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Little is known about how changes in chain length, ionic strength, and pH affect the binding of perfluoroalkyl substances to biomolecules. Using a convenient and quantitative analytical method, this work identifies substantial differences in these interactions for different proteins. Such information can guide the development of biomolecular removal strategies and improve our understanding of the biological effects of these compounds.

ARTICLE

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Proteins as Adsorbents for PFAS Removal from Water

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As industries replace perfluorooctanoic acid (PFOA) and perfluorooctanoic sulfonic acid (PFOS) with short-chain perfluoroalkyl substances (PFAS), these more water-soluble short-chain compounds are emerging as persistent environmental contaminants. Like PFOA and PFOS, health effects on humans and wildlife also occur with these industrial alternatives. However, their removal from water is more challenging due to their increased hydrophilicity. Herein, we describe a material discovery approach using commercially available proteins, molecular weight cutoff (MWCO) filter devices, and Liquid Chromatography Mass Spectrometry (LCMS) to identify proteins capable of extracting shorter chain compounds. Depletion with PFOA was first attempted to establish a reliable screening method. The method developed identified proteins that could deplete hexafluoropropylene oxide dimer acid (HPFO-DA), perfluorobutyric acid (PFBA), and perfluoro butane sulfonic acid (PFBS) from water. Bovine serum albumin (BSA) and lysozyme performed the best at 84 – 99% removal of PFOA, HPFO-DA, PFBA, and PFBS from relevant water matrices with starting concentrations of 250 ppb. In addition to identifying new candidates for the removal of PFAS, this approach allowed for comparative protein analysis to arrive at protein properties contributing to PFAS binding.

Introduction

PFOA and PFOS (**Fig. 1a**) have been phased out of use in the U.S. since 2006, yet their high stability makes their presence in the environment an ongoing issue.[1–3] Furthermore, their replacements, like short chain (< C6) perfluorocarbons (HPFO-DA) (**Fig. 1b**), have raised new concerns for possible health risks.[4,5] In fact, there are over 5000 perfluoroalkyl compounds still in use, many with toxicological effects still unknown.[6] Moreover, shorter-chain compounds (**Fig. 1b**) provide a greater removal challenge due to their increased water solubility over traditional C8 PFAS,[7,8] and the known PFOA/PFOS removal strategies are not sufficiently active to reduce the compound concentrations to target levels. This creates a growing need for different ways to remove these new contaminants, particularly assays that can rapidly evaluate their binding to biomolecules.[9,10]

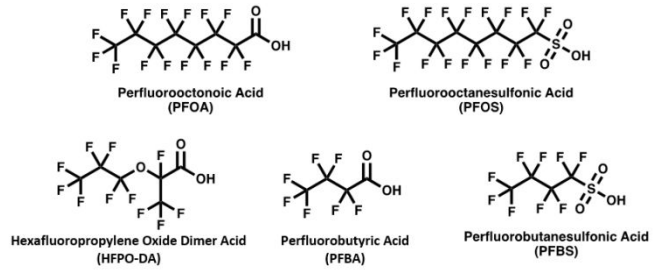

Fig. 1 a) Long-chain PFAS pollutants. b) Short-chain PFAS pollutants.

Common remediation practices to reduce PFOA and PFOS concentrations include sorption strategies using granulated activated carbon (GAC), polymer resins or chitosan beads,[11] and filtration strategies using nanopore membranes.[12,13] Additional methods, such as sonochemical destruction,[14,15] bacteria- or fungi-mediated biological degradation,[16,17] MXenes,[18] MOFs,[19] and zero valent iron composites[20] also show promise. Although 90% of PFAS can be removed by the most effective strategies,[14] shorter-chain contaminants have proven to be more difficult to remove by methods dependent on PFAS hydrophobicity. Instead, removal strategies exploiting other chemical properties are more promising, such as positively charged covalent organic frameworks (COFs) capable of electrostatically removing negatively charged short chain PFAS from water with > 90% efficiency.[7,21] However, the chemical diversity of PFAS compounds is unlikely to be addressed by any single remediation method. In addition, samples may be found in complex matrices, including background ions and humic acid, varied pH values, and other factors that can interfere with a given removal process.[22,23] New methods will need to rely on multiple chemical properties to be widely applicable to diverse compounds and diverse matrices.

