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Partition coefficients of four perfluoroalkyl acid alternatives between bovine serum albumin (BSA) and water in comparison to ten classical perfluoroalkyl acids†

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Perfluoroalkyl acids (PFAAs) are persistent, ubiquitous environmental contaminants and their long-chain representatives are bioaccumulative. The phase-out of these compounds (*e.g.* PFOA and PFOS) shifted the production to alternatives. However, little is known about the bioaccumulative behaviour of the alternatives, which are still highly fluorinated. PFAAs are predominantly detected in blood, where they bind to the transport protein serum albumin. This sorption can be described by the albumin/water partition coefficient. It is unclear whether the partition coefficients of the alternatives are lower than or in the same range as those of classical PFAAs. We determined albumin/water partition coefficients for seven perfluoroalkyl carboxylates, three perfluoroalkane sulfonates and four alternatives by dialysis experiments in a physiologically representative system. Quantification was done by LC-MS/MS and a mass balance approach. Logarithmic albumin/water partition coefficients for PFAAs range from 2.8 to 4.8 [L_{water} $kg_{albumin}^{-1}$] and increase with increasing chain length. Perfluorinated sulfonates sorb more strongly than their carboxylate counterparts. The albumin/water partition coefficients for the alternatives (HFPO-DA, DONA, 9Cl-PF3ONS and PFECHS) are in the same range as for classical PFAAs. Structural modifications such as the introduction of ether groups into the chain do not reduce sorption to albumin, whereas the chlorine atom in 9Cl-PF3ONS seems to even increase the sorption to albumin. We further investigated whether the sorption strength could be affected in the presence of medium- or long-chain fatty acids. Binding competition between medium-chain fatty acids and PFAAs appeared to be possible. However, the presence of physiologically more relevant long-chain fatty acids should not alter the albumin/water partition coefficients of PFAAs.

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Environmental significance

Increasing numbers of structurally modified but still highly fluorinated alternatives to perfluoroalkyl acids are entering the environment and subsequently organisms. Little is known about their bioaccumulative behaviour. The bioaccumulation potential of these chemicals depends (among other factors) on their affinity to the main transport protein in blood, serum albumin. We found that four alternatives bind similarly strongly to albumin as classical PFAAs. We therefore conclude that the alternatives are, based on their sorption to albumin, not less potent to bioaccumulate than PFAAs. Understanding of the bioaccumulative behaviour enables authorities to regulate the production of new alternatives before they can enter the environment and not in retrospect, like it was the case for the long-chain perfluoroalkyl acids PFOA and PFOS.

Introduction

Perfluoroalkyl acids (PFAAs) have been produced since the 1950's as processing aids for fluoropolymer production or as surfactants in firefighting foams. They may enter the environment directly, from impurities or from unintentional side products, leading to an ubiquitous distribution through water and air.[1–3] They were found to be persistent and long-chain PFAAs are also bioaccumulative in a variety of organisms[4,5] and suspected to be developmental and organ toxic to humans.[6,7] A characteristic of PFAAs is the acidic head group attached to an alkane tail of varying length in which every

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hydrogen is substituted by a fluorine atom.[8] The high electronegativity of fluorine is responsible for their strong acidic nature (pK_a is estimated to be below 1)[9,10] as well as for the extremely stable C–F bonds. This, together with their surface active properties leads to their widespread use. Until now authorities and researchers mainly focused on the impact of the broadly used compounds perfluorooctanoate (PFOA) and perfluorooctanesulfonate (PFOS), which led to regulatory actions for the use of PFAAs and an increased awareness of the risk of PFAAs.[5,11,12] Manufacturers responded precautiously with a voluntary phase-out of long-chain PFAAs like PFOA and PFOS during the last few decades,[11] shifting the production at the same time towards alternative compounds. These alternatives comprise shorter-chain PFAAs but also a whole new variety of chemicals that are structural modifications of the 'classical' PFAAs and still highly fluorinated. Four representatives of these alternatives are in the focus of this work and shown in Fig. 1. Indeed, perfluoro-4-ethylcyclohexanesulfonate (PFECHS) has not been introduced in the course of the phase-out of PFAAs (the production goes back to the 1990s),[13] but is included in the study as a structural alternative. While it was shown that these modified compounds are still persistent,[14–17] their bioaccumulative and toxic potency compared to long-chain PFAAs is still under discussion.[3,14,15,18,19]

The accumulation of a compound in an organism depends on several toxicokinetic factors involving the absorption, distribution, metabolism and excretion (ADME). Physiological based toxicokinetic (PBTK) models of various complexities are used to describe and predict concentration–time curves based on the ADME processes. To this end, equilibrium partition coefficients $K_{phase1/phase2}$ between various biological phases (*e.g.* lipids and proteins) and water need to be known. While the required partition coefficients can be predicted quite well for most neutral chemicals,[20] such predictions become much more difficult for ionisable chemicals and even more so for perfluorinated acids.[21,22] The partition behaviour of PFAAs differs strongly from other compounds due to their unique chemical properties. Highly fluorinated compounds have much smaller van der Waals interactions compared to non-fluorinated molecules of the same size.[23,24] Hence, to improve the prediction of the partition behaviour for the whole substance class and consequently to contribute to valid PBTK models, the experimental determination of partition coefficients to relevant phases for some of these compounds is essential.[25–27] Often, the octanol/water partition coefficient K_{ow} (or in the case of ions an adjusted K_{ow}) is used as an estimation for protein/water partitioning. Partitioning of an analyte in a two-phase system is influenced by intermolecular interactions occurring between the analyte and each phase. Octanol as a solvent cannot mimic the interaction possibilities (*e.g.* ionic interactions and H-bonding) of a protein, as a result the K_{ow} is by no means an appropriate substitute for a protein/water partition system.

