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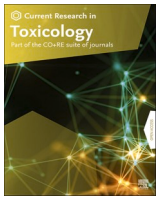

Detail study on the interaction between perfluorooctanoic acid (PFOA) with human hemoglobin (Hb)

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ABSTRACT

Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) are often referred to as legacy perfluoroalkyl substances (PFAS). Human exposure to PFAS leads to severe negative health impacts including cancers, infertility, and dysfunction in the kidneys. Steady-state absorbance, fluorescence, and circular dichroism (CD) methods were used to study the interactions between PFOA and Hb. The results demonstrate the presence of multiple PFOA binding sites on the Hb protein. The detailed analysis of the ferric hemoglobin protein (met Hb) absorbance data as a function of PFOA concentration indicates the presence of at least two binding sites with equilibrium dissociation constants of $0.8 \pm (0.2) \times 10^{-6}$ M and $63 \pm (15) \times 10^{-5}$ M. A competitive binding study with 1,8-ANS showed PFOA can bind to the same binding site as 1,8-ANS on the Hb protein. The titration curve for PFOA binding to Hb in its CO bound form (CO-Hb) yields a single equilibrium dissociation constant of $139 \pm (20) \times 10^{-6}$ M. PFOA binding at low concentrations occurs at the high-affinity sites leading to the destabilization of the protein structure as reflected by changes in the CD spectrum. PFOA interactions with Hb also interfere with the kinetics of CO association to this protein. The rate for CO association to Hb increases at low PFOA concentrations, whereas at elevated PFOA concentrations, the ligand association is biphasic as a new kinetic process with a different rate constant was observed. Overall, this study provides a detailed explanation of PFOA-induced structural and conformational changes to the Hb protein based on the spectroscopy data.

Introduction

Per and poly-fluoroalkyl substances (PFAS) including perfluorooctanoic acid (PFOA) and perfluoro sulfonic acid (PFOS) are notorious pollutants with powerful negative health impacts (Sunderland et al., 2019). PFAS have received tremendous attention due to their detection in an extensive number of environmental and biological systems (Weiss-Errico et al., 2018). PFOA (Fig. 1), one of the most recognized PFAS, was used on industrial scales from 1940 s until its usage was recently phased out (Weiss-Errico et al., 2018; Trudel et al., 2008). PFOA is synthesized via the degradation of fluorotelomer precursors and used in various applications, including food packaging, paints, non-stick coating on cooking pans, and firefighting foams (Verma et al., 2021; Weiss-Errico et al., 2018). Due to its chemical structure with hydrophobic perfluoro-*n*-octyl chain and hydrophilic carboxylate head, PFOA possesses strong surfactant-like properties. PFOA also has high thermal stability because of the unusual strength and unreactive nature of the C-F bonds (Blotevogel et al., 2023). In general, PFAS including PFOA are extremely stable in the environment and accumulate inside the human body where they have a residence half-life of 3.8 years (Li et al., 2022). The use of several problematic PFAS was restricted or banned in several countries including the USA (Trudel et al., 2008). PFOA production in the USA was phased out by the major manufacturers in the year 2015 (Steenland et al., 2018). Yet these compounds continue to be detected in water systems and inside the human body. As reported in 2018, it is estimated 98 % of the general population in the USA has detectable levels of PFAS in their blood (Carignan et al., 2023). However, due to its phenomenal persistence in the environment and the associated health risks, PFOA still represents a serious health concern (Pelch et al., 2019).

Human exposure to PFAS such as PFOA has been associated with numerous health impacts. PFOA is known as a developmental and immune system toxicant (Hines et al., 2009) which can also exert adverse

Abbreviations: PFAS, Perfluoroalkyl substances; PFOA, Perfluorooctanoic acid; PFOS, Perfluoro sulfonic acid; CD, Circular dichroism; Hb, Hemoglobin; SDS, Sodium dodecyl sulfate; 1,8-ANS, 8-anilinonaphthalene-1-sulfonic acid; CO-Hb, Hb in CO bound form; metHb, Ferric hemoglobin protein; oxy-Hb, oxygen bound Hb; Trp, Tryptophan; Tyr, Tyrosine.

