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Interaction of ionic liquids with human serum albumin in the view of bioconcentration: a preliminary study

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Abstract

Bioaccumulation potential is critical in PBT and risk assessment of chemicals. However, for ionic liquids (ILs), this aspect remains neglected. It is especially important to fill this gap, because for this group of compounds, existing data confirm their risk of being environmentally persistent and toxicity. Moreover, considering preliminary reports on the interactions of ILs with lipids, it may be assumed that ILs have a higher potential for bioaccumulation than indicated by previous estimations built upon octanol–water partition coefficients. Moreover, the bioconcentration of ionizable chemical compounds may also be strongly related to plasma protein contents. Therefore, in this work, the affinity of a set of imidazolium cations and organic anions, and their combination to human serum albumin (HSA) was determined. The obtained results reveal that both cations and anions can be strongly bound to HSA, and blood proteins might play an important role in overall bioaccumulation. Furthermore, it was observed that HSA binding properties towards IL cations depend on the hydrophobicity of cations. The obtained data also provide indication that cation–anion interaction may affect ILs ions affinity to HSA.

Keywords Protein binding · Bioaccumulation · Bioconcentration · Ionic liquids · Human serum albumin

Introduction

The accumulation of chemicals in biota is of major concern for environmental hazard and risk assessment. For this reason, bioaccumulation assessment is a part of international efforts to identify and control chemicals of environmental concern. The European regulation on the Registration, Evaluation, Authorisation and restriction of Chemicals (REACH), for all the compounds which are produced or imported in the amount of more than 10 tonnes requires that chemicals have to be screened and evaluated for their

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overall Persistence (P), Bioaccumulation (B) and Toxicity (T)—PBT behaviour (Reach 2006). Although using aquatic species (e.g. OECD TG 305) is the most reliable approach because the results obtained in such tests can be directly compared with the B criterion (Gramatica et al. 2015), due to several concerns, e.g. cost, time and ethical issues, in vitro experiments should be supplanting in vivo tests in bioconcentration assessment.

Generally, membrane and storage lipids have been considered as underlying compartments in the bioaccumulation of organic chemicals (Geisler et al. 2012; Endo et al. 2011). In the assessment of bioaccumulation potential by using octanol–water partition coefficients ($LogK_{OW}$), the assumption held is that the bioaccumulation of substances is determined mainly by the partitioning between lipid and aqueous phases (Mackay et al. 1982, Meylan et al. 1996). However, it has been already demonstrated that in case of permanently charged and ionogenic compounds, such models are not reliable, because they do not take into account the ion–macromolecule interactions of charged compounds with charged phospholipids (Klamt et al. 2008).

Moreover, the bioconcentration/bioaccumulation potential of chemicals is not only defined by lipid partitioning. Knowledge regarding the partitioning of these compounds to

proteins is equally important, because it may be an essential sorption phase for this group of compounds (Armitage et al. 2017). In addition, it has been shown that some chemicals (e.g. PFAS) deviate from classical bioaccumulation behaviour, and their accumulating potential within organisms arises from interaction with a number of proteins (Ng et al. 2013; Lu et al. 2021).

Although almost half of the 143,000 preregistered compounds in REACH are ionic chemicals, knowledge of their potential for bioconcentration/bioaccumulation is very limited (Franco et al. 2010). Ionic liquids (ILs) have gained much attention among scientists and industry due to their unique properties. Furthermore, due to the fact that ILs are purely ionic compounds, there is the possibility to design an enormous number of cation and anion combinations with different, specific properties. Although they have been hailed as "green solvents" not long ago, significant drawbacks, related mainly to their toxicity and persistence, have already been noticed (Samori et al. 2007; Steudte et al. 2014; Steudte et al. 2012; Stolte et al. 2008; Jeremias et al. 2021; Yue et al. 2021; Mena et al. 2020; Khan et al. 2021). However, data on their potential for accumulation in living organisms are still limited (Kowalska et al. 2021).

Taking into consideration the in vitro studies conducted so far on the bioconcentration potential of ILs, partitioning experiments using phosphatidylocholine have been carried out. The study revealed that ILs may bind to the phospholipid membrane to a great extent (much greater than assumed before on the basis of the logKow) and hence can have a potential for bioaccumulation (Dołżonek et al. 2017). Therefore, as a complementary study, it is necessary to assess their interaction with proteins so as to obtain more data for true bioconcentration estimations. For this reason, the aim of this study was to evaluate the affinity of selected cations and anions of ILs (Table 1) to proteins by using the ultrafiltration (UF) technique.

Human serum albumin (HSA) is the main component of blood plasma synthesized in the liver. It reaches a blood concentration of about 0.6 mM (Artali et al. 2005). The modular structural organization of HSA provides a variety of ligand binding sites. For this reason, HSA is the best known for its extraordinary ligand binding capacity. Research on pharmaceuticals and endogenous chemicals has shown that this protein has binding potential towards a broad spectrum of chemicals, especially hydrophobic anions of medium size. Moreover, albumin is responsible, to a great extent, for the distribution of chemicals after they enter the organism (Fanali et al. 2012). Therefore, HSA was chosen as the model protein in our research.

Due to the fact that the structure of long-chain imidazolium ionic liquids is similar to typical surfactants, and they possess surface-active properties and can form micelles in an aqueous solution (Łuczak et al. 2008). ILs in a water solution exhibit surface activity, and their micellization process is dependent on the length of the alkyl chain. This is easier for more hydrophobic compounds (Cornellas et al. 2011; Jungnickel et al. 2008; Dong et al. 2008). It has also been shown that the presence of inorganic or organic electrolytes promotes micelle formation in ILs (Dong et al. 2008; Jiao et al. 2013). In addition, the formation of aggregates can potentially have an influence on the binding of ILs to protein. Therefore, we additionally determined the critical micelle concentration for selected ILs in a phosphate saline buffer (PBS), using two techniques: ITC and conductometry. The available procedures have been refined in order to measure the CMC values for ILs in PBS.