One of the most relevant sorbing phases for PFAAs is serum albumin,[28–30] which is the major transport protein in blood with concentrations ranging between 35 and 50 g L^{-1}.[31] It contains several binding sites for various endogenous ligands such as fatty acids, bilirubin, heme and thyroxine.[32,33] Moreover, three of these binding sites are involved in the interaction with various drugs and other chemicals, such as warfarin, diazepam or ibuprofen, among others.[34–36]

So far association constants to serum albumin for the most prominent PFAAs were determined with a variability up to four orders of magnitude for single compounds depending on the method and ligand : protein mole ratio used.[28,29,37–45] The first consistent data set of albumin–water partition coefficients of a series of perfluoroalkyl carboxylic acids (PFCAs) and three perfluoroalkane sulfonic acids (PFSAs) was provided by Bischel *et al.* (2011). They found log $K_{albumin/water}$ ranging from 3.3 to 4.3 [L kg^{-1}] (K_a values: 1×10^5 to 1×10^6 M^{-1}) by means of equilibrium dialysis experiments.[46]

In this study we determined albumin/water partition coefficients for four alternatives to classical PFAAs by dialysis experiments,[22] thereby investigating the effect of their structural modifications on their sorption behaviour. Additionally, we determined the albumin/water partition coefficients of the PFAAs by the same method to ensure that we compare results within one consistent data set. We discuss our albumin/water partition coefficients for the PFAAs in comparison to literature values, which were determined by different approaches.

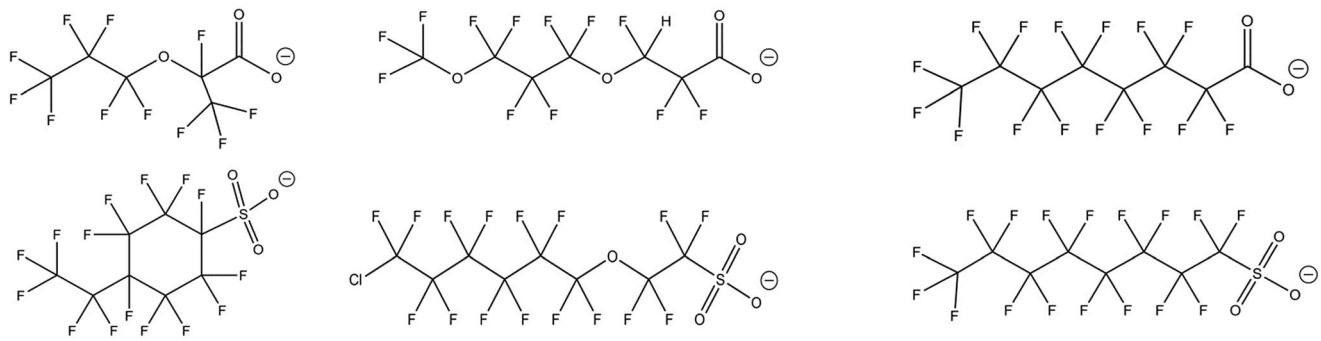

Fig. 1 Alternative compounds to long-chain PFAAs in comparison with two prevailing classical PFAAs. Above: tetrafluoro-2-(heptafluoropropoxy)-propanoate (HFPO-DA) and 4,8-dioxa-3*H*-perfluorononanoate (DONA), compared to perfluorooctanoate (PFOA). Below: perfluoro-4-ethylcyclohexanesulfonate (PFECHS) and 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate (9Cl-PF3ONS) compared to perfluorooctanesulfonate (PFOS).

In the literature it is often claimed that PFAAs compete with other ligands such as fatty acids for specific binding sites on serum albumin.[28,30] We additionally tested whether a competition for specific binding sites can be demonstrated, which would affect the partition coefficients of PFAAs. Since the PFAA chain length may affect this competitive behaviour,[29,47] we decided to investigate short-chain and long-chain PFCAs (the latter defined as $C_nF_{2n+1}COOH$, $n \geq 7$).[8]

Experimental

Chemicals and reagents

Sodium dodecafluoro-3*H*-4,8-dioxanonanoate (DONA, correct abbreviation NaDONA‡), 2,3,3,3-tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy-)propanoic acid (HFPO-DA/GenX), potassium 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate (9Cl-PF3ONS, the main component of F-53B), potassium perfluoro-4-ethylcyclohexanesulfonate (PFECHS) and a mixture of PFAA calibration standards (PFAC-MXB) and internal standards (MPFAC-MXA) were supplied by Wellington Laboratories (Ontario, Canada).

Perfluorobutanoic acid (PFBA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), and perfluoroundecanoic acid (PFUnDA), as well as three sulfonic acid salts – tetrabutylammonium perfluorobutanesulfonate (PFBS), potassium perfluorohexanesulfonate (PFHxS) and potassium perfluorooctanesulfonate (PFOS) were purchased individually from various manufacturers (see ESI-1.1†). For each compound, a stock solution was prepared in methanol and stored at −18 °C. Lyophilized powders of bovine serum albumin (BSA) were supplied by Sigma Aldrich with the product numbers A3803 (heat shock fraction, essentially fatty acid free, ≥98%) and A7906 (heat shock fraction, pH 7, ≥98%). Partition coefficients presented in Table 1 were measured only with batch A3803, essentially fatty acid free. A3803 was the same batch used in experiments where the partition coefficients of other anionic compounds were determined.[22]

Dialysis experiments

Dialysis cells were used as described in detail before.[22,48] The individual poly- and perfluorinated compounds were diluted in Hank's balanced salt solution (HBSS; pH 7.4, see ESI-1.2.1†) to a concentration of 20 ng mL^{-1}. Where possible, polypropylene containers were used to avoid adsorption to glass surfaces. The concentration of BSA in solution was dependent on the compound investigated and ranged from 0.025–3 g L^{-1}. The molar ratio of compound to BSA was held below 0.1 to avoid ligand oversaturation of the protein binding sites. Compounds were measured individually in an assembly of two glass chambers separated by a membrane (see ESI-1.2.2†). The membrane (Spectrum Laboratories Inc., Por 4) has a cutoff for molecules greater than 12–14 kDa allowing permeation of the analytes (poly- and perfluorinated compounds) with the retention of BSA (66.5 kDa). 5 mL of the respective poly- and perfluorinated compound solution were added to one chamber of the dialysis cells. Into the other chamber either 5 mL of BSA solution (measurement cells) or 5 mL of pure HBSS (reference cells) was pipetted. The dialysis cells were incubated at 37 °C under constant agitation. BSA-free chambers of both measurement and reference cells were sampled on two consecutive days at least and partition coefficients were determined. When both values matched, equilibrium could be assumed. Most of the compounds required an equilibrating time of 72 h, except for longer-chain PFAAs like PFDA, PFUnDA and PFOS, which needed 96 h to equilibrate. For each compound, a setup of three measurement cells and three reference cells was prepared.

