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Direct monitoring of bicarbonate transport by fluorescence spectroscopy

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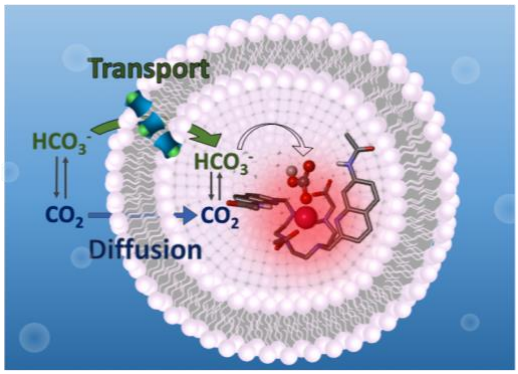

Abstract

The transmembrane transport of bicarbonate is a key step in many important biological processes, and problems with bicarbonate transport are at the origin of various diseases. This warrants efforts to develop synthetic transporters for bicarbonate. However, the mechanisms of bicarbonate transport by synthetic receptors are not fully understood and reliable assays to report on bicarbonate directly are needed. Here we present an assay that allows the kinetics of bicarbonate transport into liposomes to be monitored directly, using fluorescence spectroscopy. The assay utilises an encapsulated europium(III) complex, which exhibits a large increase in emission intensity upon binding of bicarbonate. Our assay offers a number of advantages over existing methodologies including a real-time read-out signal and high sensitivity. These enable the mechanisms of bicarbonate transport to be determined, various antiport and uniport processes to be compared, and low concentrations of anionophores to be used. We have found that mechanisms involving CO_2 diffusion and the dissipation of a pH gradient can lead to an increase in bicarbonate concentration within liposomes, without transport of the anion occurring at all. This potential mechanism should be considered when developing and studying bicarbonate transporters for applications in physiological studies or therapies, and the assay presented here can be used to distinguish this alternative mechanism from actual bicarbonate transport.

Introduction

The transport of bicarbonate is crucial to many biological processes, such as the regulation of pH[1,2] and the removal of metabolic waste.[3] The development of synthetic HCO_3^- transporters could contribute to the study and understanding of various diseases linked to mutations in HCO_3^- transporting proteins, such as haemolytic anaemia, renal diseases, congenital chloride diarrhoea, and glaucoma.[3] Furthermore, HCO_3^- transporters have potential therapeutic applications and were reported to restore the properties of airway surface liquid in cystic fibrosis airway epithelial tissue.[4,5]

Despite the importance of bicarbonate transport in health and disease, most research on synthetic anion transporters to date has focussed on chloride transport.[6-10] This is mainly driven by the ease by which Cl^- transport can be studied compared to that of HCO_3^-, rather than by a difference in biological relevance between these two transport processes. Whereas Cl^- transport through the membranes of liposomes can be readily monitored by fluorescent probes or by ion selective electrodes (ISE),[8] no equivalent methods for the study of HCO_3^- transport exist. The pH sensitive probe HPTS has been widely used to study transport of many different anions and cations;[11] however, this method cannot be readily adapted for the study of HCO_3^- transport, due to the inherent pH variations in HCO_3^- solutions over time.

Monitoring HCO_3^- transport across lipid membranes remains a significant challenge. In the homeostasis of biological systems, the transmembrane transport of HCO_3^- anions and the spontaneous diffusion of CO_2 through membranes are two closely associated processes, which have clearly distinct roles.[3] In model systems such as unilamellar vesicles (LUVs), it is not possible to distinguish between the actual transport of HCO_3^- and mechanisms based on CO_2 diffusion using current assays. Consequently, the exact mechanism(s) by which synthetic HCO_3^- transporters operate remains ambiguous. Nonetheless, numerous reports on HCO_3^- transporting anionophores exist,[12-20] of which the first was on a series of isophthalamides and the natural compound prodigiosin by J. T. Davis, Gale, Quesada and co-workers in 2009.[12] However, indirect methods were employed to study the kinetics of HCO_3^- transport by these and other compounds. In most cases either the efflux of Cl^- out of liposomes was monitored with a chloride selective electrode,[12-16] or the influx of Cl^- was followed with the fluorescent probe lucigenin[17-19] or SPQ.[20] From the observed Cl^- transport it was concluded that an antiport process with HCO_3^- must have taken place. Consequently, these methods are restricted to the study of Cl^-/HCO_3^- antiport only, and do not permit the study of exchange with any other anions, nor HCO_3^- uniport. This limits the possibilities of studying and understanding HCO_3^- transport.

There is only one existing method for the direct study of HCO_3^- transport. This method uses ^{13}C NMR spectroscopy in combination with $NaH^{13}CO_3$ and a paramagnetic species, to distinguish interior from exterior isotopically labelled bicarbonate.[12-14,20,5] The major disadvantage of this method is the difficulty in monitoring transport processes over time (requiring 3-5 minutes per NMR spectrum), which precludes the accurate measurement of transport kinetics when using standard instrument configurations.[5] More recently, an osmotic assay was reported where the efflux of HCO_3^- by an anionophore is accompanied by the efflux of a cation (by a cationophore), resulting in an osmotic efflux of water, which can be observed as a change in the scattering intensity of the liposome dispersion.[21] This is a promising strategy for studying HCO_3^- uniport; however, the assay suffers from a low sensitivity and requires relatively large concentrations of transporter to be present in the membranes (~10 mol%).

The limitations associated with current methods clearly call for a new assay that can report on HCO_3^- transport directly, accurately and with high sensitivity. A fluorescence-based assay in which the influx of HCO_3^- can be monitored directly would surmount these limitations, enabling an accurate comparison and quantification of rates of HCO_3^- transport, and verification of the results obtained by indirect methods. Crucially, it would enable the mechanisms of transport to be elucidated unequivocally, including 1) exchange processes of HCO_3^- with different anions (antiport), 2) uniport of HCO_3^-, and 3) identification of actual transport of HCO_3^- versus mechanisms based on CO_2 diffusion. Such an assay

requires a water soluble probe whose emission intensity changes in response to HCO_3^- levels, while the emission should not be affected by the presence of other anions and cations in the assay.