Materials and methods

Chemicals

Human serum albumin (in the form of lyophilized powder with purity $\geq 97\%$) and benzalkonium chloride were purchased from Sigma-Aldrich (Darmstadt, Germany). Acetonitrile of HPLC grade, potassium dihydrogen phosphate ($KH_2PO_4, \geq 99.5\%$), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O, > 99\%$) and potassium chloride ($KCl, > 99\%$) were obtained from POCH (Poland). Sodium chloride ($NaCl > 99\%$) was acquired from STAN-LAB (Poland). Orthophosphoric acid ($H_3PO_4, > 99.9\%$) was procured from CHEMPUR (Poland). 1-decyl-3-methylimidazolium chloride ([IM1-10]Cl, $\geq 95.5\%$), 1-octyl-3-methylimidazolium chloride ([C(8)mim]Cl, $\geq 97\%$), potassium tetracyanoborate ($K[B(CN)_4], > 98\%$) and lithium bis(trifluoromethylsulfonyl)imide $Li[(CF_3SO_2)_2 N, 99.95\%]$ were secured from Merck KGaA (Germany). 1-hexyl-3-methylimidazolium chloride ([C(6)mim]Cl, $> 98\%$) and 1-butyl-3-methylimidazolium chloride ([C(4)mim]Cl, 96%) were purchased from Iolitec GmbH (Germany). 1-dodecyl-3-methylimidazolium sulphate ([C(12)mim]HSO$_4$) and 1-dodecyl-3-methylimidazolium chloride ([C(12)mim]Cl) were synthesized at the Center for Environmental Research and Sustainable Technology (UFT) of the University of Bremen according to a procedure presented elsewhere (Nowicki et al. 2016).
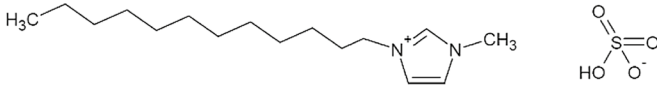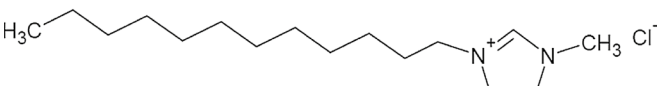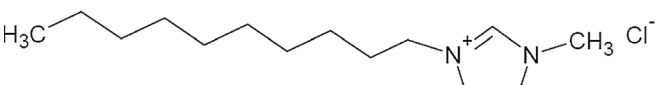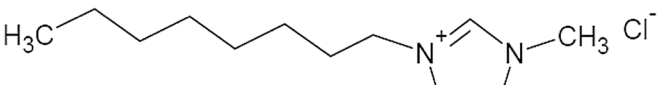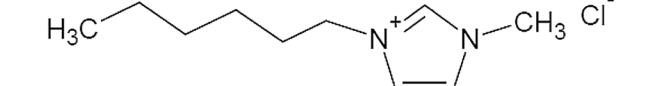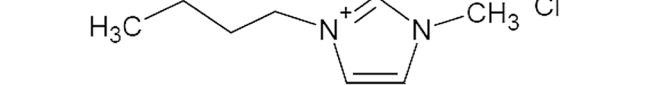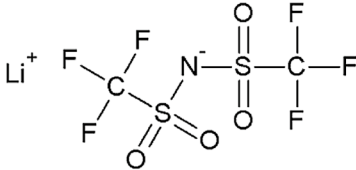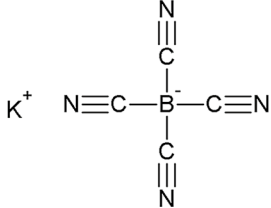
Materials

Ultrafiltration VIVASPIN 500 tubes were acquired from Sartorius Stedim Biotech (Germany).

Instrumental analysis

All cations were analysed by means of an HPLC—DAD system: Shimadzu Nexera XR. A Multhospher® 100 RP18

Table 1 Names and structures of studied ILs

Ionic Liquid	Structure
1-dodecyl-3-methylimidazolium sulphate [C(12)mim]HSO$_4$	
1-dodecyl-3-methylimidazolium chloride [C(12)mim]Cl	
1-decyl-3-methylimidazolium chloride [C(10)mim]Cl	
1-octyl-3-methylimidazolium chloride [C(8)mim]Cl	
1-hexyl-3-methylimidazolium chloride [C(6)mim]Cl	
1-butyl-3-methylimidazolium chloride [C(4)mim]Cl	
lithium bis(trifluoromethylsulfonyl)imide Li[(CF$_3$SO$_2$)$_2$N]	
potassium tetracyanoborate K[B(CN)$_4$]	

chromatographic column–5 µm, 4, 6 × 120 mm, purchased from CS-Chromatographie Service GmbH (Germany) was used in the case of most of the analytes. The mobile phase was composed of acetonitrile (HPLC grade) and a buffer containing 20 mM KH$_2$PO$_4$ and 3.9 mM H$_3$PO$_4$. The proportions of acetonitrile and the buffer were as follows: [C(12) mim]HSO$_4$ and [C(12)mim]Cl: 50:50 (v/v), [C(10)mim]Cl:

40:60 (v/v), [C(8)mim]Cl: 35:65 (v/v), [C(6)mim]Cl: 20:80 (v/v), [C(4)mim]Cl: 5:95 (v/v). The analytical wavelength was 212 nm. The flow rate was 1 ml/min. The temperature was 30 °C. The methods are characterized by very good linearity, confirmed by correlation coefficient values greater than 0.99. The accuracy of the method was in an acceptable range from 90 to 120%, and the values of coefficients of variation (< 3%.)

confirmed very good precision of the applied analytical methods. The limit of quantification (LOQ) was 5 μM, and the limit of detection (LOD) was 1.67 μM in all cases.

