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In Vitro Interactions of Biological Nucleophiles with Fluorotelomer Unsaturated Acids and Aldehydes: Fate and Consequences

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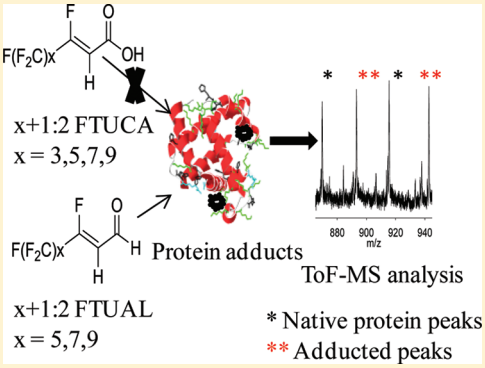
Supporting Information

ABSTRACT: Fluorotelomer unsaturated aldehydes and acids (FTUALs and FTUCAs) are intermediate metabolites that form from the biotransformation of fluorotelomer-based chemicals. FTUALs and FTUCAs have been previously suggested to contribute to the toxicity associated with human exposure to fluorotelomer compounds by covalently binding to biological nucleophiles. However, the extent of their reactivity has only been assessed with glutathione. The purpose of the present study was to assess the reactivity of these intermediate metabolites with a series of nucleophilic amino acids and model proteins. In vitro experiments were carried out in an aqueous buffer system to determine the reactivity of nucleophilic amino acids with FTUCAs and FTUALs having varying fluorinated chain lengths. Using ^{19}F NMR spectroscopy to monitor the disappearance of the FTUCAs and FTUAL signals and the production of a fluoride signal, reaction rate constants were determined under pseudo-first-order conditions. The FTUCAs reacted only with cysteine with the following second order rate constants: 3.63 ($\pm$1.37) $\times$ 10^{-5} min^{-1} mM^{-1} (4:2 FTUCA), 1.19 ($\pm$0.91) $\times$ 10^{-5} min^{-1} mM^{-1} (6:2 FTUCA), and 4.56 ($\pm$0.94) $\times$ 10^{-5} min^{-1} mM^{-1} (8:2 FTUCA). The FTUALs were significantly more reactive than any of the FTUCAs with reactivity decreasing in the following order: cysteine $\gg$ histidine > lysine $\gg$ arginine. The following second-order rate constants were obtained: 5.7 ($\pm$4.2) $\times$ 10^{-4} min^{-1} mM^{-1} (histidine), 4.3 ($\pm$1.4) $\times$ 10^{-4} min^{-1} mM^{-1} (lysine), and 1.4 ($\pm$0.73) $\times$ 10^{-4} min^{-1} mM^{-1} (arginine). FTUCAs and FTUALs were also reacted with model proteins to assess their potential for forming covalent adducts. Electrospray ionization mass spectrometry (ESI-MS) was used to investigate the stoichiometry of FTUCAs and FTUALs covalently bound to apomyoglobin (ApoMg) and human serum albumin (HSA). FTUCAs were not reactive, whereas two measurable FTUAL adducts were formed with both ApoMg and HSA at each of the FTUAL chain lengths (6:2, 8:2, and 10:2). This is the first study to probe the reactivity of FTUALs and FTUCAs with nucleophiles other than glutathione, further elucidating possible FTUAL and FTUCA fate within biological systems.



INTRODUCTION

A class of fluorotelomer-based compounds that contribute to the perfluorocarboxylate (PFCA) load found in humans is polyfluoroalkyl phosphate diesters (diPAPs). DiPAPs are used as grease- and water-proofing agents in food packaging materials[1] as well as in personal care and cosmetic products.[2,3] These chemicals have been observed in American blood sera at low ng/mL concentrations,[4,5] and biotransform to PFCAs in rats and microbial systems.[6−8] Another significant source of exposure to PFCAs might be from the inhalation of fluorotelomer alcohols (FTOHs). FTOHs are found as residual chemicals in commercial fluorotelomer-based surfactants and polymers.[9] The biotransformation of an FTOH to a PFCA was first reported by Hagen et al.[10] They observed PFOA as one of the major metabolites produced from the biotransformation of 8:2 FTOH in rats.[10] Additional research has shown PFCAs from FTOH biotransformation in microbial systems,[11−14] rats,[15−18] mice,[17,19,20] and rainbow trout.[17]

PFCAs are the final stable metabolite in the biotransformation of fluorotelomer-based materials. Indirect human exposure to PFCAs from labile precursor materials has generated scientific interest due to the ubiquity and persistance of PFCAs in humans, with PFCA concentrations at ng/mL levels within human sera[7,21] and a reported serum half-life of 3.5 years for the C8 congener, perfluorooctanoate (PFOA).[22] Thus, attention has focused on elucidating the carcinogenic, hepatotoxic, and developmental effects of PFCAs.[23,24] Recent research has also been done to determine the toxicity of PFCA precursor metabolites formed from the biotransformation of fluorotelomer-based compounds.

Fluorotelomer unsaturated carboxylic acids and aldehydes (FTUCAs, $F(CF_2)_7CF{=}CHCOOH$; and FTUALs, $F(CF_2)_7CF{=}CHCHO$, respectively) are metabolic intermediates formed through the biotransformation of diPAPs and FTOHs.[11,15,17,25] FTUCAs and FTUALs were suggested to be responsible for the observed lesions in rat liver following exposures to FTOHs, and contributed to cytotoxicity through

