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Influence of the Insertion Method of Aryl-Extended Calix[4]pyrroles into Liposomal Membranes on Their Properties as Anion Carriers

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Dedicated to Professor Pablo Espinet on the occasion of his 70th anniversary

Abstract: We disclose the results of our investigations on the influence that the insertion method of aryl-extended calix[4]pyrrole into liposomal membranes exerts on their properties as anion carriers. We use the standard HPTS assay to assess the transport properties of the carriers. We show that the post-insertion of the carrier, as DMSO solution, assigns better transport activities to the "two-wall" α,α-aryl-extended calix[4]pyrrole **1** compared to the "four-wall" α,α,α,α-counterpart **2**. Notably, opposite results were obtained when the carriers were pre-inserted into the liposomal membranes. We assign this difference to an improved incorporation of carrier **2** into the membrane when delivered by the pre-insertion method. On the other hand, carrier **1** shows comparable levels of transport independently of the method used for its incorporation. Thus, an accurate comparison of the chloride transport activities featured by these two carriers demands their pre-incorporation in the liposomal membranes. In contrast, using the lucigenin assay with the pre-insertion method both carriers displayed similar transport efficiencies.

Introduction

The efficient transport of anions across lipid bilayers is fundamental to sustain a great variety of biological processes, such as, cellular pH regulation, maintenance of cell volume, or electrical signalling.[1] Dysfunction of the natural proteins (i.e. ion channels) responsible of this transport is associated with several human diseases, which are collectively known as channelopathies (e.g. cystic fibrosis, renal diseases).[2–4] In this area, the development of synthetic small molecules capable to function as anion transporters has attracted significant attention.[5–11] The knowledge gained with synthetic carriers also contributes to the further understanding of the functioning of electrophysiological transport processes in natural systems and might bring significant advances in biomedical science and its potential therapeutic applications (e.g. disruption of cellular ion homeostasis).[12]

Anion transport using vesicle models has been assessed using different techniques. A common method is the use of commercially available ion-selective electrodes (ISEs).[13] Alternatively, the entrapment of fluorescent dyes in the inner cavity of large unilamellar vesicles (LUVs) has been widely employed to study anion transport. The well-established HPTS assay measures the rate of pH-gradient dissipation and serves to evaluate the anion-transport activity of synthetic carriers by simply monitoring the change of fluorescence of the entrapped pH-sensitive dye. In a typical experimental set-up, LUVs containing the pH-sensitive fluorophore 8-hydroxy-1,3,6-pyrenetrisulfonate (HPTS) are prepared and suspended in an aqueous ionic media. Next, the vesicles were exposed to an extravesicular base pulse (e.g. NaOH), followed by the addition of the transporter to the media (typically as DMSO solution). It is worth noting that the standard HPTS assay involves the post-insertion of the carrier on the lipid bilayer, that is, the incorporation of the transporter after the assembly of the vesicles.

Using other fluorescent probes, such as lucigenin (halide sensitive dye), Davis and co-workers have demonstrated that the transport activity of the synthetic carrier is highly dependent on the insertion method used for its incorporation in the

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liposomes: post-insertion (added as a solution in, for example, DMSO or methanol, after the liposomes' self-assembly) or pre-insertion (incorporated into the vesicles' membranes during self-assembly).[14] Davis and others have suggested fast precipitation and/or non-homogeneous insertion of some carriers as putative causes for the lower activities displayed when added to the LUVs suspensions as external DMSO solutions.[15,16] In order to overcome this limitation, the pre-insertion of the carriers during the self-assembly of the LUVs preparation was used. To the best of our knowledge, related comparative studies of post- versus pre-insertion of anion carriers using HPTS assays are not reported in literature.

Calix[4]pyrroles are known receptors for the binding of anions and ions pairs, as well as efficient anion carriers through model phospholipid bilayers.[17–19] In 2008, Quesada, Gale and co-workers demonstrated, using LUVs, that *meso*-octamethyl-calix[4]pyrrole functioned as co-transporter for CsCl.[20] In the same work, different *meso*-substituted calix[4]pyrroles (Et, *n*Pr, *n*Bu, cyclohexyl) were also tested and showed a dramatic loss of transport activity compared to the parent octamethyl calix[4]pyrrole. The authors did not provide any immediate explanation for the results. However, they propose the possibility that the reduced solubility of the lipophilic derivatives in water was responsible for the observed reduction in transport activity.