Proteins are a material class capable of addressing many of these challenges.[24–28] Whether purchased, isolated, or engineered, proteins provide unrivaled levels of diversity and compound specificity that are difficult to achieve in GAC or synthetic materials. They are active in aqueous matrices relevant to environmental remediation. In addition, many proteins can be produced on very large scale at low cost. Indeed, there are reports of binding

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interactions between C8 PFAS and proteins, such as human serum albumin (HSA) and bovine serum albumin (BSA).[29–33] However, very little is known about the interactions of other proteins with these contaminants. Moreover, even serum albumins have not been evaluated for their ability to remove PFOA and PFOS from water samples, and their ability to bind to shorter chain PFAS (<C8) has not been explored. To evaluate their binding abilities in the context of a wider range of pollutants, and to determine whether other proteins can also sequester these compounds, we report herein a reliable screening approach for identifying protein-based PFAS absorbates in realistic water samples. This approach revealed which proteins were the most appropriate for PFAS removal from a given water source, suggesting the potential of this screening method for discovering new material candidates for future applications. These results show that BSA interacts with a number of PFAS, and that the chain length affects binding. These studies have also identified lysozyme as a second candidate for the removal of short chain PFAS from tap water. In both cases, these proteins could serve as components of environmentally benign materials with enhanced abilities to remove these persistent compounds from a variety of water sources.

Results and Discussion

Protein and Analyte Candidates. Serum albumins are known to bind to both PFOA and perfluorooctane sulfonic acid PFOS, enabling their prolonged circulation in humans and animals.[26, 29-35] Chen et al. observed that PFOA binds preferably to sub-domain IIA in BSA, while PFOS binds preferably to sub-domain IIIA.[39] Also, PFAS accumulates and distributes differently across several species, [2,40,41] while ionic strength and pH can modulate PFAS binding to these proteins, too.[41]

We leveraged this knowledge of PFAS binding preferably to serum albumins under physiological conditions towards PFAS removal from water. Specifically, we sought to develop an approach to identify protein/PFAS/matrix condition combinations in which removal of these contaminants occurred. PFAS pollutants are found in a wide variety of environmental contexts, and thus it is important to evaluate their binding ability in solutions with varied ionic strengths and compositions. Several different water types were screened: 18.2 MΩ•cm (25°C) purified water, local tap water, a creek water sample, buffered solutions at pH 7.2 and pH 8.0, an ocean water sample, and high-ionic strength water at pH 7.2 (60 mg/mL NaCl), and pH 8.0 (60 mg/mL NaCl). These solutions reflected commonly accepted sources and conditions where PFAS can accumulate.[41,42] Initial "contaminated" water samples were prepared with starting PFAS concentrations of 250 ppb.

In addition to BSA, casein, egg white albumin, lysozyme, and RNase A were screened. These proteins are readily available at low cost and represented a range of potentially useful properties for PFAS binding. Casein can form micelle structures with an inner region that could be desolvated and where fluorinated contaminants could accumulate.[42] Egg white albumin is an alternative to serum-based sources. Lysozyme (pI = 11) and RNase A are positively charged at relevant pH values[44,45] and therefore may be able to interact electrostatically with the many anionic PFAS. Lysozyme also has a demonstrated utility in biomaterial development.[11,45]

Contaminants studied included PFOA, HPFO-DA, PFBA, and PFBS. PFOS was excluded due to assay incompatibilities (see below). As PFOS and PFOA are discontinued, HPFO-DA and similar compounds have become more prevalent.[22,46,47] Shorter-chain (< C8) PFAS, like PFBA and PFBS, were chosen because their removal with traditional methods has proven ineffective.[12] Their carboxylate and sulfonate functionalities also provided a useful comparison point, allowing for further rationalization of the depletion differences.

