FINAL REPORT - Phase II

Protein sorbents for PFAS-contaminated water treatment: focused sorption kinetics, protein degradation, and thermal regeneration testing

ER18-1417

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This project aimed to (1) quantify kinetics of PFAS sorption to select proteins, which is needed to assess viability of protein sorbents to effectively treat AFFF-contaminated groundwater under realistic treatment train conditions, (2) assess protein stability and sorption performance over time under groundwater treatment-relevant conditions, and finally (3) investigate thermal regeneration of the protein sorbents and post-regeneration sorption effectiveness. Detecting and quantifying sorption at low PFAS concentrations during this one year follow-on effort proved challenging, suggesting a pre-concentration step would be needed for current suite of PFAS and proteins tested. Mammalian protein sorbents were not stable under ambient conditions for extended time; microbial- and plant-derived proteins may hold promise. Surface plasmon residence-determined binding kinetics and affinity for PFAS-protein pairs reported herein constitute a significant contribution to science.

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LIST OF ACRONYMS

3D	3-dimentional
AFFF	Aqueous film-forming foam
$\Delta G°$	Free energy of binding
FA1 – FA7	Fatty acid binding site 1 through 7 (on serum albumin)
GAC	Granular activated carbon
GPU	Graphics processing unit
H-FABP	Heart fatty acid binding protein
HSA	Human serum albumin
I-FABP	Intestinal fatty acid binding protein
ISCO	*In situ* chemical oxidation
K_A	Equilibrium association constant
K_D	Equilibrium dissociation constant
LC-MS/MS	Liquid chromatography tandem mass spectrometry
L-FABP	Liver fatty acid binding protein
MD	Molecular dynamics
NAPL	Non-aqueous-phase liquid
OSU	Oregon State University
PDB	Protein data bank
PFAAs	Perfluorinated alkyl acids
PFAS	Per- and polyfluorinated alkyl substances
PFBA	Perfluorobutanoic acid
PFBS	Perfluorobutane sulfonic acid
PFHxA	Perfluorohexanoic acid
PFHxS	Perfluorohexane sulfonic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
PPAR	Peroxisome proliferator-activated receptor proteins, α and γ
TCE	Trichloroethylene
UP	University of Pittsburgh

ACKNOWLEDGEMENTS

This report represents the results and conclusions from a collaborative effort between scientists and engineers at U.S. Army Engineer Research Development Center, Oregon State University and University of Pittsburgh. This project was funded by the Strategic Environmental Research and Development Program (SERDP), with the goal of exploiting known per- and polyfluorinated alkyl substances (PFAS) interactions with proteins to develop novel bio-inspired sorbents for PFAS water treatment. The project team wishes to thank Dr. Andrea Leeson and the support staff from the SERDP program office for their help and guidance throughout this limited-scope project.

ABSTRACT

Objectives: Per- and polyfluorinated alkyl substances (PFAS) are a diverse group of chemicals that have been used as components of aqueous film-forming foam (AFFF) for decades. Early formulations used perfluorooctane sulfonate (PFOS) in large proportion. Unfortunately, long-chain PFAS like PFOS have since been found to be bioaccumulative and toxic, prompting voluntary phase-outs, an establishment of drinking water guidelines by the US EPA and many states, and the need to remediate contaminated water for the protection of environmental and human health. Areas where AFFF were routinely deployed during firefighting exercises, including DoD sites, have accumulated a variety of PFAS in their groundwater, and are now in need of cost effective and efficient remediation solutions. Many PFAS eventually degrade to form perfluorinated alkyl acids, which are extremely persistent and have varying levels of water solubility. Because of this, *ex situ* treatment technologies may be most suitable. However, currently available technologies such as adsorption with activated carbon cannot effectively treat both short- and long-chain PFAS. The objective of this follow-on to our limited-scope project was to evaluate the kinetics of protein-PFAS binding in order to better understand sorbent performance and test sorbent stability and regeneration.

Technical Approach: First, candidate protein sorbents identified from our first limited-scope project as having high affinity for certain PFAS were evaluated under a time-resolved dialysis experiment. Following dialysis, we evaluated two alternative approaches to directly measure binding kinetics: isothermal titration calorimetry (ITC), carried out by our partners at ERDC, and surface plasmon resonance (SPR), carried out in collaboration with ERDC at the University of Pittsburgh. Column experiments were planned to evaluate breakthrough kinetics, but did not proceed based on the time-resolved equilibrium results. Finally, additional sorbent candidates for future work were identified through molecular screening.

Results: Pilot tests and a range-finding experiment employing the time-resolved dialysis approach indicated high variability and lack of binding at low PFAS concentration for all proteins evaluated (bovine serum albumin (BSA), liver fatty acid binding protein (L-FABP), and PPAR-alpha). Proteins from different sources were tested to evaluate whether a quality control issue was responsible, and a greater range or protein-PFAS mole ratios than used in the original ER181417 studies was tested to confirm the binding affinities. This expansion to test L-FABP binding with PFOS helped confirm the K_Ds determined in our previous work, suggesting that for lower-affinity binding, the removal of PFAS at low concentrations from feed streams could be a limiting factor. As a back-up to dialysis, ITC experiments again showed limitations. Initial experiments with BSA indicated no observable binding for PFBS, but a K_D was calculated for PFOS in good agreement with reported ITC values. For L-FABP, the commercially provided protein concentration was too low for the volumes needed for ITC; no association constant could be derived. Further development would require access to a micro-ITC system. Pivoting to explore a novel localized SPR approach, we were able to show that non-specific binding of PFOA and PFOS to the surface of the analysis chip could be disrupted using NaOH, and that the target protein L-FABP could be successfully concentrated on the chip surface and attached. Experiments are ongoing to determine optimal conditions to measure binding with PFAS. High concentration injections of PFOS (1mM) detects (likely non-specific) binding to L-FABP, but running buffers, chip chemistry, and ligand immobilization parameters need further optimization to successfully detect lower affinity binding, e.g. with shorter-chain PFAS. Finally, further simulation-based screening identified three promising targets for further research: a phospholipase from rice, a fluoroacetyl co-enzyme A from *Streptomyces cattleya*, and a thermophilic esterase from *Thermogutta terrifontis*. The origins of these newly identified sorbent candidates (plants and microbes) may lead to greater robustness in the environment vs. mammalian proteins. In addition, these small proteins should be easier to produce and may provide clearer signals upon binding with the small-molecular-weight PFAS (e.g. in SPR).

Benefits: While this one-year project experienced substantial and ongoing disruptions in laboratory access, personnel availability, and the scientific supply chain due to COVID19, we were able to identify promising new approaches to sorbent evaluation (SPR) and collect data on the critical factors around protein availability and binding affinity that could limit the application of certain approaches for evaluating binding kinetics. Ongoing method development will substantially contribute to our knowledge base on the intersection of PFAS with biological systems and on leveraging these interactions for PFAS capture.