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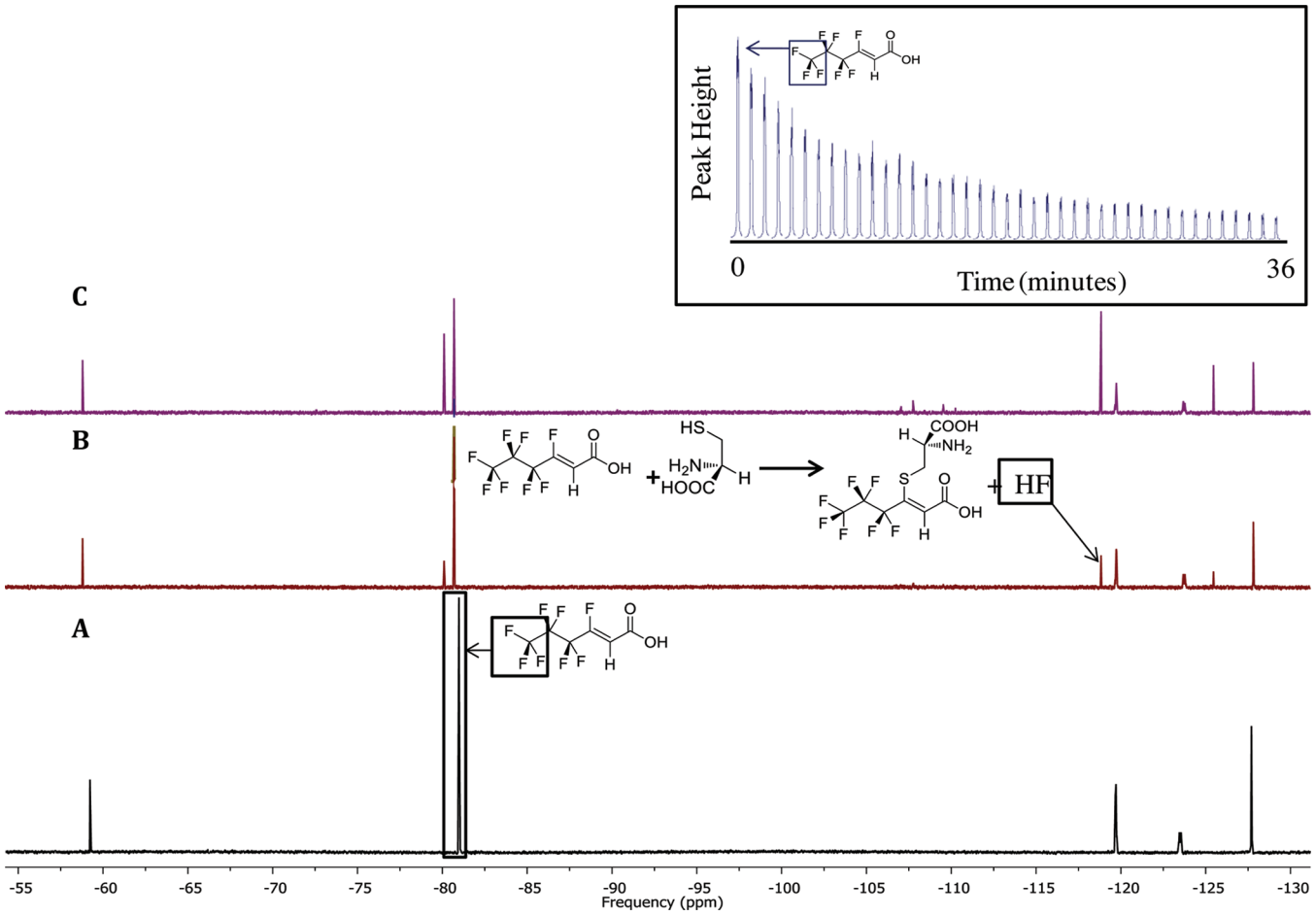

Figure 1. ^{19}F NMR spectra of the 4:2 FTUCA reaction with cysteine at a concentration ratio of 27:1 (cysteine:4:2 FTUCA). (A) ^{19}F NMR spectrum of the unreacted 4:2 FTUCA control, showing the CF_3 group signal (approximately −81 ppm). (B) ^{19}F NMR spectrum of 4:2 FTUCA after 4 min, showing a new peak corresponding to the fluoride product ion (approximately −119 ppm). (C) ^{19}F NMR spectrum of 4:2 FTUCA reaction with cysteine after 36 min. The inset in the black box shows the preacquisition delay ^{19}F NMR experiment with loss of the CF_3 peak corresponding to the 4:2 FTUCA reacting with cysteine. Peak height was normalized to the height of the internal standard, 4-TFMeAc (approximately −58 ppm), to determine the pseudo-first-order rate constant, k_{obs}.

protein carbonylation.[15,16] The FTUCA and its corresponding saturated acid (FTCA) have also been shown to be 1−4 orders of magnitude more toxic than PFCAs to fresh water organisms, with notable toxicity thresholds in the μM range.[26,27] Acute toxicity depends on the length of the fluorinated chain, where tested species were more sensitive to telomer acids with chain lengths of ≥8 fluorocarbons, a trend that was similar to the toxicity trend exhibited for PFCAs.[28,29]

Although FTUCAs and FTUALs might be short-lived intermediates, evidence of their reactivity with biological nucleophilies has been observed; FTUCAs and FTUALs have been shown to form conjugates with glutathione (GSH) through a proposed 1,4-Michael addition reaction.[15,25] The GSH conjugates conserved the initial α,β-unsaturation of the FTUCA and FTUAL structure, which might also pose risk for toxicity from chemical reactivity.[25] Based on their reactivity with GSH, Martin et al. hypothesized that FTUCAs and FTUALs might react with other cellular nucleophiles, including cysteine, lysine, histidine, and nucleic acids.[16,25]

The findings from Martin et al. encouraged our interest in examining the fate of PFCA precursors when determining the risk associated with exposure to fluorinated compounds. The present study explores the potential for these intermediate metabolites to interact with biological functional groups from cysteine, serine, lysine, histidine, and arginine, respectively. There is a general paradigm that relates protein covalent binding of endogenous reactive intermediates to cellular toxicity.[30] This study examines the capability and extent to which these intermediate metabolites covalently bind to two model proteins: apomyoglobin (ApoMg) and human serum albumin (HSA).