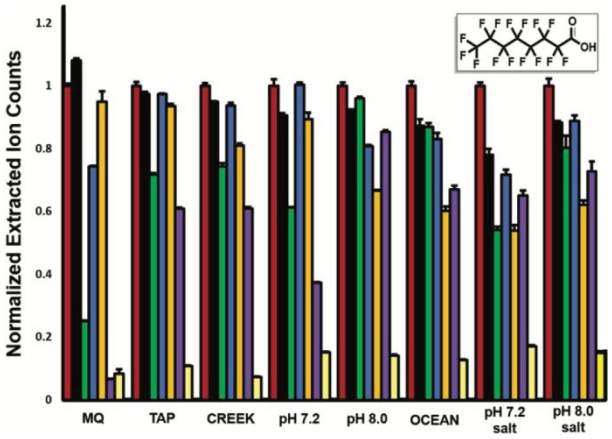

Fig. 2 Normalized extracted ion count (EIC) for PFOA $[M-H]^-$: 412-414 Da. Starting concentrations were 250 ppb with 3.5 µM protein. Red: starting sample, no protein (no filter); Black: MWCO filter only, no protein; Green: RNase A; Blue: casein; Orange: egg white albumin; Purple: lysozyme; Yellow: BSA. EICs reflected the amount of PFOA present in solution after incubating with a protein and passing solution through the MWCO filtration device. Normalized to mean ion counts of starting sample (no filter), the control where PFOA did not pass through the filtration device (std. error, n=3).

Inert Filtration Device. PFAS binding was assessed by adding proteins to a given protein and incubating overnight. Following this, the proteins were removed using a molecular weight cutoff (MWCO) filtration device that would not interfere with the target analytes. The filtration device consisted of a sample reservoir and a high-density polypropylene receiving vial. A medical-grade rubber O-ring formed a water-proof seal between the polycarbonate reservoir wall and the acetal reservoir base. A filter (regenerated cellulose) was housed between the O-ring and base. To determine which (if any) device components caused unintended signal loss, solutions of PFAS (250 ppb) were incubated individually with device components (100 µL, RT, 30 min) and analyzed by LCMS. The filtration device was inert to PFOA, HPFO-DA, PFBA, and PFBS, but PFOS showed background binding to the polycarbonate and was not evaluated further.

PFOA Removal. BSA (3.5 µM) removed 83% - 92% PFOA from all matrices, including creek water and ocean water (**Fig. 2**). Although PFOA is negatively charged, the presence of high concentrations of cationic spectator ions proved to have little influence. It is notable that the binding ability of BSA was not altered by increases in ionic strength (to 60 mg/mL) or changes in pH. Thus, BSA binding these compounds in non-physiological conditions suggested that BSA and engineered congeners can work as viable candidates for downstream, water remediation efforts.

In Milli-Q water, lysozyme (3.5 µM) worked comparably to BSA (93% removal of PFOA) but otherwise proved less effective in different water conditions. Despite this weakening, lysozyme worked reasonably well at pH 7.2 (62% removal) and bound less PFOA as the pH increased to 8.0 (14% removal). Increased ionic strength at pH 7.2 and 8.0 also reduced depletion, suggesting that electrostatic effects between the positively charged protein and chloride in solution may interfere. Similarly, RNase A removed 75% of PFOA from Milli-Q water but was much less effective in different aqueous solutions. In tap and creek water, depletions between 25-28% were observed. Under buffered conditions, PFOA binding decreased with increasing pH (39% removal at pH 7.2, 19 % at pH 8.0) (**Fig. 2**).

Casein demonstrated slight depletion in Milli-Q water and only achieved similar levels of removal in buffered (pH 7.2), high salt conditions (28% removal). Egg white albumin was most effective at removing PFOA from saltier, buffered conditions (37%-47%), but failed to remove any from pure or tap water (**Fig. 2**). Depletion improved in less pure water sources, like creek water, while remaining unchanged in tap water.

HPFO-DA Removal. BSA removed HPFO-DA to comparable levels as PFOA, but only with 100-fold more protein (350 µM). We are unaware of any prior evidence of HPFO-DA binding to BSA and are thus

