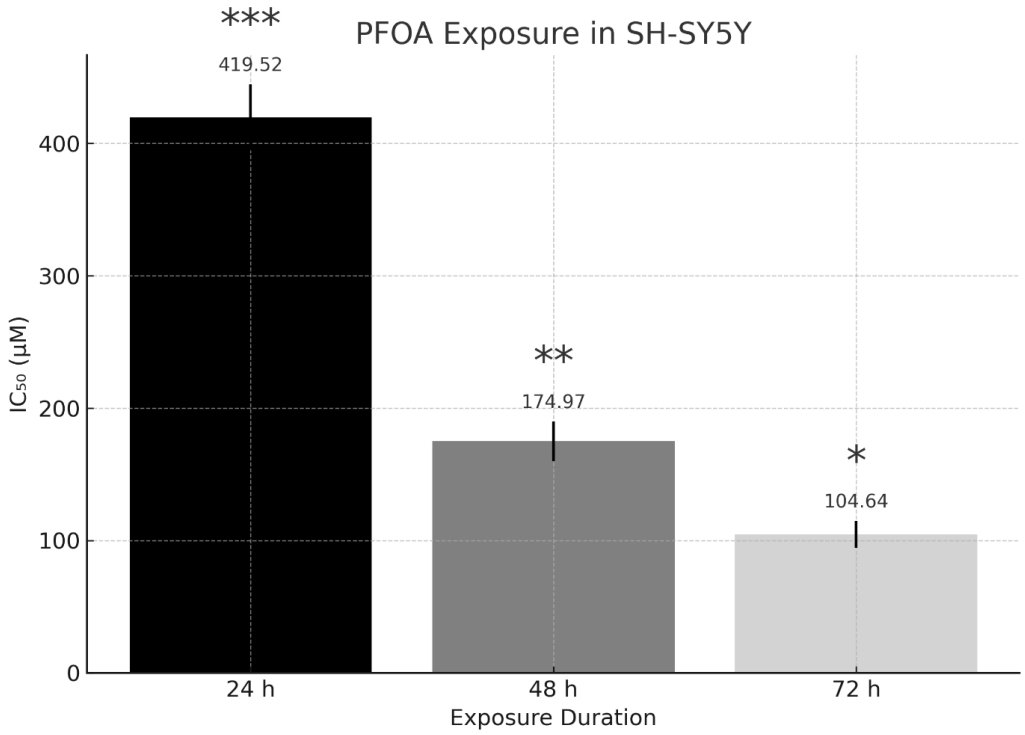

Figure 1. **IC_{50} values for PFOA exposure in SH-SY5Y cells at 24, 48, and 72 h. Data are expressed as mean ± SD. The symbols ***, *, and * reflect the relative magnitude of the IC_{50} values, where *** corresponds to the highest value (24 h), ** to the intermediate value (48 h), and * to the lowest value (72 h). Statistical significance was evaluated using one-way ANOVA with post-hoc comparison.*

Declaration of Interest

The authors declare that there is no conflict of interest.

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