1.0 INTRODUCTION

1.1 PROJECT OVERVIEW

This project was a one-year follow-on from Limited-Scope Project ER181417, focused on the development of protein-based sorbents for the removal of per- and polyfluoroalkyl substances (PFAS), especially short-chain PFAS, from contaminated water. PFAS are a diverse group of chemicals that have been used as components of aqueous film-forming foam (AFFF) for decades. Early formulations used the long-chain perfluorooctane sulfonate (PFOS) in large proportion, whereas current formulations use shorter-chain PFAS, a transition prompted by the bioaccumulative and toxic nature of PFOS. Both legacy and current AFFF formulations also included a number of polyfluorinated substances that could degrade to form a suite of shorter-chain perfluorinated acids which have proven difficult to remediate with standard technical approaches. Long-term accumulation of these substances in areas where AFFF were routinely deployed during firefighting exercises, including DoD sites, has led to a need for cost efficient remediation solutions. Many PFAS are extremely persistent and have varying levels of water solubility. Because of this, *ex situ* treatment technologies may be most suitable. However, currently available technologies such as adsorption with granular activated carbon (GAC) cannot effectively treat both short and long-chain PFAS. Limited-scope project ER181417 sought to address this gap by exploiting the propensity of PFAS to bind with proteins, and used a combination of molecular modeling and batch testing to confirm that proteins efficiently adsorb a variety of PFAS, with sorption capacities similar to (or in some cases much higher than) conventional sorption materials like GAC. This one-year follow-on project aimed to (1) quantify kinetics of PFAS sorption to select proteins, which is needed to assess viability of protein sorbents to effectively treat AFFF-contaminated groundwater under realistic treatment train conditions, (2) assess protein stability and sorption performance over time under groundwater treatment-relevant conditions, and finally (3) investigate thermal or pH regeneration of the protein sorbents and post-regeneration sorption effectiveness. Screening by molecular modeling was also employed to identify future promising targets for experimental evaluation.

1.2 TECHNICAL OBJECTIVE

ER181417 reported strong associations of biomolecules with short-chain PFAS, raising both prospects for treatability and potential concern about toxic effects, as described in the publication that resulted from that project (Khazaee *et al*. 2021, Toxics). Moreover, the binding capacities we calculated from protein-PFAS data for some short-chain PFAS were substantially higher than with either granular or particulate activated carbon, illustrating the potential of our approach for targeting difficult to treat PFAS. Based on those observations, we selected four PFAS-protein pairs for targeted follow-on analysis of sorption kinetics and protein regeneration in this project: PFHxS and PFBS with L-FABP, PFHxA with PPAR-α, and PFOS with PPAR-γ. The objective of this one-year follow on project was to address the following key properties: 1) sorption kinetics for conceptual treatment design; 2) protein stability under realistic treatment conditions and timeframes; and 3) effectiveness of thermal or pH regeneration of the protein sorbent for repeated use. Finally, ongoing molecular screening is used to broaden the list of candidate sorbents to be pursued experimentally.

1.3 COVID-RELATED DISRUPTIONS AND CHANGES TO PROPOSED TASKS

This one-year follow-on project unfortunately coincided with the outbreak of the COVID-19 pandemic, which led to substantial disruptions in the timeline, in the accessibility of laboratory facilities to conduct experiments, and in the availability of standard laboratory supplies. This led to a no-cost extension until the end of October 2021, and required us to make some changes to the proposed Tasks. Changes are described in detail within the reporting for each proposed and, where applicable, modified Task, below.

2.0 TECHNICAL APPROACH

2.1 TASK 1: TIME-RESOLVED EQUILIBRIUM DIALYSIS AND ISOTHERMAL TITRATION CALORIMETRY

Initial batch tests were performed to establish the relative speed of PFAS-protein equilibration. These time-resolved dialysis experiments were meant to aid design and selection of columns for more refined establishment of kinetics. A similar approach was used as in the equilibrium dialysis (EqD) batch tests featured under ER181417, in which proteins were excluded by dialysis membranes with molecular weight cutoffs smaller than the protein size while PFAS could freely equilibrate in the buffer on both sides of the dialysis system. The PFAS-spiked buffer solution (not in contact with the proteins) were sampled at several time points (Table 1).

Table 1: Time points and sample designations for triplicate time-resolved equilibrium dialysis experiments. 'Spike' referes to PFAS controls (no protein binding).

TIME POINT (HR)			
0	spike	spike	spike
0.25	T1a	T1b	T1c
0.5	T2a	T2b	T2c
1	T3a	T3b	T3c
2	T4a	T4b	T4c
4	T5a	T5b	T5c
8	T6a	T6b	T6c
12	T7a	T7b	T7c
24	T8a	T8b	T8c
36	T9a	T9b	T9c
48	T10a	T10b	T10c
72	T11a	T11b	T11c
PROTEIN ONLY CONTROL	T11pc	T11pc	T11pc
DENATURED PROTEIN CONTROL	T11dp	T11dp	T11dp
TOTAL NUMBER OF SAMPLES	42		

Pilot tests: BSA and Batch Tests. An initial pilot test of time-resolved dialysis was performed using bovine serum albumin (BSA). The stock solution of BSA was prepared using a phosphate-buffered saline (PBS) buffer at a pH of 7.4. PFOA solutions were prepared from a 10 ppm stock. The BSA concentration was 1 µM, and a PFOA:BSA molar ratio of 1 was considered in this experiment. At each of the total of 11 time points (see Table 1), triplicate samples were prepared using 1280 µl of PFOA and 20 µl of BSA. In addition, 3 PFAS control spike samples were used where 1.2 ml of PFOA was added to 1.5 ml tubes and 100 µl was added to the dialysis cup. Three protein control samples were also prepared with no PFOA. Finally, three denatured protein control samples were prepared by adding the same concentrations and volumes of BSA and PFOA mentioned above. To denature BSA, the protein was placed in a water bath in which the temperature was increased to 55 °C. The protein was maintained in the water bath for 10 mins to be completely denatured and then cooled to room temperature.

Results from this pilot study indicated high variability across triplicates and a lack of binding over the time points. Indeed, the protein-containing samples had higher concentration than the PFAS controls, indicating a potential issue either with the PFAS controls or with contamination on the

protein. The denatured protein spike showed a concentration more consistent with PFAS being bound to BSA for the other time point samples (Figure 1).

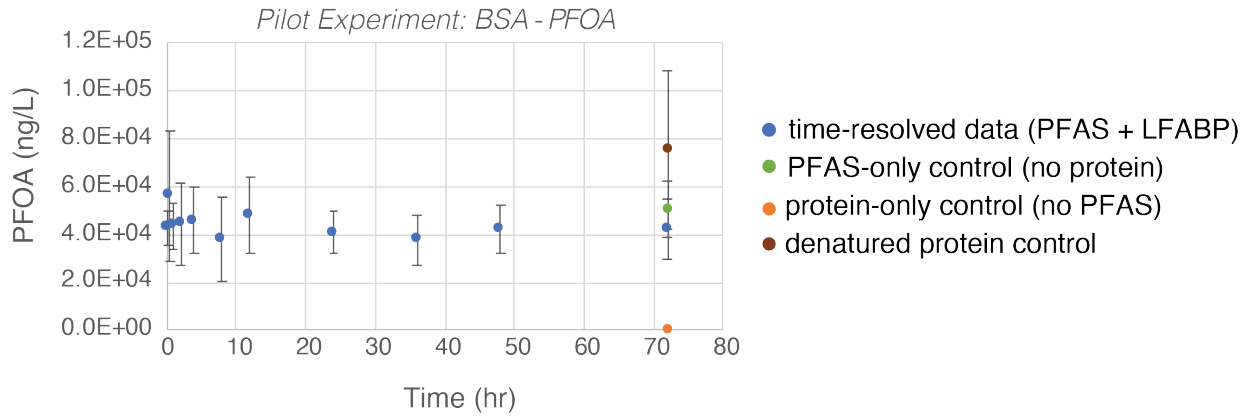

Figure 1: Pilot time-resolved dialysis experiment using bovine serum albumin (BSA) and perfluorooctanoic acid (PFOA). No binding was observed