The anions were analysed by employing a Metrohm 881 compact IC system (Metrohm, Herisau, Switzerland) with a conductometric detector. A Metrosep A Supp ion exchange column (50×4.0 mm ID and 5 μm mean particle size) coupled with a Metrosep A Supp 4/5 guard and a Metrosep RP guard was also exploited. The mobile phase was composed of acetonitrile and a buffer containing 3.2 mM Na_2CO_3 and 1 mM $NaHCO_3$. The proportions of acetonitrile and the buffer were as follows: $[(CF_3SO_2)_2 N]^-$: 30:70 (v/v), $[B(CN)_4]^-$: 33:67 (v/v) (Stolte et al. 2011). The limit of quantification (LOQ) was 5 μM, and the limit of detection (LOD) was 1.67 in both cases.

Modulation of non-specific binding in protein binding studies using the ultrafiltration method

The separation of the analytes and the tested protein was carried out in ultrafiltration (UF) tubes with polyethersulfone (PES) membranes, with the molecular weight cut-off of 10 kDa. UF is a very simple and rapid method, but its major disadvantage is the non-specific binding (NSB) of compounds on filter membranes. When the studied compound binds widely to the filtration membrane, it may deviate from the true free concentration (Lee et al. 2003). Due to the strong possibility of NSB between imidazolium ionic liquids and the UF membrane, a method for NSB minimizing was tested. For this purpose, two substances: benzalkonium chloride (BAC) and polysorbate 20 (TW20) were used, because of their different properties–BAC is a cationic surfactant and TW20 is non-ionic surfactant. This allowed ascertaining which type of interaction between the ILs and the membrane is dominant and thus which of the surfactants will be more effective in NSB reduction. A detailed description of these methods is presented in the Supporting Information.

Quantitative determination of non-specific binding

In order to determine the non-specific binding (NSB) of ILs to filter membranes, 20 μL of the respective IL at a concentration of 1000 μM, and 380 μL of PBS were introduced into the upper compartment of the UF tube and then centrifuged (10 min, 16,000 g). The obtained filtrates were analysed by HPLC–DAD. At the same time, a solution of IL at a concentration of 50 μM without centrifugation was analysed as a reference. NSB was calculated according to the following equation (Lee et al. 2003):

$$NSB = (C_{BD} - C_{BF})/C_{BD} \qquad (1)$$

C_{BD} represents the concentration of the IL solution in the reference sample, while C_{BF} is the concentration of IL in the obtained filtrate after centrifugation.

Determining the equilibrium time of IL partitioning to protein

380 μL of HSA solution at a concentration of 0.1 mM and 20 μL of $[IM1-12]HSO_4$ ($c = 1000$ μM) were placed into seven Eppendorf tubes. The samples were subsequently incubated in a thermostated shaker (37 °C, 1000 rpm) for 10, 20, 30, 40, 60, 100 and 120 min. Afterwards, the samples were transferred to the upper compartments of ultrafiltration tubes (prepared according to procedure number 2) and centrifuged at 16,000 g for 15 min. The obtained filtrates were analysed using HPLC–DAD.

Critical micelle concentration

Conductometric measurements

Conductometric measurements were carried out on the Cerko Lab System microtitration unit fitted with a 5-mL Hamilton syringe and a CD-201 conductometric cell (HYDROMET). A weight calibration method was used for the syringe. The conductometric electrode was standardized with conductivity standards (aqueous KCl solutions) of conductivity, 84 and 200 μS/cm (Hamilton Company). The measurements were taken at 298.15 ± 0.10 K, controlled by a Lauda E100 circulation thermostat. The 1 mM buffered solutions of the appropriate IL (5 mL) were conductometrically titrated with buffered IL solution (35 mM). The titrant was added in 0.02-mL increments and after a 45 s pause.

Isothermal titration calorimetry

All ITC experiments were performed at 298.15 K by exploiting an AutoITC isothermal titration calorimeter (MicroCal Inc. GE Healthcare, Northampton, the USA). Details of the measuring devices and experimental setup were described previously (Wyrzykowski et al. 2013). [C(10)mim]Cl and [C(12)mim]Cl were dissolved directly into the PBS buffer solution (pH = 7.4). The experiment consisted of injecting 10.02 μL (29 injections, 2 μL for the first injection only) of the buffered solution of IL at a concentration of 35 mM into the reaction cell containing 1 mM of the buffered solution of IL. All solutions were degassed prior to titration. The titrant was injected at 4 min intervals to ensure that the titration peak returned to the baseline before the next injection. Each injection lasted 20 s. For homogeneous mixing in the cell, the stirrer speed was kept constant at 300 rpm.

Protein binding

Determining the percentage of ionic liquid bound to protein

In order to quantitatively determine the cation and/or anion of the IL bound to protein (% PB), 380 µL of the HSA solution at a concentration of 0.1 mM and 20 µL of the respective ionic liquid solution at a concentration of 1000 µM were added to Eppendorf tubes leading to a final concentration of 50 µM. Samples prepared in this way were incubated for 20 min at 37 °C and 1000 rpm. Afterwards, the samples were transferred to UF tubes (prepared according to procedure 2) and centrifuged at 16,000 g for 15 min. Simultaneously, the ultrafiltration process was also carried out for a solution containing 20 µL of the ionic liquid at a concentration of 1000 µM and 380 µL of the PBS buffer in order to determine NSB and thereby confirm the efficiency of minimizing NSB by procedure 2. The obtained filtrates and the reference IL solution at a concentration of 50 µM (not filtrated using UF tubes) were analysed using HPLC–DAD. Additionally, the amount of the compound bound to protein for a nominal HSA concentration of 0.63 mM (42 g/L) was assessed, due to the fact that this is the average physiological concentration of HSA in blood (Barnaby et al. 2011).

