Measuring anion binding at biomembrane interfaces

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Abstract: Molecular interactions at biomembrane interfaces[1] are ubiquitous in many biological processes and underpin several mechanisms of drug actions.[2-5] Despite the important biological regulatory roles of transmembrane anion transport, fundamental knowledge of anion binding to natural or synthetic molecules within lipid bilayers is lacking[6] in contrast to the better-understood solution-phase studies.[7-10] We here bridge this knowledge gap by making anion binding measurable within lipid bilayers. This was achieved using a macrocycle that has a record aqueous SO_4^{2-} affinity among neutral receptors which increases fluorescence on SO_4^{2-} binding. We show that in lipid bilayers the determinants of anion binding are extraordinarily different from those expected that govern anion binding in solution. Charge-dense anions $H_2PO_4^-$ and Cl^- that prevail in DMSO fail to bind to the macrocycle in lipids. In stark contrast, ClO_4^- and I^- that hardly bind in DMSO show surprisingly significant affinities for the macrocycle in lipids. We have revealed a lipid bilayer anion binding principle that depends on anion polarisability and bilayer penetration depth of complexes leading to unexpected advantages of charge-diffuse anions. These insights enhance our understanding of how biological systems select anions and guide the design of functional molecular systems[11] operating at biomembrane interfaces.

Lipid bilayers have remained as an uncharted territory for studying anion binding by molecular receptors despite significant advances in anion receptor chemistry[12] and the knowledge that some anionophores can carry anions across cell membranes for putative therapeutic applications.[4,5] While in bulk solvents, anion binding affinities are usually determined by NMR titration techniques,[10] technical difficulties including poor solubility, signal broadening, and weak anion affinities have so far impeded the application of NMR techniques to elucidating anion binding in native lipid bilayer environments. These difficulties are now circumvented by a fluorescent anion-binding macrocycle **1** (Fig.1a). This highly symmetric macrocycle has a 3.5 Å cavity composed of a perfectly aligned array of nine strong NH hydrogen bond donors pointing inwards. This ensures hydrogen bonding interactions with large anions such as SO_4^{2-} to be sufficiently strong and measurable in extremely competitive lipid bilayer environments (*vide infra*).

While the structure of trimeric macrocycle **1** was initially proposed in a theoretical study in 2017,[13] no synthetic progress was subsequently made despite the successful synthesis of the dimeric[14] and the tetrameric[15] analogues. We have developed a remarkably simple one-pot SO_4^{2-}-templated synthesis to access the uniquely flat and pre-organised trimeric macrocycle **1** (Fig.1a). The crystal structure of **1**-SO_4^{2-} (Fig.1b) shows a D_{3d}-symmeric complex with the central bound SO_4^{2-} ion interacting with all urea motifs of **1** via six short NH···O contacts of 2.01 Å and with carbazole NHs via longer NH···O contacts of 2.61 Å. The macrocycle was slightly buckled (Fig.1b right, dihedral angles between carbazole units measured to be 171.91°) to accommodate the large tetrahedral SO_4^{2-} ion.

