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Characterization of the binding of per- and poly-fluorinated substances to proteins: A methodological review

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ABSTRACT

Per- and poly-fluorinated substances (PFASs) are known to have the potential of binding to certain proteins. Protein binding is important to the understanding of cellular toxicities, biotransformation pathways, and the fate of selected PFASs. In this work, we provide a systematic review of major approaches to characterizing PFAS-protein binding, including the techniques based on separation, calorimetry, spectroscopy, mass spectrometry, surface plasmon resonance, molecular docking, and surfactant nature of PFASs. These approaches enable qualitative and/or quantitative characterization of binding parameters, such as the binding affinity constant and binding stoichiometry. For each approach, we review its principles and evaluate inherent strengths and limitations. In addition, the main methodologies for the identification of binding sites and target proteins are reviewed. Through the integrated review and identification of knowledge gaps and challenges, our work will guide the selection of methodologies for better characterizations of the mechanisms, kinetics, and adverse effects of PFAS-protein binding.

1. Introduction

Per- and poly-fluorinated substances (PFASs) are anthropogenic chemicals with more than 3000 sub-classes, among which perfluoroalkyl carboxylates (PFCAs) and perfluoroalkyl sulfonates (PFSAs) are most widely studied [1]. Due to their chemical inertness and surfactant properties, PFASs are widely used as surfactants, polymers, or as components of pharmaceutical, lubricant, and insecticide mixtures [2,3]. Massive usage has resulted in global occurrences of PFASs, even in polar regions, demonstrating their environmental persistence and long-range transport potential [4]. Selected PFAS chemicals have been widely reported in fish, wildlife and humans, representing considerable bioaccumulation potencies and potential biological effects [2,5]. Reported toxicities of PFASs include developmental effects, hepatotoxicity, endocrine disruption, and immunotoxicity [6]. Consequently, perfluorooctane sulfonic acid (PFOS), its salts, and its precursor perfluorooctane sulfonyl fluoride (PFOSF) have been added to the Stockholm Convention on Persistent Organic Pollutants and subjected to environmental surveillance in many regions of the world [7].

Interactions of proteins with various ligands (e.g., fatty acids and drugs) have been investigated for decades. The structures of PFSAs and PFCAs contain a highly hydrophobic perfluorocarbon tail paired with a strongly polar carboxylate or sulfonate headgroup, respectively. These structures resemble those of fatty acids and facilitate both hydrophobic and ionic interactions with proteins [8]. It is well known that PFCAs and PFSAs are mainly distributed in protein-rich body compartments, such as plasma, liver and kidney [9–11]. The PFAS-protein interactions were firstly investigated in the 1950s, but the purposes of early studies were to protect bovine serum albumin (BSA) against denaturation through its interaction with perfluorooctanoate acid (PFOA) and subsequent precipitation [12–14]. After organofluorine chemicals were reported in human plasma [15], and later PFASs were recognized as global contaminants, interactions between PFASs and proteins recaptured researchers' attentions.

To date, the characterization of PFAS-protein binding was mainly conducted for the investigation of PFASs' toxicity via direct or competitive binding assays. Some studies employed direct binding assays to explore the effects of interactions on protein structures and functions as well as the underlying mechanisms. Zhang et al. reported that the binding of PFOS to human serum albumin (HSA) changed secondary conformation and inhibited

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transport functions of HSA [16]. PFOS-BSA interactions have been shown to impair BSA's secondary and tertiary structures and decrease its esterase-like activity [17]. Other studies used competitive binding assays to investigate the interfering or competitive displacement of endogenous ligands (e.g., fatty acids and amino acids) by PFASs. Such competitive binding may further disrupt normal functions of proteins, such as endocrine and immunological functions [18,19].

In addition to toxicity evaluations, the PFAS-protein binding was also investigated for understanding the bioaccumulation, biotransformation and elimination of PFASs [20–22]. For example, the fate of fluorotelomer unsaturated aldehydes and acids was explored by investigating their reactivity with model proteins [23]. The mechanism for sex-dependent elimination of PFOA in rats was also investigated by characterizing the binding of PFOA to liver- and kidney-form α2u-globulins [21].

In light of the need for studying PFAS-protein binding as summarized before, the present study aims to provide a systematic review on the approaches to characterizing PFAS-protein binding and the methodologies for identifying selected PFASs' target proteins and binding sites. In this review we describe the principles of each method and its applications, discuss their advantages and limitations, and identify knowledge gaps as well as future perspectives.

2. Methodologies for characterizing PFAS-protein binding

Available techniques used to investigate PFAS-protein interactions are summarized in Fig. 1. These approaches allow qualitative and quantitative characterization of the interactions via several main techniques, including the separation of free and protein-bound fractions of ligands and the detection of perturbation in the physicochemical properties of PFASs, binding protein, or the PFAS-protein complexes [24]. These characterizations further illustrate the type of binding, binding affinity, binding capacity, binding sites, or the effects of binding on both the structures and activities of proteins for different study aims (Table 1).