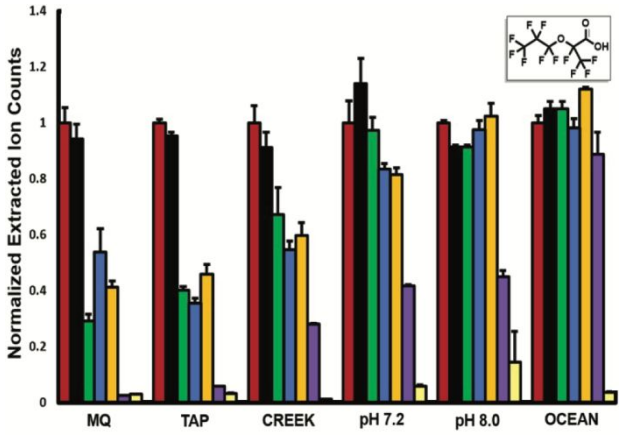

Fig 3 Normalized extracted ion count (EIC) for HPFO-DA $[M-H]^-$: 328-330 Da. Starting concentrations were 250 ppb with 350 µM protein. Red: starting sample, no protein (no filter); Black: MWCO filter only, no protein; Green: RNase A; Blue: casein; Orange: egg white albumin; Purple: lysozyme; Yellow: BSA. EICs reflected the amount of HPFO-DA present in solution after incubating with a protein and passing solution through the MWCO filtration device. Normalized to mean ion counts of starting sample (no filter), the control where HPFO-DA did not pass through the filtration device (std. error, n=3).

not surprised that such high concentrations are required to achieve binding. Like PFOA, removal from water occurred consistently across all water types (**Fig. 3**), possibly suggesting a stabilization in protein structure in all matrices, like PFOA. Lysozyme removed HPFO-DA comparably to BSA in Milli-Q water, at 96% and 91%, respectively. Removal also happened readily in tap water with 91% removal (**Fig. 3**). However, in less pristine matrices, like creek water, BSA outperformed lysozyme by 23%. RNase A worked reasonably well in Milli-Q and tap water (69% and 53%, respectively), but depletion worsened in high pH and salt concentrations. The same trend was seen with BSA and lysozyme. Casein and egg white worked similarly across water types with depletions between 35% and 57% (**Fig. 3**).

Short-chain PFAS. Current remediation methods, like the use of charcoal and resins, have struggled to remove short-chain contaminants (< C8-chain length) when compared to PFOA or PFOS.[6,12,48,49] This difference may be explained by the greater solubility and dispersity in the environment observed for short-chain PFAS.[50] These challenges therefore highlighted a need for improved water remediation approaches. Proteins could provide an attractive possibility for this due to the large number of candidates that can be purchased, engineered, or isolated from natural sources. To test the feasibility of short-chain removal, proteins studied for PFOA and HPFO-DA were also screened for removal of PFBA and PFBS. The parameters and concentrations chosen were based on those used for long chain depletion experiments to allow data comparison and make conclusions from a consistent set of results. In addition, concentrations were chosen to be within the sensitivity range of available instrumentation.

PFBA Removal. Interestingly, BSA generally did not remove PFBA as well as it did PFOA and HPFO-DA. Like HPFO-DA, a higher concentration of protein (350 µM) was required to achieve depletion values comparable to that of BSA/PFOA. In Milli-Q water, lysozyme performed significantly better, removing 78% of PFBA while BSA only removed 23%. The trend held for tap water, and comparable depletions were observed in creek water for both proteins (**SI-Fig. 19**). Removal of PFBA was appreciably hindered in high ionic strength environments, such as ocean shore water or high-salt buffers, generally for all proteins studied. RNase A worked second best in Milli-Q water and performed slightly worse in tap water, creek water, and buffers. Egg white albumin and casein overall removed the least amount of PFBA (**SI-Fig. 19**). For the set of proteins and contaminants studied, PFBA proved the most challenging to remove. Increased water solubility, relative to PFOA and HPFO-DA, may help explain these results. Additionally, since the compound is smaller, a high affinity binding pocket may not exist under the conditions tested. As a result, PFBA removal may be more sensitive to the presence of other contaminants in water (i.e., ionic species, buffers, and salts).

PFBS Removal. Like PFBA, lysozyme removed more PFBS (82%) relative to BSA (70%) in pure water, but the relative performance flipped in tap water (75% removal by BSA, 68% by lysozyme). BSA also performed the best in creek water when compared to lysozyme. As the pH and ionic strength increased, removal with BSA improved, achieving 93% removal at pH 8.0 and high salt (**Fig. 4**). Depletion levels reduced in creek water, salt-free buffer, and ocean shore water for lysozyme (41%-60% removal). In saline buffer, lysozyme removed between 78-80% of PFBS from solution. For RNase A, removal occurred between 21% and 41%. The greatest removal happened in saline pH 7.2; otherwise, in high ionic strength environments, RNase A depleted to similar levels to salt-free environments. Casein and egg white albumin comparably depleted the least (**Fig. 4)**.