The cationic europium complex $[Eu.L^1]^+$ previously developed by Butler (Fig. 1c), satisfies these requirements. $[Eu.L^1]^+$ binds reversibly to HCO_3^- in aqueous solution and shows an increase in Eu(III) emission intensity upon binding, particularly within the emission band centred at 615 nm. In contrast, $[Eu.L^1]^+$ has negligible responses to Cl^- and NO_3^- and this made it an ideal candidate for the development of the transport assay.[22] We present here the use of this emissive probe encapsulated in liposomes, to directly monitor the transport of HCO_3^- across the lipid bilayers by fluorescence spectroscopy. We have used this new assay to study HCO_3^- transport by a series of highly potent synthetic anionophores (**1-3**, Chart 1) and natural product prodigiosin (**4**), for which transport was previous observed indirectly using the lucigenin assay (Fig. S1).[19,17,23] This novel HCO_3^- assay allows the study of the kinetics and mechanisms of HCO_3^- transport by these ionophores in unprecedented detail, as well as the comparison of various antiport and uniport processes. We have established that transporters **1**-**4** operate in different ways, and that only **1** is a "pure" HCO_3^- carrier, transporting the anion without interference from other processes. Our results raise the distinct possibility that reported HCO_3^- transporters might not transport the HCO_3^- anion, but rather dissipate the pH gradient induced by CO_2 diffusion. The assay represents a significant step forwards for identifying pure HCO_3^- transporters and providing the mechanistic insight required to develop their potential biological applications.

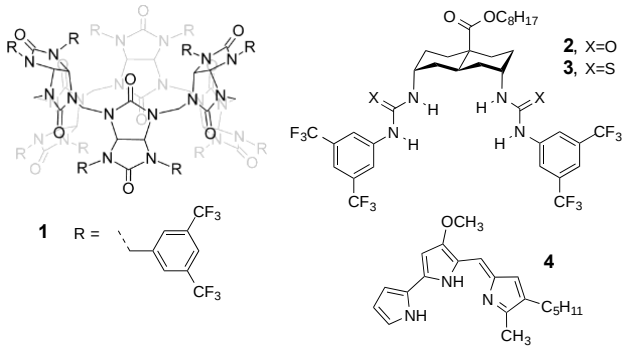

Chart 1 Structures of anionophores **1-4**.

Results & Discussion

Assay to monitor transport of bicarbonate directly

The cationic Eu(III) complex $[Eu.L^1]^+$ (Fig. 1c) is based on a 1,4,7,10-tetraazacyclododecane (cyclen) scaffold possessing two pendant quinoline arms that absorb UV light around 330 nm and transfer energy efficiently to the central Eu(III) ion, which emits red light in the range 570-720 nm.[22] The Eu(III) probe has an open coordination site, occupied by a single water molecule in aqueous solution which quenches the Eu(III) emission significantly. In the presence of HCO_3^-, the coordinated water molecule is displaced upon binding of the hard oxyanion, resulting in a large enhancement in emission intensity (especially around 615 nm) and changes in spectral form (Fig. 1a). The probe responds to physiologically relevant (millimolar) concentrations of HCO_3^- and exhibits high selectivity over poorly coordinating anions that are commonly used in anion transport assays, including Cl^- and NO_3^-.[22] The complex is also sensitive to hydroxide ions, and thus to pH, but this can be controlled with the use of a buffer (see ESI).

In order to use $[Eu.L^1]^+$ to monitor the transport of HCO_3^-, we encapsulated this probe into large unilamellar vesicles (LUVs) consisting of the lipids POPC and cholesterol in a 7:3 ratio and extruded these liposomes through a membrane with 200 nm pores, to obtain LUVs with an average hydrodynamic diameter of 183 nm (Fig. S2). Liposomes of this diameter are routinely used for transport experiments by fluorescence spectroscopy and can be prepared reliably with a high degree of unilamellarity, in contrast to much larger vesicles, as used in the ^{13}C NMR assay.[12] An aqueous solution of 225 mM NaCl was present both interior and exterior to facilitate HCO_3^-/Cl^- exchange (antiport), which also contained 5 mM HEPES buffer to adjust the pH to 7.0 (Fig. 1c). Anionophore **1** was preincorporated in the

membrane of the LUVs and a $NaHCO_3$ solution was added to create a HCO_3^- concentration gradient of 10 mM (Fig. S3). An increase in the intensity of the different emission bands of $[Eu.L^1]^+$ was observed (Fig. 1a) upon the addition of $NaHCO_3$. We chose to monitor the $\Delta J = 2$ emission band around 615 nm (see Fig. 1b), as this showed the largest increase (Fig. 1a), in agreement with observations in titrations of $[Eu.L^1]^+$ with HCO_3^-.[22] From here on, we will refer to these experimental conditions as the EuL1 assay.