The percentage of ionic liquid bound to protein was calculated according to the equations presented below (Lee et al. 2003):

$$f_u = C_{SF}/(1 - NSB)/C_{SD} \tag{2}$$

$$\%PB = 100 \times \left(1 - f_u\right) \tag{3}$$

f_u: free fraction of IL; C_{SF}—IL concentration in the filtrate; C_{SD}—IL concentration in the reference solution; NSB—value of non-specific binding (determined in accordance with Eq. 1); % PB—concentration of ionic liquid bound to protein.

Evaluation of the influence of the anion on the affinity of the IL cation to protein

To assess whether the concentration of the anion affects the affinity of the IL cation to protein, mixtures of [C(12)mim]Cl (constant concentration of 50 µM), and K[B(CN)$_4$] at different concentrations (1200, 1000, 800, 500, 80, 20 µM) were tested. The concentration of HSA in this experiment was 0.1 mM. In parallel, a control sample containing only the PBS buffer, without protein, was investigated. The samples were incubated for 20 min at 37 °C and 1000 rpm. Afterwards, both kinds of samples (the solution containing protein, as well as the reference sample (PBS without

protein)) were transferred to ultrafiltration tubes and centrifuged for 15 min at 10,000 rpm. The obtained filtrates and the reference solution (not centrifuged using UF tubes) were analysed by HPLC–DAD and ion chromatography with conductometric detection in order to quantitatively determine both the cation and anion remaining in the water phase at the partitioning equilibrium.

Isothermal titration calorimetry

The thermodynamic parameters (K, ΔH) of the HSA interactions with the investigated anions, namely [B(CN)$_4$]$^-$, [(CF$_3$SO$_2$)$_2$ N]$^-$, and [(C$_2$F$_5$)$_3$PF$_3$]$^-$, were calculated based on the ITC data processed with Origin 7 from MicroCal. The experiments consisted of injecting 10.02 µL (29 injections, 2 µL for the first injection only) of 1 mM (or 0.5 mM) buffered solution of an appropriate salt into a reaction cell that initially contained 0.05 mM of a buffered solution of HSA. The titrant was injected at 5-min intervals. For each experiment, a blank was performed by injecting the titrant solution into a cell filled only with the buffer. This blank was subtracted from the corresponding titration to account for the heat of the dilution.

The enthalpy change (ΔH) of the HSA interactions with selected ILs ([C(4)mim]Cl, [C(6)mim]Cl, [C(8)mim]Cl, [C(10)mim]Cl and [C(7)mimCOOH]Br) was measured according to the procedure described previously (Borowik et al. 2019). The titrant was HSA at a concentration of 0.1 mM, whereas in the reaction cell, the appropriate IL was at a concentration of 1–10 mM ([C(4)mim]Cl, [C(6)mim]Cl, [[C(7)mimCOOH]Br, [C(10)mim]Cl: 5 mM, [C(8)mim]Cl: 10 mM, [C(12)mim]HSO$_4$: 1 mM).

Results and discussion

Determining the equilibrium time of IL partitioning to protein, NSB and CMC

In the first stage of experiments (details shown in Supporting Information), the incubation time was investigated and set to 20 min. In order to reduce NSB in the case of IL cations, procedure 2 (detailed description in Supporting Information) was chosen, in which the cationic surfactant benzalkonium chloride (BAC) was used. Through the application of this method, it was possible to reduce the value of NSB from approximately 90% to 41 ± 2%. In the case of the investigated IL anions, the membrane was pre-treated only with PBS. The ITC and conductometric titration measurements showed that the critical micelle concentration (CMC) values of [C(10)mim]Cl and [C(12)mim]Cl in a PBS buffer (pH = 7.4) are significantly higher than the concentration of

Table 2 Amount of IL binding to protein at concentrations of 0.63 mM

IL cation (with Cl^- or HSO_4^- as counter ion)	% PB of cation
$[C(12)mim]^+$	85 ± 0.1
$[C(10)mim]^+$	73 ± 0.3
$[C(8)mim]^+$	17 ± 4
$[C(6)mim]^+$	5 ± 1
$[C(4)mim]^+$	nb

nb no binding observed

ILs used in the study of partitioning to protein, so micelles could not be formed under the experimental conditions.

Protein binding

Determining the percentage of ionic liquid bound to protein

For all cations, the albumin concentration was at 0.63 mM. This mimics physiological conditions. In the case of selected cations: $[C(12)mim]^+$, $[C(6)mim]^+$ and $[C(4)mim]^+$, experiments were also carried out with HSA at concentrations of 0.1 mM in order to ascertain whether HSA concentration may affect the PB.

The results presented in Table 2 show that $[C(12)mim]^+$ and $[C(10)mim]^+$ exhibited the highest affinity to HSA. The % PB values are definitely lower for the other cations: $[C(8)mim]^+$ and $[C(6)mim]^+$. Furthermore, in the case of $[C(4)mim]^+$, no significant binding could be determined. The obtained results indicate that the binding of ionic liquids to HSA strongly depends on the structure of the cation and increases with the length of the hydrocarbon chain (Table 2).

A similar trend was observed by Huang et al. who showed that the binding strength of $[C(4)mim]Cl$, $[C(6)mim]Cl$ and $[C(8)mim]Cl$ to bovine serum albumin (BSA) is dependent on the length of the carbon chain (Huang et al. 2013). According to Zhong et al. the binding affinity between PFAS and HSA is also closely related to the hydrophobicity, and their bioaccumulation ability increases with an increase in perfluoroalkyl carbon chain length (Zhong et al. 2019). Moreover, Henneberger et al. demonstrated that the additional $-CH_2$ group in the structure of various ionic compounds brings about an increase in the logarithmic value of the partition coefficient between BSA and water (log $K_{BSA/w}$) from 0.28 to 0.62 (Henneberger et al. 2016). However, this average range relates mainly to anions, as only four cations were investigated in this study.

For cations, Henneberger et al. obtained very weak binding, though a high concentration of albumin was used (50 g/L). In contrast, a stronger binding of long-chain imidazolium cations on albumin was reported by Zhang et al.