Chemical quantification

The analysis of PFAAs and alternatives was performed using ultra performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) (Xevo TQ-S Waters Corporation) as described elsewhere.[49,50] Aqueous samples were mixed with methanol containing 4 mM ammonium acetate and methanol extracts were mixed with 4 mM aqueous ammonium acetate before analysis. Internal standards were added to all samples (to a final concentration of 1 ng mL^{-1}) before injection (see ESI-1.3.2† for the assignment of internal standards to analytes). Separation was achieved with an ACQUITY UPLC BEH Shield C18 column (1.7 μm particles; 2.1 mm × 50 mm). A "PFC isolator column" (Waters) was used to retain and separate method blanks originating from the mobile phases or the HPLC system. The detector was operated in negative electrospray ionization mode (ESI^-), followed by multiple reaction monitoring for quantification. Instrumental parameters and MS/MS transitions are reported in ESI-1.3.† Data acquisition, processing and analysis were done by using Water's proprietary software MassLynx (version 4.1). Quantification was achieved by the internal standard method using an external linear calibration curve to determine relative response factors.

Determination of partition coefficients

The albumin/water partition coefficient K was calculated as

$$K_{\mathrm{i,albumin/water}} = \frac{c^*_{\mathrm{i,albumin}}}{c^*_{\mathrm{i,water}}} \left[\mathrm{L_{water}\ kg_{albumin}}^{-1}\right] \quad (1)$$

where i refers to the compound of interest and * represents the equilibrium condition. $c_{\mathrm{i,albumin}}$ is the bound concentration to albumin [g $kg_{albumin}^{-1}$] and $c_{\mathrm{i,water}}$ is the concentration in water [g L_{water}^{-1}].

$c_{\mathrm{i,bound}}$ cannot be measured directly. Therefore, a mass balance approach was used to determine $c_{\mathrm{i,bound}}$ indirectly. For each compound a fixed amount of serum albumin was added into measurement cells. The mass of the compound bound to BSA was determined as

$$m_{\mathrm{i,bound}} = m_{\mathrm{i,total}} - m_{\mathrm{i,free}} \quad (2)$$

‡ ADONA (ammonium dodecafluoro-3*H*-4,8-dioxanonanoate) is often used instead. We use DONA as the name for the anion.

Table 1 Albumin/water partition coefficients (log $K_{albumin/water}$ [L_{water} $kg_{albumin}^{-1}$]) determined for ten PFAAs and four alternative compounds. Mean and standard deviation SD were taken from six measurements. HFPO-DA and DONA are not fully fluorinated; therefore no number is assigned. Fraction bound $f_{i,bound} = m_{i,bound}/m_{i,total}$. K_a is the association constant ([PL]/([P] × [L])) with protein P and ligand L and is displayed to facilitate comparison with literature values

Number of perfluorinated carbons	Analyte	log $K_{albumin/water}$ [L kg^{-1}]	SD	K_a[a] [M^{-1}]	SD	f_{bound}	SD	Recovery (incl. extracts)	c_{BSA} [g L^{-1}]
PFCAs									
3	PFBA	2.52	(0.11)	2.3×10^4	(4.8×10^3)	34%	(5%)	98%	3×10^0
5	PFHxA	3.43	(0.04)	1.8×10^5	(1.6×10^4)	58%	(2%)	98%	1×10^0
6	PFHpA	4.02	(0.08)	7.2×10^5	(1.4×10^5)	34%	(4%)	99%	1×10^{-1}
7	PFOA	4.20	(0.05)	1.1×10^6	(1.1×10^5)	44%	(3%)	94%	1×10^{-1}
8	PFNA	4.32	(0.07)	1.4×10^6	(2.2×10^5)	37%	(4%)	99%	5×10^{-2}
9	PFDA[b]	4.73	(0.04)	3.6×10^6	(3.1×10^5)	57%	(2%)	57% (106%)	5×10^{-2}
10	PFUnDA[b,c]	4.60	(0.30)	3.1×10^6	(1.4×10^6)	35%	(13%)	40% (115%)	2.5×10^{-2}
	HFPO-DA	3.06	(0.03)	2.3×10^4	(5.0×10^3)	64%	(1%)	96%	3×10^0
	DONA	3.93	(0.03)	1.8×10^5	(4.7×10^4)	30%	(2%)	93%	1×10^{-1}
PFSAs									
4	PFBS	3.20	(0.11)	1.1×10^5	(2.8×10^4)	44%	(6%)	101%	1×10^0
6	PFHxS	4.81	(0.05)	4.4×10^6	(3.5×10^5)	62%	(2%)	92%	5×10^{-2}
8	PFOS[b]	4.67	(0.07)	3.2×10^6	(4.9×10^5)	37%	(4%)	78% (104%)	2.5×10^{-2}
8	9Cl-PF3ONS[b]	5.01	(0.04)	6.9×10^6	(6.2×10^5)	56%	(2%)	75% (106%)	2.5×10^{-2}
8	PFECHS	4.55	(0.03)	2.4×10^6	(1.4×10^5)	66%	(1%)	95%	1×10^{-1}

[a] Note that the association constant is converted from $K_{albumin/water}$ at one molar ratio and not determined by measuring the binding isotherms. [b] Extraction step included due to glass sorption of the analyte. [c] Only mean of five measurements was taken.