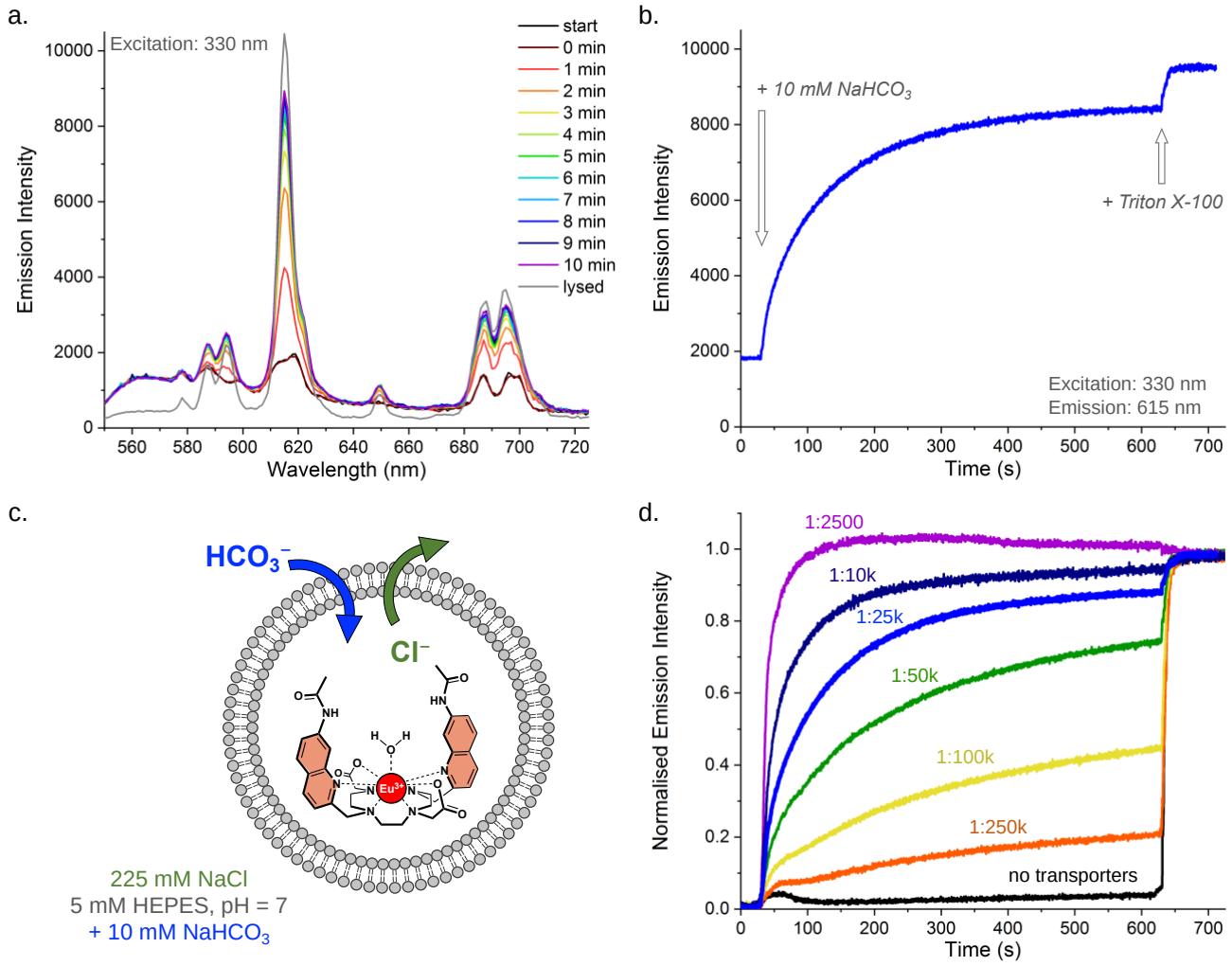

Fig. 1 Transport of HCO_3^- by anionophore **1** preincorporated in LUVs with the probe $[Eu.L^1]^+$ encapsulated (50 μM), suspended in 225 mM NaCl with 5 mM HEPES at pH 7.0 (interior and exterior), upon addition of 10 mM $NaHCO_3$ after 30 seconds and lysis of the LUVs after 10 minutes. a. Emission spectra of $[Eu.L^1]^+$ recorded during the transport by **1** (at 1:25k transporter to lipid ratio); b. Emission intensity at 615 nm monitored over time for the transport as in a.; c. Schematic representation of EuL1 assay to study transport of HCO_3^-; d. Normalised transport curves for anionophore **1** preincorporated at various anionophore to lipid ratios.

The increase in the emission intensity over time following the addition of $NaHCO_3$ indicates that HCO_3^- has entered the liposomes. Since hardly any change in the emission intensity was observed in the absence of anionophore **1** (Fig. 1d, black curve), we can conclude that bambusuril **1** transports HCO_3^- into the liposomes and that the new EuL1 assay allows this process to be monitored. The concentration of **1** was varied (Fig. 1d) and a clear increase in the rate of transport was observed for increasing concentrations of anionophore **1**. This shows that the EuL1 assay is highly sensitive and can be used to study the kinetics of HCO_3^- transport. Furthermore, these results reinforce our previous findings that bambusuril **1** is a very potent HCO_3^-/Cl^- transporter,[19] showing activity even at 1:250,000 ratio, which corresponds to 1.6 nM concentration and an average of two bambusurils per LUV.

Differentiating the mechanisms of bicarbonate transport

The processes by which actual and 'apparent' HCO_3^- transport can occur are schematically represented in Figure 2. The simplest mechanism for HCO_3^- transport is the antiport process with another anion, such as Cl^- (Fig. 2, mechanism A). However, we should consider that addition of a pulse of $NaHCO_3$ to the exterior of the liposomes at pH < 8 does not only create a gradient of HCO_3^-, but also of its conjugate acid H_2CO_3[21] and of CO_2, formed upon dehydration.[24] At equilibrium the concentration of CO_2 is almost 1000-fold higher than that of H_2CO_3 in aqueous salt solutions.[25] Furthermore, it is well known that CO_2 can diffuse spontaneously across the membranes of cells that play important roles in HCO_3^- homeostasis,[26] such as red blood cells and renal epithelial cells.[3] Upon the addition of the HCO_3^- pulse, CO_2 could thus diffuse across the membranes of our liposomes. This increase in the concentration of CO_2 inside the liposomes would result in an acidification of the interior, causing a pH gradient to build up, which would stop the diffusion of CO_2. However, when transporters that can dissipate pH gradients are present in the membrane, the diffusion of CO_2 can continue, leading to a net increase in HCO_3^- concentration inside the liposomes, without this anion crossing the membrane (Fig. 2B-D).