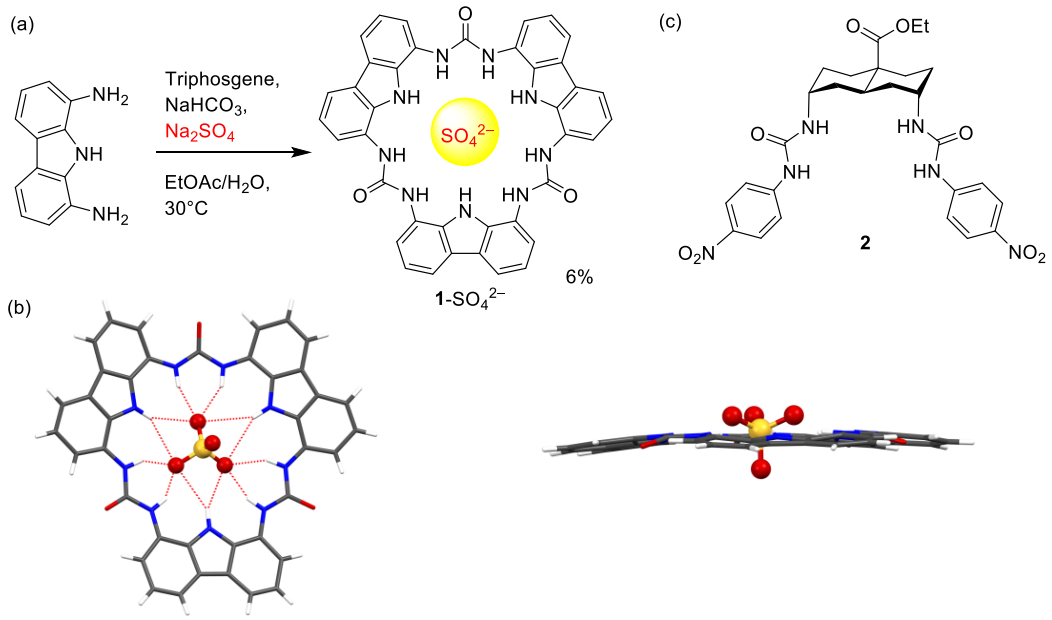

Fig.1 (a) Synthesis of macrocycle **1**. The SO_4^{2-} template could be removed by EtOAc/H_2O extraction, which, however, led to partial degradation of the macrocycle. The free macrocycle with 80–90% purity was used in ^{1}H NMR titrations in DMSO-d_6/0.5% H_2O. The pure SO_4^{2-} complex was used in fluorescence studies in water where the complex completely dissociated at 50 nM due to the competitive aqueous conditions. (b) Crystal structure of **1**-SO_4^{2-} complex (CCDC: 2128483) with the counterion and disorder omitted for clarity. (c) Reference bis-urea anion receptor **2**.

We conducted ^{1}H NMR titrations of **1** with tetrabutylammonium (TBA^+) salts of SO_4^{2-}, $H_2PO_4^-$, Cl^-, Br^-, NO_3^-, I^- and ClO_4^- in DMSO-d_6/0.5% H_2O (Table 1). Macrocycle **1** has an exceptionally strong SO_4^{2-} affinity in the sub-nanomolar range, which necessitates the use of a $BaSO_4$ precipitation method for quantification (SI). For monovalent anions, the anion binding affinity decreases in the order of $H_2PO_4^- > Cl^- > NO_3^- > Br^- > I^- > ClO_4^-$. Compared with Davis's acyclic bis-urea **2** (Fig.1c),[16] the macrocyclic receptor confers a modest 1–2 fold affinity enhancement for Cl^-, Br^- and I^-, but a significant 33-fold enhancement for NO_3^-. Geometrical optimisations of the anion complexes suggest that **1** has a perfect size and shape complementary fit for NO_3^- resulting in a precisely flat and D_{3h}-symmeric complex (Fig.S4b).[13] By contrast, the macrocyclic cavity is slightly too large for I^- (Fig.S4c) and consequently much too large for Br^- (Fig.S4d) and Cl^- (Fig.S4e). Notably, here without using **2** as a reference receptor for comparison, the macrocycle's structural preference for NO_3^- would have been blurred in the binding constant data in DMSO, as charge-dense[17] monovalent anions $H_2PO_4^-$ and Cl^- have much greater affinities than NO_3^- which only narrowly edges out Br^-. Thus, in DMSO the anion binding affinities are dominated by strength of electrostatic interactions leading to favourable binding of charge-dense anions.

Table 1 Anion binding and transmembrane anion transport properties of **1**, along with literature values of anion hydration free energies and anion binding properties of **2** and PC vesicles. Errors represent SD from at least two experiments.