Other calix[4]pyrroles have been investigated as chloride transporters and their transport mechanisms studied in detail.[21] Recently, Ko et al. reported the use of a series of diamide strapped calix[4]pyrroles to facilitate the chloride transport in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposomal models and cells. The authors not only describe the facilitated anion transport across the membranes exerted by the calix[4]pyrrole carriers but also their ability to trigger cell apoptosis.[22]

In 2013, one of us evaluated the anion transport activity of a series of "two-wall" α,α-aryl-extended calix[4]pyrroles using lipid-based lamellar membranes and the HTPS assay.[23] The obtained results showed that the transport activities correlated well with the electronic nature of the *meso*-aromatic walls of the carrier. This observation supported the importance of anion–π interactions in the transport mechanism.[24]

In this work, we undertook the evaluation of "four-wall" α,α,α,α-aryl-extended calix[4]pyrroles as chloride carriers mainly using the HTPS assay. In the cone conformation, "four-wall" aryl-extended calix[4]pyrroles display a well-defined polar aromatic cavity suitable for the inclusion of a chloride ion. We demonstrate that using the post-insertion methodology of the carrier (as DMSO solution) the "four-wall" α,α,α,α-aryl-extended calix[4]pyrroles, **2** and **3**, display significantly lower chloride transport activities than the reported for the "two-wall" analogue **1** (Figure 1). Conversely, using the pre-insertion methodology of the synthetic aryl-extended transporters, the "four-wall" carriers out-performed to "two-wall" counterparts. When switching to the lucigenin assay, maintaining the pre-insertion methodology, the "four-wall" and the "two-wall" carriers displayed almost identical transport efficiencies.

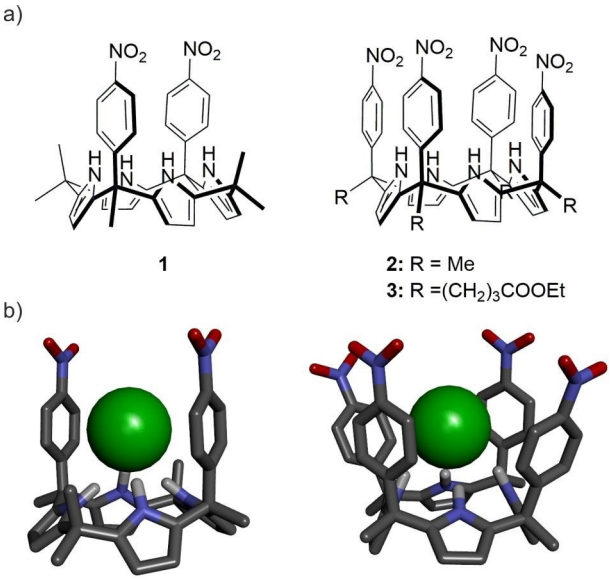

Figure 1. a) Molecular structures of "two-wall" and "four-wall" calix[4]pyrrole receptors **1**–**3** used in this study. b) MM3 energy minimized structures of complexes $Cl^-\subset$**1** (left) and $Cl^-\subset$**2** (right). Calix[4]pyrrole receptors **1** and **2** are shown in stick representation and Cl^- anion is displayed as CPK representation. Non-polar hydrogens have been omitted for clarity.

Results and Discussion

Standard HPTS assay

Compound **1**[25,26] was selected as representative of "two-wall" aryl extended calix[4]pyrrole carriers. Compound **1** bears two π-acidic *meso*-aromatic substituents, binds chloride with high affinity in acetonitrile solution and showed good anion transport activities.[23] Receptors **2** and **3** are "four-wall" analogues of **1**. While **2** and **3** feature identical substitution at their upper rims, they are equipped with different substituents at the *meso*-position of their lower rims, methyl and 3-(carboxyethyl)-butyl, respectively.[27,28]