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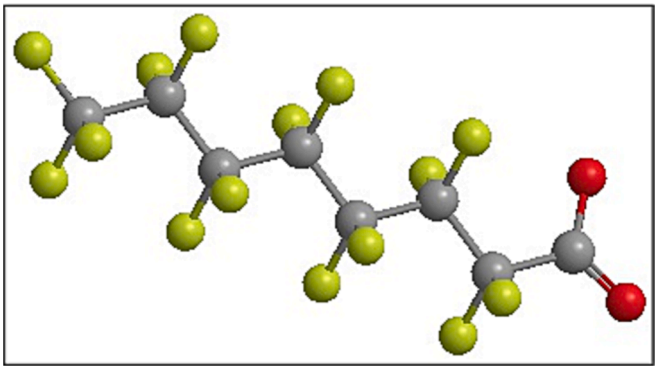

Fig. 1. The structure of perfluorooctanoate ($C_8F_{15}O^-$).

alteration to hormone levels inside the human body and induce suppression of a broad range of genes, thus leading to the classification of PFOA as a genotoxic compound (Nakamura et al., 2016).

While PFOA primarily enters the body through ingestion, the highest accumulation levels are recorded in breast milk and the bloodstream with concentrations up to 0.005 μM (Völkel et al., 2008) with human serum albumin representing the potential molecular target for PFOA and related compounds. PFAS have great potential to interact and penetrate cell membranes because of their amphiphilic characteristics (Qin et al., 2011).

Due to the elevated presence of PFOA in the bloodstream, we chose to investigate the interactions between PFOA and hemoglobin (Hb). Hb is an iron-containing metalloprotein in red blood cells of vertebrates that exists as a tetramer of globin chains with two α and two β subunits, with each subunit, carrying a heme prosthetic group (Waks et al., 1973). Hb is the primary oxygen-transporting protein in the blood but is also critical in transporting other gases including carbon monoxide and nitric oxide (Perutz, 1990). Recent studies have suggested that in addition to the physiological ligands, CO and O_2, Hb also has the ability to bind a diverse set of molecules including physiological allosteric effectors, synthetic effectors, and small hydrophobic molecules such as acridines (Hlastala et al., 1976; Chatterjee and Kumar, 2016). Hb strongly bind detergents including sodium dodecyl sulfate (SDS) with an equilibrium association constant equal to $4 \times 10^5\ M^{-1}$ (Bordbar et al., 1996). The binding of SDS promotes the formation of so-called hemichrome, a six-coordinate heme iron with both distal and proximal histidine in the position of axial ligands (Bordbar et al., 1996). Given the presence of a negative charge with a long hydrophobic chain, and the surfactant-like properties of both SDS and, we hypothesize PFOA can bind to and alter the structural properties which could affect the critical biological function of the Hb protein. With this in mind, steady-state and time-resolved spectroscopic techniques were employed to investigate the PFOA interactions with Hb and to characterize the structural properties of the Hb: PFOA complex.

Material and methods

Chemicals

PFOA, 8-anilinonaphthalene-1-sulfonic acid (1,8-ANS), sodium dithionate, and Hb were purchased from Sigma-Aldrich. Dibasic sodium phosphate and monobasic sodium phosphate were purchased from Fisher Scientific. Argon gas was purchased from Airgas. All chemicals were used without further purification.

Sample preparation

For the fluorescence spectroscopy and circular dichroism (CD) experiments, stock solutions of PFOA (1.0 mM), and Hb (1.0 mM) were prepared in a 50 mM sodium phosphate buffer (pH 7.4) and stored in polypropylene tubes. The stock solutions were mixed well and filtered using microfilters of 0.45 μM pore size.

Hb in CO bound form (CO-Hb) was prepared by purging ferric hemoglobin protein (met Hb) samples placed in a sealed quartz cuvette with argon gas for 20 min. The heme iron was then first reduced to deoxy-Hb by adding a small amount of freshly prepared sodium dithionite dissolved in 50 mM sodium phosphate buffer. The transition to deoxy-Hb was confirmed by observing the shift of the Soret band from 405 to 430 nm. The sample was then purged with CO gas to obtain the CO-Hb form which was confirmed by the UV–Vis spectra.

For the preparation of oxygen-bound samples, Hb protein in the deoxy form was purged with air, and the formation of oxygen-bound Hb (oxy-Hb) was confirmed by the shift of the Soret band maximum from 430 to 415 nm.

