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Perfluorodecanoic Acid Binding to Hemoproteins: New Insights from Spectroscopic Studies

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ABSTRACT: Perfluorodecanoic acid (PFDA), a representative of the perfluoroalkyl acids, poses a great threat to humans and animals via food and other potential sources. In this work, we determined the effects of PFDA binding to two hemoproteins, bovine hemoglobin (BHb) and myoglobin (Mb). Using fluorescence spectroscopy, we found that PFDA greatly enhanced the fluorescence intensity of both hemoproteins, while perfluorooctanoic acid (PFOA) and perfluoropentanoic acid (PFPA) have minimal effects on the fluorescence. UV−vis absorption (UV) spectroscopy showed that PFDA induced the unfolding of the hemoproteins accompanied by exposure of the heme pocket and facilitating the formation of hemichrome. Additionally, as shown by the circular dichroism (CD) data, PFDA altered the secondary structure of both BHb and Mb. This work elucidates the interaction mechanism of PFDA with two hemoproteins.

KEYWORDS: perfluorodecanoic acid, hemoprotein, hemichrome, toxicological evaluation

INTRODUCTION

Perfluoroalkyl acids (PFAAs) are synthetic surfactants with a charged moiety and a carbon backbone saturated with fluorine, rendering them both hydrophobic and lipophobic. These physical properties of PFAAs are why these chemicals are widely used in consumer and industrial applications, ranging from surfactants and emulsifiers to textiles, carpets, and paper products.[1] The direct and indirect emissions of PFAAs during the manufacture, use and disposal of these products have led to their widespread distribution in the environment.[2] Extensive amounts of data have recently become available describing the concentrations of PFAAs in the environment, wildlife and human tissues in many different geographic locations throughout the world.[3,4] This has led to efforts to better understand the hazards that may be inherent in these compounds. Much attention has been focused on understanding the toxicology of the most common PFAAs, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), and previous research found that they could induce various adverse effects in test organisms like decreased body weight, increased liver weight, liver peroxisomal proliferation, and increased intracellular free fatty acids and free cholesterol in the liver.[5−7] Limited toxicity data are available regarding avian sensitivity to PFAAs of chain lengths greater than C8, which are of increasing environmental relevance following the phase-out of PFOA and PFOS.[8]

PFAAs have been found mainly in liver, serum and blood in animals living anywhere from the polar regions to industrialized areas, and they are slow to clear from the body because of their long half-lives.[9−11] In recent years, some research activity on the significant changes of conformation, function, and dynamic properties of proteins has developed, exploring the biological toxicity of contaminants at the functional macromolecular level.[12,13] In our previous work, we studied the impact of carbon chain length on binding of PFAAs to bovine serum albumin by spectroscopic methods.[14] The data revealed that the longer chained perfluorodecanoic acid (PFDA) had a greater impact on the conformation of BSA than perfluorooctanoic acid, PFOA. Although a likely cause of oxidative stress and peroxisome proliferation are seen with PFDA,[7,15] yet little has been observed concerning its potential adverse effects on hemoproteins. Because of the hydrophobic group and the polar end, PFDA has great potential to cross membranes and to enter red blood cells.[16]

Hemoproteins are those containing a heme prosthetic group, either covalently or noncovalently bound to the protein itself. Hemoglobin (Hb) and myoglobin (Mb) are two of the most important representatives. Hb plays a major part in oxygen transport and is important in many clinical diseases, such as anemia, leukemia and heart disease.[17] Environmental pollutants may impact the protein conformation, and thus affect its normal physiological function. Hb exists as a tetramer of globin chains, consisting of two α and two β subunits. Myoglobin, Mb, is a structurally well characterized globular protein with a comparatively simple function.[18,19] It is a monomeric heme protein found mainly in muscle tissue, where it serves as an intracellular storage site for oxygen. In both hemoproteins, the amino acid groups that are packed into the interior of the molecules are predominantly hydrophobic in character, while those exposed on the surface are generally hydrophilic, thus making the two molecules relatively water-soluble. The heme prosthetic groups, however, are inserted into a hydrophobic cleft of the protein, and each of them contains one central iron atom that is normally in the ferrous state. Oxidation of the iron to its ferric state renders the molecule incapable of normal oxygen binding.[20]

In the present investigation, we made a special attempt to examine the interaction of PFDA with two hemoproteins (BHb and Mb) by means of fluorescence, synchronous fluorescence, UV−vis absorption (UV), and circular dichroism (CD) under simulative