Freely dissolved analyte mass $m_{i,free}$ was measured from the samples of the measurement cells when equilibrium was reached, see above. Two different approaches were used to determine the total mass m_{total} of the compound in the cells. PFAAs and their alternatives sorb to the glass[51] and membrane surfaces as determined in pre-tests. For compounds where less than or equal to 10% of the $m_{i,total}$ sorbed to the surface, reference cells were used, which did not contain any protein. For compounds that sorb substantially (>10%) to the surfaces of the dialysis cells, reference cells could not be taken as an appropriate reference for the mass balance due to the variability of glass surfaces of the handmade cells. Substantial glass sorption was observed for longer-chain PFAAs. For these compounds an extraction step was added to fulfil the total mass balance criterion. To this end, all fluids were discarded after the last sampling and the interior of the cells was extracted with 2 mL of methanol. Subtraction of the mass found in the extracts from the initially used dilution of the analyte leads to $m_{i,total}$. Overall recoveries after correction (masses from the dissolved fraction and the extracts of the reference cells in comparison to the initially used dilution of each compound) ranged from 92 to 115% (see Table 1).

For the determination of the partition coefficient of a compound, mean and standard deviation of six measurements were taken. We performed a test to determine week-to-week precision for the determination of the partition coefficient for PFNA. For that, dialysis experiments were carried out three times within seven months based on the setup described above, each time with three replicates. The precision was calculated by taking the standard deviation of nine partition coefficients into account.

Competition experiments with fatty acids

We conducted dialysis experiments with PFHxA or PFNA adding known amounts of two different fatty acids (tridecanoic acid (TDA, Fluka) or heptadecanoic acid (HDA, Fluka)) to test whether free fatty acids and PFAAs would compete for specific albumin binding sites. For that, essentially fatty acid free BSA (batch A3803) was first equilibrated with the respective fatty acid for 24 h before adding the perfluorinated compound. Each setup had a specific molar ratio of fatty acid to albumin with approximate [fatty acid] : [albumin] ratios of 2.5, 1 and below 1, respectively. Samples of the BSA-free chambers were taken separately for fatty acid analysis and PFAA analysis at the same time points (after 72 h and 96 h).

Results and discussion

Experimental design

Albumin/water partition coefficients were determined by dialysis experiments for each compound individually. Experiments were conducted with seven perfluoroalkyl carboxylic acids (PFCAs), three perfluoroalkane sulfonic acids (PFSAs) and four per-/polyfluorinated alternatives to long-chain PFAAs. We expect similar partition coefficients for BSA and human serum albumin (HSA) and used the cheaper BSA in our studies. BSA and HSA share a sequence homology of 76%.[52] They differ in length by two amino acid residues and fold into structurally similar globular tertiary structures.[31] Previously it was shown that sorption to BSA compared to HSA is in the same order of magnitude[53] for compounds like fatty acids as well as for PFOA and PFNA.[40,54]

For each compound concentrations of BSA were adjusted in the dialysis experiments. The amount of protein (the water volume is fixed at 10 mL by the glass dialysis cells) plays a critical role in determining the partition coefficient. It has an impact on the freely dissolved concentration of the compound and therefore on the fraction analysed. The albumin concentration was adjusted for each compound to ensure that the fraction of the analyte sorbed to albumin ($m_{i,bound}/m_{i,total}$) is between 20 and 80%. Below 20% or above 80%, already small variations due to the measuring uncertainty could shift partition coefficients up to 1–2 log units (see ESI-2.1†). Simultaneously the molar ratio of respective compound to BSA was held below 0.1 to work within the linear range of the sorption isotherm as shown before.[46,55,56] Recovery from dialysis experiments was >90% for the majority of the compounds. The $m_{i,total}$ from reference cells and $m_{i,free}$ from the measurement cells were used to calculate $m_{i,bound}$ and the respective partition coefficient. For PFOS, PFDA, PFUnDA and 9Cl-PF3ONS sorption to the glass surface of the dialysis cells (recovery < 90%) was observed. For these compounds glass cells were extracted with methanol after the dialysis to calculate $m_{i,total}$, $m_{i,free}$ and $m_{i,bound}$ with respect to the loss through surface sorption. Due to error propagation this additional correction led to greater uncertainties in the resulting albumin/water partition coefficients, especially for long-chain compounds like PFUnDA (where 60% of the analyte was sorbed to the glass surface). For this reason, we were not able to determine the partition coefficients of longer chain-length analogues of the PFCAs.

Additionally, we performed a test with PFNA to assess week-to-week precision for the determination of partition coefficients (see ESI-2.2†). PFNA was chosen as the test compound since it is a long-chain PFAA but still with less than 10% sorption losses to cell surfaces. It was shown that within one experimental setup the standard deviation is smaller than that between the week-to-week setups, where standard deviation was determined to be 0.1 log units.

Logarithmic albumin/water partition coefficients of the PFAAs

Experimentally determined partition coefficients of PFAAs are summarized in Table 1 and Fig. 2 and 3 (see also ESI-2.3†). Logarithmic albumin/water partition coefficients range from 2.5 to 4.8 ($K_a = 2.3 \times 10^4$ to 4.4×10^6 M^{-1}). When considering the sorption behaviour of other organic anions to albumin, the partition coefficients of PFAAs are comparably high but in the same range as those of ibuprofen and 2-naphthaleneacetic acid (log K of 3.9 and 4.8, respectively).[22] Furthermore, PFCAs sorb more strongly to albumin than their hydrocarbon counterparts (C4–C11, measured at pH 7.6 and 23 °C).[38,57]

The partition coefficients of the PFCAs increase with increasing chain length, *i.e.* number of perfluorinated carbons. PFBA (3 perfluorinated carbons) and PFDA (9 perfluorinated carbons) have logarithmic albumin/water partition coefficients of 2.5 and 4.7, respectively. It cannot be concluded whether this trend continues between PFDA and PFUnDA from our experiments, due to the relatively higher uncertainty in the measurement for PFUnDA (SD > 0.3 log units). Among the