Anion	ΔG_{hydr} / kJ mol^{-1} [a]	Association constant K_a / M^{-1}					Transport rate by **1** / anions s^{-1} carrier^{-1} [h]
		in DMSO-d_6/0.5% H_2O		in C12E8 micelles[d]	in POPC vesicles[d]	PC vesicles	
		1	**2** [c]	**1**	**1**		
SO_4^{2-}	–975	(7.4 ± 1.1) × 10^9 [b]	> 10^5	54000 ± 3000	370 ± 10 [e]	n.d.	0.042 ± 0.006
$H_2PO_4^-$	–473	> 10^5	46000	140 ± 20	< 1	n.d.	0.031 ± 0.010
Cl^-	–344	2000 ± 100	670	19 ± 1 [e]	< 1	0.2 [f]	0.082 ± 0.016
Br^-	–318	200 ± 10	70	29 ± 1 [e]	2.6 ± 0.6 [e]	2 [f]	0.097 ± 0.018
NO_3^-	–286	340 ± 10	10	210 ± 10 [e]	24 ± 4 [e]	2.8 [f]	2.1 ± 0.1
I^-	–280	6.1 ± 0.6	3	200 ± 10 [e]	24 ± 2 [e]	32 [g]	2.0 ± 0.4
ClO_4^-	–229	< 1	n.d.	32 ± 1 [e]	45 ± 9 [e]	115 [g]	0.83 ± 0.10

[a]Compiled by Marcus.[18] [b]Determined using a $BaSO_4$ precipitation method. [c]Reported by Jurček et al.[16] [d]Surface potential effects corrected. [e]Ionic strength fixed at 0.2 M. [f]Reported by Tatulian, using egg PC vesicles.[19] [g]Reported by Rydall and Macdonald, using POPC vesicles.[20] [h]Determined at an anion concentration of 20 mM.

To evaluate the anion binding strength of **1** in water, we next switched the medium from DMSO to non-ionic octaethyleneglycol monododecyl (C12E8, Fig.2b) micelles dispersed in water,[21] as neither **1** nor its anion complex is soluble in pure water. Gratifyingly, **1** can be solubilised at sub-μM concentrations in C12E8 micelles and demonstrates a fluorescence enhancement response to SO_4^{2-} (Fig.S17), giving a remarkable SO_4^{2-} binding constant of 5.4 × 10^4 M^{-1}, which is > 20 times greater than a bis-cyclopeptide,[22] previously the highest affinity neutral SO_4^{2-} receptor in water.[9]

Apart from $H_2PO_4^-$ that induced a fluorescence response similar to SO_4^{2-} (Fig.S21), other anions produced either negligible fluorescence responses (Cl^-) or fluorescence quenching responses (Br^-, NO_3^-, I^- and ClO_4^-) partly attributable to dynamic quenching (Fig.S23), rendering direct fluorescence titrations unfeasible. Instead, we conducted competitive titrations of SO_4^{2-} in the presence of these anions and calculated the affinities based on attenuation of SO_4^{2-} affinity using a competitive binding model (SI). In these analyses, it was necessary to correct the binding constant values against Boltzmann factors as anions adsorb to micellar surfaces[23] leading to a negative surface potential (estimated by electrophoretic measurements) which then reduces the SO_4^{2-} concentration at the surface by a Boltzmann factor compared with in the bulk solution.