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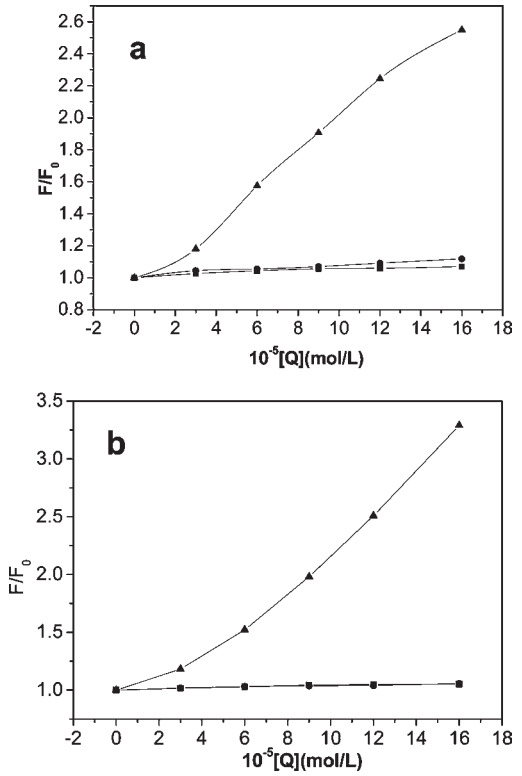

Figure 1. Normalized fluorescence intensity of (a) BHb and (b) Mb monitoring at 340 nm in the presence of different concentrations of (■) PFPA, (●) PFOA and (▲) PFDA. Conditions: PFPA, PFOA, PFDA ($\times 10^{-5}$ mol/L), 0, 3, 6, 9, 12, 16; BHb, 3×10^{-6} mol/L; Mb, 3×10^{-6} mol/L; buffer, $NaH_2PO_4-Na_2HPO_4$, pH = 7.40.

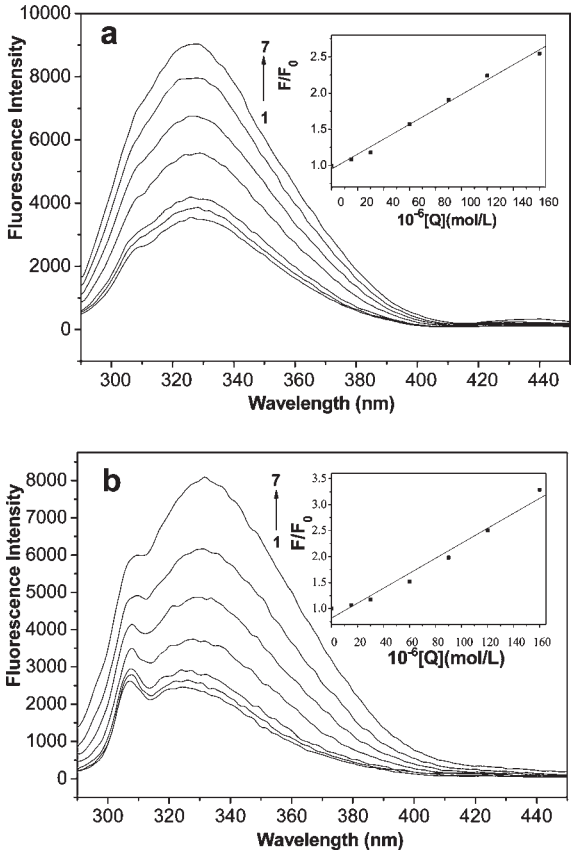

Figure 2. Fluorescence spectra of BHb (a) and Mb (b) in the presence of PFDA. Conditions: PFDA ($\times 10^{-5}$ mol/L) 1−7, 0, 1.5, 3, 6, 9, 12, 16; BHb, 3×10^{-6} mol/L; Mb, 3×10^{-6} mol/L; buffer, $NaH_2PO_4-Na_2HPO_4$, pH = 7.40.

physiological conditions. We also compared the effect of PFDA on the fluorescence of hemoproteins with two other PFAAs, perfluoropentanoic acid (PFPA) and PFOA. The results may be helpful to complement the limited toxicity literature for PFDA, and will also provide a new approach to probe the toxicity of PFAAs at the molecular level.