MATERIALS AND METHODS

Materials. A list of all standards and reagents used in this study is provided in the Supporting Information (SI). FTUCAs, FTUALs, and the 6:2 FTAL were synthesized by methods described in the SI. Purity of the synthesized materials was determined using ^{1}H and ^{19}F NMR spectroscopy, as described in the SI.

Determination of 8:2 FTUAL and FTUCA Half-Lives Using ^{19}F NMR Spectroscopy. It was important to establish potential reactive centers in proteins by assessing the reactivity of free amino acids with FTUCAs and FTUALs. ^{19}F NMR spectroscopy was chosen to examine the interaction of FTUCAs and FTUALs with amino acids, because it can simultaneously monitor the CF_3 signal corresponding to the FTUCA and FTUAL reactants, the growth of the CF_3 signal corresponding to the bound products, and the growth of the

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signal corresponding to the production of the fluoride ion released. An example of this is shown in Figure 1, where ^{19}F NMR spectra were acquired during the course of a reaction with 4:2 FTUCA and cysteine.

Pseudo-first-order rate constants (k_{obs}) were obtained from monitoring the loss of the FTUCA CF_3 peak, normalized to the height of the internal standard (Figure S1) while keeping the molar concentrations of amino acids at least 10-times greater than the concentrations of the FTUCAs. The 10:1 ratio of amino acids to FTUCA or FTUAL is needed in order to make a valid determination of the rate constant. Although the amino acid, FTUCA, and FTUAL concentrations used for the NMR experiments were likely higher than normal physiological concentrations, the purpose of these experiments was to elucidate the relative reactivity of FTUCAs and FTUALs with amino acids. The second-order rate constants would change with FTUCA or FTUAL concentration, but the relative reactivity should be conserved. For the present study, five amino acids were chosen due to the presence of nucleophilic functional groups in their structures, highlighted in red in Figure S2: cysteine, serine, lysine, histidine, and arginine. As the thiol in GSH has been previously shown to form conjugates with FTUCAs and FTUALs,[15,17,25] cysteine, which also contains a nucleophilic thiol group, was hypothesized to interact similarly. The FTUCAs included the 4:2, 6:2, and 8:2 congeners. NMR studies were performed with the 8:2 FTUAL; the 6:2 and 10:2 FTUAL were not used due to instability and solubility issues, respectively.

Stock solutions of the 4:2 and 6:2 FTUCA were made in phosphate buffer (pH = 7.4), while the 8:2 FTUCA and 8:2 FTUAL were dissolved in methanol. The final concentration within the NMR tube was 10 mM. Each amino acid in phosphate buffer was added to an NMR tube containing the fluorotelomer compound and 10% (v/v) D_2O immediately before NMR analysis, so that the molar ratio of amino acid:fluorotelomer compound was ≥10:1. Due to low aqueous solubility 20% (v/v) MeOH was used for the 8:2 FTUCA and 8:2 FTUAL reactions. Final concentrations were adjusted using phosphate buffer, bringing the total reaction volume of the 8:2 FTUAL and 8:2 FTUCA solutions to 0.77 mL, and the 4:2 and 6:2 FTUCA solutions to 0.60 mL. NMR parameters used for individual experiments are given in Table S1, including relaxation delay times, scan numbers, and total experiment times. Spectra were obtained at 25 °C on a Varian 400 NMR spectrometer with an ATB8123-400 autoswitchable probe tuned to ^{19}F (376.14 MHz).

To determine the k_{obs} values, the normalized height of the CF_3 signal and that corresponding to the fluoride product were monitored. A preacquisition delay experiment was carried out to acquire spectra at repeated time intervals of 0.5 s. The acquisition time was 1 s. Chemical shifts were recorded relative to a 1000 mg/L solution of 4-trifluoromethoxyacetanilide (4-TFMeAc) in methanol ($\delta = -58.1$ ppm), spiked into a capillary tube. 4-TFMeAC was also used as an internal standard to normalize the reactant and product signal heights produced from the reaction. The scanning width chosen was +100 to −230 ppm. To confirm the identity of the fluoride product, aqueous sodium fluoride was spiked into the NMR tube, giving a signal that overlapped with the product from the reaction with amino acids (Figure S3)

In Vitro Modifications of Human Serum Albumin (HSA) and Apomyoglobin (ApoMg) by FTUCAs and FTUALs.[31,32] Fatty acid- and globulin-free HSA (250 μg/mL) and ApoMg (30 μg/mL) were incubated in an aqueous solution, buffered with 10 mM K_2HPO_4 adjusted to pH 7.4. Both proteins were chosen for their structural rather than physiological importance. ApoMg lacks a free thiol group and was used to probe whether other nucleophilic groups react with FTUCAs and FTUALs. HSA contains several nucleophilic residues, including one free thiol (Cys34) which reacts to endogenously generated α,β-unsaturated aldehydes[31,33] and has been used as an exposure biomarker to electrophiles.[34,35] In separate experiments, the 4:2−10:2 FTUCAs and 6:2−10:2 FTUALs were added at the following protein:fluorotelomer compound final molar ratios: 1:(0.25, 0.5, 1, 5). Although actual physiological concentrations of fluorotelomer intermediates have never been reported in human sera, the highest plasma concentration of 8:2 FTUCA as reported by Fasano et al. was approximately 200 ng/mL after a 125 mg/kg dose of 8:2 FTOH.[18] In comparison, concentrations of 8:2 FTUCA used in these experiments were higher, ranging from 200 ng/mL to 4 μg/mL and 400 ng/mL to 8 μg/mL for the ApoMg and HSA binding experiments, respectively.