$$HCO_3^- + H^+ \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2 + H_2O$$

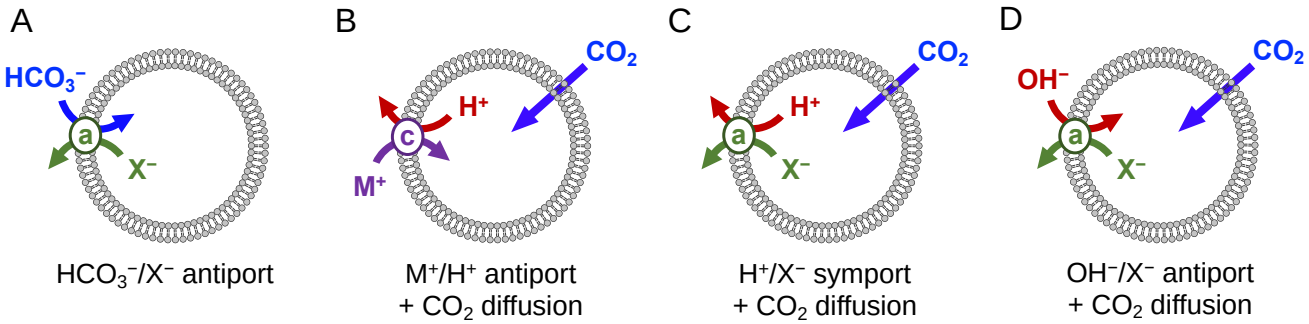

Fig. 2 Different mechanisms by which apparent transport of HCO_3^- could occur. In mechanism A, anionophore (a) exchanges HCO_3^- for another anion – we refer to this as actual HCO_3^- transport . Mechanisms B-D rely on the diffusion of CO_2 coupled to transport of H^+ or OH^- by cationophores (c) or anionophores to result in the net increase in HCO_3^- concentration, without this anion crossing the membrane.

We indeed found that the addition of the cationophore monensin (H^+/M^+ antiporter)[27,28] to liposomes with $[Eu.L^1]^+$ encapsulated gave a clear response upon addition of a HCO_3^- pulse (Fig. 3, red curve). A similar response was observed when the combination of K^+ transporter valinomycin[29] and the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP)[30] were added (Fig. 3, green curve), while those transporters added individually gave no significant response. Further experiments showed that monensin gives similar transport curves in MCl, MNO_3, and M_2SO_4 solutions (where M^+ is Na^+ or K^+, see Fig. S5 and 4a), in agreement with the anion independent CO_2 diffusion mechanism B (Figure 2). No systematic differences were observed between the experiments using either sodium or potassium salts. This could mean either that monensin performs H^+/Na^+ and H^+/K^+ antiport at identical rates, or that the formation[24] and diffusion of CO_2 are rate limiting in mechanism B. To distinguish between these possibilities, we varied the concentration of monensin. While decreasing the monensin to lipid ratio from 1:1000 to 1:10,000 gave a lower rate of transport, increasing to a ratio of 1:100 did not significantly impact the rate of transport (Fig. S6). This confirms that the diffusion of CO_2 is rate limiting in the observed increase in HCO_3^- concentration within the liposomes and not the H^+/M^+ antiport by monensin.

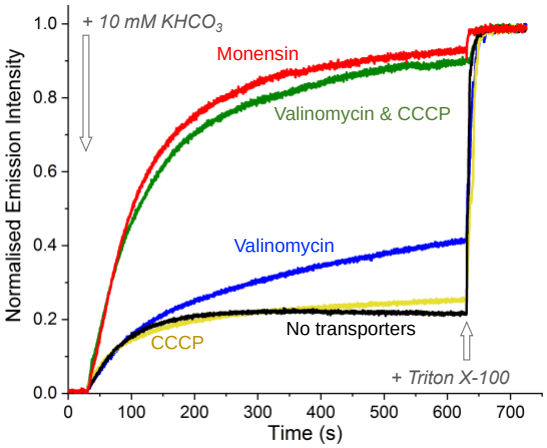

Fig. 3 Increase in interior HCO_3^- concentration as monitored by the EuL1 assay in 112 mM K_2SO_4 with 5 mM HEPES at pH 7, upon addition of 10 mM $KHCO_3$ after 30 seconds. Different cation transporters were added to the LUVs at a transporter to lipid ratio of 1:1000.

To verify if the pH inside the LUVs changes as expected upon diffusion of CO_2 (in the absence of ionophores) or upon dissipation of the pH gradient by monensin, transport experiments were performed in which the pH sensitive probe HPTS was encapsulated instead of the bicarbonate sensitive probe $[Eu.L^1]^+$. All other conditions were identical to those used in the standard EuL1 assay (*i.e.*, 225 mM NaCl, 5 mM HEPES, pH 7.0). The results in Fig. 4d indeed show that the addition of 10 mM $NaHCO_3$ to LUVs with monensin (1:1000 ratio) results in a rapid increase of the pH to 7.4 (red curve), indicating the equilibration of the pH gradient caused by the addition of the basic solution of $NaHCO_3$. In contrast, addition of $NaHCO_3$ to LUVs without transporters results in an acidification of the interior (black curve), in agreement with the formation of carbonic acid upon diffusion of CO_2. LUVs with a very low concentration of monensin (1:50,000) show an initial acidification of the interior due to CO_2 diffusion, followed by a slow increase of the pH due to the H^+/Na^+ antiport by monensin. These experiments with HPTS confirm that the apparent transport of HCO_3^- by monensin can be attributed to mechanism B. Furthermore, these data show that the pH equilibration by monensin at 1:1000 ratio (Fig. 4d) is much faster than the apparent HCO_3^- transport revealed by the EuL1 assay (Fig. 4a), which is further evidence that CO_2 diffusion (and/or formation[24]) is rate limiting in the apparent transport of HCO_3^- by monensin (at 1:1000 ratio).