2018 (Zhang et al. 2018). This work tallies well with our findings. In addition, Zhang et al. by using molecular modelling, have indicated enhanced binding of imidazolium ILs with the increase of the number of carbon atoms of the hydrophobic chain. Still, at the same time, electrostatic interaction of the imidazolium ring with the positive charge of selected amino acids cannot be excluded (Zhang et al. 2018).

Although more studies regarding the influence of ILs on HSA have been presented in the literature, they focus mainly on the denaturation effect of ILs on protein and do not quantitatively evaluate the degree of binding of ILs to this protein (Page et al. 2009; McCarty et al. 2008; Kumar et al. 2012; Sasmal et al. 2011; Chowdhury et al. 2012; Heller et al. 2013; Akdogan et al. 2011a, b; Baker et al. 2009; Rawat et al. 2012). The available literature data in unison, however, confirm that ILs may destabilize the secondary structure of albumin, and the concentration that initiates this process decreases with the increasing hydrophobicity of the compound (Page et al. 2009; McCarty et al. 2008; Kumar et al. 2012; Sasmal et al. 2011; Chowdhury et al. 2012; Heller et al. 2013; Akdogan et al. 2011a, b; Baker et al. 2009; Rawat et al. 2012).

The probable reason for this phenomenon is the increasing contact surface area of the cation due to the increasing length of the alkyl chain (Silva et al. 2014). The concentration that may cause the destabilization of albumin equals 35, 2 and 0.2% (w/v) for $[C(2)mim]^+$, $[C(4)mim]^+$ and $[C(8)mim]^+$, respectively (Rawat et al. 2012; Akdogan et al. 2011b; Page et al. 2009). Therefore, on the basis of the available literature data, we could assume that it was unlikely that the protein structure was destabilized during this experiment, since the used concentration of 50 μM corresponded to a concentration not exceeding 0.002% (w/v), depending on the molar mass of the respective IL. Moreover, for long-chain imidazolium ILs, e.g. 1-tetradecyl-3-methylimidazoliumbromide ($[C(14)mim]Br$), previous research has demonstrated that at concentration below CMC, $[C(14)mim]Br$, barely affects the secondary structure of bovine serum albumin (BSA), whereas a destabilizing effect is observed above concentrations corresponding to CMC (Yan et al. 2012; Geng et al. 2010).

Table 3 PB values of ILs anions and different HSA concentrations

IL anion with Li^+ or K^+ as counter ion	Albumin concentrations (mM)	%PB of anion
$[(CF_3SO_2)_2 N]^-$	0.1	93 ± 2
$[(CF_3SO_2)_2 N]^-$	0.01	36
$[B(CN)_4]^-$	0.1	75 ± 11
$[B(CN)_4]^-$	0.01	14 ± 3

The results of the assessment of the affinity of different anions of ILs to albumin (at two concentrations) are summarized in Table 3.

At the albumin concentration of 0.1 mM, the anion $[(CF_3SO_2)_2 N]^-$ has a higher affinity to protein than the analysed cations (Table 2). Furthermore, the PB of $[B(CN)_4]^-$ is slightly lower (75%) than the PB value determined for $[C(12) mim]^+$ (85%). In the case of anions, albumin concentration has an impact on their protein binding. Higher % PB values were determined for the anion $[(CF_3SO_2)_2 N]^-$. However, in the case of both anions, the value of % PB decreases significantly with the decrease in albumin concentration (Table 3), which indicates that the protein undergoes saturation by the analysed ligand at this condition.

The available literature data on the affinity of organic bases to albumin show that benzoic, naphthoic and phenoxy acids strongly bind to this protein (> 90%) (Henneberger et al. 2016). In this work, the value obtained for $[(CF_3SO_2)_2 N]^-$ (93%) is similar to the PB of PFAS, for which high binding to BSA (> 95%) was observed. Based on the aforementioned literature and the values of % PB for $Li[(CF_3SO_2)_2]$ and $K[B(CN)_4]$ that we obtained, it can be assumed that the hydrophobic anions of ILs can strongly interact with HSA. This effect may affect transport and distribution processes in living organisms.

All the dependencies described above concerning the degree of binding of ILs with HSA result from its binding properties and its multidomain structure. This comprises three homologous domains (I, II, III), each of which is divided into two subdomains (A and B) (Artali et al. 2005). The binding mostly occurs at two well-known drug-binding sites, i.e. Sudlow's site I—which locates at subdomain IIA of HSA, and Sudlow's site II—found at the subdomain IIIA. However, other binding sites with similar function exist (Artali et al. 2005).

The fatty acids binding sites have some significance with regard to this effect, as HSA is able to bind seven equivalents of fatty acids (FAs). Currently, the binding sites in HSA for ILs are unknown. However, compounds of similar structure are mechanistically better understood. It has been shown that perfluorooctanylsulfonyl acid (PFOS) shows affinity for two binding sites of the HSA. The first is located at Sudlow site II, and the second is at the FA6 binding site. In addition, previous studies have revealed that the strongly polar sulfonyl group of PFOS may bind to hydrophilic residues of HSA, while the fluorinated carbon chain interacts with adjacent hydrophobic protein residues (Luo et al. 2012).

The literature indicates that biomolecule stability at very low IL concentrations (< 0.01 M) is predominantly driven by electrostatic interactions (Jha et al. 2018). However, our study, which including a relatively broad spectrum of imidazolium cations, clearly revealed the higher affinity of long-chain ILs to HSA, and hence, their binding at even lower IL

concentration might be related also to interaction with the hydrophobic domains (IIA and IIIA) of HSA. The fact that ILs interact with the protein through both electrostatic and hydrophobic interactions has been confirmed by other studies (Zhang et al. 2018; Geng et al. 2009, 2010; Mester et al. 2010; Pei et al. 2012; Yan et al. 2012; Cao et al. 2014; Wang et al. 2012; Zhu et al. 2011; Singh et al. 2012; Bharmoria et al. 2014).