To dissolve the FTUALs and FTUCAs, acetonitrile and methanol were used, respectively, making up ≤1% of the total reaction mixture. Samples were incubated for 2 h at 37 °C, after which $NaBH_4$ (final concentration 5 mM) was added and incubated for an additional hour to reduce and stabilize Michael or Schiff base adducts with protein nucleophilic sites.[36] The ApoMg incubation mixtures (∼ 1 mL) were immediately desalted using Supelco disposable PD-10 columns (Sigma-Aldrich, Oakville, ON, Canada). The sample volume was increased to 2.5 mL and eluted with 3.5 mL of 0.1% HCOOH to remove unbound analytes and buffer salts. The HSA incubation mixtures (∼ 1 mL) were filtered using Amicon 10 kDa Ultra-4 centrifugal filter units (Millipore, Billerica, MA). The samples were then centrifuged for 10 min at 4180*g* using a VWR Clinical 200 centrifuge (Radnor, PA) and desalted by adding 4 mL of 0.1% HCOOH. This procedure was repeated four times using the centrifuge conditions previously stated. Prior to mass spectrometry analysis, samples were lyophilized and reconstituted in H_2O:ACN:HCOOH (70:30:0.1). Spike and recovery results of the two desalting procedures described previously were obtained using buffered solutions containing 250 μg HSA or 30 μg/mL ApoMg, and were quantified using matrix-matched external calibration. Recovery results for $n = 3$ samples were 107.9 ± 4.6% and 92.1 ± 15.8% for the ApoMg and HSA method, respectively.

Time of Flight Mass Spectroscopic Analysis. To establish changes in protein mass and to determine the number of adducts formed after the reaction, native and treated protein samples were analyzed by manual direct injection (10 μL injection loop, flow rate 100 μL/min) on an AB/Sciex QStar mass spectrometer (Concord, Ontario, Canada) in ESI positive-ion mode. Details regarding mass spectroscopic parameters, including mobile phase, scan range, capillary temperature, and declustering potential, are provided in the SI. Native and adducted protein masses were obtained using Bayesian Protein Reconstruct processing incorporated in the Analyst software, the parameters of which are found in the SI. Mass averages and 95% confidence intervals for the native and adducted proteins were obtained using $n \geq 4$ replicate injections.

Determination of the Strength of Adducts. MS/MS product ion scans were carried out in order to confirm the formation of covalent protein adducts. An m/z value

corresponding to the native proteins was selected and the decrease of intensity was monitored as the collision energy was increased from 15 to 40 V. For example, m/z 998.2 (z = +17) was selected to monitor the loss of intensity corresponding to the native ApoMg control. Next, an m/z value for each adducted protein was selected and fragmented in the same manner as the native protein. For example, m/z 1023.1 (z = +17) was selected as a representative ApoMg adduct with 8:2 FTUAL. Since the collision energy needed to fragment the adducted protein was similar to the native protein, the strength of the protein adducts was attributed as covalent. Noncovalent bonds would be expected to fragment at lower collision energies because they are weaker and thus easier to break. Further details for this method are reported in the SI.

TOF-Combustion Ion Chromatography Analysis. Concentrations of total organic fluorine (TOF) in the HSA-8:2 FTUAL samples were determined using ion chromatography (IC),[37] the details of which are presented in the SI. Briefly, HSA-adducted samples were combusted in a furnace (Automatic Quick Furnance (AQF-100), Mitsubishi Chemical Analytech, Japan) at 900−1000 °C. Sample combustion converted organofluorines into hydrogen fluoride (HF) which was transferred into an absorption unit where HF dissolved into H^+ and F^-. The concentration of F^- in the solution was analyzed using IC (ICS-2100, Dionex Co. Ltd., Sunnyvale, CA, USA). Methanesulfonic acid (CH_3SO_3H) was used as an internal standard. Sample solutions were prepared using 70:30:0.1 H_2O:ACN:HCOOH and sample replicates (n = 3) were achieved at each molar ratio.

RESULTS AND DISCUSSION

^{19}F NMR Pseudo-First-Order Kinetics of FTUCAs and FTUALs with Nucleophilic Amino Acids. All FTUCA congeners were reactive with cysteine, based on the observed loss of the CF_3 peak after comparison to the blank controls. Values of k_{obs} were plotted against varying cysteine concentration to obtain the second-order rate constant and corresponding half-lives (Figures S4−S6). The half-lives, determined based on the 10 mM FTUCA concentration and the second-order rate constants (the equation of which is shown in the SI), were as follows: 57.5 ± 6.4, 80.5 ± 11.7, and 32.6 ± 3.0 h for the 4:2, 6:2, and 8:2 FTUCA, respectively. Due to production of an NMR singlet signal at approximately −120 ppm which corresponded to the fluoride product ion (Figure S3), the proposed mechanism of reactivity was the 1,4-Michael addition followed by elimination of HF to restore the α,β-unsaturated system, as expected from the hard−soft acid−base principle.[38] This principle has been thoroughly addressed in terms of thiol reactivity to α,β-unsaturated aldehydes, ketones, and methyl esters by Bohme et al. who observed a significant difference between the reactivity of varying compound classes with GSH.[39] Differences between reactivity also affect the toxic mode of action associated with exposure to the chemical.[39,40] This mechanism was also observed by Martin et al. from the reaction of the 8:2 FTUCA and FTUAL with GSH.[25] No reactivity was observed with arginine, histidine, lysine, and serine for any of the FTUCAs (Figure S7).

The 8:2 FTUAL reacted with cysteine much more than what was observed for the FTUCAs; determination of the pseudo-first-order rate constant was not possible due to the near-instantaneous decline of the ^{19}F NMR 8:2 FTUAL signal, presented in Figure S8 of the SI. Again, the appearance of the fluoride peak (inset, Figure S8) was consistent with the involvement of the Michael-addition mechanism. This reaction was also confirmed from the absence of the fluoride peak in the controls (8:2 FTUAL and solvent mixture); the appearance and growth of the fluoride peak was observed upon the addition of the amino acid, indicating that the fluoride ion is a product from reaction with cysteine and 8:2 FTUAL.