Determining the transport mechanisms of different anionophores

Following experiments with bambusuril **1**, monensin, and other cationophores, we studied the HCO_3^- transport by urea **2**, thiourea **3**, and prodigiosin **4** in the EuL1 assay in NaCl (blue curves in Fig. 4c and S9). A clear increase of the emission intensity was observed for all anionophores, even at the relatively low concentration of 1:25,000 (transporter to lipid ratio). A key question we wished to address was whether the observed increase in HCO_3^- in the liposomes is due to HCO_3^-/Cl^- antiport transport mechanism A, or rather by CO_2 diffusion and pH gradient dissipation, as in mechanisms C and D (Fig. 2). Ureas and thioureas with acidic N-H groups have been reported to not only transport anions, but also H^+ (or OH^-), and prodigiosin **4** is a known H^+Cl^- transporter as well.[31] Indeed, rapid pH equilibration was observed for anionophores **2**-**4** (blue curve in Fig. 4f and S21). In contrast, bambusurils have an electron deficient cavity formed by twelve polarised methine C-H groups, which can neither be readily deprotonated nor interact strongly with OH^-.[32] Upon addition of $NaHCO_3$ to liposomes with bambusuril **1**, a gradual increases in pH is observed, resembling the kinetics of the transport of the basic HCO_3^- anion into the LUVs (blue curve in Fig. 4e vs 4b). This result concurs with our previous finding that **1** is unable to dissipate pH gradients by HCl symport or Cl^-/OH^- antiport.[19] This excludes mechanisms C and D for this compound, leaving HCO_3^-/Cl^- antiport (A) as the only possible transport mechanism for **1**.

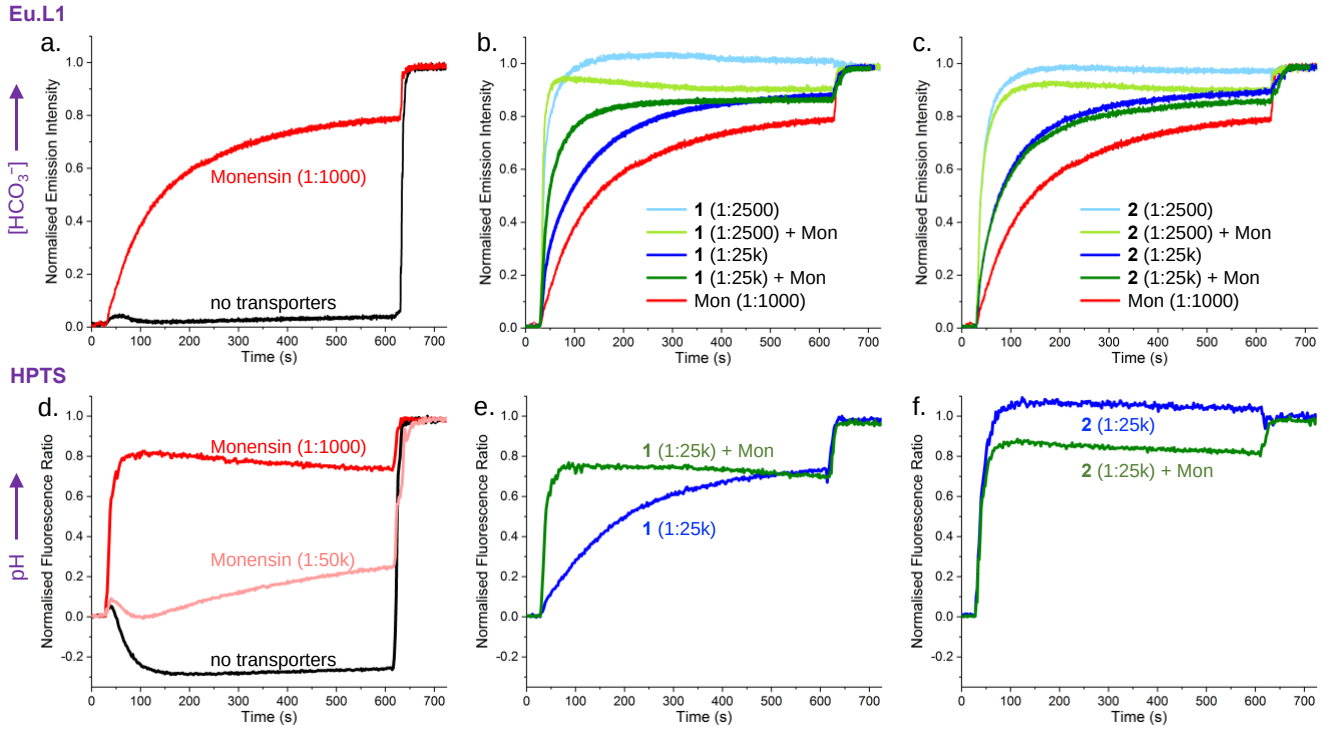

Fig. 4 Increase in interior HCO_3^- concentration as monitored using the EuL1 assay (a-c) or change of the interior pH as monitored using the probe HPTS (d-f) in 225 mM NaCl with 5 mM HEPES at pH 7, upon addition of 10 mM $NaHCO_3$ after 30 seconds and lysis of the LUVs 10 minutes after that, to study transport by monensin (a,d), bambusuril **1** (b,e) and urea **2** (c,f). Monensin (1:1000 transporter to lipid ratio) was added to the experiments with anionophores **1** and **2**.