2.1. Separative methods

Equilibrium dialysis (ED) is a widely used separative method for the characterization of protein-ligand binding. It is based on the establishment of an equilibrium state between the protein solution and buffer solution containing PFASs separated by a membrane permeable to PFASs but impermeable to the proteins and their complexes [8,16,25]. Although it is considered as a standard approach to characterizing binding affinities over a wide range of ligand to protein (L:P) mole ratios, this method has low throughput since the time to reach equilibrium is often long (typically 6–24 h; 2–4 h in some cases) [26]. As a rapid approach alternative to ED, ultrafiltration is also based on a semipermeable membrane which is only permeable to ligands. However, potential membrane effects limit broad applications of both the ultrafiltration and equilibrium dialysis approaches. Han et al. indicated that the failure of using an ultrafiltration system to characterize PFOA-rat serum albumin (RSA) binding was attributed to the non-specific binding of PFOA with the membrane [27].

Another two approaches to separating free PFASs from bound forms are size-exclusion chromatography (SEC) and desalting column separation. In two SEC studies, rat plasma or blood samples containing dosed ^{14}C-PFOA were loaded on the chromatographic column and then eluted with phosphate buffered

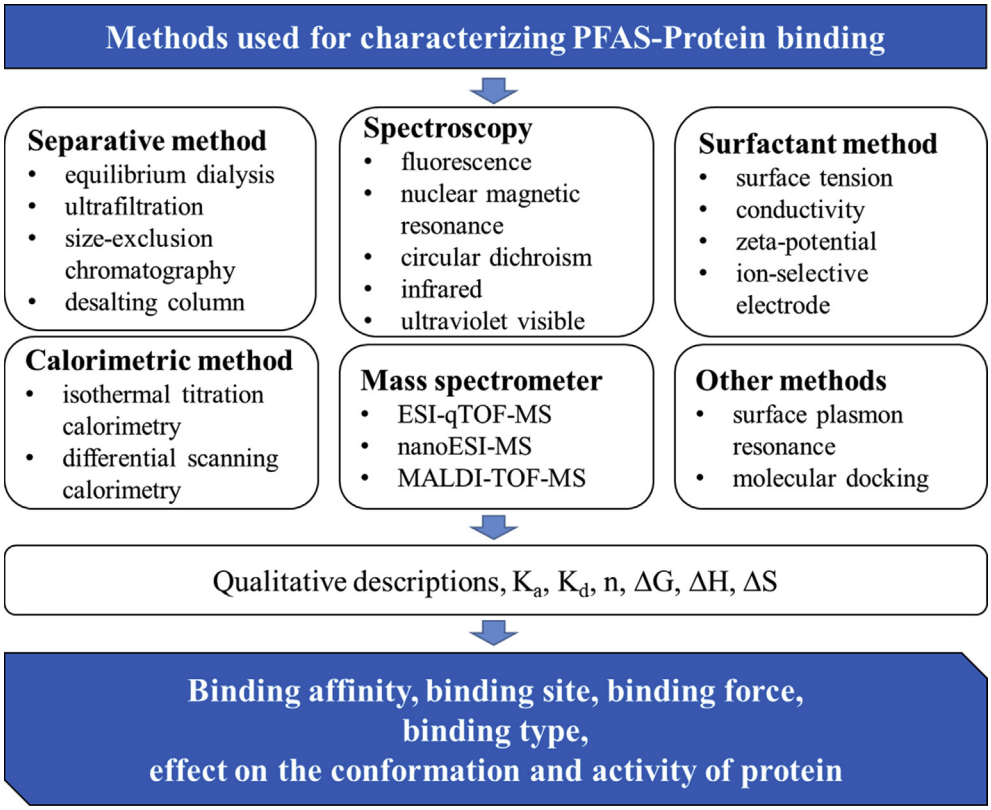

Fig. 1. A summary of the main methodologies used to characterize PFAS-protein binding. ESI-qTOF-MS, electrospray ionization quadrupole time of flight mass spectrometry; MALDI-TOF-MS, matrix assisted laser desorption/ionization time of flight mass spectrometry; n, stoichiometry; ΔG, Gibbs free energy; ΔH, the enthalpy of the binding reaction; ΔS, the change of entropy.

Table 1
Examples of available studies on the binding of PFASs to different proteins.