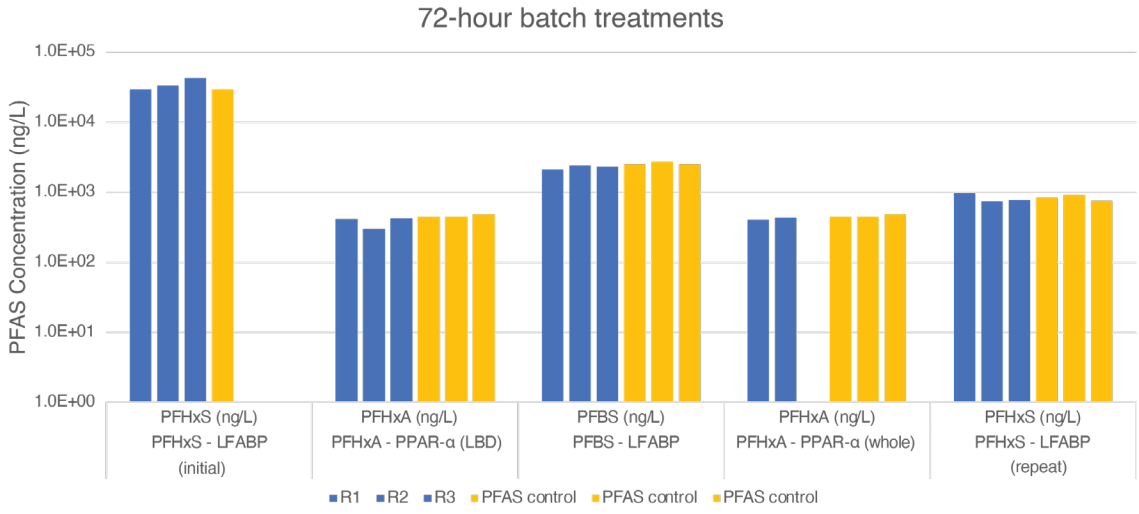

Figure 2: Batch experiments to test for binding across PFAS-protein combinations.

In these experiments, as with the BSA pilot study, the 1:1 molar ratio of PFAS to protein was used that had been employed in our earlier study[1]. No evidence of binding was determined in an initial test with PFHxS and L-FABP, so the batch study was repeated with other proteins and PFAS and with lower initial PFAS concentrations. Again, no difference was found between the PFAS-only controls and the incubations with proteins, for either LFABP or PPAR-α.

PFHxS-LFABP. Based on the lack of binding observed for the concentrations and PFAS-protein molar ratios employed in the batch tests, we next performed a range-finding experiment to identify a molar ratio at which binding was detected for L-FABP with PFHxS (Figure 3A). We followed this range-finding exercise with two time-resolved experiments at L-FABP concentrations of 1 µM and 3 µM (Figfure 3B and 3C, respectively). The protocol adopted in these experiments were similar to the BSA pilot: samples were collected at time points between 15 mins and 72 hrs to reach equilibrium. Again, triplicates of each time point were collected, in addition to three PFHxS-only control samples, 3 buffer-only control samples, and 3 denatured protein control samples. Unfortunately, no significant decrease in the free PFHxS concentration was observed throughout the time course in either experiment.

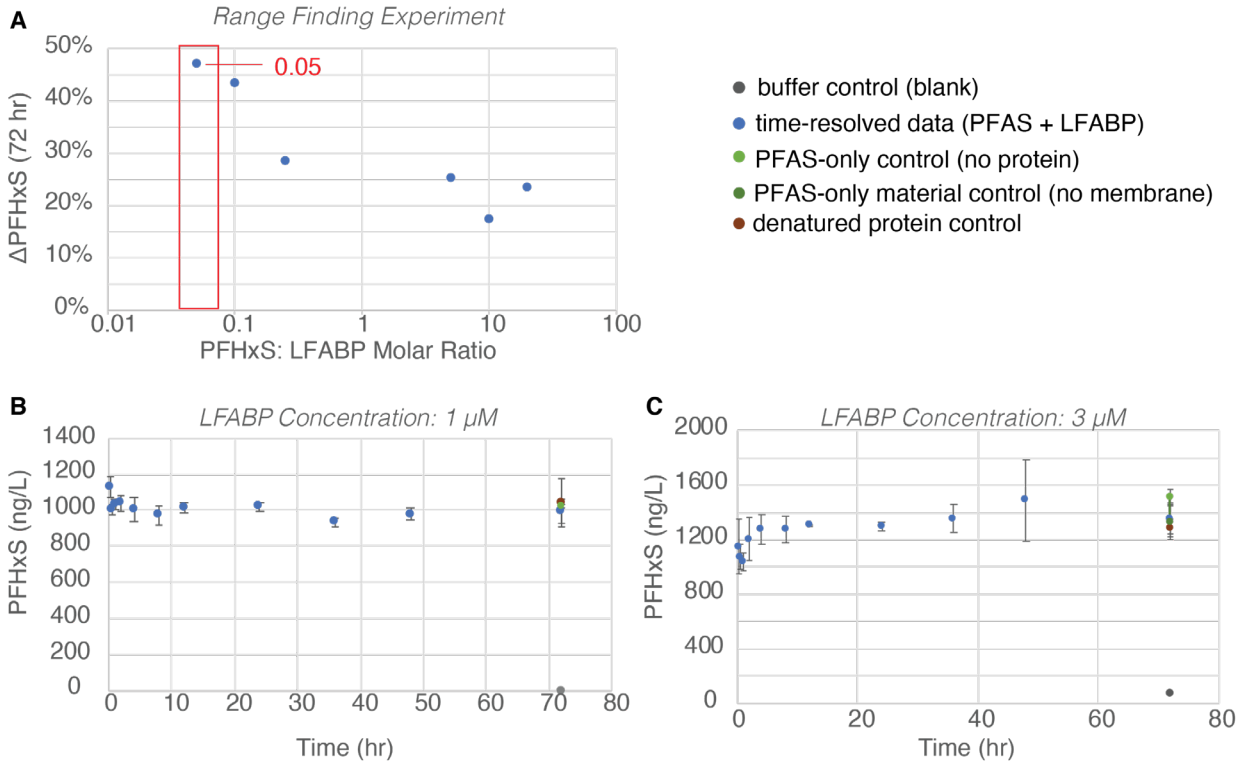

Figure 3: Range-finding and time-resolved experiments for L-FABP with PFHxS

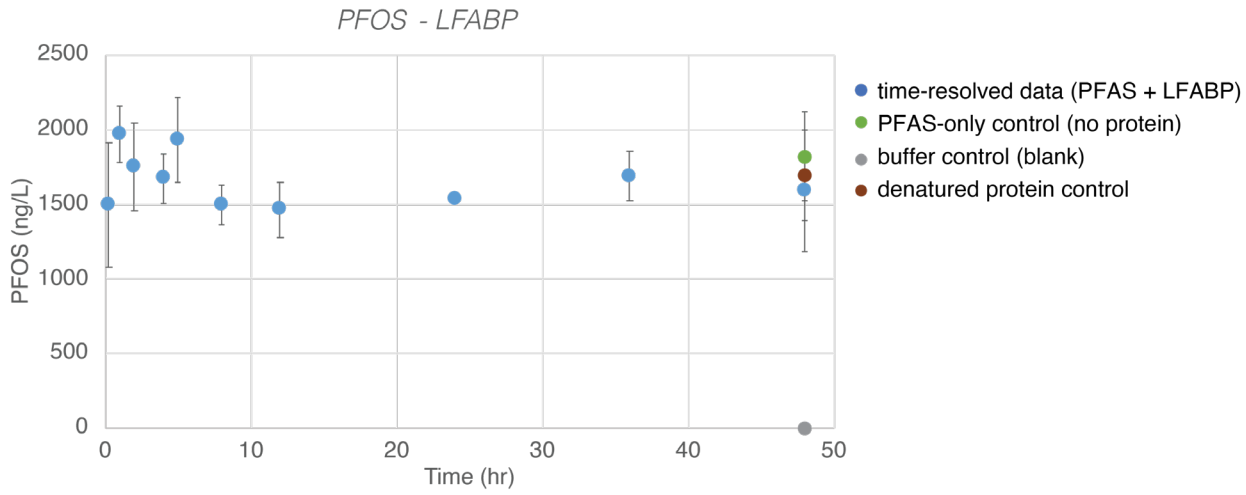

Figure 4: Time-resolved experiments using high (3µM) L-FABP concentration with PFOS

Ongoing Equilibrium Testing. Experiments are ongoing using the original equilibrium dialysis procedure (multiple PFAS concentrations, one equilibrium time point) to resolve problems with the time-resolved experiments. Proteins from different sources have been selected to evaluate whether there is a quality control issue with the proteins, and a greater range or protein-PFAS mole ratios are being explored than in the original ER181417 studies to confirm the binding affinities. A recent experiment using PFOS and L-FABP as a broader set of PFOS concentrations (0.05 – 50 molar ratio) helps to confirm the range of K_Ds determined in our previous work (Figure 5).