To distinguish the mechanisms involved in the apparent HCO_3^- transport by the other compounds, we have made use of the limited rate of CO_2 diffusion in the EuL1 assay, as observed from the experiments with monensin. For bambusuril **1**, addition of monensin gives a clear increase in the rate of transport as seen from the comparison of the green to the blue curves in Fig 4b and S11. This increase can be understood from the combined effect of mechanism A by **1** and mechanism B by monensin, leading to a higher rate of apparent transport of HCO_3^- than by either of these two processes alone. In contrast, addition of monensin to LUVs with anionophores **2**-**4** did not increase the rate of transport, as shown for **2** in Fig. 4c and for **3** and **4** in Fig. S9. Because pH equilibration by these compounds is nearly instantaneous (Fig 4f and S21), the addition of a second pathway to dissipate the pH gradient (by monensin) will have no effect, as the overall rate of (apparent) HCO_3^- transport will remain limited by CO_2 diffusion. From this observation we can conclude that anionophores **2**-**4** primarily act via mechanism C or D.

To test if urea **2** and thiourea **3** can perform any HCO_3^-/Cl^- transport (mechanism A), we have increased the concentrations of **2** and **3** in the membranes of the liposomes to 1:2500 (transporter to lipid ratio). The light blue curves in Fig. 4c and S9a show that this ten-fold increase in transporter concentration leads to a significantly faster rate of (apparent) HCO_3^- transport, and that this overall rate clearly exceeds rates of transport that are limited by CO_2 diffusion (as observed in the curves for monensin ≥ 1:1000 ratio, see also Fig. S10). From this we can conclude that HCO_3^-/Cl^- antiport mechanism A also takes place. These compounds dissipate the pH gradient faster than they transport HCO_3^- and as a result C or D is the main mechanism, up to the point that CO_2 diffusion becomes rate limiting, after which mechanism A contributes to the apparent HCO_3^- transport. It is clear from these data that bambusuril **1** is the only "pure" HCO_3^- transporter studied, which functions without interference from other processes.

Quantification of rates of transport

To verify the qualitative trends and comparisons described above, we have fitted the transport data from the EuL1 assay with single and double exponential functions, to obtain half-lives and initial rates

respectively (see ESI for details). Due to the slight differences observed in the equilibration levels of the different transport curves after normalisation and the effect of pH on the fluorescence levels (see ESI for a discussion), half-lives are more reliable to compare transport data, as these values indicate the time required to reach half of the final transport level and are thus a measure of how fast equilibrium is reached, independent of absolute emission values. The obtained values for the half-lives are given in Table 1 (see Table S1 for initial rates and additional concentrations of **1**). The comparison of half-lives of transport by **1** with and without monensin clearly shows that equilibrium is reached much faster in the presence of monensin (Table 1 and Fig. S12), confirming the additivity of mechanisms A and B as discussed above. In contrast, the half-lives by **2**-**4** are nearly identical in the presence and absence of monensin.

Table 1 Performance of anionophores **1**-**3** and prodigiosin in the EuL1 assay in NaCl and $NaNO_3$.

Salt	**Anionophore**	**Concentration (anionophore:lipid)**	**Half-life (s)[a] (without monensin)**	**Half-life (s)[a] (with monensin)**
NaCl	None		*	82
	1	1:2500	10	4
		1:25k	64	21
	2	1:2500	12	11
		1:25k	51	50
	3	1:2500	12	11
		1:25k	46	47
	4	1:25k	59	74
$NaNO_3$	None		*	81
	1	1:2500	*	67
		1:25k	*	85
	2	1:2500	45	40
		1:25k	89	85
	3	1:2500	16	14
		1:25k	65	59
	4	1:25k	61	68
KGluc[b]	None		*	124
	1	1:25k	83	38
	2	1:25k	140	45
	3	1:25k	180	42
	4	1:25k	*	n.d.

[a] Calculated from a single exponential fit of the transport curve, see ESI for details.
[b] Transport in KGluc was studied in presence of valinomycin.
* Transport was absent or too slow to quantify.
n.d. = not determined

Table 1 also shows that the overall half-lives obtained from the apparent HCO_3^- transport by anionophores **1**-**4** (in absence of monensin) are rather similar. However, the different pH profiles could affect this comparison (see ESI Section 2.6) and it would thus be better to compare the different transporters in the presence of monensin. Under those conditions, CO_2 diffusion based mechanisms contribute to the transport for all the compounds, but as this process has a limited and thus constant rate, the differences in half-lives between anionophores **1**-**4** (in presence of monensin) can be attributed to the differences in rates of HCO_3^-/Cl^- antiport (mechanism A) by the anionophores. In this comparison, bambusuril **1** is clearly the most active ionophore for HCO_3^-/Cl^- antiport. Bis-urea **2** and bis-thiourea **3** show similar rates of transport and are slightly more active than prodigiosin **4**, for which the half-life is very close to that of transport by monensin alone.

Bicarbonate uniport and antiport with nitrate

After demonstrating that our EuL1 assay can distinguish between different HCO_3^- transport mechanisms, and enables quantitative analysis of anionophores during HCO_3^-/Cl^- exchange, we used the assay to study the exchange of bicarbonate with other anions, or uniport of HCO_3^-. Commonly employed indirect methods to study HCO_3^- transport rely on the monitoring of Cl^- concentrations, preventing their use for studying exchange between HCO_3^- and NO_3^-, or the uniport of HCO_3^-. In contrast, $[Eu.L^1]^+$ can operate in various salt solutions to study other processes than HCO_3^-/Cl^- exchange. Hence, we utilised the EuL1 assay in $NaNO_3$ solution, to monitor HCO_3^-/NO_3^- exchange (Fig 5a), and in a potassium gluconate (KGluc) solution in the presence of the K^+ cationophore valinomycin, to study the uniport of HCO_3^- (Fig 5b).