PFASs	Target Proteins	Methodologies	Interaction Characterization	Ref
PFOA	HSA, rat serum albumin	Size-exclusion chromatography,^{19}F-NMR, ESI-qTOF-MS, ligand blotting, microdesalting column separation	Number of binding sites, K_d	[27]
PFBS, PFHS, PFOS, PFBA, PFOA, PFDA	BSA	qTOF-MS	Binding capacity	[19]
PFOA	2u-globulins	Ligand blotting, ESI-qTOF-MS, fluorescence	K_d	[21]
PFOA	HSA	UV–vis, CD, ion-selective electrodes	Binding capacity, number of ligand sites, Hill binding constant, Hill coefficient	[34]
PFOA	HSA	Surface tension measurements, UV–vis, electrophoretic mobility	Conformational changes	[32]
PFOA with different counterions	β-lactoglobulin	CD, fluorescence, conductivity,^{19}F- NMR	Conformational changes	[33]
PFOA, PFDA	Catalase	Electrophoretic mobility, UV–vis	Number of binding sites, conformational changes	[37]
PFOA, PFBA, PFBS, PFOS, PFDoA	HSA	Fluorescence	Binding sites and K_a	[38]
PFOA	HSA	Equilibrium dialysis, fluorescence, ITC, CD	Thermodynamic properties of interaction, conformational changes, binding sites	[25]
PFOS	Serum albumin	Equilibrium dialysis, fluorescence, ITC, CD	Thermodynamic properties of interaction, conformational changes, binding sites	[16]
PFOA, PFNA	BSA, HSA	Equilibrium dialysis, nanoESI-MS	K_a, binding stoichiometry	[8]
PFOA, PFHxA	HSA	^{19}F- NMR	Binding sites	[53]
PFHpA, PFOA, PFNA, PFDA, PFUnA, PFHxS, PFOS	HSA	Fluorescence	Hill binding constant, Hill coefficient	[40]
PFOA, PFNA, PFDA, PFUnA	BSA, HSA	Surface tension measurements, fluorescence,^{19}F- NMR	K_a	[31]
PFDA, PFOA, PFPA	Bovine hemoglobin, myoglobin	Fluorescence, UV–vis, CD	Conformational changes, secondary structure changes	[42]
Fluorotelomer unsaturated acids and aldehydes	Apomyoglobin, HSA	ESI-qTOF-MS,^{19}F- NMR	Complex stoichiometry	[23]
PFBA, PFBS, PFOA, PFOS, PFDoA, PFTA	Estrogen receptor	surface plasmon resonance, molecular docking	K_d, K_a, relative binding affinity	[48]
Fluorotelomer unsaturated aldehydes and carboxylic acids	HSA, BSA	ESI-qTOF-MS	Adducts stoichiometry	[20]
PFOA	β-lactoglobulin	Fluorescence, ITC, differential scanning calorimetry, infrared spectroscopy	Conformational changes, effects on thermal stability	[29]
PFBA, PFPA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTA, PFHxDA, PFOcDA, PFBS, PFHxS, PFOS, 6:2 FTOH, 8:2 FTOH	Liver fatty acid binding protein	Fluorescence, CD, molecular docking	K_d, structure changes, binding strength	[41]
PFBA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFOcDA, PFTA, PFBS, PFHxS, PFOS, 6:2 FTOH, 8:2 FTOH, 10:2 FTOH	Thyroid hormone transport proteins	Fluorescence, CD, molecular docking	Relative potency compares with natural ligand thyroxine, K_d	[50]
PFOS	BSA	Fluorescence, CD, UV–vis, molecular docking	Conformational changes, effects on thermal stability, binding sites	[17]
PFOS	Hemoglobin	Fluorescence, CD, UV–vis, molecular docking	Conformational changes, effects on thermal stability, binding sites	[39]
PFOA	HSA	Electrophoretic mobility, UV–vis	Number of binding sites, binding energy, conformational changes	[36]
PFOA	Lysozyme, hemoglobin, catalase	Electrophoretic mobility	Number of binding sites	[35]
PFOA, PFOS	Serum albumins	ESI-qTOF-MS, fluorescence, molecular docking	Complex stoichiometry, Ka, conformational changes, binding sites	[45]
Perfluoroalkane sulfonyl fluorides	HSA	Fluorescence, MALDI-TOF-MS	Binding sites, complex stoichiometry	[44]

PFBA: perfluorobutanoic acid; PFPA: perfluoropentanoic acid; PFHxA: perfluorohexanoic acid; PFHpA: perfluoroheptanoic acid; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid; PFDA: perfluorodecanoic acid; PFDoA: perfluorododecanoic acid; PFTA: perfluorotetradecanoic acid; PFUnA: perfluoroundecanoic acid; PFOcDA: perfluorooctadecanoic acid; PFOS: perfluorooctane sulfonic acid; PFBS: perfluorobutane sulfonate; PFHxS: perfluorohexane sulfonate; 6:2 FTOH: 6:2-fluorotelomer alcohol; 8:2 FTOH: 8:2-fluorotelomer alcohol; 10:2 FTOH: 10:2-fluorotelomer alcohol; HSA: human serum albumin; BSA: bovine serum albumin; ESI-qTOF-MS: electrospray ionization-quadrapole time of flight-mass spectrometry; CD: circular dichroism; NMR: nuclear magnetic resonance spectroscopy; ITC: isothermal titration calorimetry; UV–vis: ultraviolet visible spectroscopy; MALDI-TOF-MS, matrix assisted laser desorption/ionization time of flight mass spectrometry.

saline. Given that free PFOA usually elutes later than protein molecules, the coelution of ^{14}C-PFOA and protein molecules, indicated by simultaneous monitoring of the radioactivity of ^{14}C-PFOA and the ultraviolet absorption wavelength of proteins, demonstrated the occurrence of ^{14}C-PFOA-protein binding [27,28]. In the desalting column method, free PFASs (PFOA for example) are retained on the column after loading samples, while the complexes can freely pass [27,28]. If the column is demonstrated to result in high recoveries for both proteins and free PFOA, the desalting column method deems reliable for quantitative measurement of binding parameters (e.g. K_d) under both high- and low-affinity binding conditions [27,28].