MATERIALS AND METHODS

Reagents. Bovine hemoglobin (BHb) and myoglobin (Mb) from equine skeletal muscle (purity ≥95%) were from Sigma and prepared at 3.0×10^{-5} mol/L and diluted as required. Perfluoropentanoic acid (PFPA, 97% purity), perfluorooctanoic acid (PFOA, 95% purity) and perfluorodecanoic acid (PFDA, 97% purity) were all purchased from Alfa Aesar (USA). Stock solutions of PFPA, PFOA and PFDA were prepared at a concentration of 1.0×10^{-3} mol/L, 1.0×10^{-3} mol/L and 2.0×10^{-4} mol/L. The 0.2 mol/L $NaH_2PO_4-Na_2HPO_4$ solution was used as the buffer and adjusted to pH 6.0, pH 7.40, pH 7.0 and pH 8.0. All other chemicals were of analytical grade. Ultrapure water obtained from the Upw ultrapure water system in the lab was used throughout the experiments.

Fluorescence Measurements. All fluorescence spectra were taken on an F-4600 fluorophotometer (Hitachi, Japan) equipped with a 10 mm quartz cell and a 150 W xenon lamp. Varied concentrations of PFAAs, 1 mL of 0.2 mol/L $NaH_2PO_4-Na_2HPO_4$ (pH 7.40) and 1 mL of BHb or Mb stock solution were added in turn to a 10 mL colorimetric tube and made up to the mark with ultrapure water. After equilibration for 20 min, the fluorescence emission spectra were scanned in the range of 290−450 nm using an excitation wavelength of 278 nm at room temperature. The excitation and emission slit widths were both set at 5 nm.

The synchronous fluorescence spectra were operated over the range 250−320 nm at $\Delta\lambda$ = 15 and 60 nm. The slits for excitation and emission scan were also 5.0 nm.

UV Spectroscopic Measurements. The UV−vis absorption spectra of BHb and Mb in the presence and absence of PFDA were recorded at room temperature on a UV−vis-2450 spectrophotometer (Shimadzu, Japan) equipped with 10 mm quartz cells in the range from 190 to 700 nm. The reference solution was a mixture of PFDA and buffer at the same concentration.

To evaluate the effect of PFDA under different pH conditions, varied amounts of PFAAs, 1 mL of 0.2 mol/L $NaH_2PO_4-Na_2HPO_4$ (pH 6.0/pH 7.0/pH 8.0) and 1 mL of BHb or Mb stock solution were added in turn to a 10 mL colorimetric tube and made up to the mark with ultrapure water. The UV−vis absorption spectra were measured after equilibration for 20 min, and the intensities of absorption peaks at 406 nm were obtained.

CD Studies. CD spectra of BHb and Mb in the absence and presence of PFDA were measured over the range of 200−250 nm on a J-810 circular dichroism spectrometer (Jasco, Tokyo, Japan) using a quartz cell with a path length of 1 mm. The scanning speed was set at 200 nm/min. Each spectrum was the average of two successive scans.

The CD results are expressed in terms of mean residue ellipticity (MRE) in degree·cm^2·$dmol^{-1}$ according to the equation[13]

$$\text{MRE} = \frac{\text{observed CD (mdeg)}}{C_p n l \times 10} \quad (1)$$

$$\alpha\text{-helix (\%)} = \frac{-\text{MRE}_{208} - 4000}{33000 - 4000} \times 100 \quad (2)$$