Table 1 demonstrates a modified anion selectivity in water/C12E8 compared with in DMSO, despite SO_4^{2-} remaining as the most strongly bound anion. While $H_2PO_4^-$, Cl^- and NO_3^- are the top-3 strongest binding monovalent anions in DMSO, Cl^- drops out of this group in the water/C12E8 system and is replaced by I^-. We rationalise the binding data on the basis of anion solvation free energies and medium polarity (Fig.2). The interfacial dielectric constant of C12E8 micelles was estimated to be ~27–35,[24] which is similar to that of MeCN and lower than DMSO, and hence anion binding should not be weakened solely on medium polarity considerations. Here the diminished affinities of charge-dense SO_4^{2-}, $H_2PO_4^-$ and Cl^- anions, and the shift of anion selectivity towards more charge-diffuse NO_3^- and I^- anions are attributed to the heavier dehydration costs of charge-dense than charge-diffuse anions. The augmented affinities of I^- and ClO_4^- in water/C12E8 than in DMSO despite the anion dehydration cost in water/C12E8 can be rationalised by the high receptor desolvation cost in DMSO (see Fig.S11 for evidence of desolvation).[9] We have also performed ^{1}H NMR of macrocycle **1** in water/C12E8 in the

presence of anions (Fig.S31), in which the observation of resonances from anion complexes provided unambiguous evidence of binding of all tested anions in the biphasic system.

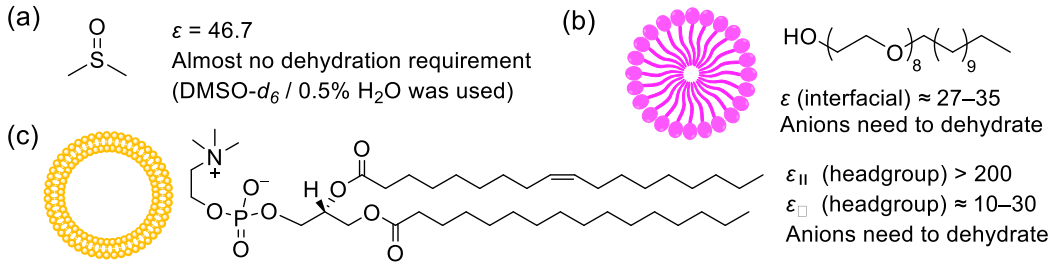

Fig.2 Schematic representation of three media used for anion binding studies in this paper. (a) DMSO. (b) C12E8 micelles. (c) POPC vesicles.

Importantly, macrocycle **1** also demonstrated a fluorescence enhancement response to SO_4^{2-} when incorporated at sub-μM concentrations in POPC (Fig.2c) vesicles suspended in water (Fig.S33). A further reduced SO_4^{2-} affinity of 370 M^{-1} was found for **1** in POPC vesicles compared with in C12E8 micelles (Table 1). This attenuation could be in part due to competitive receptor binding to the phosphate headgroup of POPC.[25] To gain more evidence for this, we performed SO_4^{2-} titrations of **1** in 10% and 20% POPC/C12E8 mixed micelles, which demonstrated 4.5-fold and 9-fold reduced SO_4^{2-} affinities (Figs.S19,S20), respectively, compared with in pure C12E8 micelles. Further evidence was provided by the direct observation of ^{1}H NMR signals corresponding to the **1**-POPC complex in POPC/C12E8 mixed micelles (Fig.S32).