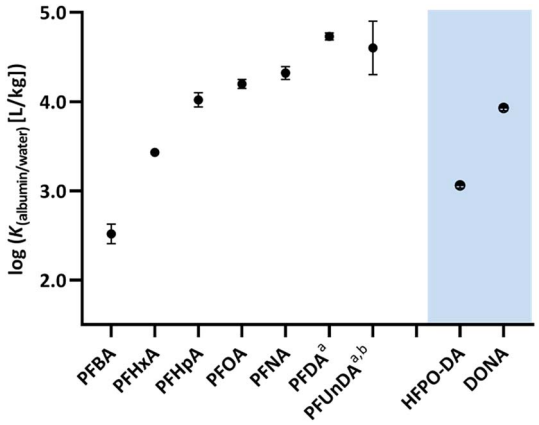

Fig. 2 Logarithmic albumin/water partition coefficients for the series of PFCAs and the two alternatives HFPO-DA and DONA with a carboxylate head group. The order of PFCAs corresponds to an increasing chain length with PFBA with 3 perfluorinated carbons and PFUnDA with 10 perfluorinated carbons. Mean and standard deviation SD were taken from six measurements. Error bars of standard deviations are partly covered by symbols of data points.

PFSAs, PFBS has the lowest logarithmic albumin/water partition coefficient of 3.2, while PFHxS and PFOS show relatively high logarithmic partition coefficients of 4.8 and 4.6, respectively. The log K values for PFHxS and PFOS are in the same order; however, a clear trend of increasing albumin water partition coefficients with increasing molecular weight, which was observed for the PFCAs up to PFDA, cannot be seen for the investigated sulfonic acids.

PFSAs have in general higher albumin/water partition coefficients compared to PFCAs with the same number of fluorinated carbons. Stronger sorption of PFSAs compared to their

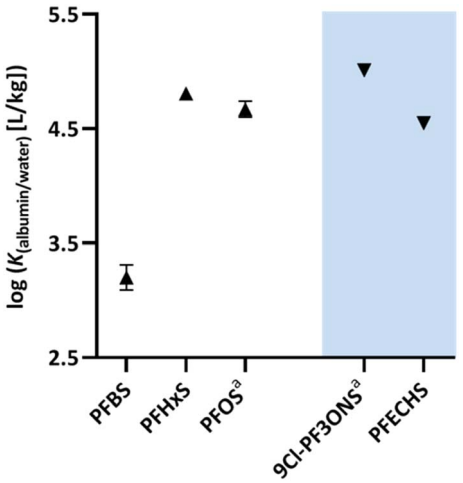

Fig. 3 Logarithmic albumin/water partition coefficients for the three PFSAs and two alternatives 9Cl-PF3ONS and PFECHS with a sulfonate head group. The order of PFSAs corresponds to an increasing chain length with PFBS with 4 perfluorinated carbons and PFOS with 8 perfluorinated carbons. Mean and standard deviation SD were taken from six measurements. Error bars of standard deviations are partly covered by symbols of data points.

PFCA counterparts is consistent with other studies.[58,59] The log K value of PFHxS is 0.8 log units higher compared to the log K of PFHpA whereas the partition coefficients of PFOS and PFNA differ by 0.4 log units. For PFBS, the corresponding carboxylic acid was not analysed. We determined 3D structures by using quantum chemical software COSMOconf/TURBOMOLE[59,60] to compare the conformers of PFHxS and PFOS, but no obvious differences were discernible. Reasons why binding of PFSAs to albumin is more favourable than PFCAs are still unclear and discussion is ongoing.[38,46,61,62]

Another batch of BSA (A7906) was tested to verify whether the determined albumin/water partition coefficients are dependent on the albumin selected for the experiments. See ESI-2.4† for details. We found differences up to 0.3 log units in the albumin/water partition coefficients especially for shorter-chain PFAAs. Approximately the same overall standard deviation was determined for PFNA with both batches of BSA. In conclusion we assume that different batches of BSA could affect the value of determined partition coefficients. Variable extraction and purification steps could probably affect the conformation of the native serum albumin which could be a reason for the observed differences in the log K values.[63]

Albumin/water partition coefficients of alternative compounds HFPO-DA, DONA, 9Cl-PF3ONS, and PFECHS

Due to their environmental significance, we included four alternative compounds to long-chain PFAAs in our analysis.[13,15,64–67] Logarithmic albumin/water partition coefficients were 3.1 and 3.9 for the carboxylic acids HFPO-DA and DONA and 5.0 and 4.6 for the sulfonic acids 9Cl-PF3ONS and PFECHS, respectively (Table 1).

The albumin/water partition coefficients are in the same range as the ones of the PFAAs. The categorization of the alternatives based on the number of perfluorinated carbons is difficult since some carbons (*e.g.* in HFPO-DA and DONA) are not fully fluorinated. Based on chemical structures HFPO-DA may be compared to PFBA or PFHxA and DONA to PFHxA or PFHpA. Both determined partition coefficients are between the partition coefficients of the respective PFAAs (Fig. 2). The two alternatives to the perfluorinated sulfonic acids, 9Cl-PF3ONS and PFECHS, can be compared directly to PFOS ($\log K_{\mathrm{albumin/water}} = 4.7$) based on their chemical structure (Fig. 1). They have two additional structural characteristics, *i.e.* the chlorine in the terminal position of 9Cl-PF3ONS and the cyclic structure of PFECHS. 9Cl-PF3ONS shows the highest albumin/water partition coefficient of all compounds analysed in our experiments ($\log K = 5.0$). The chlorine substituent on the terminal carbon in 9Cl-PF3ONS appears to cause an increase in sorption. Reasons could be its larger atom radius (99 pm compared to 72 pm of fluorine) and subsequently increased interaction possibilities at specific binding sites (sterically favoured) or favourable van der Waals interactions with amino acid residues of the albumin with the chlorine atom. PFECHS was provided as a mixture of *cis*/*trans*-isomers of the *para*-substituted cyclohexane ring. Due to analytical limitations, we could not differentiate between these isomers. Since PFECHS and PFOS have similar albumin/water partition coefficients, we conclude that the cyclic structure of PFECHS does not affect the interactions with albumin (*e.g.* by steric hindrance).