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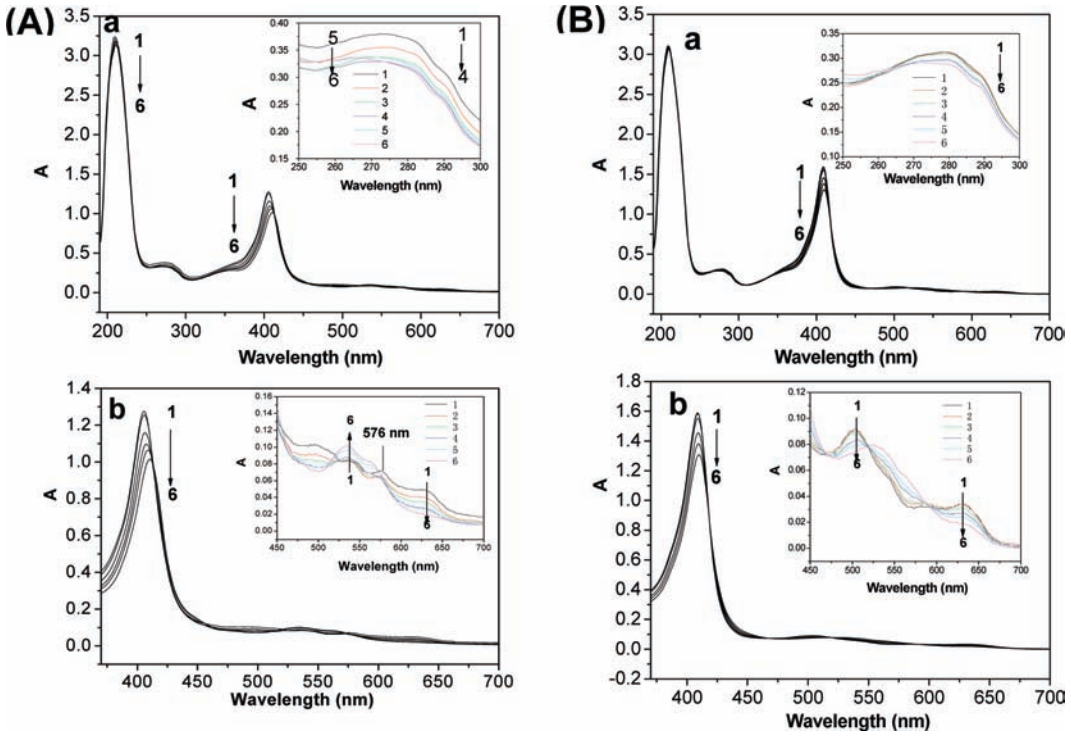


Figure 3. Absorption spectra of BHb (A) and Mb (B) as a function of PFDA. (a) Overall spectra of the hemoprotein in the presence of PFDA. (b) Expanded region of the heme Soret band in the presence of PFDA. Conditions: PFDA ($\times 10^{-5}$ mol/L) 1−6, 0, 3, 6, 9, 12, 16; BHb, 3×10^{-6} mol/L; Mb, 1.2×10^{-5} mol/L; buffer, NaH_2PO_4−Na_2HPO_4, pH = 7.40.

where C_P is the molar concentration of the protein; n is the number of amino acid residues and l is the path length.

RESULTS AND DISCUSSION

Fluorescence Spectra of BHb and Mb. The fluorescence spectra of two hemoproteins in the presence of PFAAs at various concentrations are demonstrated in Figures 1 and 2. The fluorescence intensities of both hemoproteins in the presence of PFPA and PFOA are essentially unchanged, which indicates that these two PFAAs have little effect on the structure of hemoproteins. In contrast, the fluorescence spectra of PFDA/BHb and PFDA/Mb systems manifest remarkable enhancement as the concentration increases. Previous studies have shown that PFAAs with longer carbon chain lengths have a longer half-life,[11] and could pose more threat to humans. The spectra indicate that PFDA binds more strongly to hemoproteins than the other two PFAAs.