UV–Vis absorbance spectroscopy

The UV–Vis absorption spectra were recorded using a double beam spectrophotometer (Varian Cary 100 Bio,UV Visible Spectrophotometer). The absorbance spectra for met Hb and CO-Hb samples were measured in the presence of increasing concentrations of PFOA. The experiments were performed in triplicate.

Fluorescence spectroscopy

The fluorescence emission spectra for met Hb in the presence of an increasing PFOA concentration (0–287 μM) were recorded employing a Cary Eclipse fluorescence spectrophotometer (Agilent technologies). Protein samples were excited using a 280 nm output from a Xe lamp and emission spectra were measured from 300 to 400 nm with a 10 nm slit width for both excitation and emission path. The experiments were performed in triplicate and the concentrations were corrected for dilution.

The titration curves for PFOA binding to Hb were constructed by monitoring the changes in the absorption or emission spectrum of met Hb and the absorption spectrum of CO-Hb with the increasing concentrations of PFOA. The titration curves were analysed using a single binding site model according to Eq. (1) or two independent binding sites according to Eq. (2)

$$\Delta S = \frac{\Delta S_{max}[\mathrm{PFOA}]}{[PFOA] + K_D} \tag{1}$$

$$\Delta S = \frac{\Delta S_{max1}[\mathrm{PFOA}]}{[PFOA] + K_{D1}} + \frac{\Delta S_{max2}[\mathrm{PFOA}]}{[PFOA] + K_{D2}} \tag{2}$$

Where ΔS represents the change in the spectroscopic signal upon PFOA binding to the protein, ΔS_{max} represents the change in the spectroscopic signal of PFOA bound Hb and PFOA free Hb and K_D is the equilibrium dissociation constant.

Competition study

Fluorescence emission spectra for met Hb (10 μM) in complex with 1,8-ANS (12 μM) were recorded using Cary Eclipse fluorescence spectrophotometer as a function of increasing concentration of PFOA. The excitation wavelength was 350 nm, and the emission spectra were collected between 410–640 nm. The slit width was 5 nm for excitation and emission. The recorded spectra were corrected for the dilution and all experiments were performed in triplicate.

Transient absorbance spectroscopy

The kinetic measurements of CO binding to Hb were carried out using a custom-built transient absorption instrument (Butcher et al.,

2017). The 447 nm output from a diode laser (MDL-III-447, Changchun New Industries Optoelectronics Tech; China) was used as a probe light. The beam was focused on the centre of a 1 × 0.5 cm quartz cuvette placed in the temperature-controlled holder. CO photo-dissociation was then triggered using a 532 nm output from Nd:YAG laser (7 ns pulse, Surelite I-10, Continuum). Changes in probe beam intensities were detected using a PDA10A photodiode (Thorlabs; Newton, NJ). Traces of an average of 100 sweeps were digitized with a Wave Surfer 42Xs oscilloscope (Teledyne LeCroy; Chestnut Ridge, NY), and the data were analysed using a two -exponential decay model, Eq. (3), employing OriginLab software.

$$A(t) = \sum_i \alpha_i e^{-k_i t} \quad (3)$$

where A(t) corresponds to the absorbance, α_i is the pre-exponential factor, k is the pseudo first order rate constant, and t is the time. The kinetic traces for CO association for Hb:PFOA complex were recorded under pseudo-first order conditions, [CO] ≫ [Hb:PFOA]. The pseudo first order rate constants, k_i, obtained by fitting the transient absorption traces using Eq. (3), were then used to calculate the bimolecular rate constants, k, for CO binding to Hb:PFOA complex, according to $k = k_i$ [CO] with[CO] = 1 mM (Astudillo et al., 2013).

CD spectroscopy

CD measurements were performed to monitor changes to protein secondary structure upon binding with PFOA using the JASCO J-815CD spectrometer. The samples for CD measurements were prepared by dissolving met Hb in 50 mM sodium phosphate buffer (pH = 7.4) to reach the final concertation of 10 μM protein in the presence of PFOA.