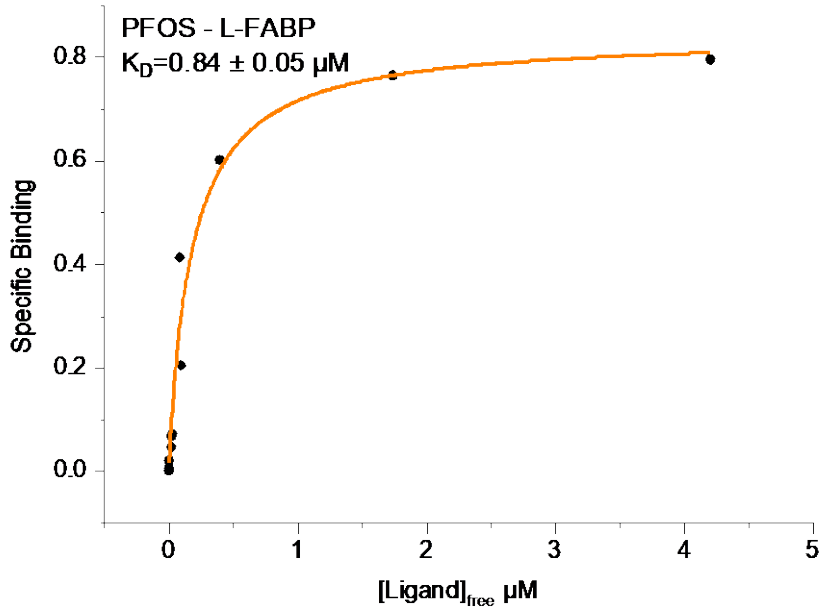

Figure 5: Calculation of K_D from equilibrium dialysis of PFOS with L-FABP

This recent experiment produced a K_D value of 0.84 ± 0.05 µM, which is within the range of our earlier study (0.18 ± 0.032 µM)[1].

Isothermal Titration Calorimetry (ITC). In parallel with equilibrium dialysis testing, pilot experiments were conducted at ERDC to evaluate the use of isothermal titration calorimetry (ITC) for protein binding studies. Two proteins and three PFAS were evaluated: PFOS and PFBS with BSA, and PFOA with L-FABP. The first experiments evaluated the abundant and inexpensive BSA protein with both PFBS, a short-chain PFAS not expected to bind strongly to BSA, and PFOS, a long-chain PFAS that should show substantial binding. The final experiment tested L-FABP and PFOA, which are known based on published literature to bind with substantial affinity.

For the BSA experiments, 2.5 mM solutions of PFBS and PFOS were made using pH 7.4 PBS buffer. In each experiment, PFAS solutions were injected 27 times via 10 µL injections to an ITC cell. The titration intervals were set to 600 and 720 s, respectively, for PFBS and PFOS. Within the (3 mL) ITC cell, the BSA protein was loaded at a concentration of 0.01 mM in PBS buffer. The temperature was set to 37 °C and the heat of binding was measured for each injection of PFAS. Raw heats were corrected by running an experiment in which the same concentration of PFAS was injected with same conditions into buffer solution with no BSA. From these experiments, no binding was observed for PFBS, but a binding affinity was calculated for PFOS and is in good agreement with reported values from similar studies (Figure 6).

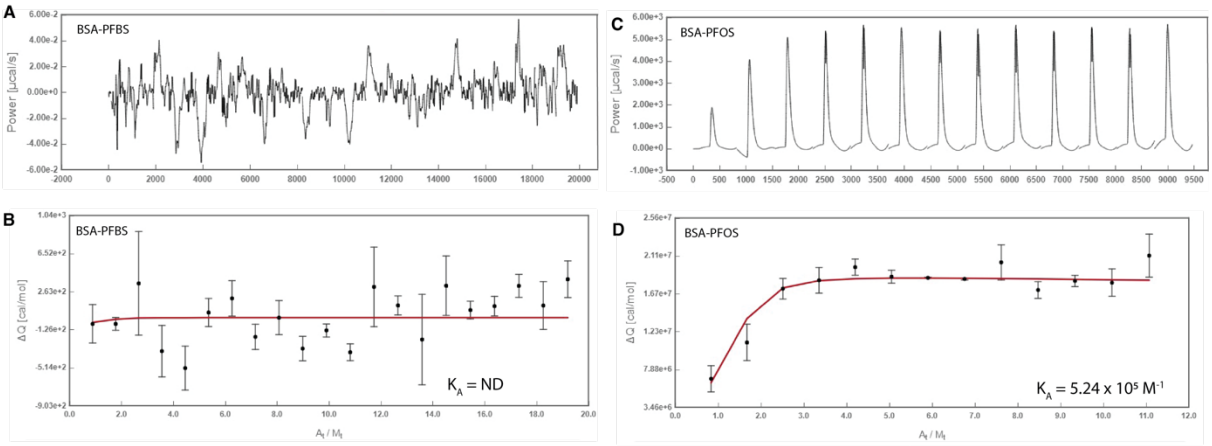

Figure 6: Isothermal titration calorimetry tests with BSA and (A, B) PFBS and (C,D) PFOS

For the PFOA-L-FABP study, the protein concentration as provided by commercial sources proved too low for the volumes needed in this ITC setup, and no association constant could be derived from the single pilot experiment that was run using 93.8 µg/mL (6.25 µM) protein and 750 µg/mL (50 µM PFOA concentrations, with a similar experimental setup in terms of the time-course of injections. We therefore determined that further development of ITC experiments with these proteins would require access to a micro-ITC system, which remains and option for future work.

2.2 TASK 4.2 (PROPOSED): COLUMN TESTING -No-go Decision Following Dialysis

Tracer Study. In preparation for the packed column kinetic testing (and in parallel with equilibrium dialysis experiments), a tracer study was performed to determine the effective bed volume of the packed columns to be used. Columns were packed with Biogel P30, size exclusion beads chosen because they would not interact with PFAS and had a nominal exclusion limit that would retain the proteins to be tested (Table 2). P-30 was gradually added to 20 mM NaCl, and allowed to settle for 12 hrs to hydrate. The

solution was then transferred to a filter flask and degassed for 5 to 10 minutes. Columns were packed with P-30 (1 g of P-30 will forms 9 mL of packed bed) and primed with a buffer of Milli-Q water + 20 mM ionic strength (NaCl) with a pH of 7. Two bed volumes of eluant (~30 mL) was passed through the column at a flow rate of 0.3 mL/min.

Table 2: Characteristic of Bio- Gel P-30

Bio-Gel	Hydrated Beads Particle Size **µM**	Typical Hydrated BV **mL/g dry gel**	Typical Flow Rates **cm/hr**	Nominal Exclusion Limit **kDa**
P-30	90-180	9	7.0-13	2.5-40

NaCl at a concentration of 100 mM was used a tracer for this experiment, since it is non-interactive with both the beads and the proteins. The pulse tracer was be added to the column with continuously flow while measuring and recording the conductivity. The spike in conductivity (Figure 7) indicated most of the tracer had left the column; following the peak, two additional bed volumes of eluant were passed through the column. Based on the tracer test, the packed bed volume was computed to be 1.5 mL.