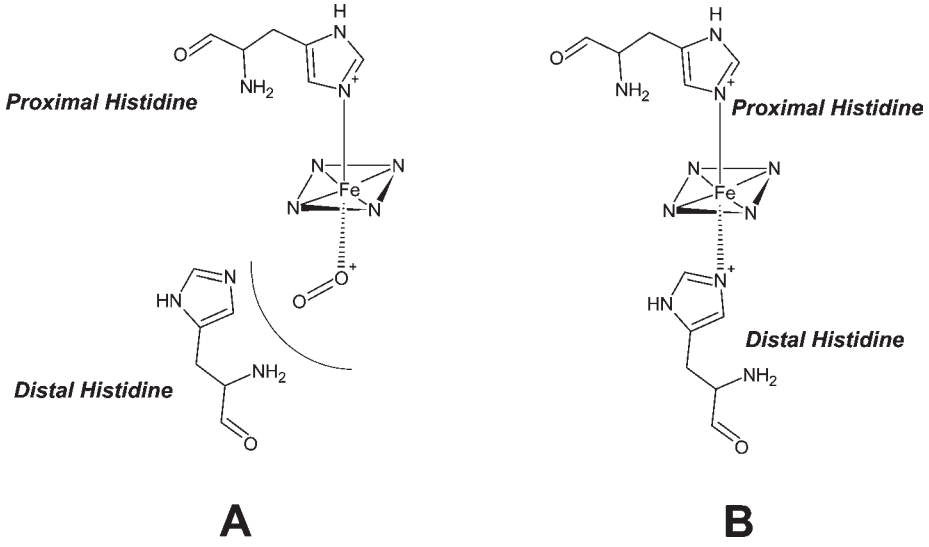
Proteins possess intrinsic fluorescence mainly due to the trytophan (Trp) and tyrosine (Tyr) residues, which are highly sensitive to their local environment. Commonly, emission spectra change in response to protein conformational transitions, or denaturation. There are six Trp residues in BHb, while there are two in Mb. But the hemoprotein exhibits a low fluorescence in water, because the efficient energy transfers from Trp residues to the heme group significantly quench the protein fluorescence.[21,22] After the addition of PFDA, however, the fluorescence of hemoproteins greatly increases (Figure 2). PFDA is a surfactant with a hydrophobic carbon chain and a hydrophilic carboxyl group. When PFDA is added to the system, the hydrophobic chain is more likely to interact with the surface of Trp through hydrophobic interaction. Furthermore, PFDA is also lipophobic enough to penetrate into the hydrophobic heme cavity, which is shown by the characteristic spectral changes in Figure 3. The hydrophobic interaction with PFDA depressed the fluorescence quenching between Trp and the heme group, which could reduce the energy loss and enhance the fluorescence quantum yield, causing the fluorescence intensity to greatly increase. Although PFPA and PFOA also contain a carboxylic acid functional group, they have little effect on the fluorescence intensity of either hemoprotein, which may be due to the lower carbon number decreasing the lipophilicity.

UV−Visible Spectra of BHb and Mb. The absorption spectra of BHb and Mb as a function of PFDA concentration in the range of 0 to 1.6×10^{-4} mol/L are presented in Figure 3.

There are three major absorption peaks in the hemoprotein spectra. The peak located at 200 to 230 nm designates the secondary structure due to the transition of $\pi \rightarrow \pi^*$ of polypepetide backbone structure C═O, while the band from 260 to 300 nm indicates the chromophore microenvironment.[12,13] The intrinsic peak around 406 nm is the characteristic absorption of the porphyrin-Soret band.[23]

Figure 3 shows that, after the addition of PFDA, the absorption peak around 210 nm is slightly changed, while the peak intensity around 280 nm is reduced. However, when the concentration of PFDA is 1.2×10^{-4} mol/L, the aromatic peak in Figure 3Aa begins to increase and the peak obviously blue shifts from 274 to 265 nm. With a further increase of PFDA, the peak begins to decrease again. We speculate that the higher concentration of PFDA results in the alteration of the microenvironment around aromatic residues from hydrophobic to polar, which is in accordance with the conclusion drawn from the fluorescence section. Also, Mb exhibits a similar trend with a decrease of absorbance and blue shift around 280 nm, indicating the alteration of the microenvironment of its aromatic residues.

Scheme 1. Proposed Mechanism for the Oxidation of the Hemoproteins Induced by PFDA



A significant decrease of absorption at the Soret band (406 nm in BHb and 409 nm in Mb) and a red shift to 411 nm are observed in Figure 3Aa and Figure 3Ba. In order to evaluate whether PFDA could induce structural changes in the hemoproteins leading to their oxidation, or even denaturation, we examined the region from 500 to 700 nm in detail (Figure 3Ab and Figure 3Bb).

In Figure 3Ab, the band at 576 nm in BHb, which is characteristic of oxyhemoglobin (oxyHb), disappears with the addition of PFDA,[24] indicating that the ability of hemoglobin to bind to oxygen is reduced by the addition of PFDA. Furthermore, BHb undergoes slight spectral alterations in the presence of PFDA, characterized by increases in the absorption peak around 537 nm, and the gradual disappearance of the absorbance around 630 nm. For Mb, the absorption around 500 nm gradually decreases and the peak red shifts with the increase of PFDA. When the concentration of PFDA reaches 1.6×10^{-4} mol/L, the absorbance increases at 537 nm. In addition, the absorbance around 630 nm shows the same declining trend as BHb. The bands at 411 and 537 nm are considered to be characteristic of hemichrome.[24,25] All the spectral changes in the heme regions reveal the occurrence of hemichrome in both BHb and Mb. Scheme 1 illustrated the detailed structures of heme and hemichrome groups.[26,27] As is known, the heme cavity in hemoproteins is stabilized by a unique balance of hydrophobic, hydrogen bonding and polar interactions. Apart from the four N atoms in the porphyrins, the remaining coordination sites around the normal Fe ion of heme are taken up by the proximal histidine and the oxygen molecule as the fifth and sixth ligands (Scheme1A). After the addition of PFDA, its carboxyl group can bind to the peptide chain (with positive charge) through electrostatic attraction, and the hydrocarbon chain can penetrate into the heme pocket through hydrophobic attraction, both of which can disturb the hydrophobic balance there, and then induce the formation of hemichrome.[22,28,29] Hemichrome is the oxidized product of hemoglobin with two histidines (proximal and distal) ligated to the hexa-coordinated low spin heme iron atom (Scheme 1B). It has been reported that hemichrome accumulation in red blood cells is related to some blood diseases and aging of erythrocytes.[17,30] Therefore, the data suggest that PFDA may affect the normal biological function of hemoproteins in transferring oxygen.