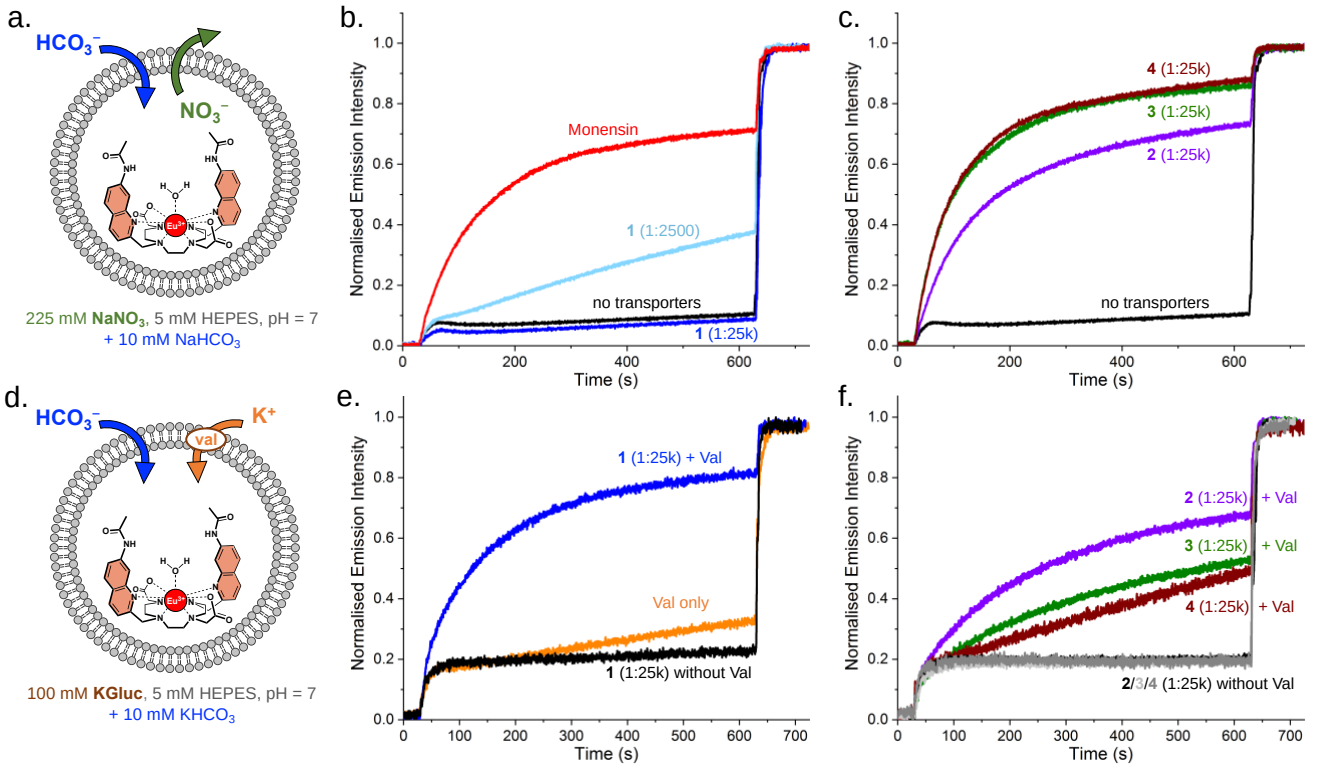

Fig. 5 Increase in interior HCO_3^- concentration in the presence of anionophores **1**-**4** monitored by the EuL1 assay in different salt solutions: a-c exchange with nitrate in 225 mM $NaNO_3$ with 5 mM HEPES at pH 7, and d-f uniport in 100 mM KGluc with 5 mM HEPES at pH 7 in presence of valinomycin. 10 mM $NaHCO_3$ (in b,c) or $KHCO_3$ (in e,f) was added after 30 seconds and the LUVs are lysed after 10 minutes. The schematic representations in a and d only show the mechanisms based on actual HCO_3^- transport, while mechanisms based on CO_2 diffusion could also take place.

Compounds **2**-**4** were found to exhibit efficient (apparent) transport of HCO_3^- in $NaNO_3$ (Fig. 5c) and the rates do not change upon addition of monensin (see Table 1), similar to the results obtained for these compounds in NaCl, indicating that the same combination of mechanisms are occuring. However, bambusuril **1** showed no transport at a 1:25,000 ratio, and only very slow transport was observed when using a 10-fold higher concentration of **1** (1:2500, Fig. 5b). This slow HCO_3^-/NO_3^- exchange by **1** resembles previous results reported for Cl^-/NO_3^- exchange, which was found to be 100-fold slower than Cl^-/HCO_3^- exchange by this bambusuril.[19] This large difference in Cl^-/HCO_3^- and Cl^-/NO_3^- exchange rates was explained by the very high affinity of **1** for NO_3^- ($K_a = 5 \times 10^{11}$ M^{-1} in acetonitrile), which could prevent the release of this anion.[19] In addition, it was proposed that simultaneous binding of a Cl^- and a HCO_3^- anion in the bambusuril could facilitate the exchange of these anions.[19] Even though the formation of an equivalent complex with NO_3^- and HCO_3^- simultaneously is possible, this does not appear to increase the rate of the exchange of these two anions by **1**. Instead, the very strong binding of NO_3^- is the most probable cause for the low rates of HCO_3^-/NO_3^- exchange by **1** (see also Fig. S19).

This was further confirmed by the HCO_3^- uniport experiment in KGluc (Fig. 5e), where HCO_3^- was efficiently transported by bambusuril **1**. In this experiment valinomycin transports K^+ to compensate for

the displacement of charge associated to the HCO_3^- uniport, while the highly hydrophilic gluconate anion is not readily transported.[21] Under these conditions, it is highly unlikely that an anion exchange process takes place and instead bambusuril **1** will have to release the strongly bound HCO_3^- and return through the membrane without an anion bound.