Results and discussion

UV–Vis absorption

UV–Vis absorption spectroscopy was used to probe the binding of PFOA by met Hb and CO-Hb. We use the met form of the protein as a model for the Hb in the T state and CO-bound Hb as a model for the protein in the R-state for the characterization of the deoxy- and O_2 bound protein is impeded by a potential autoxidation of the heme iron. The changes in UV-spectra of met Hb and CO-Hb as a function of PFOA concentrations are shown in Figs. 2 and 3, respectively. The absorption spectrum of the met Hb exhibits the characteristic Soret band at 405 nm (Fig. 2(a)). Upon PFOA addition the intensity of the Soret band decreases. The shape and position of the Soret band are important for the spectral mapping of the structural properties of the heme binding cavity as Soret band distortion is an indication of increased conformational heterogeneity in the heme vicinity (Boffi, 1994; Mahato et al., 2010). The decrease in the Soret band intensity was used to construct a titration curve for PFOA binding (Fig. 2(b)). The analysis of the binding curve using a two binding site model (Eq. (2)) provides equilibrium dissociation constants of 0.8 ± (0.2) × 10^{-6} M and 63 ± (15) × 10^{-5} M indicating the presence of at least two binding sites for PFOA on the Hb surface. The presence of the multiple binding sites, i.e. presence of multiple absorbing species in the sample, is also evident in the absence of an isosbestic point in Fig. 2(a).

The addition of PFOA to CO-Hb also resulted in a decrease in the Soret band absorbance at 420 nm (Fig. 3(a)). The overlay of the absorption spectra for CO-Hb at increasing [PFOA] reveals two isobestic points at 405 and 435 nm. The presence of isosbestic points is consistent with two absorbing species in the sample: CO-Hb and CO-Hb:PFOA complex, pointing towards a single PFOA on the surface of CO-Hb. Analysis of the titration curve for PFOA binding to CO-Hb using Eq. (1) yields an equilibrium dissociation constant of 139 ± 20 μM (Fig. 3 (b)).

The changes in the UV–Vis characteristics of met Hb and CO-Hb upon the addition of PFOA illustrate alterations to the electronic structure of the heme group indicating the PFOA binding sites are near the heme-binding pocket.

The PFOA exhibits a lower affinity of PFOA for CO-Hb than for met Hb indicates that the presence of the diatomic ligand in the heme distal site and repositioning of the distal histidine side chain restricts the association of PFOA into the heme-binding pocket and decreases the number of binding sites available for PFOA.

Steady-State fluorescence emission

Fluorescence spectroscopy is an important tool to probe the structural changes induced by small molecules binding on the quaternary structure of the proteins containing intrinsic fluorophores (Trp or Tyr residues). Changes in the emission spectra reflect environmental and structural changes close to the fluorophore environment and can be used to determine the affinity for binding of small molecules. Thus, to probe the impact of PFOA on Hb quaternary structure, we monitored the changes in the Trp emission spectra upon PFOA addition.

Hb contains six Trp residues, each with a fluorescence emission maximum at ~334 nm (De-jia et al., 2003). In general, the Trp residues in heme proteins shows a low fluorescence quantum yield due to the

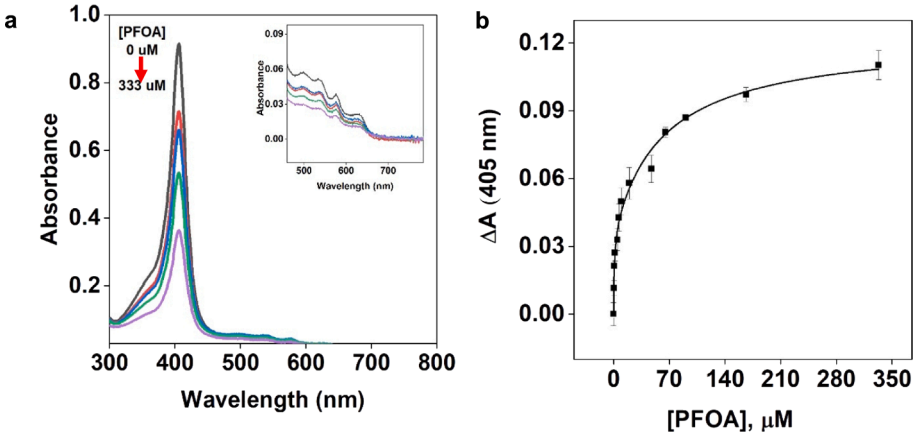

Fig. 2. (a) Absorption spectrum of metHb (10 μM) as a function of [PFOA]. 0 μM PFOA (black trace), 5 μM PFOA (red trace), 10 μM PFOA (blue trace), 90 μM PFOA (green trace), and 233 μM PFOA (purple trace). The insert visulizes the absorbance in the visible part of the spectrum (450 nm to 800 nm). (b) Change in absorbance of the Soret band as a function of increasing [PFOA] (0–333 μM). The solid line corresponds to the fit of the experimental data using Eq. (2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