The reactivity of 8:2 FTUAL with arginine, histidine, lysine, and serine was also quantified by determining the second-order rate constants and corresponding half-lives. As presented in Figure S9, k_{obs} values for reactions of 8:2 FTUAL with arginine, histidine, and lysine were obtained by monitoring the height of the product fluoride peak rather than the loss of the CF_3 peak since the aldehyde reacted with the water to form a hydrate, observed from a shift in peak frequency (δ = −83.5 ppm vs −81.7 ppm for the CF_3 groups corresponding to a hydrate and aldehyde functional group, respectively). Additionally, the signal corresponding to the product aldehyde was not detected, possibly due to its instability in solution arising from the reactivity of the aldehyde with the aqueous solvent mixture. By monitoring the production of the fluoride peak, characterized by the addition of the aqueous NaF solution (Figure S10), it was possible to determine the second-order rate constant corresponding to the Michael addition reaction. The half-lives, calculated from the k_{obs} plots shown in Figures S11−S13, were as follows: 2.9 ± 0.7 h (histidine), 3.9 ± 0.3 h (lysine), and 11.8 ± 0.5 h (arginine) (n = 3). The relative difference in reactivity can be rationalized based on the speciation of each amino acid, since speciation will change nucleophilicity. Speciation depends on the pK_a of each amino acid, where pK_a = 6.5, 10.0, and 12.0 for histidine's imidazole ring, lysine's primary amine, and arginine's guanidinium group, respectively. At pH 7.4, the unprotonated histidine neutral molecules would outnumber the protonated ions by approximately 10-fold. In comparison, both lysine and arginine predominated in their protonated ionic forms, with arginine being approximately 100× more protonated than lysine. Therefore, arginine reacted the slowest with 8:2 FTUAL, and was the worst nucleophile to undergo a 1,4-Michael addition reaction. Histidine, which had a higher fraction of the neutral species at pH 7.4, was the best nucleophile.

The rate of the 1,4-Michael addition generally depends on the nature of the nucleophile; whether the nucleophile is considered "soft" (large, diffuse orbitals, interacting with electrophiles through orbital overlap) or "hard" (tightly held orbitals, interacting with electrophiles electrostatically) greatly determines the reaction mechanism.[38] Cysteine has a "soft" thiol functional group and was presumed to react more quickly via a 1,4-Michael addition, in comparison to lysine, arginine, and histidine, which contain "harder" nucleophilic groups.[31,40] This was consistent with the rapid reaction between cysteine and 8:2 FTUAL that was observed by NMR spectroscopic analysis. Histidine, arginine, and lysine were also observed to react with 8:2 FTUAL by way of a 1,4-Michael addition. In contrast, 8:2 FTUAL was not reactive with serine. At molar concentration ratios of 10:1−27:1 serine:8:2 FTUAL, no difference was observed between the slope of the reacted 8:2 FTUAL CF_3 peak height and that of the control peak (p = 0.952), suggesting low or lack of reactivity between 8:2 FTUAL and serine (Figure S14). These observations might be important when considering the relative reactivity of the FTUALs with amino acid residues in proteins, where cysteine would be the most reactive, followed by histidine, lysine, arginine (although accessibility and pKa's of the nucleophilic

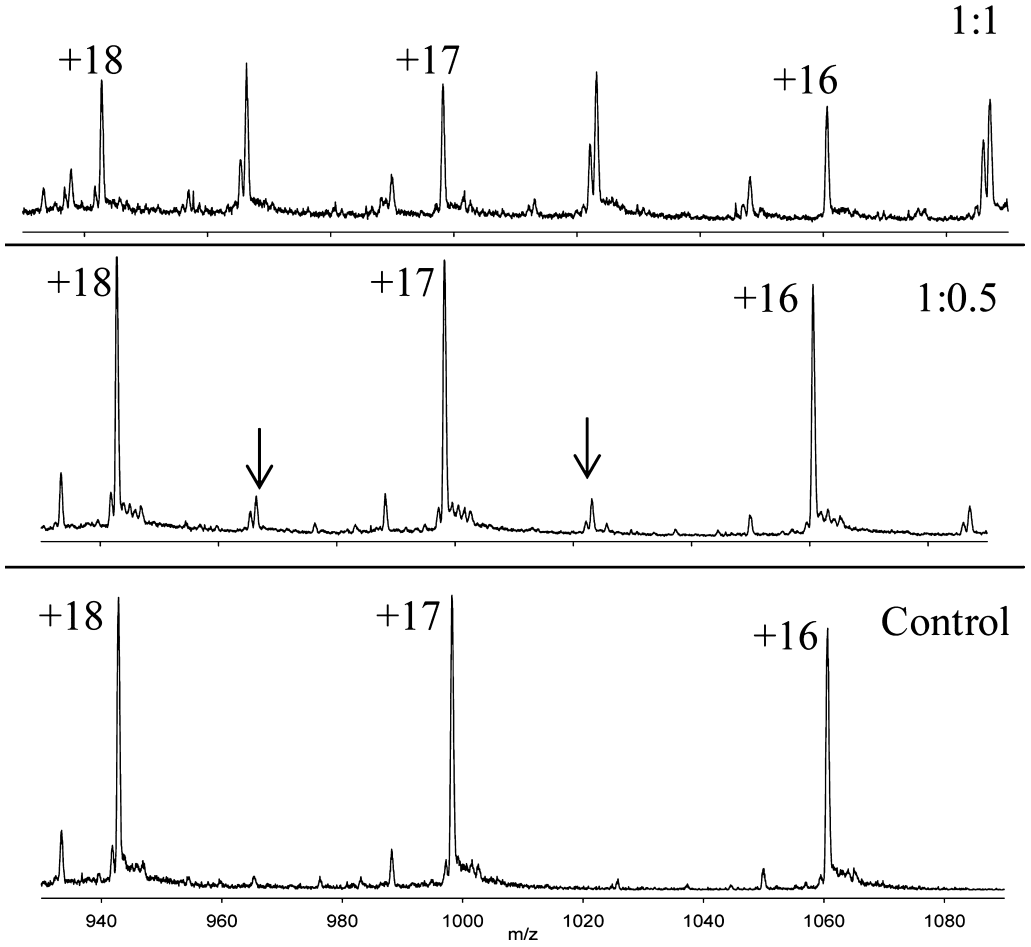

Figure 2. ESI-ToF mass spectra of apomyoglobin and stoichiometry of 8:2 FTUAL adduct formation. Apomyoglobin was incubated for 2 h at 37 °C in the absence (control) and presence of 8:2 FTUAL at the following apomyoglobin:8:2 FTUAL molar ratios: 1:0.5 and 1:1. The arrows in the 1:0.5 spectrum indicate the presence of adducts, which increase in intensity with increasing molar ratio.