2.2. Calorimetric techniques

Two calorimetric approaches are used to study the PFAS-protein binding: isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) [29]. The ITC method is based on one of the fundamental molecular characteristics, i.e., thermodynamics is involved in molecular interactions. In the ITC method, PFAS solution is injected into the protein solution at a low constant speed and under a constant temperature, during which the change of thermodynamic parameters (e.g., reaction enthalpy and heat capacity) is measured. The ITC is not suitable for very high- or very low-affinity systems, as in both processes it is unlikely to derive accurate thermodynamic information from calorimetric data [30]. The ITC also has low throughput as it takes a relatively long time to run a full titration experiment. The DSC approach employs a reference cell and a sample cell heated at a controlled rate. Proteins are denatured along with temperature increase, while the addition of PFOA to the sample cell might shift the denaturation process. Comparisons of the DSC thermograms of proteins with and without the presence of PFOA and measurement of the shift in denaturation enthalpy qualitatively determine binding affinity constants (e.g., association constant K_a). However, DSC is an indirect method since the shift is conducted on the folded and unfolded proteins rather than the complexes themselves.

Calorimetric methods demonstrate an advantage over other methods in their ability to provide a relatively full picture of thermodynamic parameters during binding reactions, including the equilibrium binding constants, the enthalpy of binding reactions (ΔH), the entropy change (ΔS), and the Gibbs free energy (ΔG). These thermodynamic data allow the exploration of not only the interaction mechanisms, but also the effects of PFASs on protein properties. For example, DSC measurements revealed that the presence of PFOA caused irreversible denaturation of β-lactoglobulin (BLG) at a room temperature [29].

2.3. Surfactant nature-based methods

A few approaches were applied to determine the PFAS-protein binding based on the nature of selected PFASs as surfactants. The surface-active properties of surfactants enable the investigation of PFAS-protein binding through the measurement of surface tension. When PFASs are bound to proteins, the surface tension of a solution will be increased since the amount of free PFASs is reduced [31,32]. Another approach is to measure the changes in the conductivity of aqueous PFAS solution with and without proteins [33]. In both methods, the critical micelle concentration (cmc) of a PFAS is selected as a critical operation condition. These methods are simple and quick, but neither of them can directly measure association constants or characterize interaction mechanisms.

Ion-selective electrode has been employed to measure PFOA-protein binding through potential measurement via a millivolt meter equipped with a $C_7F_{15}COO^-$-ion-selective electrode and a saturated calomel electrode [34]. As the electromotive force value (emf) is related to free $C_7F_{15}COO{-}Na^+$ concentrations, the amount of $C_7F_15COO^-$ bound to HSA, binding kinetics, as well as K_a, stoichiometry and ΔG, can be determined via emf data.

Electrophoretic mobility (zeta-potential) measurement has been used to determine the effect of electrostaticity and hydrophobicity of PFOA on its interaction with proteins [35–37]. The measured electrophoretic mobilities of a complex are used to calculate zeta-potential based on the Henry equation. Data of zeta-potential with an increase of PFAS concentration to a final concentration near its cmc can give both quantitative and qualitative information on binding, such as binding affinity constants and binding energy, thus facilitating a better understanding of PFAS-protein interaction mechanisms.

2.4. Spectroscopy

Spectroscopic techniques represent the most widely used approaches for PFAS-protein binding characterizations, which usually include fluorescence spectroscopy, nuclear magnetic resonance spectroscopy (NMR), circular dichroism, infrared spectroscopy, and ultraviolet visible (UV–vis). In addition to the measurement of binding affinity constants, spectroscopic approaches also facilitate the exploration of binding mechanisms by providing insight into three-dimensional protein structure and complementary structural/conformational changes of protein molecules resulting from complex formation.

Fluorescence spectroscopy finds broad applications in PFAS-protein binding studies (Table 1) by offering a variety of measurements, including emission spectra, excitation spectrum fluorescence intensity, and fluorescence lifetime. Qualitative and quantitative information of PFAS-protein interactions can be achieved through these measurements. Shift of the maximum emission wavelength [38,39], decrease of fluorescence intensity [25,31], and decrease of fluorescence lifetime [31] have been observed when PFOA or PFOS was added to a protein solution. The great sensitivity of intrinsic fluorescence of tryptophan or tyrosine residues results in a high signal-to-noise ratio and allows the method to be applicable to a wide range of L:P mole ratios. Three-dimensional fluorescence spectroscopy further enables more efficient observation of conformational changes of proteins (e.g., BSA) following the interaction with PFOS [17]. Mathematical models, such as the Scatchard plot, Lineweaver-Burk, and Stern-Volmer plots, are applied in fluorescence spectroscopy for the measurement of binding affinity constants [31,38]. However, a primary limitation of these methods arises from the inability of fluorescence spectroscopy to directly measure free PFASs. In these models, concentrations of free PFASs are usually substituted by the total concentrations of free and bound forms [31], which may deviate binding affinity measurements [40]. Another potential limitation of these models is their oversimplification of ligand attachment to binding sites by fitting curvilinear plots with straight lines. In addition to the mathematical models based on direct measurement of fluorescence intensity, indirect displacement assays or competitive binding assays have been used more frequently to measure binding constants [18,21,41]. Small molecule acids (e.g., dansyl undecanoic acid) are often used as fluorescence probes. After PFASs are added to the probe-protein system and measured by fluorescence spectroscopy, the dissociation constant (K_d) of a PFAS can be estimated with the below equation [21]:

$$K_{d\_PFAS} \,/\, K_{d\_Probe} = IC_{50\_PFAS} \,/\, [\text{Probe}] \quad (1)$$

where IC_{50_PFAS} is the 50% binding inhibitory concentration of a PFAS, [Probe] is the probe concentration, and K_{d_PFAS} and K_{d_Probe} represent the K_d for PFAS and the fluorescence probe, respectively.