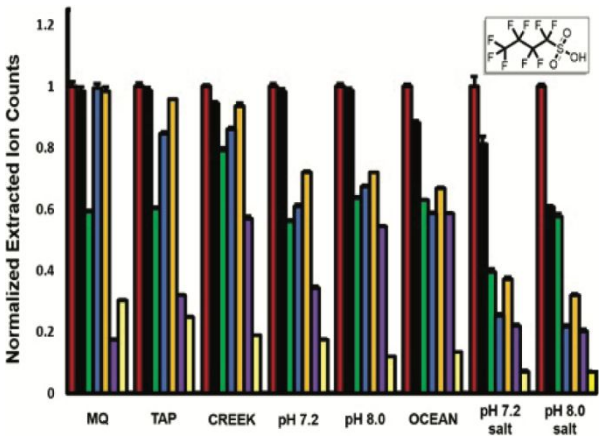

Fig. 4. Extracted ion count (EIC) for PFBS $[M-H]^-$: 298-300 Da. Starting concentrations were 250 ppb with 350 µM protein. Red: starting sample (MWCO (no filter)); Black: MWCO filter only, no protein; Green: RNase A; Blue: casein; Orange: egg white albumin; Purple: lysozyme; Yellow: BSA. EICs reflected the amount of PFBS present in solution after incubating with a protein and passing solution through the MWCO filtration device. Normalized to mean ion counts of starting sample (no filter), the control where PFBS did not pass through the filtration device (std. error, n=3).

We found that BSA could remove HPFO-DA effectively when used at higher protein concentrations (350 µM protein; 99% and 94% removal in tap and creek water occurred, respectively). Shorter chain compounds proved more challenging but were still removed by both BSA and lysozyme. Moderate depletion of PFBA from Milli-Q and tap water (78% and 64% removal, respectively) was achieved with lysozyme. Both BSA and lysozyme removed about half of the PFBA from creek water. For PFBS, lysozyme performed the best in Milli-Q water (82 % removal), but BSA depleted more in tap and creek water (75% and 81% removal, respectively). Removal efficiencies by other proteins were more dependent on pH/or ionic strength. Lysozyme also exhibited sensitivity to these conditions, but consistently worked better than casein, egg white albumin, and RNase A, particularly at removing PFBA.

The list of proteins and analytes used in this study represented a sampling of the possible contaminant and biomolecule combinations that could be explored. Key information such as electrostatic effects and hydrophobic interactions can be tested by selecting desired contaminants and proteins to compare. In our study, our selections suggested a new material candidate for PFAS removal (lysozyme), especially for more difficult to remove PFBA. This protein was previously not identified for PFAS binding, which most likely benefited from the electrostatic interactions with the contaminant, and it is used extensively as a cost-effective candidate in material design. BSA worked well even in high salt concentrations, suggesting that competing ions did not interfere and hydrophobic interactions play a strong role. However, for most proteins binding was reduced at high concentrations of salt, suggesting that binding of PFAS most likely resulted from electrostatic interactions. Although selectivity was not the focus of this study, the use of creek water presented opportunities to confirm that representative concentrations of competing binders did not hinder in PFAS binding.

Due to the large number of PFAS contaminants present in the environment, the vast chemical space available with proteins, we believe that rapid and accessible screening approaches for protein/PFAS binding, like the one presented here, will continue to grow in relevance.

Conclusions

In this report, we describe an accessible approach for the screening of proteins for PFAS removal from water. By using commercially available filtration devices, proteins, and LCMS, candidates for extracting PFOA, HPFO-DA, PFBA, and PFBS were found. BSA worked the best at removing PFOA, corroborating literature accounts of its binding to these compounds. However, the results described herein add a rich layer of information pertaining to BSA stability toward its application in removing PFOA from tap (89% removal) and creek water (92% removal). This new approach thus facilitates exploration of previously untested proteins and PFAS contaminants for unidentified binding interactions.

Conflicts of interest

There is no conflict of interests to report.

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