Examination of binding constants of other anions in POPC vesicles (determined by competitive binding experiments with surface potential effects corrected, Table 1) have however revealed a trend that cannot be explained solely by competitive headgroup binding which does not impact anion selectivity. Strikingly, while the divalent SO_4^{2-} always remained the strongest binding anion, the top-three monovalent anion group changed again compared with in C12E8 micelles, with $H_2PO_4^-$ being knocked out by ClO_4^- which joins NO_3^- and I^-. In POPC vesicles, $H_2PO_4^-$ and Cl^- no longer showed appreciable affinities (K_a < 1 M^{-1}). This trend also cannot be explained by anion dehydration costs alone, as anion binding is subject to the dehydration requirement in both water/C12E8 and water/POPC systems. The dielectric property of the lipid bilayers, on the other hand, could provide a clue to understanding the enhanced selectivity for charge-diffuse anions in POPC vesicles than in C12E8 micelles. Previously molecular dynamics simulation studies have estimated the dielectric constant of the zwitterionic headgroup region to be higher than that of bulk water ($\varepsilon_{||}$ > 200, where $\varepsilon_{||}$ is the dielectric constant component parallel to the bilayer surface and affects half of the NH binding sites on average assuming perpendicular insertion of the macrocycle; the perpendicular component $\varepsilon_{\perp}$ was estimated to be ~10–30, which affects the remaining half of the NH sites).[26-28] Flood and coworkers have shown in a Cl^- binding macrocycle that with higher solvent dielectric constant, the energetic contribution of electrostatic interactions reduces while non-electrostatic induction and dispersion contributions start to dominate.[8] We thus reason that should anion binding occur at the high dielectric constant headgroup region, charge-dense anions SO_4^{2-}, $H_2PO_4^-$ and Cl^- that mainly rely on electrostatic interactions to bind would be disadvantaged over large charge-diffuse anions ClO_4^- and I^- that have favourable induction and dispersion terms due to their polarisabilities. This effect would add to the chaotropic preference that arises from dehydration cost alone as we have already seen in C12E8 micelles.

Key information on the location of anion binding in POPC vesicles was then obtained by fluorescence penetration-depth studies using spin labelled lipids to quench the fluorescence of **1** at different

locations (Figs.S45–S48).[29] Without anions, the most probable location of macrocycle **1** was determined to be 19 Å from the bilayer centre, corresponding to the headgroup region. This is consistent with the lipid headgroup binding hypothesis.[25] Macrocycle **1** remained at the headgroup region upon binding to SO_4^{2-}, but upon binding to ClO_4^- penetrated deeper (16 Å) into the carbonyl/glycerol region with a lower dielectric constant of 3–4.[26,27] Here we have confirmed that the binding of SO_4^{2-} occurs at the high dielectric constant headgroup region, supporting the hypothesis that binding of charge-dense anions are subject to severe electrostatic screening which diminishes their affinities (note that the SO_4^{2-} selectivity persists but is much weaker than in DMSO and in C12E8 micelles). We have shown an additional benefit for charge-diffuse anions such as ClO_4^- that their complexes (and likely also the free anions[30,31]) can penetrate deeper into a more hydrophobic microenvironment where anion binding is enhanced.

It is of interest to compare the anion binding properties of macrocycle **1** in lipids against anion binding by lipids themselves. Lipid bilayers preferentially adsorb charge-diffuse anions and exhibit a Hofmeister selectivity pattern of $ClO_4^- > I^- >> NO_3^- > Br^- > Cl^- > H_2PO_4^-$ (see also Table S3).[19,20] Table 1 shows that macrocycle **1** binds Br^-, I^- and ClO_4^- with similar or weaker affinities than lipids, but binds NO_3^- ~8 times more strongly than lipids, again manifesting the perfect size and shape matching of the macrocycle for NO_3^- (Fig. S3b). The ability of lipids to preferentially accumulate ClO_4^- below the headgroup region,[30,31] on the other hand, has overwhelmed the macrocycle's preference for NO_3^-.

Further information came to light when we compare the abovementioned phenomena to cation binding to lipids and to the cation receptor/carrier valinomycin in lipids. The cation affinities of PC lipids among alkali metal cations from Li^+ to Rb^+ are very similar,[32] while being far weaker than lipids binding charge-diffuse anions.[19,20] For valinomycin, although cation binding affinities dropped by several orders of magnitude when the medium switched from organic solvents to lipids, no drastic alteration of cation selectivity was found in lipids[33] in contrast to the behaviour of "anti-valinomycin" **1**. As shown by previous theoretical investigations, the great polarisability of large charge-diffuse anions[34] is central to their strong interfacial adsorption[35,36] and in the cases of water/lipid interfaces, this then benefits anion binding to an anion receptor embedded in lipids due to increased local anion concentrations, in addition to highly polarisable anions having favourable induction and dispersion interactions with an anion receptor. This effect is absent in cation binding because of the poor polarisabilities of cations.