Owing to high-resolution signals, the NMR techniques are able to characterize binding interactions in more detail, including the stoichiometry, kinetics and conformational properties of a complex. The ^{19}F-NMR is frequently used in PFAS-protein binding studies (Table 1). Determination of K_a, K_d, and other quantitative parameters can be achieved by measuring PFASs' resonance shift before and after binding to proteins. However, NMR is limited by its inability to characterize binding at a low L:P mole ratio. This reduces the technique's physiological relevance, as the levels of PFASs in human bodies are much lower compared with those of proteins [31].

Circular dichroism (CD) spectroscopy provides information on possible conformational changes of proteins [39,41,42]. Its spectrum characterizes the percentages of the major components of a protein's secondary structure (e.g., α-helica, β-sheet, and β-turn). Thus, the changes of the percentages with the presence of PFASs indicate conformational alteration of proteins and the occurrence of PFAS-protein interactions [17,34]. In theory circular dichroism enables the determination of protein-ligand binding parameters (e.g., K_a). However, this technique has rarely been used to quantitatively characterize PFAS-protein binding, probably because the binding of PFASs to most proteins is in a low to medium affinity range, while CD is more reliable for high-affinity binding interactions [43].

The UV–vis spectrometry has also been employed to study PFAS-protein binding (Table 1). The outcome of this method is expressed as the differences in UV absorption spectra of protein molecules with or without the interaction with PFASs [17,39]. Infrared spectrometry was also combined with other spectrometric methods to investigate how PFOA binds with the BLG and whether the binding affects BLG's thermal stability [29]. The spectra of PFOA/BLG complexes were markedly different from those of pure BLG, thus indicating the occurrence of binding from a qualitative view.

2.5. Mass spectrometry (MS)

Mass spectrometry offers a straightforward and efficient approach to measuring the stoichiometry and molecular weight of the complexes during the ligand-protein interaction. Analytical advantages of MS measurements include rapid characterization, ability to measure binding stoichiometry, and simultaneous evaluation of multiple equilibria and different complexes. In PFAS-protein binding studies, two soft ionization techniques, electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI), have been used [8,23,44], which in combination of time-of-flight (TOF) or quadrupole TOF (qTOF) MS also allows for high resolution measurements of interaction complexes (Table 1).

The ESI-MS allows the detection and quantification of free and PFAS-bound protein ions based on the mass differences between the molecular ions of unadducted protein and the complexes. The K_a is determined from the ratio of the total abundance of protein-PFAS complex ions to free protein ions and known initial concentrations of proteins and PFASs in the solution [8,45]. Although the transformation of ions from liquid to gas phase will largely preserve the interaction, the most controversial aspect of this approach is the extrapolation from the gas-phase measurements to solution-phase behavior [46]. This is because the relative abundance of the protein-PFAS complex ions to free protein ions may change during the evaporation and droplet fission processes, possibly causing incorrect measurements of K_a and binding stoichiometry.

Since most PFAS chemicals are found to be weak ligands when binding to proteins [18,19,25], the successful implementation of ESI-MS requires the complexes present in a solution to be well preserved during the ESI process and in the gas phase. Operational parameters of the mass spectrometry, such as capillary temperature and collision energy, may have effects on not only the sensitivity of the mass spectrometry, but also the structural stability of a complex. Han et al. reported that the ions corresponding to the complex of PFOA and α2u-globulins disappeared at cone voltages higher than 30 V [21]. On the other hand, if the complex dissociates after entering into MS by changing instrument conditions (e.g., increasing collision energy), this will permit the detection of free PFASs and then measurement of binding capacity. However, it works only if free proteins and PFASs are completely filtered out before entering into the MS system [19]. Another challenge limiting the widespread implementation of this method is the contradictory effects of "physiological" buffer system on the protein stability and the sensitivity and reproducibility of mass spectrometric analyses. Jones et al. investigated the influences of different buffer systems on the sensitivity and reproducibility of the PFOS-BSA binding. Results revealed that no ideal buffer could simultaneously maintain proteins stable while enhancing the sensitivity of the proteins during mass spectrometric analyses [19].

NanoESI-MS was employed to overcome some disadvantages of ESI-MS (e.g., low sensitivity and high sample consumption) for the investigation of noncovalent bindings between PFAS and BSA [8]. A particular nanoESI-MS system (i.e., the Nanomate) functions as a combination of autosampler and nanoelectrospray with a nL/min flow rate, offering softer ionization than conventional ESI-MS [47]. Compared with the results from equilibrium dialysis and other approaches, nanoESI-MS was demonstrated with the capability of measuring K_a and binding stoichiometry over a wide range of L:P mole ratios. However, some challenges to conventional ESI-MS, such as poor reproducibility and the risk of complex disassociation, also exist for nanoESI-MS.