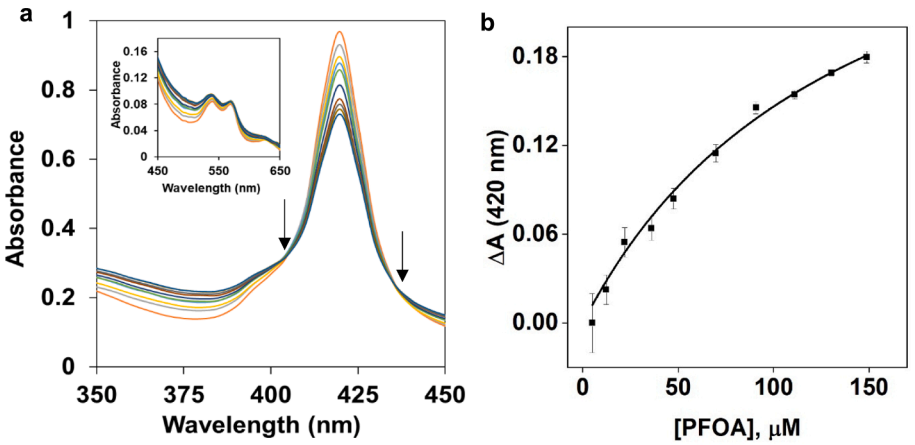

Fig. 3. (a) Absorption spectra of CO-Hb (10 μM) as a function of increasing concentration of PFOA (0—160 μM). The isosbestic points are indicated using black arrows. The inset presents absorption spectra in the visible range, from 450 nm to 650 nm. (b) Changes in absorbance of the Soret band (420 nm) with the increasing concentration of PFOA. The solid line corresponds to the fit of the experimental data using a single binding site model, Eq. (1).

energy transfer from excited Trp residues within the heme group. The emission spectrum of met Hb in the absence of PFOA has a maximum wavelength of 330 nm. As shown in Fig. 4(a), the addition of PFOA to the metHb leads to the shift of the fluorescence emission maximum from 334 to 317 nm. Initially, the fluorescence emission intensity of metHb decreases at PFOA concentrations below 50 μM (Fig. 4(b)). Further addition of PFOA leads to a increase in fluorescence emission intensity, likely due to larger structural changes and possibly protein unfolding that eliminates quenching of Trp emission due to the increase in the distance between the heme group and the fluorophore.

A hypsochromic shift in the maximum emission wavelength in the presence of an increasing concentration of PFOA indicates either a change in the protein conformation that causes a less polar Trp environment or a direct interaction between hydrophobic PFOA chain and Trp residues. The plot of the changes in the maximum emission wavelength as a function of PFOA concentration, shown in Fig. 4c, was analysed using a single binding site model (Eq. (1)) and provided the equilibrium dissociation constant of 60 ± 10 μM that is similar to that