Finally, to gain a fundamental understanding of the biomedically relevant topic of carrier-mediated anion transport[4,5] based on our findings, we studied macrocycle **1** as an anion transporter in POPC vesicles.[37] Macrocycle **1** functions as an H^+/anion$^-$ symporter but not as an anion uniporter (Fig.S55) presumably due to the strong headgroup binding that inhibited transmembrane diffusion of the free receptor.[25] An anion transport selectivity of $NO_3^- \approx I^- > ClO_4^- > Br^- > Cl^- > SO_4^{2-} > H_2PO_4^-$ was observed (Table 1, Fig.S53), which correlates with, but is not identical to the anion binding selectivity in lipids. Carrier-mediated ion transport rates depend both on the ion binding affinity and the rate of ion-carrier complex diffusion through the membrane,[38] the latter being clearly unfavourable for the doubly charged SO_4^{2-}. While NO_3^- and I^- can be fully embedded into the macrocyclic plane (Figs.S4b,S4c), ClO_4^- has an exposed oxygen atom after binding to **1** (Fig.S4a), likely slowing down ClO_4^- transport than NO_3^- and I^-. In light of the ClO_4^-, $I^- >> NO_3^-$ transport selectivity commonly observed for structurally simple hydrogen bond-based anion transporters following Hofmeister series,[39] here the clear $NO_3^- > ClO_4^-$ transport selectivity of **1** again reflects macrocycle's structural fit for NO_3^-, which, however, is insufficient to confer a significant $NO_3^- > I^-$ selectivity due to the preference of the lipid environment for the more hydrophobic I^-. Our results in lipid bilayers thus explain the difficulty[39] of overcoming the Hofmeister bias to facilitate selective membrane transport of more hydrophilic anions

such as Cl^- which shows a deceptively strong affinity of 2000 M^{-1} for **1** in DMSO. In addition, as high-efficacy anion transporters typically have Cl^- affinities in the range of 10^2–10^4 M^{-1} in DMSO,[5] our results imply that those systems should operate far from ion binding saturation when transporting Cl^- under physiologically relevant conditions.

In summary, we have gained access to the intricacies of anion binding at biomembrane interfaces taking advantage of an extremely strong SO_4^{2-} binding macrocycle **1** showing fluorescence perturbation upon binding SO_4^{2-} at interfaces. We show that in organic solvents such as DMSO, electrostatic effects dominate leading to preferential binding of charge-dense anions. In biphasic systems with a moderate interfacial polarity such as non-ionic micelles, both electrostatic and dehydration effects operate such that a range of anions across the Hofmeister scale can bind. Contrastingly, we show that anion binding in lipid bilayers behaves markedly differently from the above two scenarios in that anion polarisability, electrostatic screening and penetration depth underlie anion binding strength/selectivity leading to surprisingly favourable binding of charge-diffuse anions, in particular ClO_4^-. In all tested media, we have seen the intrinsic size/shape matching selectivity of **1** for NO_3^- struggling to manifest itself amid the electrostatic, solvation and polarisability effects characteristic of the anions and medium conditions. The elucidation of anion binding principles at lipid bilayers is important to diverse research topics ranging from ion interactions with membrane-embedded proteins/peptides to the development of drug delivery vehicles and synthetic receptors/transporters/assemblies functioning at biomembrane interfaces.

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Author contributions

X.W. and P.A.G. conceived the project and X.W. carried out the synthesis and analytical studies. P.W. contributed to the synthetic work. W.L. conducted the crystallographic studies. P.A.G., Y.B.J. and X.W. obtained the funding for the project. P.A.G. supervised and directed the research. The manuscript was written by X.W. and revised by P.A.G. and Y.B.J.

Data availability

The data that support the findings of this study are available within the paper and its Supplementary Information or are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing interests.

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