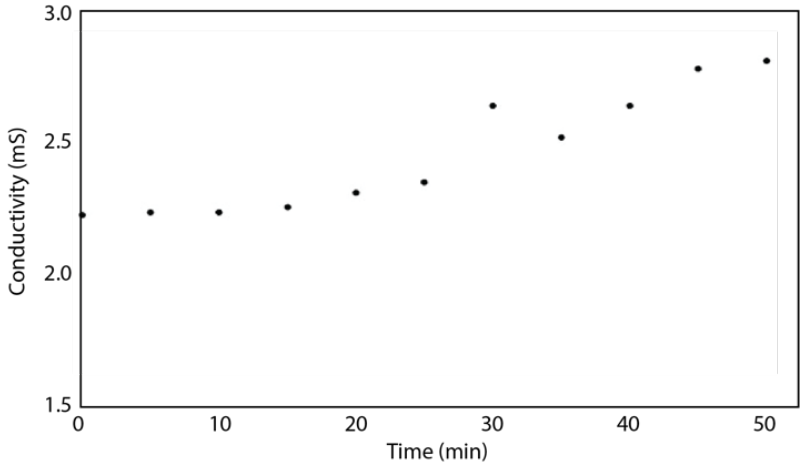

Figure 7: NaCl tracer study with P30 beads

Protein retention study. In order to test that the protein will be indeed retained by the beads, concentration of 1 µM of L-FABP was passed through the column, at a rate of 0.3 mL/min. Then, 2 bed volumes of buffer were passed. The sample was collected and was analyzed in 4 replicates using Qubit 3.0 Fluorometer. The results of all the 4 replicates indicated that the protein concentrations in the sample was lower than LOD (< 0.1 µg/mL). This serves as indication that the P-30 Bio-gel is successful in retaining the protein and can be used in subsequent experiments.

No-go Decision. Due to the unsuccessful completion of the time-resolved dialysis, the kinetic column experiments were not performed, and a decision was made to focus on development of the surface plasmon resonance (SPR) method to evaluate the binding kinetics of proteins and PFAS directly.

2.3 TASK 4.2 (REPLACEMENT TASK): SURFACE PLASMON RESONANCE METHOD DEVELOPMENT:

The following method development work replaced the originally proposed column experiments to determine the binding kinetics of PFAS with selected proteins. It should be noted that SPR has not been previously used to test PFAS-protein binding, so this constitutes novel method development, and should result in an impactful publication from this project.

Principles of SPR. The localized surface plasmon resonance approach our team has selected as a novel method to determine PFAS-protein binding and to serve as a parallel method to our efforts with equilibrium dialysis depends on immobilizing a tarted protein on a gold nanosensor chip and detecting the deflection of the protein as a test ligand (i.e., PFAS) binds to it (Figure 8).

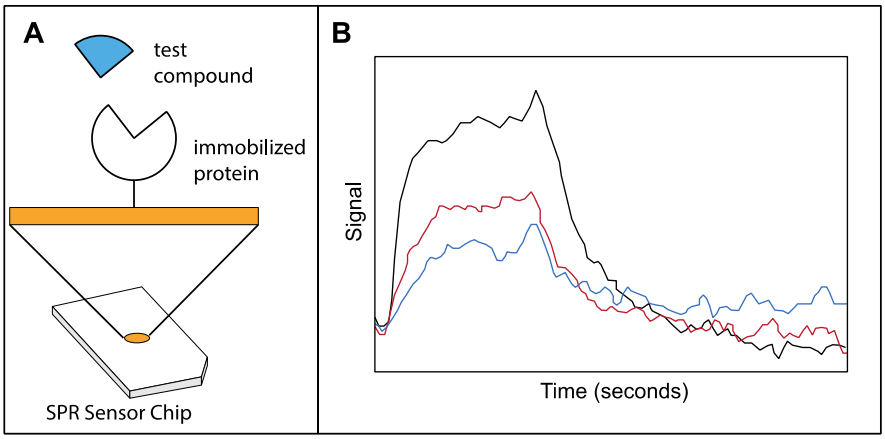

Figure 8: Schematic of SPR experiment. (A) Protein-ligand system on gold nanosensor chip. (B) Signals generated by chemical-protein binding at different concentrations.

In order to carry out a successful experiment that can provide both the strength of that binding interaction and the kinetics, the method must first be optimized to: (i) minimize non-specific binding between the PFAS and the unmodified sensor surface (which could lead to false positive binding signal); (ii) maximize the coverage of the sensor chip by the protein of interest (to collect as much signal as possible upon binding events) and (iii) determine the appropriate concentrations of PFAS and protein and the appropriate solutions compositions to regenerate the system by removing binding). The experiments can then be run at multiple PFAS concentrations to generate binding curves. During this project period we successfully minimized non-specific binding of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), the two PFAS expected to have the strongest binding among the PFAS included in our project (all other PFAS are shorter-chain substances with generally weaker binding to surfaces). We also tested the pre-concentration and binding of liver fatty acid binding protein (L-FABP) to the carboxyl sensor. A high-sensitivity carboxyl sensor was chosen for all initial SPR experiments because it does not require the use of a specifically tagged protein (it is versatile) and the high sensitivity allows for detection of binding between two molecules of very different size (as is the case with the large protein and relatively small PFAS). Experiments are ongoing with nitrilotriacetic acid (NTA) chips that covalently bind to His-tagged proteins to evaluate if they provide superior performance.

L-FABP-PFOS Experiment. To illustrate the method development for measuring PFAS-protein binding by SPR, we report here the results of each step in a full kinetics experiment using PFOS and L-FABP with a high-sensitivity carboxyl chip.

1. Checking Non-Specific Binding of PFOS to Sensor Surface:

We previously reported on a PFOA non-specific binding test which showed substantial binding. Here, a similar procedure was used to test for non-specific binding with PFOS. As before, HCl is used to activate the chip, and NaOH is used to disrupt non-specific binding (if found) after PFOS injection (Figure 9).

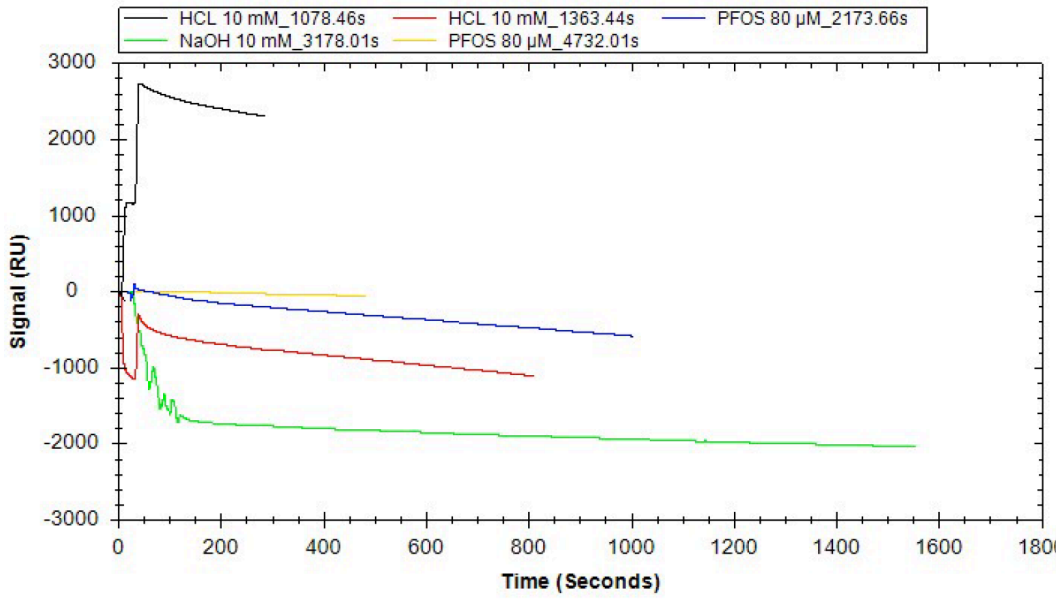

Figure 9: Evaluation of non-specific binding of PFOS on high-sensitivity carboxyl chip

Monitoring the response of the reference channel (Ch 1), a steady response was found, indicating that there was no non-specific binding of PFOS to the carboxyl chip. NaOH was used as a regeneration solution to remove any bound PFOS to the chip, and can also be used to reduce non-specific binding, if any.