amino acids within proteins might also impact reactivity). Lysine, which has a primary amine functional group, might also react directly with the carbonyl group of FTUALs to produce a carbinolamine intermediate first, which upon rearrangement and water loss would yield a Schiff base (imine) (Figure S15). 4-Hydroxy-2-nonenal (HNE), an α,β-unsaturated aldehyde produced from the oxidation of fatty acids, has a chain length similar to 8:2 FTUAL; past studies investigating covalent protein conjugation of HNE have observed reactivity with lysine residues through both a 1,4-Michael addition and to a lesser extent, a Schiff base mechanism.[31,32,41−44] Here, the formation of a Schiff-base adduct with lysine can not be ruled out. This merits further investigation into the reactivity of FTUALs with native proteins.

ToF-MS of FTUCA and FTUAL Adducts with ApoMg. ApoMg was chosen as the model protein for this binding study due to its relatively small size (16951.49 Da) and ease of accurate mass determination using ToF-mass spectrometry. ApoMg contains 36 basic residues, including 12 histidines, 19 lysines, 4 arginines, and the N-terminus. Results from the kinetic experiments showed a lack of reactivity between FTUCAs and amino acids, except for cysteine, which was also observed to react the fastest with 8:2 FTUAL. To establish whether FTUCAs and FTUALs form covalent adducts with lysine, histidine, and arginine residues, ApoMg was an optimal protein to use as it lacks cysteine residues.

ESI-ToF mass spectra were obtained at varying molar ratios of ApoMg:FTUCAs and FTUALs where the concentration of ApoMb was held constant (1:0.25, 1:0.5, 1:1, and 1:5) to determine the stoichiometry of adduct formation at each concentration ratio. The mass spectrum of the native protein contained multiple charge states, each of which corresponded to a protonated site. The adduct stoichiometry was obtained by measuring the increase in protein mass per adduct at each charge state. The deconvoluted spectrum of the native ApoMg gave an average molecular weight of 16 951.90 (n = 8 replicate injections) with a mass accuracy of 24.2 ppm. Additional masses were detected in the native ApoMg spectra, including that which corresponded to the mass of the native protein minus a water molecule (18.01 Da) (16 934.01 Da, mass accuracy = 31.3 ppm).

Spectra for the 4:2−10:2 FTUCAs, even at a molar ratio of 1:5 ApoMg:FTUCA, indicated no new peaks compared to the m/z peaks corresponding to ApoMg control. The lack of reactivity with ApoMg observed here was not surprising, as FTUCAs were not reactive toward any of the amino acids except for cysteine.

The 6:2, 8:2, and 10:2 FTUAL were reacted with ApoMg at varying molar ratios. As shown in Figure 2, two quantifiable adducts were detected at ApoMg:FTUAL molar ratios of 1:0.25, and 1:0.5 for all FTUAL analytes. The synthesis and purification of the 6:2 FTUAL proved to be difficult due to the

instability of the compound[45] which could potentially result in inaccurate quantification of mass adducts with the protein. Since the ^{1}H NMR spectrum of the 6:2 FTAL showed the presence of minor amounts of 6:2 FTUAL as an impurity, and because the FTAL did not react with ApoMg given the mass of the adducts, the 6:2 FTUAL impurity (approximately 10% of the 6:2 FTAL sample) was used to assess binding with ApoMg to produce adducts with the native protein and the ApoMg-H_2O.

The primary adduct for the 6:2 FTUAL had a mass of 17 275.63 ± 0.04 Da (CI 95%, n = 6 replicate injections), which gave a mass difference of 323.73 ± 0.06 Da with respect to the native mass of ApoMg. In relation to the mass of 6:2 FTUAL (MW 342.12 g/mol), the mass difference was equal to 18.39 ± 0.06 Da which likely corresponded to the loss of a fluoride ion (theoretical mass of 18.998 Da) as the leaving group in the 1,4-Michael addition. At the 1:0.5 molar ratio, a second, less-abundant adduct appeared having a mass of 17 257.68 ± 0.03 Da which is presumed to be an adduct formed from the ApoMg-H_2O, due to the resulting mass difference of 323.85 ± 0.04 Da and a difference from 6:2 FTUAL of 19.43 ± 0.03 Da. The same trend was observed for the 8:2 and 10:2 FTUAL where one primary adduct formed in relation to the mass of the native ApoMg, having an adducted mass of 17 375.21 ± 0.04 Da and 17 475.36 ± 0.04 Da, corresponding to one 8:2 FTUAL and one 10:2 FTUAL molecule forming an adduct per molecule of ApoMg, respectively. Each adduct also had a fluoride leaving group with masses of 18.65 ± 0.06 and 18.51 ± 0.04 Da for the 8:2 and 10:2 FTUAL, respectively. Like the 6:2 FTUAL, 8:2 and 10:2 FTUAL also formed an additional adduct with respect to the ApoMg-H_2O mass.

From these experiments, it was concluded that the mechanism of adduction for FTUALs was through a 1,4-Michael addition observed from the loss of a fluoride ion. Bruenner et al. established that, for HNE, greater than 99% of protein modification occurs via a 1,4-Michael addition,[46] therefore it is suggested that the FTUALs might proceed to form protein adducts in the same manner.