Additionally, many authors have observed that the process of the binding of imidazolium ILs to protein is mostly hydrophobic in nature and mainly controlled through the alkyl chains of cations (Huang et al. 2013; Geng et al. 2009; Yan et al. 2012; Cao et al. 2014; Wang et al. 2012; Singh et al. 2012). Nevertheless, it has been demonstrated that the secondary structure of albumin is destabilized with the increasing concentration of ILs, and that this effect is brought about by the hydrophobic interaction of the IL with the protein and can lead to denaturation (Geng et al. 2009).
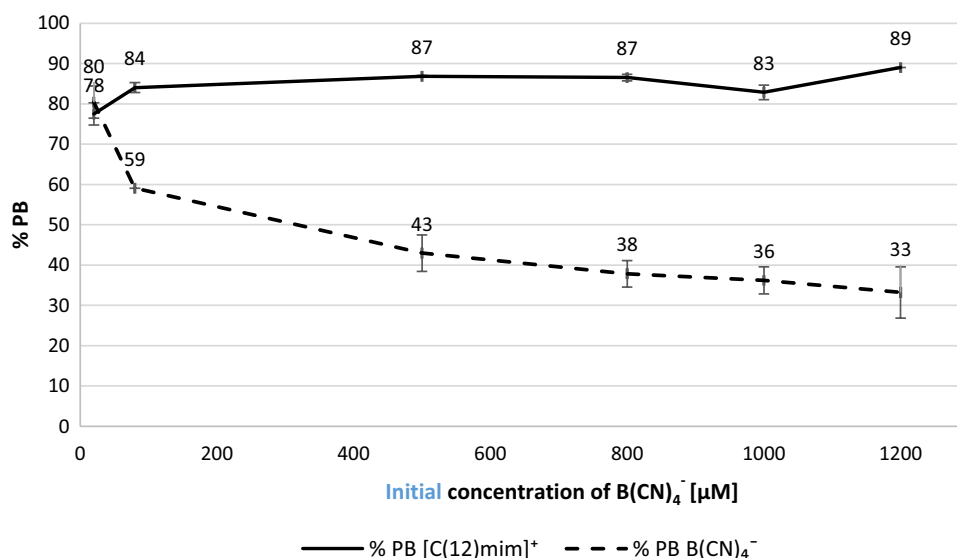
That we conclude the existence of a high affinity of anions (in comparison to cations) to HSA is justified because protein generally prefers binding to small, negatively charged hydrophobic molecules (He et al. 1992). Moreover, the obtained results may be attributed to the chaotropicity of the studied anions, leading to a great affinity towards chaotropic amide in the structure of the protein (Harada et al. 2018).

Evaluation of the influence of anions on the affinity of cations of ionic liquids to protein

As it has been recently shown, the membrane-water partition coefficient (log K_{MW}) of the cation is altered by the concentration of anions $[B(CN)_4]^-$ and $[(FSO_2)_2 N]^-$ indicating stronger partitioning of $[C(12)mim]^+$ probably due to ion pairing, reduction of repulsive interaction with choline by anion adsorption on bilayer surfaces, neutralization of the charge of choline and/or changes of lipid bilayer integrity (Dołżonek et al. 2017). Furthermore, according to the study by Figueiredo et al. (2013) organic anions are less hydrated compared to simple inorganic anions, and thus, their availability to interact with proteins could be higher. The ionic strength of the solution is also not negligible for interactions with proteins. At low ionic strength, the anion may have a greater tendency to associate with the cation, which in turn increases their tendency to dehydrate the protein surface. This may translate into a higher affinity of the anion for the protein (Figueiredo et al. 2013).

Therefore, we aimed to ascertain whether the affinity of the cation $[C(12)mim]^+$ to HSA depends on the concentration of the tetracyanoborate anion $[B(CN)_4]^-$. In this experiment, cation $[C(12)mim]^+$ at concentration of 50 μM was used with the addition of various concentrations of the tetracyanoborate anion added as $K[B(CN)_4]^-$. Apart from the quantitative determination of the cation, the obtained

Fig. 1 The graph showing the % PB for $[C(12)mim]^+$ (50 μM) in the presence of different concentrations of $[B(CN)_4]^-$ and % PB for different concentrations of $[B(CN)_4]^-$ in the presence of $[C(12)mim]^+$ (50 μM)

samples were subjected to additional analyses in order to determine the concentration of the anion remaining in the water phase after the experiment. The % PB of the anion $[B(CN)_4]^-$ is presented in Fig. 1.

The obtained results clearly show that the anion $[B(CN)_4]^-$ binds to HSA. Therein, the values of % PB for $[B(CN)_4]^-$ decrease along with an increase in the initial concentration of the anion; thus, the gradual saturation of the protein with the studied anion as its concentration increases has been observed (Fig. 1). Additionally, while the concentration of the anion $[B(CN)_4]^-$ bound to HSA increases, the amount of the cation $[C(12)mim]^+$ bound to albumin is also slightly higher. This may give an indication that the affinity of the IL cation to albumin was enhanced by the previously bound anion. Thus, the probable formation of ion-pairs between $[C(12)mim]^+$ and $[B(CN)_4]^-$ may result from the nature of both ions, likely to be chaotropic.

Although the interpretation of Hofmeister effects at the molecular level is difficult to elucidate, especially for ions more complex than simple inorganic ions (Harada et al. 2018; Constantinescu et al. 2010), some studies have tried to assign a chaotropic/kosmotropic character to selected IL cations and anions. Schröder (2017) suggested that imidazolium cations act more and more chaotropic when the alkyl side chains are prolonged (Schröder 2017). Other studies also indicate the chaotropic behaviour of imidazolium cations (Akdogan et al. 2011a; Reslan et al. 2018).