L-FABP Preconcentration: Optimizing Buffer and pH. The aim of this component of the experiment was to increase the local concentration of LFABP at the COOH chip surface. A concentration of 1 µM protein solution was used, prepared in 4 solutions of standard PBS with different pH values: 4, 4.4, 5 and 5.5 (Figure 10).

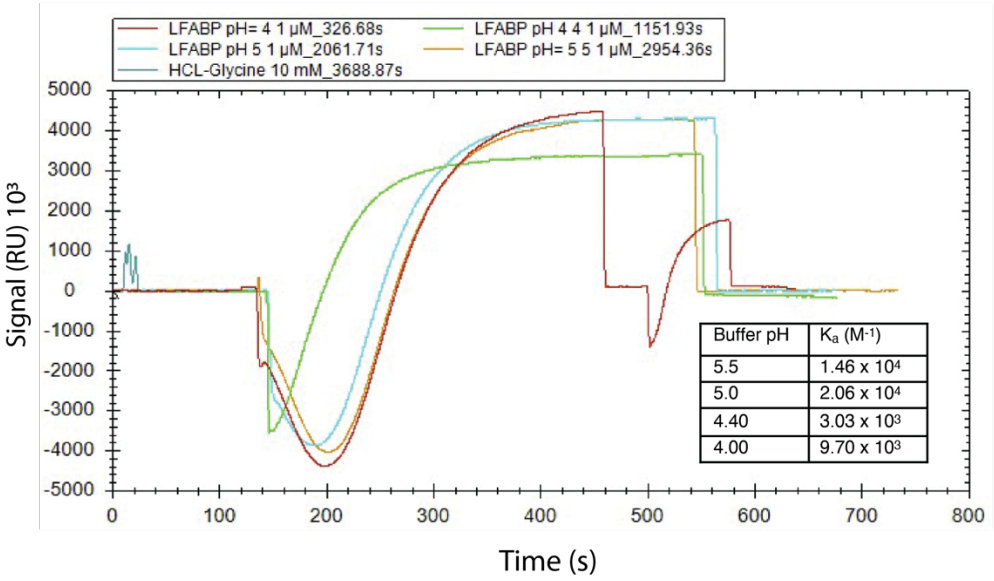

Figure 10: Optimizing PBS buffer pH for concentration of L-FABP at carboxyl chip surface.

L-FABP Preconcentration with Acetic Acid-Acetate Buffer. A different buffer was selected to evaluate whether it could provide a better signal by using the same buffer for the running buffer and protein buffer. Similar to the previous experiment, an LFABP concentration of 1 µM was used, prepared in 4 solutions of acetate of different pH values: 4, 4.4, 5 and 5.5 (Figure 11).

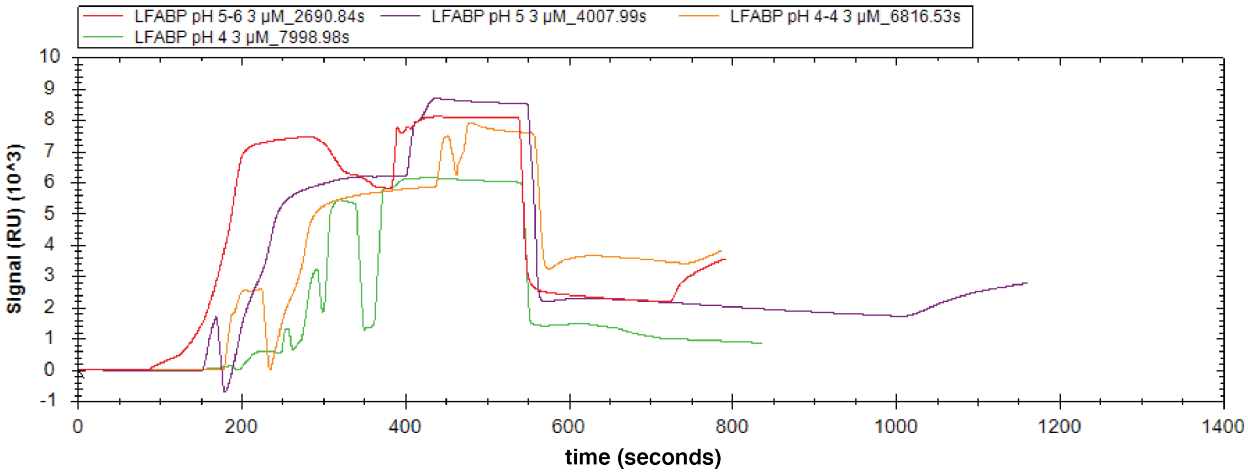

Figure 11: Optimizing acetate buffer pH for concentration of L-FABP at COOH chip surface.

For this acetate buffer system, a pH 4.0 buffer gave the highest K_a but with a poor non-linear fit. Therefore, pH 4.4 was selected as optimal.

L-FABP immobilization on carboxyl sensor chip. Once good pre-concentration of the protein was achieved near the chip surface, immobilization was evaluated using recommended reagents. 10 mM HCl was injected to activate the sensor surface. Then a mixture of EDC-NHS ((N-ethyl-N′-(3-(dimethylamino)propyl)carbodiimide/N-hydroxysuccinimide) was injected to activate the carboxyl groups

on the sensor surface and form an amine-reactive O-acylisourea intermediate. This intermediate reacts with primary amines on the protein to form an amide bond in addition to an isourea byproduct. The intermediate is unstable in aqueous solutions; failure to react with an amine will cause hydrolysis of the intermediate, and the regeneration of the carboxyl group. The L-FABP was then injected in channel 2 and directly immobilized on the sensor chip (Figure 12). A blocking solution was used to ensure L-FABP remained on the chip during further experiment. For optimum solvent-matching of signals through the sensor chip, L-FABP was diluted in the same acetic acid-acetate buffer used for working solutions.

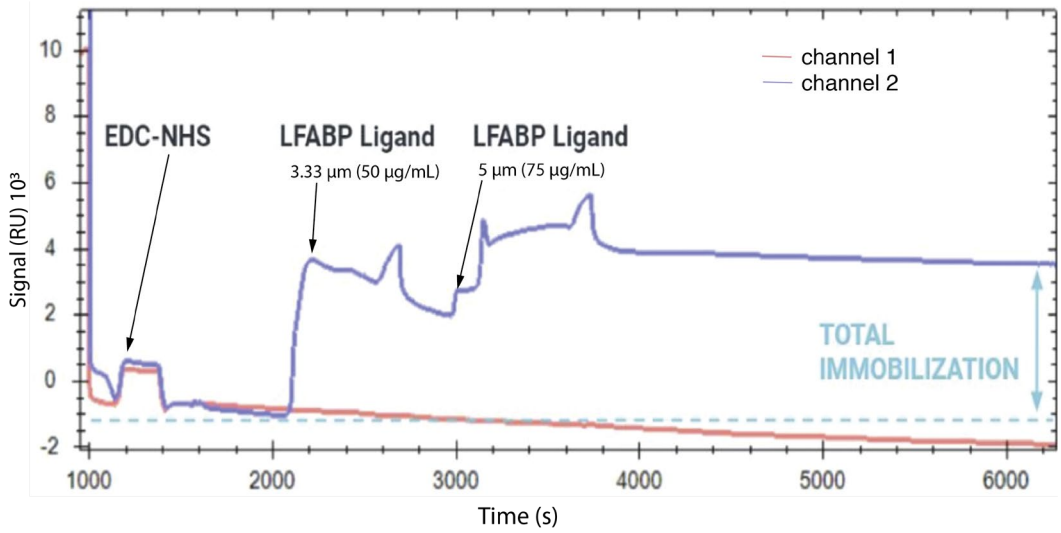

Figure 12: Immobilization of L-FABP on high-sensitivity carboxyl chip using EDC-NHS solution.