To investigate the structural characteristics of the four alternatives, especially the ether linkages, we used TURBOMOLE software included in COSMOconf[59,60] as a quantum-chemical tool to calculate and visualize the conformer of a compound with the most favourable (*i.e.* lowest) energy in 3D. For each conformer, the so-called sigma surfaces or COSMO surface polarization charge densities are provided describing the abilities to undergo various intermolecular interactions, such as electrostatic, hydrogen bonding and van der Waals interactions. 3D structures with the corresponding sigma surfaces are shown in Fig. 4 and colours are assigned to the charge densities of the surface regions. The compound di-*n*-butylether was included for comparison.

Surprisingly, there are no negative charge densities (red colour) to be seen around the ether groups of HFPO-DA, DONA and 9Cl-PF3ONS as compared to di-*n*-butylether which acts here as a representative of typical ethers. Instead the surface charge densities of the perfluorinated chains that possess one or two ether functionalities look quite similar to those of PFOA without any ether functionality. Within the $-CF_2-O-CF_2-$ group, fluorine has the highest electronegativity and induces a strong positive dipole at the neighbouring carbons. For this reason, the dipole moment of the carbon–oxygen bond for the PFAAs should be smaller compared to the dipole moment of di-*n*-butylether, resulting in a lower electron density at the oxygen in the ether bond. This can explain the rather surprising experimental finding that the inclusion of ether functionalities into the perfluorinated chain did not make these molecules more polar and less sorbing.

Comparison of albumin/water partition coefficients to literature values

Over the last 20 years association constants especially for PFOA were determined by electrophoresis,[42] ^{19}F NMR,[28,40] surface tension[42,45] and dialysis[37,39,46] and vary from 10^2 to 10^6 [M^{-1}].[37–40,42] Furthermore, studies using fluorescence spectroscopy included other PFAAs, deriving site-specific association constants on albumin.[43,44,61] Discrepancies were shown to exist between different methods, and determined association constants also varied substantially within one method.[28,39,40,42] Reasons are probably indirect measuring methods and inconsistent experimental settings, such as temperature variations[37,38,40,43] and the highly variable molar ratios of [PFAA] : [albumin] (often $\gg 1$).[28,38–40,42] Sorptive losses to laboratory devices affecting the determination of K_a were checked or reported only rarely.[37,40] Additionally, the reported fractions bound were often greater than 80%, a range where the propagated error in K could amount to one or two log units (ESI-2.1†). Our association constants for PFOA and PFNA were 1.1×10^6 M^{-1} and 1.4×10^6 M^{-1}, respectively. Oversaturation can lead to an overestimation of the freely dissolved concentration of the compound and hence to an underestimation of the partition coefficient. In comparison, approximate physiological PFOA/

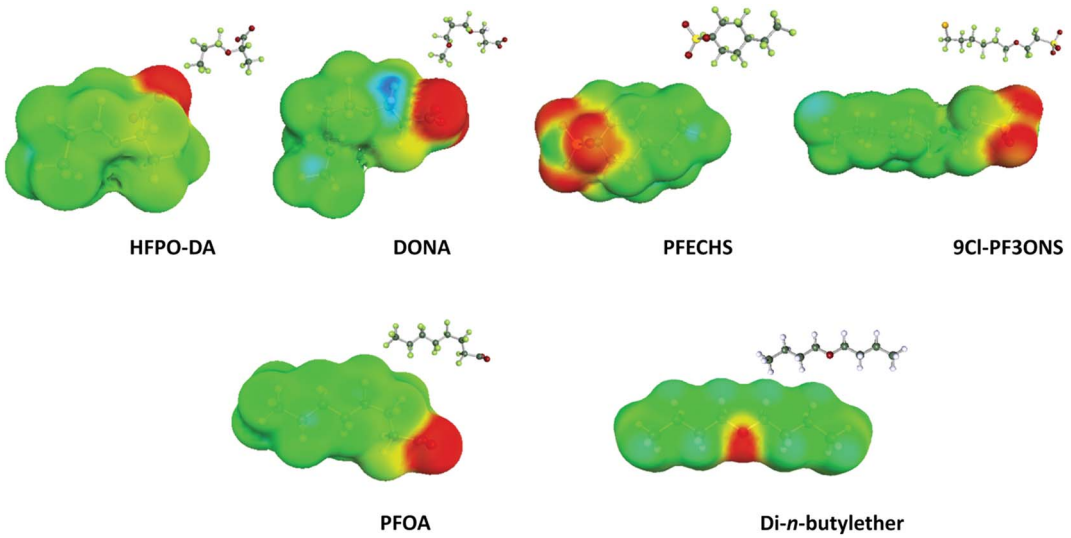

Fig. 4 Sigma surfaces of lowest-energy conformations of the target compounds determined by using COSMOconf/TURBOMOLE.[57,58] Colours are assigned to the charge densities (possible interaction site) of the surface region: green: neutral charge density; red shades: negative charge density; blue shades: positive charge density. Colour code in conformers: carbon is shown in dark green, fluorine in bright green, oxygen in red, sulfur in bright yellow, chlorine in dark yellow, and hydrogen in white.

albumin molar ratios in a worst case situation (with a serum albumin concentration of 35 g L^{-1}) would be 9×10^{-5} in general population and 2×10^{-3} in a high exposed population, *i.e.* in both cases far below a saturation of serum albumin, even when a mixture of PFAAs is taken into account.[68–71] For DONA for example the molar ratio would be far lower at 7×10^{-5} (maximal plasma concentration of a population living in the vicinity of a production plant 2015).[65] Considering this, we conclude that other methods may underestimate the sorption of PFAAs to albumin in biological systems.