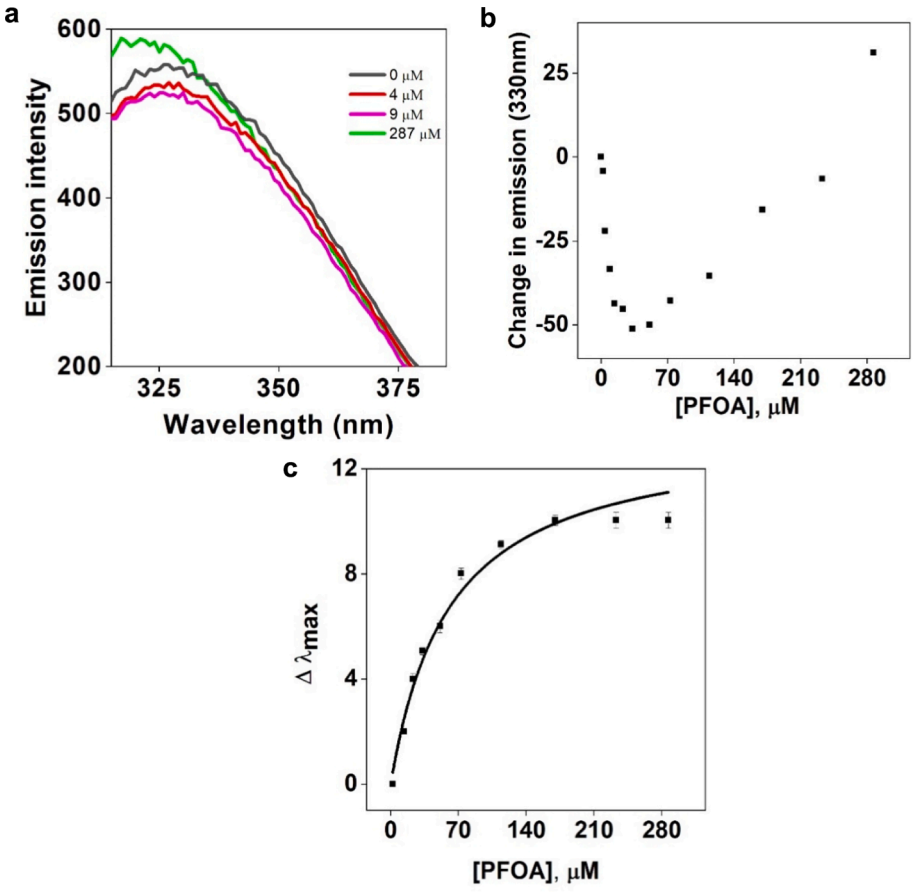

Fig. 4. (a) Emission spectrum of metHb in the presence of PFOA (b) metHb maximum emission intensity at 330 nm as a function of PFOA concentration (c) Changes in maximum emission wavelength or metHb with the increasing concentration of PFOA (0 – 287 μM).

determined in absorption study. In addition to the changes in Hb tertiary structure, the observed alteration of Hb emission may also indicate the alteration of the protein quaternary structure due to a dissociation of the Hb tetramer into individual dimers. However, De-jia et al. (2003) reported that the Hb dissociation into individual dimers leads to a bathochromic shift in Trp emission spectrum inconsistent with the observed spectral changes.

Competition binding study

To further probe the binding of PFOA to Hb, we have tested if PFOA can displace 1,8-ANS, bound to Hb. Previous studies established that 1,8-ANS binds to at least two distinct sites on Hb surface and the Hb central cavity was proposed to be one of the binding sites (Syakhovich et al., 2004). Under the experimental conditions, the emission spectrum of the metHb:1,8-ANS complex exhibits an emission maximum at 470 nm. Upon the addition of PFOA up to 28 μM, the 1,8-ANS emission intensity decreases with an insignificant shift in the emission maximum. Continued addition of PFOA leads to an increase in the intensity of 1,8-ANS emission and the maximum emission wavelength of the Hb:1,8-ANS shifts to 455 nm (Fig. 5(a) and (b)). These results suggest that at lower concentrations, PFOA competes with 1,8-ANS for the same binding site on the metHb protein surface. However, in the presence of high concentrations of PFOA, the changes in the protein structure facilitate the binding of additional 1,8-ANS molecules to new sites of a partially destabilized protein, leading to an increased emission intensity.

CD titration

CD spectroscopy is an excellent tool to determine the secondary structure content and the folding properties of proteins. CD measurements were performed to investigate the changes to the secondary structure of metHb upon PFOA addition. The presence of α-helical secondary structures in the protein is evident by ellipticity minima at 208 and 220 nm. As shown in Fig. 6, the CD spectrum of metHb upon PFOA addition (up to 178 μM), the binding of PFOA has a negligible impact on Hb secondary structure whereas with increased PFOA concentration (395 μM) the binding of PFOA leads to a decrease in α-helical content and the destabilization of the secondary structure of Hb. CD results are consistent with the fluorescence spectroscopic studies that show a distinct impact on Hb structure at low and high PFOA concentrations. Specifically, at low PFOA concentrations, the PFOA molecule(s) binds to a specific binding site(s) either in the heme distal cavity or its vicinity without a significant impact on the overall protein structure. At PFOA concentrations above 100 μM, additional binding of the PFOA molecule