2.6. Other techniques

2.6.1. Surface plasmon resonance (SPR) technique

Gao et al. [48] proposed a SPR-based biosensor method to characterize PFAS-induced conformational changes of estrogen receptor (ER). Estrogen receptor is firstly immobilized on the sensor surface, and then bound to PFASs with different chain lengths and acid groups (e.g., PFOA, perfluorobutyric acid, perfluorobutane sulfonic acid, PFOS). If the PFOA-ER binding induces a conformation state in the sensor surface, the SPR response signals will be changed and recorded. Mathematical treatment of these signals yields binding data, such as K_a and K_d. This quick and real-time method provides both kinetic and affinity information. However, its applications are limited by low throughput and time-consuming method development.

2.6.2. Molecular docking

As a computer-aided tool, molecular docking complements experimental measurements by providing insight into PFAS-protein interactions from structural characteristics [41,45,49,50]. Based on known three-dimensional structures of both the proteins and ligands, this approach predicts the structure of the formed complexes via computer programs with a combination of sampling algorithms and scoring functions. The sampling algorithms reproduce the experimental conformations by putting the ligand into an active site of the protein, followed by a rank of all generated conformations through a scoring function [51]. This method yields a number of parameters describing the interactions, including the binding energy, hydrogen-bonding interaction, and the length of ligand. Although this method is quick and effective, it might result in deviations in the prediction of *in vivo* or *in vitro* interactions, possibly due to the metabolism and other biological processes in a living organism [52].

3. Methodologies for identifying binding sites and target binding proteins

3.1. Identification of binding sites

Methods for determining binding sites differ in principles from the aforementioned approaches in the characterization of various binding parameters (e.g., binding affinity and stoichiometry). Fluorescence and NMR spectroscopy are two indirect methods used to identify binding sites, mainly via competitive displacement

measurements (Fig. 2). In brief, after proteins are bound with site-specific probes, the displacement of some binding sites on the probes by PFAS molecules results in subsequent changes in the fluorescence and NMR spectra (e.g., decrease of fluorescence intensity). Selection of sensitive probes for target ligands is critical to these methods. Based on the known fatty acid binding sites of HSA, Chen and Guo used dansylamide and dansyl-L-proline as fluorescence probes for two specific sites to investigate the binding of PFOA and PFOS to HSA [38]. Dansyl-L-proline and warfarin were used as fluorescence probes in another study for the determination of the binding sites of PFOSF on HSA [44]. D'eon et al. used $^{13}C_1$-oleic acid sitting in different binding sites as the probe to study PFOA/perfluorohexanoic acid (PFHxA)-HSA binding during ^{13}C-NMR measurements [53]. In the same study, two drugs (i.e., ibuprofen and phenylbutazone) with known binding sites were also used as oleic acid's competing ligands to further confirm binding sites [53].

3.2. Identification of target binding proteins

Identification of target proteins is critical to a comprehensive characterization of PFAS-protein binding. It also broadens the knowledge of PFAS-induced toxic effects. Ligand blotting assay has been used to identify PFOA's target proteins in plasma and liver cytosol [27,28]. After being transferred onto a polyvinylidene fluoride membrane, proteins are incubated in a buffer before blotting. After blotting, the membrane is washed and then autoradiographed by the phosphor image technique. The radio-labeled band in gel autoradiogram indicates principle proteins targeted by PFOA and the relative binding capability. However, during this method nonspecific binding could occur and hamper the identification of actual target proteins.

Two novel NMR techniques, including the heteronuclear saturation transfer difference NMR (H-STD-NMR) and the reverse heteronuclear saturation transfer difference NMR (RH-STD-NMR), have been employed to explore target proteins of PFOA/PFHxA in human serum [53]. For a traditional STD-NMR experiment, target proteins are selectively saturated, while no ligand resonances are irradiated, which is referred to as the on-resonance spectrum. Saturation will then be transferred to ligands if they interact with the proteins. By contrast, neither the ligand nor the protein resonances are irradiated in the off-resonance spectrum. This technique measures the difference spectrum by comparing on and off-resonance spectra, and only signals from saturated ligands will remain. PFASs contain two nuclei, ^{19}F and ^{1}H, which are well taken advantage of by H-STD-NMR and RH-STD-NMR. In the H-STD-NMR method, conventional ^{1}H detection is replaced with ^{19}F detection. The ^{1}H-nuclei of proteins are selectively saturated, and the saturation is then transferred to PFOA or PFHxA closely contacting with the proteins. The RH-STD-NMR is a reversed process, during which the ^{19}F-NMR signals of PFASs are firstly saturated and then transferred to ^{1}H-nuclei of any interacted components. Therefore, in difference spectrum only signals from saturated proteins that interact with PFOA or PFHxA will remain. All other components that do not interact with PFOA/PFHxA are not saturated and will be absent from the difference spectrum. Comparing the spectra of RH-STD-NMR with those of suspected components (e.g., HSA) subsequently identified HSA as the most possible target protein for PFOA or PFHxA [53]. By using the H-STD-NMR, the specificity and binding orientation of PFOA to human serum were determined and used for further confirmation of HSA as the binding protein.