Bischel *et al.* (2010 and 2011)[37,46] determined the albumin/water partition coefficients of the same series of PFAAs (PFPeA–PFDoDA and PFBS, PFHxS and PFOS) by a comparable equilibrium dialysis approach by means of direct quantification (LC-MS/MS). They used a molar ratio of [PFAA] : [albumin] <1, but did their experiments at room temperature. According to their measurements, the highest logarithmic albumin/water partition coefficient among the tested PFCAs was shown for PFHpA ($\log K = 4.2$) and among the tested PFSAs for PFHxS ($\log K = 4.3$) (ESI-2.5†). For higher analogues they describe a decrease of $\log K_{\text{albumin/water}}$ with increasing number of perfluorinated carbons (*e.g.* PFUnDA: $\log K = 3.7$ and PFOS: $\log K = 4.1$). In contrast to Bischel *et al.* we found that the albumin/water partition coefficients of PFCAs increase with increasing number of perfluorinated carbons up to at least PFDA, which is also plausible on theoretical grounds.[23,46,72] Since the $\log K$ values of Bischel *et al.* were measured with very high fractions bound (>95%), their results are more likely affected by the measuring error (as discussed before). Bischel *et al.* (2011)[46] supported their observed trend by the following mechanistic explanation: PFCAs are supposed to undergo a conformeric change when the number of perfluorinated carbons exceeds six. While PFCAs with fewer than seven perfluorinated carbons show a zig-zag geometry, longer-chain PFCAs adopt a helical twist in their structure.[73] However, we did not see such a difference in the respective 3D-structures of PFHxA and PFOA generated by the mentioned quantum chemical calculations with COSMOconf/TURBOMOLE.[59,60] Figures are included in ESI-2.6.† Our finding of a positive correlation with increasing number of perfluorinated carbons for PFCAs is supported by several other studies (including only a few PFAAs), although they were performed at very high [PFAA] : [albumin] molar ratios.[29,40,43,61] Other organic ions also show an increase in the albumin/water partition coefficient with increasing alkyl chain length.[22] A similar sorption behaviour was also observed for other compound classes such as saturated long-chain fatty acids to HSA.[74,75]

Influence of fatty acid binding

In the literature it is often postulated that PFAAs compete for the specific albumin binding sites with fatty acids.[44,47] Serum albumin is the major transport protein for especially long-chain fatty acids and the structural similarity of perfluoroalkyl acids to fatty acids suggests a potential competition. D'eon *et al.*[30] showed that PFHxA is able to displace oleic acid even at low concentrations whereas this effect does not occur for PFOA, stressing that competing potency could be dependent on the chain length.

There are two different cases for a potential competition: (A) binding of the fatty acids to serum albumin is influenced by the presence of PFAAs and (B) binding of the PFAAs to serum albumin is influenced by the presence of fatty acids. Case (A) is not reasonable; the partition coefficients of the fatty acids are higher compared to the partition coefficients of the PFAAs and the concentration of fatty acids in blood is higher compared to the concentration of PFAAs in the blood. For these reasons, a quantitative competition as described for case (A) and

investigated by D'eon *et al.*[30] could not occur in humans. However, the sorption of PFAAs to serum albumin could be affected by fatty acids, which are present at high concentrations in blood (case B). To investigate this potential competition and to answer the question whether our experimentally determined partition coefficients (with purified serum albumin) are representative of a physiological situation, where fatty acids are present in higher concentrations, we used PFHxA and PFNA as examples for PFCAs and tridecanoic acid (TDA) and heptadecanoic acid (HDA) as examples for fatty acids. Both fatty acids have an uneven number of carbons and therefore do not occur naturally. For this reason, dialysis experiments and analysis could not be affected by impurities of ubiquitous fatty acids. HDA is a long-chain fatty acid and has higher physiological relevance (medium chain fatty acids are barely measurable in human blood),[31] while TDA with its higher solubility and a lower sorption capacity to serum albumin was more suitable for the experimental approach and analysis.

The concentration of free fatty acids in human blood ranges from 0.2 to 1 mM.[31,76] The blood concentration of the fatty acids is thus considerably higher compared to the concentrations of PFAAs in general population (1–50 nM).[70,71] We adapted the albumin concentration for the determination of the partition coefficient for PFHxA and PFNA, respectively. Fatty acid concentrations were subsequently adjusted to the physiological concentration in human blood ([fatty acid] : [albumin] ≈ 2.5 : 1) and two smaller concentrations to meet approximate equimolarity and below saturation (*i.e.* a ratio below 1) criteria. All other concentrations in the setup were kept constant (with [PFCA] : [albumin] molar ratio <0.1). First, albumin and TDA or HDA were equilibrated for 24 h before the respective PFCA was added. After 72 h and 96 h, samples were taken and albumin/water partition coefficients were determined. The results are displayed in Fig. 5 and in Table ESI-2.7,† where albumin/water partition coefficients for PFHxA and PFNA are shown in dependence of different amounts of TDA (A) or HDA (B).

There is a slight decrease in the logarithmic albumin/water partition coefficients for PFHxA with increasing concentration of TDA from 3.4 (no TDA added) to 2.9 ([TDA] : [albumin] ratio of 2.3). When applying a linear regression, the slope is −0.22. Considering our inter-week precision of 0.1 log units from former dialysis setups with PFNA, we repeated the set of TDA/PFHxA-experiments (except for the lowest [TDA] : [albumin] ratio of 0.6 : 1) for confirmation of the trend. The results of a log *K* of 3.4 with no TDA added and a log *K* of 3.0 at the highest molar ratio confirm the described trend (slope: −0.14 of 3 values) and the slight influence of the present TDA concentration on the PFHxA partition coefficient. For PFNA the logarithmic albumin/water partition coefficients are 4.4 (no TDA added) and shift to 4.6 at the [TDA] : [albumin] ratio of 0.3 and slightly decline (similar to PFHxA) to a log *K* of 4.3 at the highest [TDA] : [albumin] ratio. The result without TDA for PFNA was taken from the precision experiments described above. This shift is difficult to interpret. One assumption is that the binding site could be different for PFNA than for PFHxA and, in the case of PFNA, the described shift could be caused by the effect of cooperative binding. Specific binding of fatty acid(s) to serum albumin can cause conformational changes in the tertiary structure of the protein. If this change occurs in binding sites, interaction possibilities could be created or facilitated resulting in an increased binding strength of the ligand. This cooperative binding effect was already found for other compounds.[77] While conformation of the binding site for PFHxA may not be affected when TDA is binding, the conformation of the binding site for PFNA may exhibit such a change at low molar concentrations of TDA. Increasing the amount of fatty acids, the competition for the binding sites may compensate for/overcome the cooperative binding effect.