L-FABP immobilization was performed twice: at a concentration of 3.33 µM and a concentration if 5 µM, to ensure enough protein for binding.

Protein activity check. An injection at the highest PFOS concentration (1 mM) was performed to confirm the activity of the protein (Figure 13). The increased signal in channel 2 indicates that the immobilized LFABP is active.

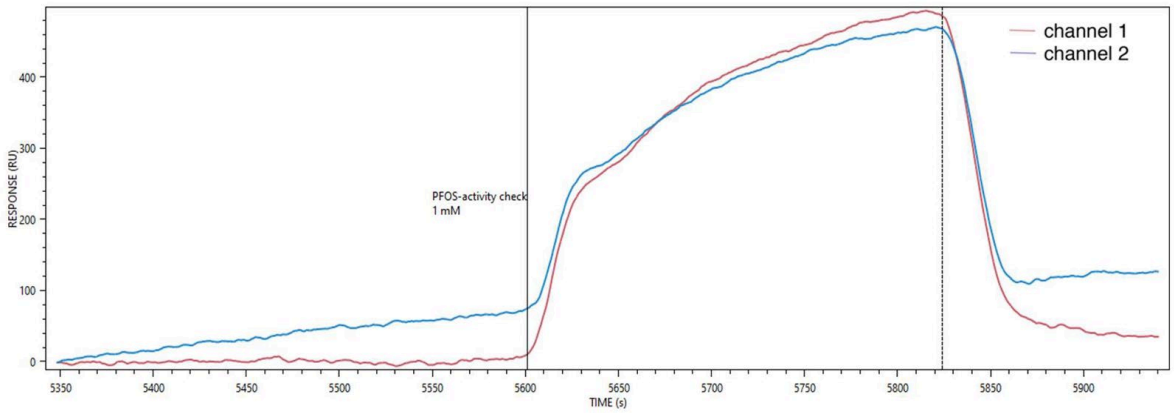

Figure 13: Binding activity of the immobilized L-FABP was confirmed by injecting a high concentration of PFOS. The shift in the signal shows binding to the protein. Similar responses in Channel 1 (reference channel) and Channel 2 (active channel) indicate that the signal is due to non-specific binding.

Evaluating PFOS binding at different concentrations. For determination of K_D and binding kinetics, multiple injections of PFOS are needed at different concentrations. However, initial tests showed minimum binding at concentrations of PFOS below 1 mM (Figure 14).

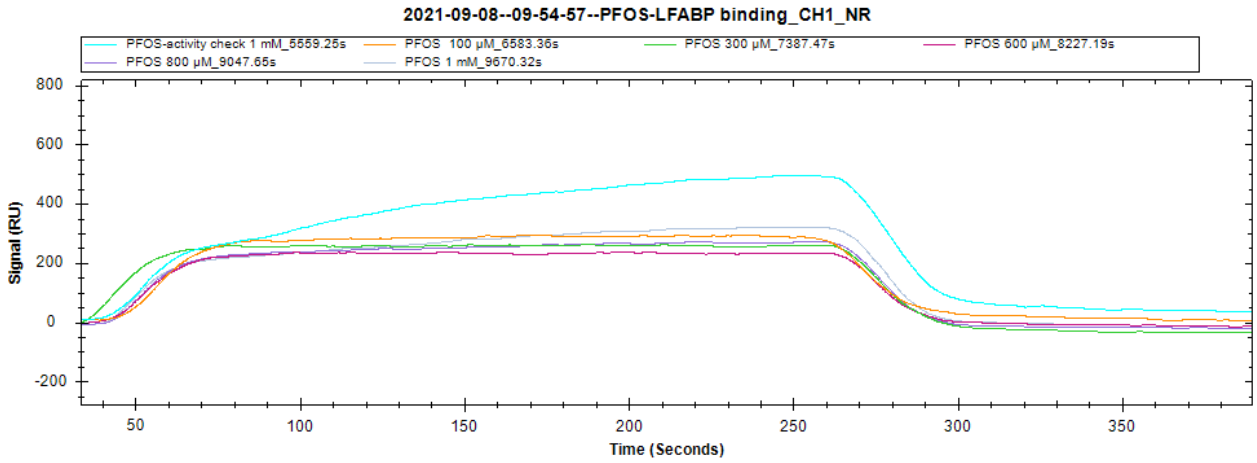

Figure 14: Binding experiment with L-FABP at multiple PFOS concentrations. Higher signal achieved at 1mM concentration due to non-specific binding.

The binding detected at 1 mM is likely due to non-specific binding and aggregation, rather than binding associated with the L-FABP binding site. This indicates that further optimization of the protocol is needed to enhance sensitivity as well as to ensure activity of L-FABP and that the PFAS injected is able to access the binding pocket. This may be an issue with non-specific immobilization of the L-FABP on the carboxyl chip surface, because some protein may be bound in a manner that blocks the binding pocket. Because of this, we are considering the use of other chip technologies, such as the NTA chip, which can covalently bond to His-tagged proteins. This covalent bonding can both provide a better platform for protein concentration and, provided the tag is placed away from the binding pocket, ensure that PFAS can access the specific binding site on the protein. Results of these ongoing experiments will be included in our final project presentation.

2.4 TASK 5.1: THERMAL REGENERATION TESTING

As this task was connected with the dialysis and column experiments, no further thermal regeneration experiments were conducted as the results would have been inconclusive in the absence of binding. Denatured protein controls included with all dialysis experiments confirmed no binding when proteins were heated to 55 °C.

2.5 TASK 5.2: pH REGENERATION TESTING

As this task was connected with the dialysis and column experiments, no further pH regeneration experiments were conducted; the results would have been inconclusive in the absence of binding. However, it should be noted that SPR method development has suggested that pH changes can indeed be used to desorb PFAS, after which the protein should be reusable once again (see Task 4.2 above). NaOH is used to

disrupt PFAS non-specific binding to the sensor surface, and we will test whether this also serves as a means to desorb PFAS from the protein for subsequent protein reuse.

2.6. TASK 6.1: STABILITY TESTING

As this task was connected with the dialysis and column experiments, no further stability testing was conducted as the results would have been inconclusive in the absence of binding. However, a sample of L-FABP has been incubated with a real (but PFAS-free) groundwater sample, and will be used to evaluate stability after several months kept at room temperature and under 4 °C refrigeration once issues with the dialysis experiments have been resolved *or* once the SPR protocol has been sufficiently developed, as either platform can be used to test protein stability.

2.7. TASK 7.1: MOLECULAR MODELING

To supplement the experimental Tasks, we performed additional molecular modeling to identify additional proteins that were promising for future experimental exploration as sorbents for short-chain PFAS. To focus on ability to bind short-chain PFAS, the Protein Data Bank (http://rcsb.org) was searched on the basis of known interactions with short-chain fatty acids. That is, proteins were first selected for their association with butyrate (four-carbon saturated fatty acid), pentanoate, hexanoate, and octanoate. From this initial screening, we further selected only proteins with molecular weights close to that of fatty acid binding proteins or smaller. Our experience in these two limited-scope projects highlighted smaller proteins as easier to produce and therefore more cost-effective. Based on these selection criteria, five proteins were selected for additional modeling (Figure 15).