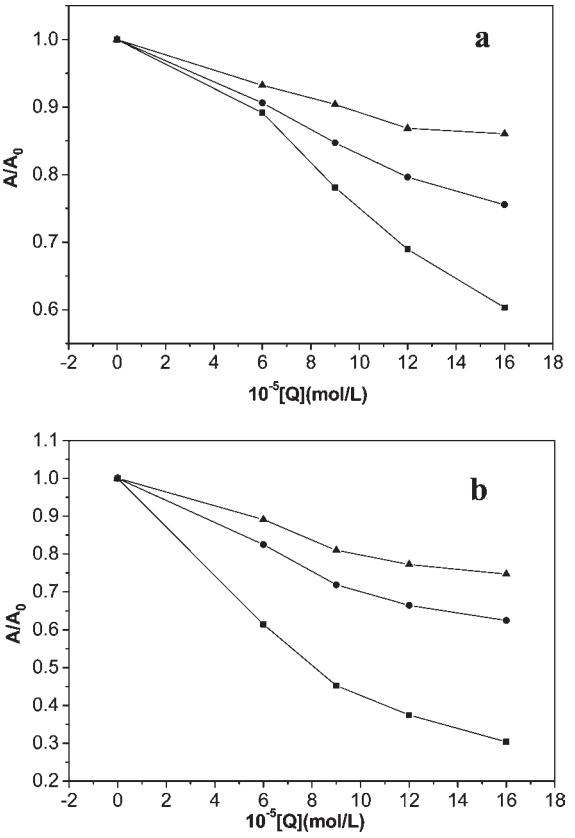


Figure 4. Effect of PFDA addition on the normalized absorption of (a) BHb and (b) Mb under different pH conditions. Conditions: PFDA ($\times 10^{-5}$ mol/L) 1−5, 0, 6, 9, 12, 16; (■) pH 6.0, (●) pH 7.0 and (▲) pH 8.0; BHb, 3×10^{-6} mol/L; Mb, 3×10^{-6} mol/L; buffer, NaH_2PO_4−Na_2HPO_4, pH = 7.40.

Like all proteins, the net charges of hemoproteins vary with pH, and the charges reduce to zero at their isoelectric point (p*I*). If there is an electrostatic interaction, the amount of PFDA bound should