For both compounds it appeared that the TDA concentration in the dialysis approach has a slight influence on the respective partition coefficient of the PFCA. TDA belongs to the group of medium-chain fatty acids which bind preferentially to lower affinity fatty acid binding sites on albumin whereas long-chain fatty acids (>C16) can be found at high-affinity fatty acid binding sites.[33] In the literature it is discussed that potential binding sites of PFCAs[46,47] are known drug-binding sites (Sudlow sites I and II and additionally a third drug-binding site next to fatty acid binding site 6) that partly overlap with well-known fatty acid binding sites.[34,62,78] From our data we cannot deduce a TDA specific binding site since we have to assume that TDA tends to sorb at all available binding sites at the given molar ratios.[31,32] A more general interpretation could be that the observed slight

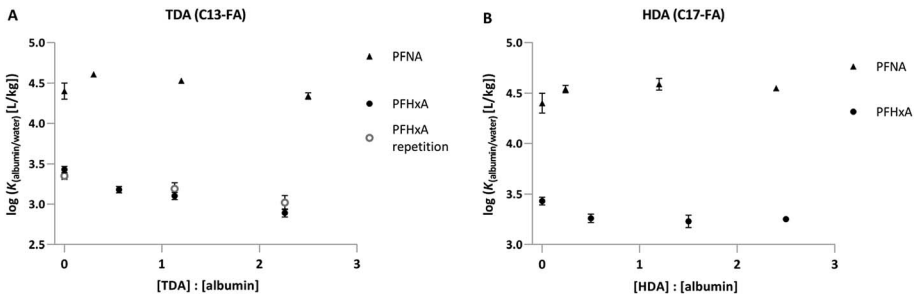

Fig. 5 Logarithmic albumin/water partition coefficients of PFNA and PFHxA in the presence of different molar ratios of the fatty acid TDA (A) or HDA (B) to albumin in equilibrium. Each data point comprises six measurements. Standard deviations are partly covered by symbols of data points. log $K_{albumin/water}$ without TDA (molar ratio: 0) for PFHxA and PFNA was taken from former experiments, and the log *K* for PFNA from precision experiments. Experiments with TDA/PFHxA were repeated once, except for the molar ratio of 0.6.

decline of the albumin/water partition coefficient of PFHxA and PFNA with increasing amount of TDA could indicate either that both compounds bind to fatty acid binding sites and that TDA is displacing the PFCA at higher concentrations, or that the respective PFCA binds to one of the drug-binding sites and the binding of TDA alters the albumin conformation thereby disrupting PFCA–albumin interaction. Both scenarios have already been described for other organic anions.[36,77] In contrast to the slight trends we observed in the experiments with TDA, almost no differences in the determined partition coefficients occurred in the presence of HDA at different adjusted [HDA] : [albumin] ratios (Fig. 5B). For PFHxA and PFNA, logarithmic albumin/water partition coefficients remain close to 3.25 and 4.55, respectively. Again, a slight difference was observed for the partition coefficient determined in the absence of HDA. It should be noted that HDA experiments for PFNA and PFHxA were each conducted using one single batch/stock solution of albumin and PFNA or PFHxA, respectively, except for the setup without HDA. This could explain the slight difference. Both values are within the standard deviation of the precision tests (PFNA) and observed deviations could not be assigned to a specific effect in this case.

Since we did not observe a concentration dependent alteration in the partitioning of PFCAs to albumin for the long-chain fatty acid HDA, we would expect that there is no significant competition of PFCAs and long-chain fatty acids in the human body (despite the slight trends we saw for the medium-chain TDA) and that the experimentally determined partition coefficients are representative of a physiological situation. But one should be aware that only two representatives were selected in our experiments (with an odd number of carbons), which does not completely cover all physiological situations.

Conclusions

The number of alternative compounds to long-chain perfluoroalkyl acids is constantly increasing. However, the bioaccumulative behaviour of both classical PFAAs and their structurally modified alternatives is still not well understood. PBTK models could help to predict the risk in humans, but such models require partition coefficients for relevant biological matrices. We determined albumin/water partition coefficients in a physiological representative system of a series of PFAAs and for four alternative compounds. Our data did not show any relevant competition between long chain fatty acids and PFAAs for sorption sites on albumin. We therefore consider our data measured for fatty-acid free albumin as physiologically relevant.

For PFCAs, our albumin/water partition coefficients increase with increasing number of perfluorinated carbons up to at least PFDA, which is consistent with most literature data. PFSAs have higher partition coefficients in comparison to their PFCA counterparts.

We demonstrated that the albumin/water partition coefficients of the alternatives are in the same range as the ones of the classical PFAAs. These results indicate that the accumulation potency of the alternatives in comparison to long-chain PFAAs is not lowered with regard to their sorption to the blood protein albumin. Serum albumin is considered as one of the preferred sorption sites for PFAAs in various organisms[26] leading to high PFAA concentrations in blood. The determination of valid partition coefficients to serum albumin is therefore decisive when describing the distribution of PFAAs in organisms by using PBTK models. PBTK models in combination with biomonitoring data facilitate valuable risk assessment. Considering the small number of studies that have monitored blood levels for alternatives in humans so far, there is demand for more data from monitoring studies. The only published study on DONA so far found rather low serum concentrations <0.2–1.7 $\mu g\ L^{-1}$ in blood donors living in the vicinity of a production site.[65] Assuming a similar intrinsic toxicity of classical PFAAs[15,66] and their alternatives and a constantly increasing exposure to the alternatives due to extended production,[79–82] it becomes clear that these compounds need further attention. Knowledge about the partitioning of these compounds will help to estimate their potency to bioaccumulate and supports the work of regulatory authorities.

Conflicts of interest

There are no conflicts to declare.

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