# PFAS Interactions with Proteins using Equilibrium Dialysis: Do's and Dont's

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## Introduction

- Per- and poly-fluoroalkyl substances (PFAS), have become pervasive environmental contaminants across the globe.
- Although the mechanisms of PFAS toxicity are not well understood, it has become clear PFAS bind to proteins with different affinities.
- Models that can predict the effects of PFAS are needed, because the large number of total PFAS (~14,000), and all of the possible combinations of those PFAS, are too great in number to allow toxicity testing of every single PFAS and their mixtures.
- Therefore, approaches that quantify the interaction of PFAS with circulating and structural proteins is needed.
- Equilibrium Dialysis (ED) is a frequently used in vitro technique for measuring compound-plasma protein binding.

## Objective and Hypothesis

- Determine the binding affinity of PFAS to the circulating protein hemoglobin using in vitro (ED) approaches.
- We hypothesized that binding affinity would be higher in long-chain sulfonic PFAS.

## Methods

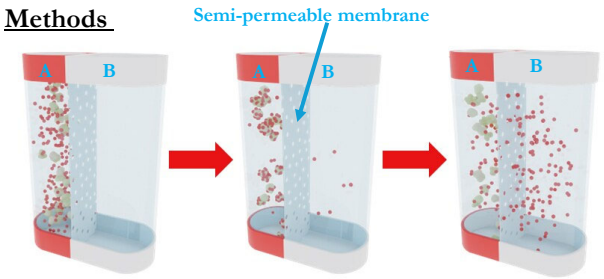


**Figure 1.** We are using the Thermo Scientific® Rapid Equilibrium Dialysis (RED) device for our studies. The insert chambers of the device are separated by a semi-permeable membrane and, when filled with sample (**A**), enable the separation of the free PFAS fraction from that of compound bound to hemoglobin (**B**).

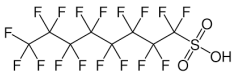

**Figure 2**. Perfluorooctane sulfonate, PFOS, 0.5 KDa.

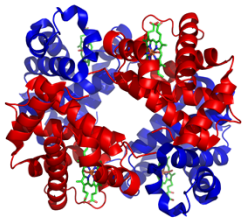

**Figure 3**. Human hemoglobin, 64.5 KDa.

### General ED Protocol

1. Select an RED device (**Figure 1**) with a membrane size larger than the molecule whose binding affinity you wish to test, but smaller than the protein that it will bind to.
2. In our case, PFOS is 0.5 Kda (**Figure 2**) and the protein of interest, human hemoglobin, is 64.5 KDa (**Figure 2**), so we are using a membrane pore size of 8KDa.
3. Add a solution containing both the protein and the PFAS to the sample cell (**Figure 1**, **A** chamber) and buffer to the buffer cell of the device (**Figure 1**, **B** chamber)
4. Incubate with agitation at 37°C.
5. Determine PFAS concentrations in **A** and **B**.
6. [PFAS] **A** = **B**, no binding.
7. [PFAS]**A** > **B**, binding.

## Human hemoglobin binds to PFOS with Ka values similar to those observed for albumin.

**Table 1.** Association constants (Ka) and binding percentage of PFOS with human hemoglobin in comparison to values reported for human albumin also using ED.

| Ligand | Protein | Ka (log $M^{-1}$) | [L]:[P] Mole Ratio Range | Reference |
|---|---|---|---|---|
| PFOS | Human hemoglobin | 1.28 - 3.14 | 0.05 - 10 | This study |
| PFOS | Human albumin | 1.799 | 0.1-2.0 | Alesio et al. 2022 |
| PFOS | Human albumin | 3.2 | <0.1 | Allendorf et al. 2019 |
| PFOA | Bovine albumin | 0.2 | 0.04-5 | Bischel et al. 2010 |