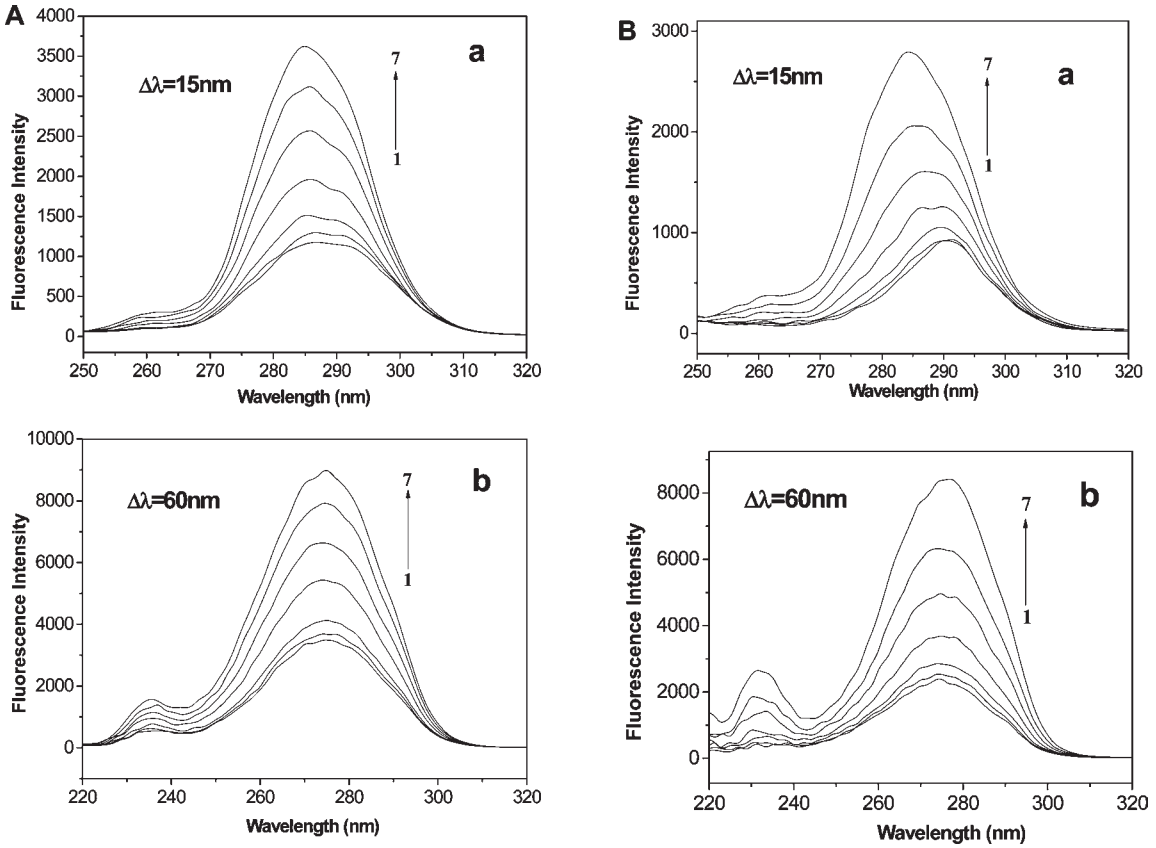


Figure 5. Synchronous fluorescence spectra of BHb (A) and Mb (B) in the presence of PFDA. Conditions: a, $\Delta\lambda$ = 15 nm; b, $\Delta\lambda$ = 60 nm; PFDA ($\times 10^{-5}$ mol/L) 1−7, 0, 1.5, 3, 6, 9, 12, 16; BHb, 3×10^{-6} mol/L; Mb, 3×10^{-6} mol/L; buffer, NaH_2PO_4−Na_2HPO_4, pH = 7.40.

change with pH. To confirm the electrostatic interaction discussed above, we studied the interactions between PFDA and BHb (Mb) under various pH conditions. The absorption changes of the Soret bands in BHb and Mb are illustrated in Figure 4. It can be seen that the curve at pH 6.0 in BHb has a greater negative slope than the other two curves, because BHb is positively charged in acidic medium with its p*I* at 6.9.[31] Hence, PFDA binding to BHb may be stronger because of the electrostatic interaction. At pH 7.0 and 8.0, however, the positive charges on the BHb surface disappear and more negative charges appear. Thus, the interaction between BHb and PFDA is weakened. The curves display the same trend in the Mb−PFDA system. Hence, the PFDA binding to hemoproteins is also likely attributed to electrostatic interaction.

Conformational Changes Shown by Synchronous Fluorescence. Generally, there is only one emission band in the normal fluorescence spectrum of a protein. However, synchronous fluorescence spectra can supply characteristic information about the Tyr or Trp residues in the vicinity of the chromospheres. The position of the spectral maximum (λmax) depends upon the properties of the environment of the tryptophanyl residues, and the fluorescence spectra depend upon the degree of exposure of the tryptophanyl side chain to the polar aqueous solvent and its proximity to specific quenching groups.[13] To separate out the emission peaks as well as to evaluate the changes in the microenvironment of both Trp and Tyr residues, the synchronous fluorescence spectra of two hemoproteins were obtained.