Proteomic methods have been introduced to directly identify target binding proteins of PFOA in liver [54]. The procedures in sequence included gel-based or MS-based competition assays,

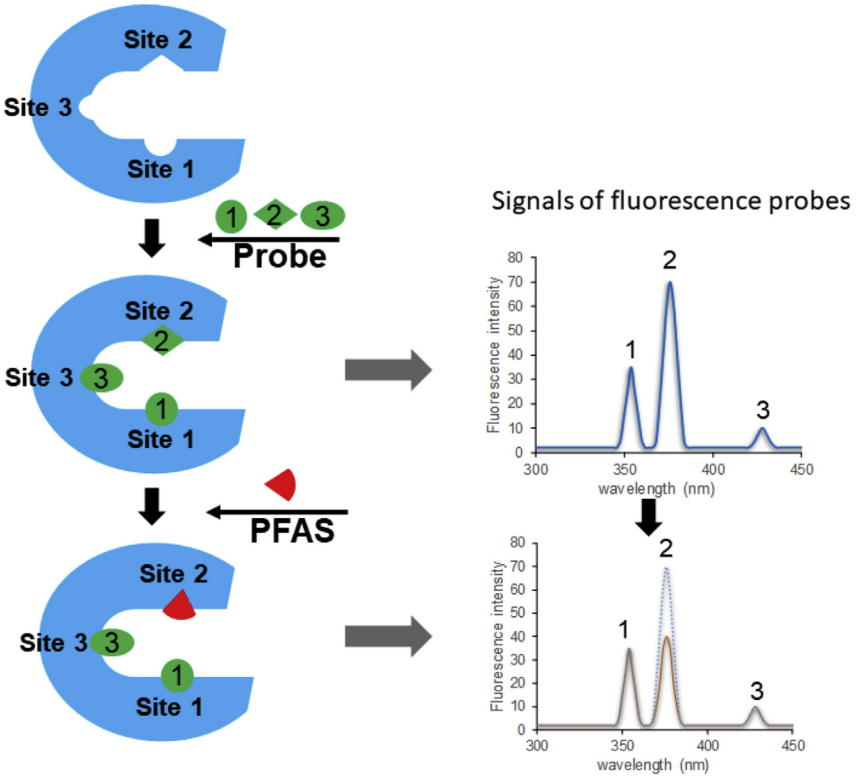

Fig. 2. Schematic presentation of displacement measurements of site-specific binding of PFASs to proteins by using fluorescence probes. Each probe is designed to specifically bind to certain sites in the protein.

parallel reaction monitoring (PRM)-based targeted proteomic strategy, thermal stability assay, and targeted metabolomics [54]. Both the gel-based and mass-based competition assays utilized two cysteine-targeting probes, i.e., iodoacetamide alkyne (IAA) and ethynyl benziodoxolone azide (EBX), since PFOA has a carboxyl group which can interact with reactive thiol groups present in cysteine. Liver samples from the control and PFOA-treated groups were pretreated with these two probes (Fig. 3). In gel-based screening, the probe-labeled proteins were tagged with a Rhodamine group prior to fluorescent analysis. Decreased fluorescence intensity in IAA or EBX group in the presence of PFOA indicates the competition of PFOA with the probes. In MS-based method, probe-labeled proteins were labeled with biotin-alkyne or biotin-azide, purified with streptavidin beads, and then digested into peptides. Subsequent liquid chromatography-tandem MS (LC-MS/MS) analysis determined intensity changes of each protein with or without PFOA, which filters out suspect proteins to be targeted by PFOA. After suspect proteins were selected via gel- or MS-based competition assays, PRM assays based on LC-MS/MS data were further used for targeted proteomic analysis and verification of target proteins. Subsequently, *in vitro* thermal stability assays were employed to demonstrate the complex interactions by examining the shift in thermal stability of suspect target proteins with the presence of PFOA. Finally, *in vivo* targeted metabolomics analysis was used for further validation of the affected functions of the target proteins. In the study by Shao et al. [54], the target binding proteins were finally validated to be two carboxylase isoenzymes via targeted metabolomics analysis, as the binding of PFOA to these two enzymes decreased their catalytic products and further interfered with fatty acid metabolism.

4. Conclusions and future perspectives

In this work, we conduct a systematic review of various methodologies for the determination of PFAS-protein binding characteristics and potential mechanisms. Given the variety of methods used, Table 2 summarizes the advantages and limitations of each technique. These factors merit careful consideration when choosing a right method for relevant studies. In most cases, a combination of different approaches may enable a better characterization of PFAS-protein binding.