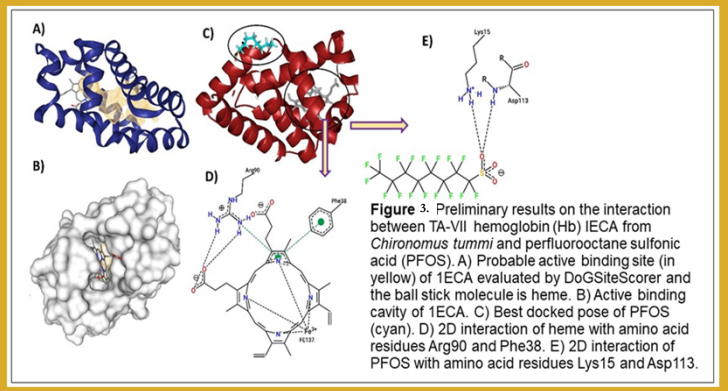


**Figure 3.** Preliminary results on the interaction between TA-VII hemoglobin (Hb) IECA from *Chironomus tummi* and perfluorooctane sulfonic acid (PFOS). A) Probable active binding site (in yellow) of 1ECA evaluated by DoGSiteScorer and the ball stick molecule is heme. B) Active binding cavity of 1ECA. C) Best docked pose of PFOS (cyan). D) 2D interaction of heme with amino acid residues Arg90 and Phe38. E) 2D interaction of PFOS with amino acid residues Lys15 and Asp113.

## Equilibrium Dialysis Experiment

- Polypropylene containers were used to avoid adsorption to glass surfaces.
- All supplies, including pipettes tips and REDs, were methanol rinsed.
- Concentration of human Hb tested ranged from 2µM to 200µM. PFOS concentration was held constant at 10µM.
- The molar ratio of PFOS to Hb ranged from 0.05:1to 10:1 PFOS to Hb.
- Samples were run in triplicate.
- PFOS only and Hb only controls were run in duplicate.
- PFOS was quantified using LC-MS/MS analyses were performed on a SCIEX Triple Quad 5500 mass spectrometer equipped with Turbo Ion Spray interface.

## PFOS - Hemoglobin Binding Model

Log(KHb/Wat) = Concentration_bound_Hb/Concentration_free_PFOS

## Results and Discussion

- We have shown that human hemoglobin binds to PFOS with Ka values ranging from 1.28 – 3.14 (**Table 1**).
- We observed significant loss of PFOS during the course of the experiment.
- We attribute this loss mostly to sorption to the chamber walls of the test cells.
- This necessitated the need for a mass-balance correction for every tests cell in order to accurately determine the final PFOS concentrations.
- Despite publications using the RED plates to study binding of proteins to PFAS, we are unaware of anyone having noted this problem before.
- We suggest the following additional modifications when testing PFAS binding using RED plates:
  - Use only gentle agitation on a shaker table rather than vortexing to decrease PFAS adsorption to the sides of the plate walls.
  - Increase incubation time from 4hr at 37°C to overnight at 37°C to allow for full mixing in the absence of vigorous agitation.
  - Measure final volumes to account for any volume loss that occurs due to condensation on the coverslip in order to perform a mass-balance correction to accurately calculate the percent of bound PFAS.

## Future Work

- We plan to test multiple different PFAS, focusing on those that our modeling predicts will have the greatest binding affinities and toxicity (**Figure 3**).
- In vivo work with midges will be done to validate the in vitro ED results.
- The ED method can be used to test the binding affinities of PFAS to other proteins to help elucidate the mechanisms of toxicity of PFAS.

## References

1. Alesio et al. 2022. Critical new insights into the binding of poly- and perfluoroalkyl substances (PFAS) to albumin protein. Chemosphere 287: 131979.
2. Bischel et al. 2010. Noncovalent interactions of long-chain perfluoroalkyl acids with serum albumin. Environ. Sci. Technol. 44: 5266-5269.
3. Allendorf et al. 2019. Partition coefficients of four perfluoroalkyl acid alternatives between bovine serum albumin (BSA) and water in comparison to ten classical perfluoroalkyl acids. Env. Sci. Process Impacts 21: 1852-1863.
4. Figure 2. The chemical structure of PFOS (PerfluoroOctaneSulfonic acid), – College of Pharmacy (uri.edu)
5. Figure 3. National Center for Biotechnology Information (nih.gov)
6. RED Device: Plasma Protein Binding Equilibrium Dialysis, RED Device: Plasma Protein Binding Equilibrium Dialysis | Thermo Fisher Scientific - US

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