Unfortunately, so far the chaotropicity of $[B(CN)_4]^-$ has not yet been studied, but taking into consideration that this anion is fairly hydrophobic and hence does not form a hydrogen bond with water molecules (is weakly hydrated), it can be considered as a 'structure-breaker', meaning a chaotrope (Tariq et al. 2011; Patel et al. 2014). This tallies with other studies indicating that cyanamide anions act as
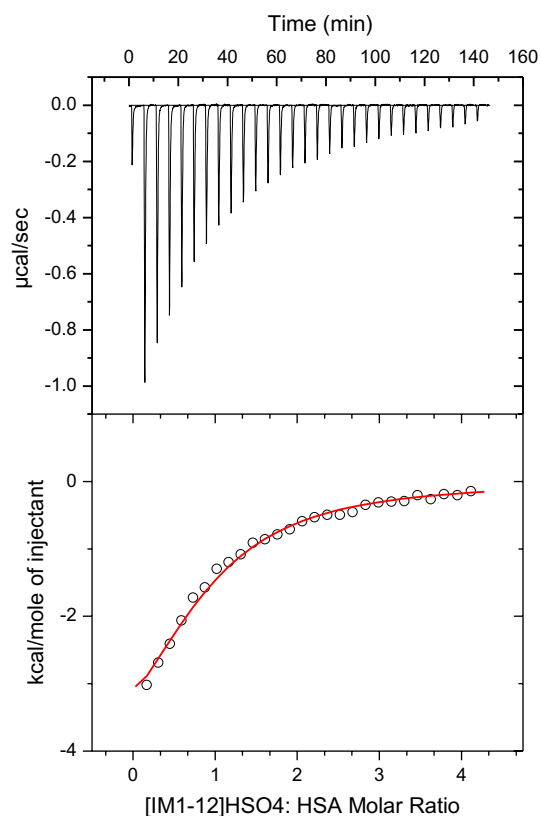
chaotropes (Schröder 2017; Mester et al. 2015). The strongest interaction between cations and anions exists if both are chaotropic (Schröder 2017). Hence, ion pairing between the investigated cation and anion could be possible and might explain the enhanced $[C(12)mim]^+$ binding to HSA in the presence of $[B(CN)_4]^-$. However, the observed increase in cation binding is too little (from 78 to 89%) to consider ion pairing as mechanistic interpretation of protein binding for IL cations and anions. Moreover, increase of $[C(12)mim]^+$ binding was lower than we expected, as it was not directly proportional to PB of $[B(CN)_4]^-$.

Unlike other experiments, the concentration of the anion was in this experiment much higher (up to 1.2 mM). Therefore, in this case, we need to consider the possible destabilizing effect upon HSA that is induced by moving the positively charged amino acid towards the anion. This could start the unfolding process and might make the repulsion to the cation stronger (Schröder 2017). Hence, it may diminish ion pairing at a high concentration of $[B(CN)_4]^-$, resulting in weaker binding of $[C(12)mim]^+$ than suspected.

Determination of the enthalpy value (ΔH) of HSA-IL interactions

The conditional parameters of the HSA interactions with $K[B(CN)_4]$, $Li[(CF_3SO_2)_2 N]$, $K[(C_2F_5)_3PF_3]$, $[C(8)mim][B(CN)_4]$ and $[C(12)mim]HSO_4$ were obtained directly from the ITC data by fitting binding isotherms, using nonlinear least-squares procedures, to a model that assumes a single set of identical binding sites. The representative binding isotherm for the $[C(12)mim]HSO_4$–(HSA) interaction is shown in Fig. 2, whereas conditional parameters (K_{ITC}, ΔH) of the interactions under study are summarized in Table 4.

Fig. 2 Calorimetric titration isotherm of the binding interaction between [C(12)mim]HSO$_4$ (1 mM) and HSA (0.05 mM) in PBS buffer of a pH of 7.4, at 298.15 K

Table 4 The condition-dependent thermodynamic data (K_{ITC}, ΔH_{ITC}) of ILs binding to HSA in PBS buffer of pH 7.4, at 298.15 K, obtained by ITC ($\pm$ Standard Deviation Values)

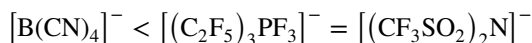| No	Ionic liquid	ΔH_{ITC} (kcal/mol)	log K_a
1	K[B(CN)$_4$]	-16.67 ± 0.82	3.79 ± 0.03
2	Li[(CF$_3$SO$_2$)$_2$ N]	-12.7 ± 3.57	4.26 ± 0.1
3	K[(C$_2$F$_5$)$_3$PF$_3$]	-12.90 ± 0.74	4.32 ± 0.06
4	[C(8)mim][B(CN)$_4$]	-17.36 ± 0.86	3.78 ± 0.03
5	[C(12)mim]HSO$_4$	-5.25 ± 0.11	4.51 ± 0.03

Due to the weaker interactions of HSA with the IL cations comprising the alkyl chain length lower than 12 ([C(4)mim]$^+$, [C(6)mim]$^+$, [C(8)mim]$^+$, [C(10)mim]$^+$, and [C(7)mimCOOH]$^+$), only binding enthalpies (ΔH) could be calculated indirectly (Woziwodzka et al. 2013). Thus, three types of experiments were carried out: (1) titration of ILs with the buffer, (2) titration of the buffer with HSA and (3) titration of ILs with HSA. The enthalpy change (ΔH) of the HSA-IL interaction was calculated by the linear regression of experimental points (the differences between the heat of titrations of ILs with HSA and the sum of the heat of ILs

Table 5 The condition-dependent binding enthalpies (ΔH_{ITC}) of IL interactions with HSA in a PBS buffer of pH 7.4, at 298.15 K, obtained by ITC ($\pm$ Standard deviation values)

No	Ionic liquid	ΔH_{ITC} (kcal/mol)
1	[C(4)mim]Cl	4.95 ± 0.08
2	[C(6)mim]Cl	6.63 ± 0.11
3	[C(8)mim]Cl	10.18 ± 0.12
4	[C(10)mim]Cl	12.62 ± 0.18
5	[C(7)mimCOOH]Br	-23.93 ± 0.22

with the buffer and of the buffer with HSA titrations) to the infinite dilution of HSA ([HSA] → 0) (Table 5).