Available research has mainly focused on PFCAs and PFSAs (Table 1). However, following the regulations of PFOS and PFOA usage and increasing environmental surveillance, additional novel PFAS chemicals have been developed as alternatives or precursors and were identified in environmental and biological matrices [55]. As alternatives, these chemicals are often structurally similar to PFCAs and PFSAs. Although several recent studies have paid attention to selected isomers and alternatives of PFOA and PFOS [49,56], possible interactions of emerging PFASs with different functional proteins warrant future studies for a better understanding of their potential toxicities and health risks. More

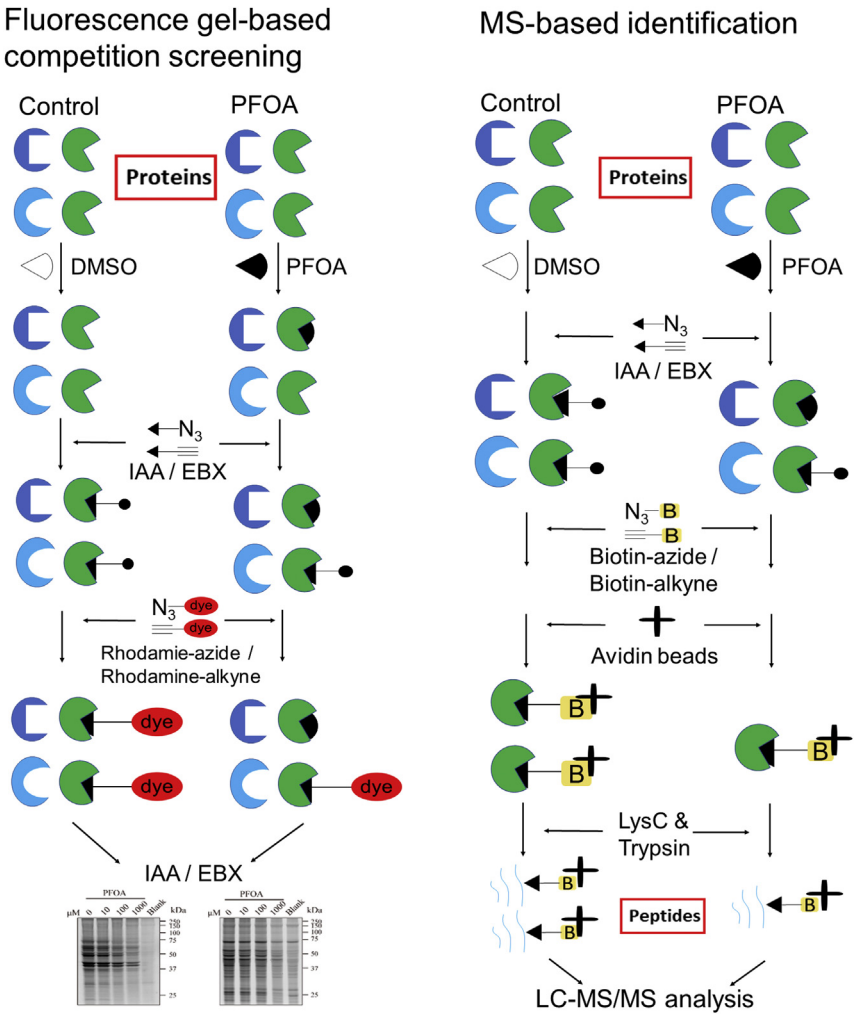

Fig. 3. Schematic description of gel-based competition screening and mass spectrometry-based competition assays, slightly modified from Shao et al. [54].

Table 2
Main advantages and limitations of different methodologies used to characterize PFAS-protein binding.

Methods	Advantages	Limitations
Equilibrium dialysis	Simplicity, inexpensive	Long equilibrium time, nonspecific binding
Ultrafiltration	Simplicity, inexpensive	Nonspecific binding, stability of binding equilibrium may be changed during separation process
Size-exclusion chromatography	Simplicity,	Low efficiency, low protein recovery
Isothermal titration calorimetry Differential scanning calorimetry	Thermodynamic information	High sample consumption, low throughput, highly pure reagent required
Fluorescence spectroscopy NMR spectroscopy Circular dichroism spectroscopy	Speed, structure information, kinetics information, low sample consumption	False negatives, not powerful for low-affinity binding, highly pure reagent required
Surface tension measurement	Simplicity	Low selectivity, poor sensitivity, unable to measure quantitative association constants
Conductivity measurement	Simplicity	Low selectivity, unable to measure quantitative association constants
Mass spectrometry	Simplicity, speed, low sample consumption, specificity, selectivity	Poor reproducibility, false negatives, risk of disassociation of complexes, limited structure information
Surface plasmon resonance	Real time, speed, kinetics information, low sample consumption	Unable to measure binding stoichiometry, time-consuming method development

attention should also be given to the binding between proteins and metabolic intermediates formed through PFAS biotransformation. Although these intermediates might be short lived, their interactions with proteins and associated toxic effects should not be overlooked.

PFAS-protein binding studies have mainly been conducted *in vitro*. Relevant techniques used in *in vivo* experiments (e.g., ED, SEC, and surface tension) were mostly conventional and relatively simple (Table 1). More efficient techniques, such as the NMR, fluorescence, and SPR, are demanded for future interaction studies *in vivo* or even in living cells. In addition, direct observation techniques, such as microscopy, have rarely been used in PFAS studies addressing binding sites [57]. Novel techniques, including in-cell NMR and cryo electron microscopy, as well as the techniques used in other ligand-protein studies, may also find useful applications in PFAS-protein binding studies [58]. These emerging techniques will greatly benefit a more comprehensive characterization of PFAS-protein binding and underlying mechanisms.

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