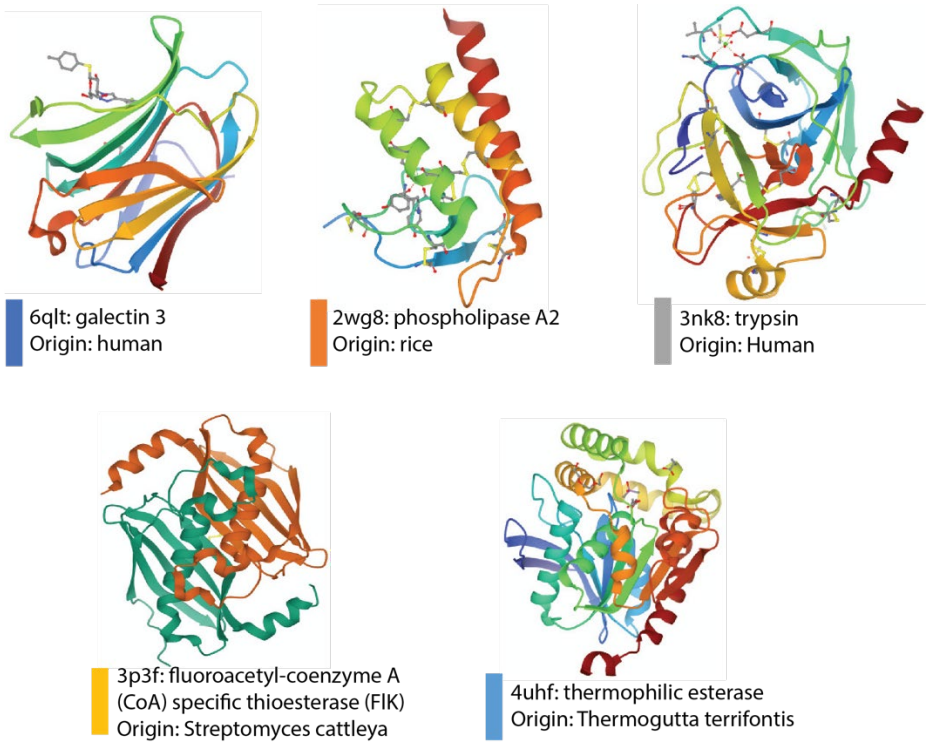

Figure 15: Proteins selected for further evaluation by molecular modeling. Column colors correspond to predicted association constant data bars in Figure 16.

Relative binding affinities of these proteins to short-chain PFAS were predicted using Autodock vina for PFBA, PFPeA, PFHxA, PFBS, and PFHxS. To benchmark these results we also included PFOS and PFOA as long-chain PFAS representatives. Autodock vina outputs, free energies of binding in kcal/mol, were converted to equilibrium association constants in M^{-1}. Several proteins were identified as promising targets for future evaluation (Figure 16). In particular, the phospholipase A2 (pdb ID: 2wg8) from the rice plant showed high predicted affinity for several short-chain PFAS, especially PFPeA and PFBS, with strong association constants (K_A) higher than 10^5 L/mol for all PFAS simulated except for PFBA. Moreover, three specific amino acid residues were identified as important for the strength of the association constant: TYR37, GLY39, and HIS-61. The latter two were key contacts for both PFPeA and PFBS. The plant origin of this enzyme may also indicate it will be easier and less expensive to isolate and/or produce than the mammalian proteins that have so far been the focus of our work. The rice PLA2 was selected due to its role in lipid metabolism and known association with fatty acids such as octanoate[2].

Nearly as promising are the fluoracetyl coA-specific thioesterase (3p3f) from *Streptomyces cattleya* (a gram-positive bacterium), and the thermophilic esterase (4uhf) from Thermogutta terrifontis, a thermophilic bacterium isolated from a terrestrial hot spring.

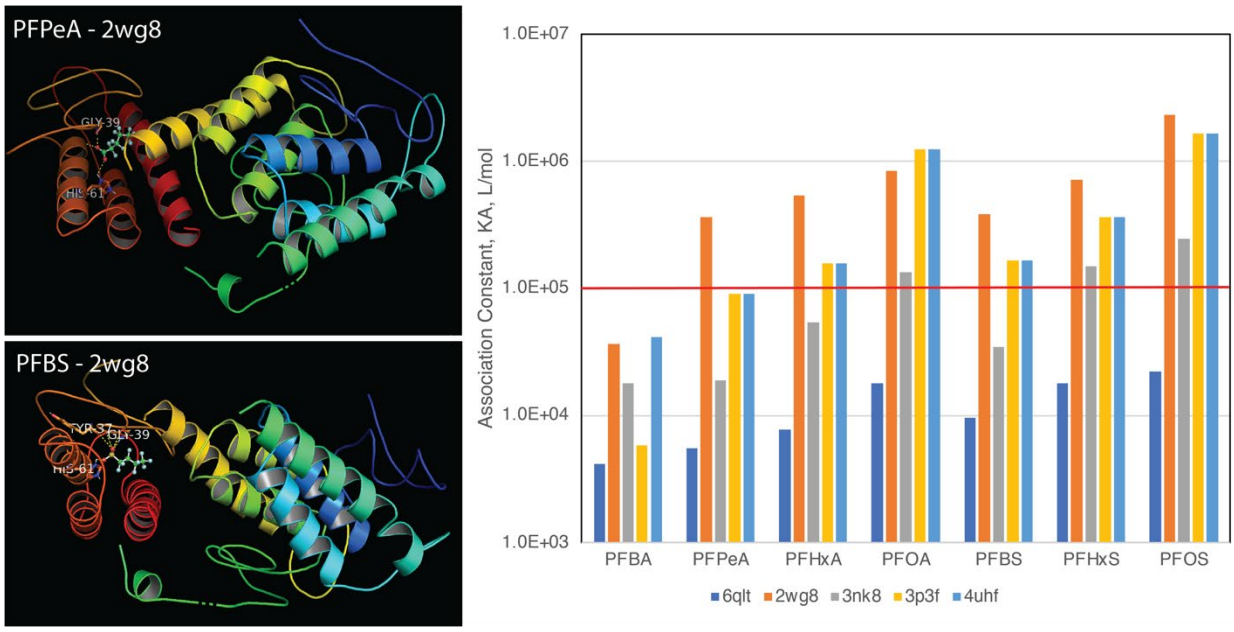

Figure 16: Molecular docking predictions for selected proteins with short- and long-chain PFAS.

These nonhuman enzymes have never been investigated for PFAS-binding activity but may point to new avenues of discovery for short-chain sorbents. Despite these successes, PFBA continues to pose a challenge to identification of sorbents as none of the tested proteins showed high predicted binding affinity for this short-chain PFAS. However, K_A was substantially higher than 10^4 for the phospholipase A2 and for the thermophilic esterase

3.0 CONCLUSIONS AND FUTURE DIRECTIONS

While this one-year limited scope project experienced substantial and ongoing disruptions in laboratory access, personnel availability, and robustness of the scientific supply chain due to the COVID19 pandemic, we were able to pivot to complementary methods and make progress in computational protein identification. While there were as yet unexplained difficulties with replicating equilibrium dialysis studies, recent dialysis results indicate (see Figure 5, with well-defined K_D that agrees with previous dialysis efforts) that some of these issues are now being resolved and work can continue to refine PFAS-protein K_Ds and determine binding kinetics. In addition, in pivoting to SPR method development when the column experiments were suspended, we have identified a promising new direction of research to directly measure PFAS-protein binding kinetics and simultaneously evaluate the stability, regenerability, and overall robustness of different protein candidates.

4.0 LITERATURE CITED

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