The heat of HSA-IL interactions (corrected for background thermal effects) is calculated as kcal mol^{-1} of the injected HSA. On the basis of the literature data (Jungnickel et al. 2008; Sadeghi et al. 2015; Wang et al. 2011; Liu et al. 2013) and our results of investigations of CMC in the PBS buffer, we can assume that ITC measurements were performed for monomers of ILs. The stability of the resulting complexes is governed by the type of anion and is enhanced with the increase of its size and the hydrophobicity in the following direction (Table 4):

$$\left[B(CN)_4 \right]^- < \left[(C_2F_5)_3 PF_3 \right]^- = \left[(CF_3SO_2)_2 N \right]^-$$

This finding is in line with the results obtained from the ultrafiltration method (Table 3). The formations of ILs—HSA complexes are exothermic processes (Table 4). Thus, it can be concluded that polar interactions as well as hydrogen bonds play an important role in the stabilization of the investigating complexes.

On the other hand, in the endothermic effects of [C(4)mim]$^+$, [C(6)mim]$^+$, [C(8)mim]$^+$ and [C(10)mim]$^+$, binding to HSA is observed (Table 5). According to the literature, this may be attributed to low degrees of saturation of HSA being related to relatively low concentrations (Juni et al. 1983) and indicates that imidazolium cations bind to HSA via ionic and hydrophobic interactions (Ross et al. 1981). Nevertheless, the delocalization of the positive charge onto the large imidazolium ring may induce weaker electrostatic interactions between HSA and imidazolium cations (Liu et al. 2013). In such a case, the formation of the complexes is an entropy-driven process. Furthermore, there is a strong dependency between the binding enthalpy and the alkyl chain length (Table 5). Herein, the binding enthalpy increases with the increase of the length of the alkyl chain presented in the structure of the cation.

A different situation is seen for [C(12)mim]HSO$_4$ which in contradiction to other investigated IL cations and interacts exothermically with HSA. The resulting [C(12)mim] HSO$_4$(HSA) complex is as stable as complexes of HSA with

the IL anions under study (Table 4). The exothermic effect of the $[C(12)mim]^+$ cation interaction with milk protein has previously been observed by Liu et al. (2013). In their work, the negative value in the binding enthalpy change was attributed to the significant contribution of hydrogen bonding in the binding of $[C(12)mim]^+$ to protein. The hydrophobic association of protein by ILs is known to contribute to a positive value of enthalpy change (Ross et al. 1981), while the formation of new non-covalent bonding, such as electrostatic (Coulombic) attraction, van der Waals interactions or hydrogen bonds, is exothermic in nature (Abraham et al. 2005).

These findings tally with the enthalpy changes measured for [C(7)mimCOOH]Br. The $[C(7)mimCOOH]^+$ cation is undoubtedly able to form an interaction with HSA via hydrogen bonding, and the heat of this interaction could be measured as exothermic (Table 5). Although our results do not confirm these types of interaction between imidazolium cations and HSA, they cannot be excluded, and a positive contribution to the overall enthalpy change may arise from the thermodynamically unfavourable release of ordered water molecules from protein (Abraham et al. 2005).

It is worth noticing that the binding constants of the $[C(8)mim][B(CN)_4]$/(HSA) and $K[B(CN)_4]$/(HSA) complexes are equal in the range of the experimental error. This suggests that the affinity of the IL anions towards HSA is higher than the IL cations comprising alkyl chain lengths lower than 12.

These results also show the utmost importance of focussing on both the cation and anion of ILs separately (as inorganic salts) when the assessment of the interaction with biomolecules, e.g. proteins, in an aqueous solution is conducted by methods like ITC or by other techniques that are not based on the quantitation of the free fraction of the cation and/or anion in the water phase, but on the other effects of protein binding (e.g. the energetic effect). Such an approach allows a proper estimation because it provides information on which ion is responsible for the measured end-point, being a reflection of the interaction with the biomolecule.

Conclusions

In this study, the binding of IL cations and anions to HSA was quantitatively evaluated in order to preliminarily assess the role of exemplary blood protein in their potential bioconcentration. Data obtained by using UF method revealed that both the cations and anions of ILs may bind to HSA, and hence, blood proteins may play an important role in the bioconcentration of ILs. The binding of ILs to HSA depends on the hydrophobicity (alkyl chain length) and structure (e.g. polar moieties) of cations and increases as the length of the alkyl chain of the cation increases (from $5 \pm 1\%$ for $[C(6)mim]^+$ to $85 \pm 0.1\%$ for $[C(12)mim]^+$), whereas polar

functional groups weaken the binding and the binding mechanism might be driven by other types of interactions (e.g. hydrogen bonding).

In contrast to previous studies indicating Coulombic forces as the main interaction responsible for the binding of imidazolium cations at low concentrations, and hydrophobic interactions for higher concentrations, our results show that hydrophobicity may be an important factor underlying the partitioning of imidazolium ILs to HSA, also at relatively very low concentrations (below 50 µM), which, however, does not exclude the role of electrostatic interactions.

Our results indicate that the type of anion has an influence on the affinity of the cation to HSA. This dependence is essential from the environmental point of view, where the chemicals do not exist as individual compounds but as a mixture. For this reason, future studies of the bioaccumulation of ionic or ionogenic compounds should consider the influence of interactions between all the ionic components of a matrix.

Due to the fact that the presented study is one of the first that focuses on the assessment of the quantitative binding of ILs to protein, future complementary studies are needed.

Declarations

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