In contrast, apparent transport of HCO_3^- by thiourea **3** and prodigiosin **4** was much slower when tested in uniport conditions (Fig 5f), compared to in the presence of Cl^- or NO_3^- (Fig. 5c and S9). In NaCl and $NaNO_3$ the apparent HCO_3^- transport by these compounds was mainly attributed to H^+/Cl^- or H^+/NO_3^- cotransport in combination with CO_2 diffusion (mechanism C, or equivalent mechanism D). The poor rates of transport in KGluc indicate that these compounds are less efficient protonophores (H^+ or OH^- transporters) than H^+/Cl^- or H^+/NO_3^- cotransporters and that they are not efficient HCO_3^- uniporters either. This result corroborates with other reports in which prodigiosin **4** was found to be a poor protonophore.[31] Rates of apparent HCO_3^- transport by urea **2** in KGluc are significantly higher than those observed for **3** and **4** and the increase in the rate of transport observed with a higher concentration of **2** (Fig. S20) indicates that **2** is able to perform actual HCO_3^- uniport (see ESI for further mechanistic discussions). Nonetheless, bambusuril **1** is clearly the most efficient HCO_3^- transporter tested (Table 1).

Discussion of the EuL1 assay compared to existing methods

The novel EuL1 assay allows monitoring bicarbonate transport directly and with high sensitivity, overcoming numerous disadvantages of existing methods, while combining their advantages, to offer a complementary tool in anion transport research. The only other direct HCO_3^- transport assay reported to date is based on ^{13}C NMR spectroscopy,[12] which suffers from low sensitivity and poor time resolution. Furthermore, we observed that the cationophore monensin also gives a positive response in the ^{13}C NMR assay for HCO_3^-/Cl^- transport (Fig. S22), which demonstrates that this assay cannot distinguish between CO_2 diffusion based mechanisms and actual HCO_3^- transport. Indirect assays, such as the commonly used ISE and lucigenin assays,[12-19] have not been able to provide mechanistic insights, nor allow comparisons between various HCO_3^- antiport and uniport processes. The osmotic HCO_3^- uniport assay is the only method reported so far that showed diffusion of neutral HCO_3^--based species in combination with H^+ transport by monensin,[21] in agreement with our findings. However, the drawbacks of the osmotic assay are the very high concentrations of ionophores required, due to the low sensitivity of the assay, and its incompatibility with the preincorporation of lipophilic transporters in the membrane during the preparation of the LUVs.

We have exploited the attractive features of the EuL1 assay to discover that bambusuril **1** can efficiently perform HCO_3^-/Cl^- antiport and HCO_3^- uniport, while bisurea **2**, thiourea **3**, and prodigiosin **4** mainly combine CO_2 diffusion and pH gradient dissipation, leading to *apparent* HCO_3^- transport. Compounds **1**-**4** have previously been shown to act as HCO_3^- transporters in the lucigenin assay[17,19] and prodigiosin **4** also in the ISE and ^{13}C NMR assays.[12] However, those experiments could not distinguish between actual transport of HCO_3^- anions and apparent HCO_3^- transport. Notably, most of the HCO_3^- transporters reported in the literature resemble compounds **2**-**4**, in that they are also able to transport H^+ or OH^-,[31] and this transport activity combined with CO_2 diffusion could be the mechanism of apparent HCO_3^- transport for many reported compounds. Furthermore, it is striking that selectivity for transport of Cl^- and NO_3^- over HCO_3^- has been reported for only two compounds, a biotinuril macrocycle and bis-triazole,[33,34] which do not have acidic protons and are thus likely to be poor H^+ and OH^- transporters.

Our results obtained by the EuL1 assay imply that only for compounds that show apparent HCO_3^- transport without dissipating pH gradients (such as **1**) and for very potent anion transporters for which the rate of total apparent HCO_3^- transport surpasses the limited rate of CO_2 diffusion (**2** and **3**), we can conclude with certainty that these can act as actual anionophores for HCO_3^-. This is to be taken into account in the future development of HCO_3^- anionophores and can then be readily verified with the EuL1 assay.

Conclusions

We have developed a new assay to directly monitor transport of HCO_3^- into liposomes by fluorescence spectroscopy, using the encapsulated europium complex $[Eu.L^1]^+$, of which the luminescence increases upon binding HCO_3^-. This assay provides a rapid and highly sensitive signal that enables anion transport kinetics to be determined and low concentrations of anionophores to be used. By combining anionophores with monensin in this direct and sensitive assay, it was possible to distinguish actual transport of HCO_3^- anions from alternative mechanisms based on CO_2 diffusion that lead to an increase of HCO_3^- concentration in the liposomes, providing an unprecedented insight into the mechanisms of HCO_3^- transport by anionophores. This mechanistic study leads to the conclusion that we should doubt if many of the reported HCO_3^- transporters are actually capable of transporting this anion, or that they rather operate by dissipating the pH gradient resulting from CO_2 diffusion. Furthermore, the versatility of the assay compared to all existing assays was demonstrated by comparing HCO_3^-/Cl^- and HCO_3^-/NO_3^- antiport and HCO_3^- uniport processes for the first time.

We are convinced that the new opportunities provided by this assay to study transport of HCO_3^- efficiently in new mechanistic detail will contribute to the further development of HCO_3^- transporters for biomedical purposes, such as channel replacement therapies.[6,35] The assay developed in this work will also inform the future design of Eu(III) probes capable of monitoring spatio-temporal HCO_3^- dynamics within living cells. Indeed, a structurally related Eu(III) complex has already been shown to enter living cells and localise to specific subcellular compartments.[36] This feature, combined with the long luminescence lifetime of $[Eu.L^1]^+$ and its derivatives augurs well for cellular imaging of HCO_3^- transport with high signal-to-noise, using time-gated fluorescence microscopy.

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