ToF-MS of FTUCA and FTUAL Adducts with HSA. HSA is the most abundant protein in blood, present at approximately 30 mg/mL in serum.[47] It is characterized by several nucleophilic amino acid residues, has been known to bind covalently α,β-unsaturated aldehydes,[31,48,49] and represents a favorable target protein for the analysis of adduct formation with FTUCAs and FTUALs. ToF-MS analysis of HSA (7.5 μM, 3.75 nmol) revealed m/z peaks corresponding to the mass of the native protein, at 66 440.55 ± 0.46 Da (CI 95%, n = 6) with an accurate mass error of 23.3 ppm (theoretical mass of 66 439 Da). One additional peak was observed, which corresponded to a mass of 66 557.95 ± 0.87 Da and was attributed to the addition of a cysteine residue through a disulfide bond, with a theoretical mass of 66 557 Da (mass error = 14.3 ppm).

It was initially hypothesized that the FTUCAs would react with HSA due to the presence of one free thiol group from the Cys34 residue in HSA. However, as was observed in the ApoMg experiments, none of the FTUCA congeners formed adducts with HSA under these experimental conditions, even at the HSA:FTUCA 1:5 molar ratio. A second experiment was conducted to determine whether FTUCA interaction with HSA depended on reaction time. Solutions having molar ratios of 1:1 and 1:5 HSA:8:2 FTUCA were left to react for 18 h under the same conditions as previously described. No formation of adducts was detected in the ToF-MS spectra, suggesting the absence of reactivity between 8:2 FTUCA and HSA. Given the results from the amino acid study, which indicated FTUCA reactivity with cysteine, and results from previous studies which showed conjugation of FTUCAs with GSH, the lack of reactivity between FTUCAs and HSA is suggestive of the relatively poor electrophilicity on the β-carbon and is preliminary evidence that FTUCAs might only react with small, thiol-containing nucleophiles.

The 6:2 FTAL (containing 6:2 FTUAL as an impurity), 8:2 and 10:2 FTUAL were reacted with HSA prior to analysis on the ToF-MS. Two adducts were detected for all FTUAL congeners, illustrated in Figure 3 with 8:2 FTUAL and HSA.

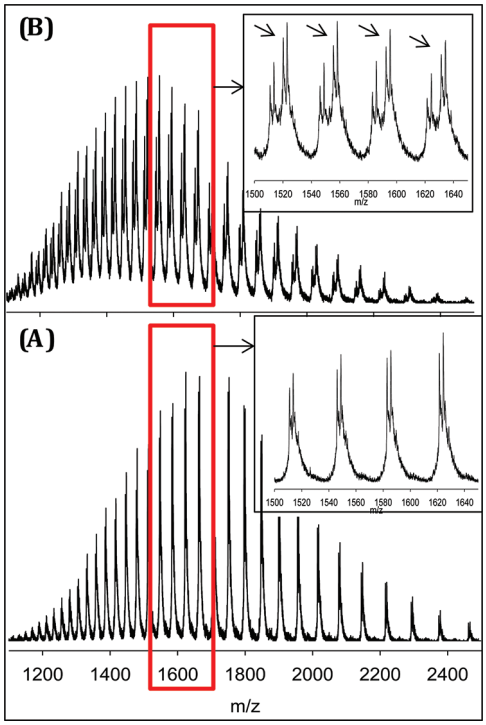

Figure 3. ESI-ToF mass spectrum of HSA-8:2 FTUAL adduct formation at the 1:0.25 (HSA:8:2 FTUAL) molar ratio compared to the native control spectrum. Panel (A) shows unadducted HSA. The inset in panel (A) is the spectrum of native HSA between m/z values of 1500−1640, showing m/z signals representing the mass of the native HSA (66 440.55 ± 0.46 Da) and the cysteinylated adduct (66 557.95 ± 0.87 Da). Panel (B) depicts the stoichiometry of 8:2 FTUAL adducts to HSA. The arrows in the inset of panel (B) indicate adducts formed from reaction of 8:2 FTUAL with HSA, with masses of +406.67 ± 2.12 and 406.18 ± 0.76 Da compared to native and cysteinylated HSA, respectively.

To ensure that the formation of the adducts was not being driven by presence of borohydride, a phenomenon that has been documented with HNE and cyanoborohydride,[50] an experiment with 10:2 FTUAL and HSA was carried out without the use of borohydride as a reducing agent. The same adducts were observed both with and without the use of borohydride, thus indicating that borohydride was not driving the equilibrium toward adduct formation.

When HSA was incubated with 6:2 FTUAL at molar ratios of 1:0.25, 1:0.5, and 1:1, two adducts were formed at +308.13 ± 1.30 Da and 305.38 ± 0.54 Da (CI 95%, n = 6) compared with native HSA and cysteinylated HSA, respectively. For 8:2 FTUAL at molar ratios of 1:0.25, 1:0.5, and 1:1, two adducts

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were observed at +406.67 ± 2.12 Da and 406.18 ± 0.76 Da (n = 6) compared with native HSA and cysteinylated HSA, respectively. Two adducts corresponding to the 10:2 FTUAL were also observed at molar ratios of 1:0.5 and 1:1; the first adduct was shifted by +522.55 ± 0.46 Da (n = 4) with respect to native HSA and the second was shifted +521.38 ± 0.38 Da with respect to cysteinylated form of HSA. In relation to the mass of 10:2 FTUAL (MW 542.12 g/mol), the mass difference was 19.71 ± 0.66 Da and 20.77 ± 0.38 Da for the native and cysteinylated adduct, respectively, which likely corresponded to the loss of a fluoride ion as the leaving group in the 1,4-Michael addition. The adducts from the reaction with 6:2 and 8:2 FTUAL had relative masses that differed from those reported for 10:2 FTUAL, and are proposed to occur either via a 1,4-Michael addition or Schiff-base formation, coupled with the loss of a water molecule from HSA (theoretical mass difference of +405 and +406 Da, respectively).