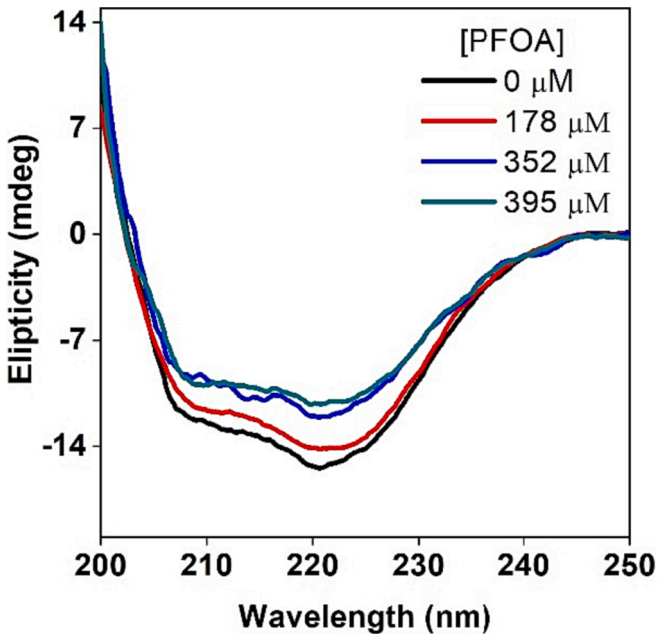

Fig. 6. CD ellipticity of metHb with the increasing concentration of PFOA.

leads to the partial destabilization of the secondary structure of Hb which facilitates binding of 1,8-ANS molecules and leads to the shift in Trp emission to shorter wavelengths.

Transient absorption

The kinetics parameters for CO rebinding to HB:PFOA complex were determined using transient absorption spectroscopy by probing the time profile of absorbance change at 470 nm and the individual transient absorption traces are shown in Fig. 7, panel a. The traces were analyzed using a two-exponential decay model, according to Eq. (3) and the determined pseudo-first order rate constant was converted into bimolecular rate constant using eq. 4. The plot of bimolecular rate constant and associated pre-exponential factors as a function of increasing PFOA concentration are shown in Fig. 7b and 7c, respectively. In the absence of PFOA, the rate constant for the bimolecular CO rebinding to Hb is 1.8 $\times$ 10^6 M^{-1} s^{-1}, which is in agreement with the previously published value of 6 $\times$ 10^6 M^{-1} s^{-1} (Turilli-Ghisolfi et al., 2023) and increases to 9.1 $\times$ 10^6 M^{-1} s^{-1} at 360 μM PFOA. Interestingly at increased PFOA concentration (>90 μM), an additional kinetic process for CO rebinding

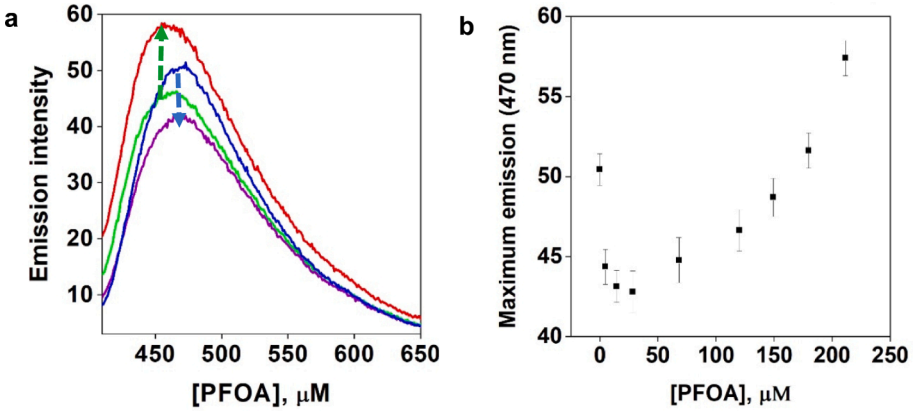

Fig. 5. (a) The fluorescence emission intensity of 1,8-ANS: metHb complex in the presence of 0 μM PFOA (blue trace), 33 μM PFOA (purple trace), 114 μM PFOA (green trace), and 233 μM PFOA (red trace). (b) Fluorescence emission of 1,8-ANS: metHb complex at 470 nm, with the increasing concentration of PFOA (0–211 μM). The concentrations of 1,8-ANS and metHb were 12 μM and 10 μM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