It is apparent from Figure 5 that the fluorescence of both residues in the two hemoproteins exhibits a great enhancement upon the addition of PFDA. The blue-shift effect of Tyr residues ($\Delta\lambda$ = 15 nm) in both hemoproteins suggests an increase of hydrophobicity in their microenvironment. Moreover, the emission peaks of Trp ($\Delta\lambda$ = 60 nm) residues in both hemoproteins exhibit a slight red shift, which indicates that the Trp residues in the protein are more exposed to water, namely, the structure of Mb becomes looser in the presence of PFDA. The above results further confirm that the conformational and microenvironmental changes occur in both BHb and Mb upon the addition of PFDA.

Secondary Structure Changes Shown by CD. CD is a sensitive physical technique for determining structures and monitoring structural changes of biomolecules.[32] To ascertain the possible influence of PFDA binding on the secondary structure of BHb and Mb, CD measurements were performed in the presence of PFDA at different concentrations. From Figure 6a and Figure 6b, an intense positive peak at about 195 nm and prominent negative bands at about 208 and 222 nm were observed. These bands are characteristic of α-helical content. The far UV CD data in the wavelength range from 190 to 250 nm provides a deep insight into the secondary structure of the two hemoproteins. It can be seen that the intensities of the negative bands at 208 and 222 nm of both BHb and Mb increased gradually with the addition of PFDA, whereas the intensity of the positive peak at around 195 nm decreased regularly with gradual mixing of PFDA.

Figure 6a shows that the proportion of α-helix decreases from 44.73% in free BHb to 42.45% and 40.53% with the increase of PFDA concentration. Besides, the amount of α-helix in Figure 6b drops somewhat from 58.74% in free Mb to 57.28% and 54.84% in the presence of PFDA. The decrease of α-helix indicates that PFDA combines with the amino acid residues of the main

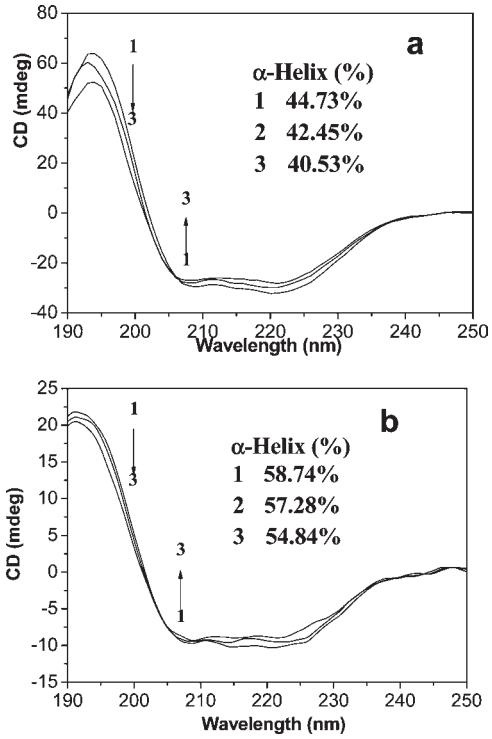


Figure 6. CD spectra of BHb (a) and Mb (b) in the presence of PFDA. Conditions: PFDA ($\times 10^{-5}$ mol/L) 1−3, 0, 6, 9; BHb, 3×10^{-6} mol/L; Mb, 3×10^{-6} mol/L; buffer, NaH_2PO_4−Na_2HPO_4, pH = 7.40.

polypeptide chain of both BHb and Mb, and destroys their hydrogen bond networks.[33] Therefore, the binding of PFDA to hemoproteins results in changes of the secondary structures of both BHb and Mb, which could lead to a loss of their normal physiological activity.

In summary, the effects of PFDA on BHb and Mb were characterized by multiple spectroscopic methods. The fluorescence results reveal that PFDA could greatly enhance the fluorescence of both hemoproteins, while PFPA and PFOA have little effect on their fluorescence. From the UV spectra, we conclude that the binding of PFDA to both hemoproteins resulted in a change of the heme group, exposing the heme group to water and allowing the formation of hemichrome. Furthermore, the secondary structures of the proteins were altered by the addition of PFDA. The conformational changes of hemoproteins could affect their normal function, and thereby pose a potential threat of toxicity. This work sheds light on the relationship of the chain lengths and functional groups of PFAAs to their molecular toxicology in vitro.

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ABBREVIATIONS USED

PFAAs, perfluoroalkyl acids; PFPA, perfluoropentanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; PFDA, perfluorodecanoic acid; Hb, hemoglobin; BHb, bovine hemoglobin; Mb, myoglobin; UV, UV−vis absorption; CD, circular dichroism; pI, isoelectric point

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