Though the observed masses for 6:2 and 8:2 FTUAL were not accurate enough to make a conclusive judgment on the reaction mechanism, it is presumed that the native form of HSA will interact with 6:2, 8:2, and 10:2 FTUAL via a 1,4-Michael addition. We propose that FTUALs will react to form an adduct with Cys34 because 8:2 FTUAL reactivity with amino acids was fastest with cysteine. Cys34 has been previously identified as a nucleophilic site and a well-known scavenger of electrophiles.[51] At physiological pH, Cys34 contains about 86% S^- (pK_a = 6.55),[33,52] further enhancing its capacity to bind to electrophiles. The second adduct was attributed to the interaction with a lysine or histidine residue with respect to the bound, cysteinlyated HSA via a 1,4-Michael addition mechanism or Schiff base mechanism (corresponding only to an adduct with lysine). Experiments using X-ray crystallography are currently underway in our lab to characterize the structure of HSA with FTUAL adducts further, and to determine any mechanistic differences among 6:2, 8:2, and 10:2 FTUAL.

When the molar ratios of the FTUALs were increased to 1:1 and 1:5 HSA:FTUAL, the intensity of the adduct and protein signals decreased. Based on this observation there might be more adducts forming, but the number of adducts is unknown. Evidence of additional adducts was confirmed using TOF-IC, where isolated HSA adducted samples were combusted to measure TOF in each sample. Results from the TOF-IC study are presented in Figure 4. A linear increase in fluoride concentration (μg/mL) was observed as the molar ratio of 8:2 FTUAL was increased, indicating that 8:2 FTUAL binds to HSA at higher molar ratios, likely via multiple adducts. The fluoride concentration resulting from the combustion of the HSA-8:2 FTUAL adducted sample was also compared to the fluoride concentration of the HSA:8:2 FTUCA (1:5 molar ratio) sample. The lack of covalent adducts from the FTUCA sample is reflected by a comparatively low concentration of fluoride (Figure S16). These findings further demonstrate that the high fluoride concentration corresponding to the HSA:8:2 FTUAL sample is likely due to the formation of several covalent adducts with HSA.

Implications. The recent detection of diPAPs in human blood[4,5] is direct evidence of human exposure to commercial fluorinated products. This is important because diPAPs and other fluorotelomer-based materials might yield reactive intermediates, such as the FTUALs, in the course of their metabolic transformations to the PFCAs. This investigation is the first to show the extent to which FTUALs react with biological nucleophiles other than GSH.[15,18,25]

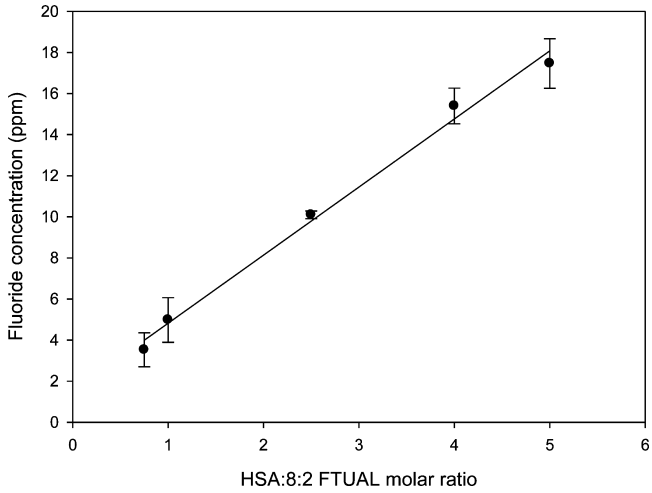

Figure 4. TOF-IC analysis showing an increase in the total fluoride concentration (ppm) as a result of combusting the protein adduct samples at varying HSA:8:2 FTUAL molar ratios (1:0.75 to 1:5). Error bars are shown for n = 3 samples.

Here, we demonstrated the reactivity of the FTUALs with several nucleophilic amino acids containing thiols and nitrogen moieties. The interaction of FTUALs with amino acids observed here suggests the potential for FTUALs to bind with other biological amine groups, such as those present within nucleic acids to form DNA adducts. FTUAL reactivity at the macromolecular level was also confirmed by the observed formation of adducts during binding studies of the FTUALs with the native ApoMg protein and its $-H_2O$ form, as well as the native and cysteinylated HSA. Previous detection of FTUAL−GSH conjugates in the bile, liver, and kidney of FTOH-exposed rats[15,25] suggests conjugation with small biological nucleophiles might be an important biotransformation mechanism of the FTUALs. The protein-binding interactions between FTUALs and ApoMg and HSA observed here demonstrate an additional pathway for the biological processing of FTUALs. A comparison between the amount of protein adduct formation relative to the formation of GSH conjugates in FTOH-exposed animals is warranted in order to approximate the importance of binding to GSH over proteins.

Although FTUALs were observed to form adducts with HSA, toxic effects associated with this mechanism might not be significant due to the much higher concentration of HSA in humans (i.e., at least 6 orders of magnitude) than the proposed ≤ppb levels of FTUALs. Since measurement of FTUAL adducts in specific tissues would help illuminate how these compounds exert their toxic effects, further work involving subcellular (i.e., microsomes) and cellular (i.e., blood plasma) systems is currently underway to investigate the fate of FTUALs within more biologically representative environments. It is not unreasonable to suggest that FTUALs might bind to other cellular constituents including the CYP2E1 enzymatic protein involved in forming FTUALs[16] with greater affinity than GSH, which might affect the resulting toxicity of the FTUALs in biological systems. Measurement of FTUAL protein adducts would also be useful as a biomarker, helping to confirm exposure and to quantify the potential absorbed dose of these reactive intermediates. Although ApoMg and HSA were used in this pilot investigation to probe the potential protein-binding reactivity of FTUCAs and FTUALs, a comprehensive survey of other protein targets susceptible to

these fluorinated intermediates at physiological meaningful concentrations is warranted before assessments of potential toxicity and implications for human exposure can occur.

■ ASSOCIATED CONTENT

Supporting Information

A list of chemicals, synthetic procedures, instrumental details, kinetic plots, ^{19}F NMR product analyses, and TOF-IC HSA-8:2 FTUAL concentration results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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