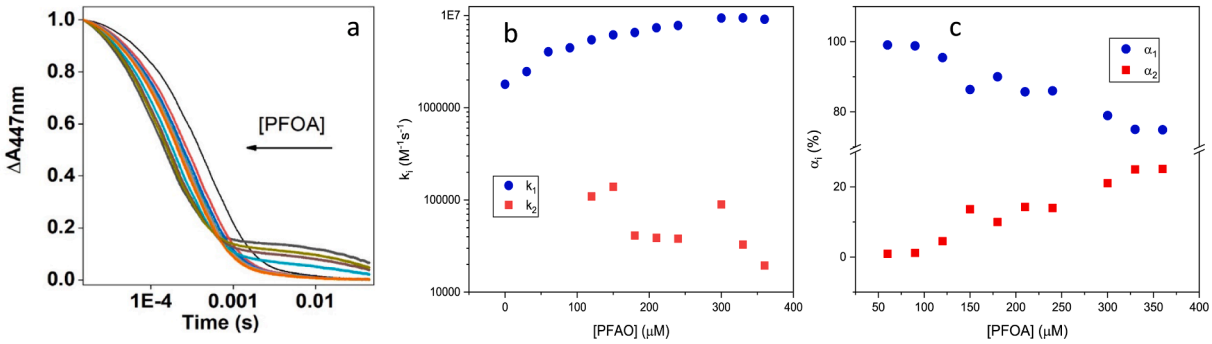

Fig. 7. (a) TA traces for CO binding to Hb as a function of increasing concentration of PFOA. The individual traces were fitted using Eg. 3. (b) The individual rate constants for the bimolecular CO rebinding to HB:PFOA complex, k_1 and k_2, (c) Associated pre-exponential factors, and corresponding pre-exponential factors α_1 and α_2.

was observed with the rate constant of 1.1×10^5 M^{-1} s^{-1} at 120 μM PFOA and 1.9×10^4 M^{-1} s^{-1} at 330 μM PFOA. The amplitude for the slower kinetics also increases at elevated PFOA concentrations as evident in the Fig. 7c. The increase in the rate constant for CO bimolecular association at low PFOA concentrations can be associated with the PFOA binding to or in the vicinity of the heme-binding pocket, opening the distal heme cavity, and facilitating CO association to the heme iron. The presence of the slow phase observed at PFOA concentrations above 90 μM suggests a larger conformational change that restricts the CO access to the heme-binding cavity. It is also possible, that binding of PFOA to deoxyHb leads to the alteration of the heme iron ligation. Previous studies on surfactant binding to heme proteins reported that the surfactant association leads to the formation of a hemichrome species with bis-histidine heme iron coordination (Mikšovská et al., 2006). Such change in the heme iron coordination, i.e. transition from the penta- to hexa -coordinate heme iron, would result in a slower CO binding kinetics, as the exogenous ligand would compete for the same binding site as the distal histidine.

We have also measured the stability of oxy Hb towards autooxidation in the presence of PFOA over a considerably long period (24 h). The addition of PFOA did not lead to autooxidation which suggests the high structural stability of oxy Hb protein (data not shown).

Conclusions

In summary, the PFOA-induced changes to the structure of Hb protein have been reported in this study using steady-state and time-resolved spectroscopic methods. The results indicated the presence of several PFOA binding sites on the Hb surface as PFOA binding impacts the heme iron electronic structure as well as the photophysical properties of Trp residues. PFOA binds to at least two binding sites on metHb with an equilibrium dissociation constant of 0.8 μM and 63 μM as observed in the absorption and emission study. At an increased concentration of PFOA, additional molecules bind to the protein, resulting in the destabilization of Hb secondary structure as evident form the decrease in the CD signal. These structural and conformational changes to Hb observed here indicate that PFOA can bind to numerous binding sites on Hb surface and affect its structure, dynamics, and reactivity of heme iron forming ligand biding. The alteration of the rate constant for CO association points towards increased reactivity and elevated equilibrium association constant for CO and potentially for other diatomic ligands such as oxygen and nitrogen monoxide, affecting efficient oxygen delivery by hemoglobin. Binding of PFOA to the central cavity at Hb tetramer structure potentially interferes Hb interactions with allosteric effectors, such as s 2,3-bisphosphoglycerate. In addition to Hb, other heme proteins, can serve a potential target for PFAS. It was reported recently, that binding of various PFAS substances can inhibit enzymatic activity of cytochrome P450 (Hvizdak et al., 2023).

Author contribution

N.L. Dilani Perera and Jovany Betancourt carried out the experiments. The manuscript was written by the contributions of N.L. Dilani Perera Jaroslava Miksovska, and Kevin E. O'Shea.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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