The binding of anions with aryl-extended calix[4]pyrrole receptors is known to occur mainly through the establishment of four convergent hydrogen bonds with the NHs of the calix[4]pyrrole core. Moreover, attractive anion–π interactions with suitable aromatic walls can also contribute to the thermodynamic stability of the complexes. The binding studies carried out with calix[4]pyrrole receptors **1**–**3** and TBA·Cl in CD_3CN solution produced binding constant values of the same order of magnitude (Table 1 and the Supporting Information).[26,27]

Chloride transport with carrier **1** was previously assessed using the standard HPTS assay.[29,23] In this assay, egg yolk L-α-phosphatidylcholine (EYPC) LUVs of 100 nm (mean diameter) are loaded with HPTS 1 mM, which functions as an internal pH reporter. Both internal and external aqueous media are initially buffered with NaCl (100 mM) and HEPES (10 mM) at pH 7.0. First, a pH gradient is generated through the membrane by the external addition of a NaOH pulse. Next, the transporter is added as a DMSO solution. The dissipation of the pH gradient between extra- and intravesicular media is monitored using a ratiometric approach between two fluorescence intensities of

Table 1. Chloride binding and transport data in 30 μM EYPC LUVs with calix[4]pyrroles **1**–**3**.

	Clog*P*[a]	K_a [M^{-1}][b]	EC_{50} [%]
1	8.9	2.7×10^{5}[b]	0.024 ± 0.018[d]
2	10.4	1.8×10^{5}[b]	0.124 ± 0.004[d]
3	12.6	2.8×10^{5}[c]	n.d.[e]

[a] Calculated using ChemDraw 16.0 software. [b] Association constants measured in acetonitrile at 298 K for 1:1 complexes of calix[4]pyrrole **1**–**2** with TBACl by means of ITC experiments (ref. 26 and 27). [c] Determined by ^{1}H-NMR competitive experiment of receptors **2** and **3** with TBACl (Supporting Information). [d] Expressed in % carrier/EYPC molar ratio. The reported value is the average of at least two Hill plot experiments. The error is given as the standard deviation. [e] n.d. EC_{50} was not determined due to the poor fitting of the experimental data to the Hill equation.

the HPTS⊂EYPC-LUVs. Finally, the addition of a solution of Gramicidin D, an effective proton channel, induces a fast equilibration of the pH at both sides of the membrane serving to calibrate the maximum change of fluorescence (100%). We performed dose-response studies with the different calix[4]pyrrole carriers. The obtained data were analysed with the Hill equation in order to calculate the Hill coefficient (a parameter that can be related to the stoichiometry of the transporter:anion active complex) and the effective concentration of transporter required to reach 50% of the maximum transport activity (EC_{50}). Using this assay, carrier **1** transported chloride anions with an EC_{50} of 0.028 ± 0.01%[30] and an *n*-value close to 1.

Using identical experimental conditions, we have now performed new dose-response experiments using carrier **1**. In the present study, the emission changes of the dye were monitored by excitation at a single wavelength (510 nm; $\lambda_{ex}=$ 450 nm).[31] The obtained results were in complete agreement with the ones previously reported using a ratiometric approach ($EC_{50}=0.024\pm0.018$%).[23] Owing to the simplicity of the single wavelength excitation methodology, which allows a continuous real time monitoring of the emission changes, we implemented it to assess the anion transport activities of transporters **2** and **3**. The results of the transport experiments were analysed using the Hill's equation in order to determine the EC_{50} values, Table 1 (see Supporting Information for details).

The simple comparison of the fluorescence versus time curves obtained in the dose-response experiments performed using carrier **2** and those measured for carrier **1** revealed marked differences in shape (Figure 2). The fluorescence time-course curves for the "two-wall" carrier **1** show a gradual increase in the emission, which is indicative of the pH equilibration. This gradual increase of emission is observed even at high carrier concentrations (i.e. 0.3% carrier/EYPC molar ratio (mr)) (Figure 2a). Conversely, the "four-wall" carrier **2** produced abrupt changes in fluorescence intensity during the initial phase of the transport experiment (< 10 s) that quickly levelled off. Moreover, the relative fluorescence values (%*F*) achieved at the end of the transport experiments of carrier **1** were superior to those registered for carrier **2** (Figure 2 and Figure S6).

It is relevant to note, that using lucigenin assays for halide transport, Davis and co-workers noticed abrupt quenching of

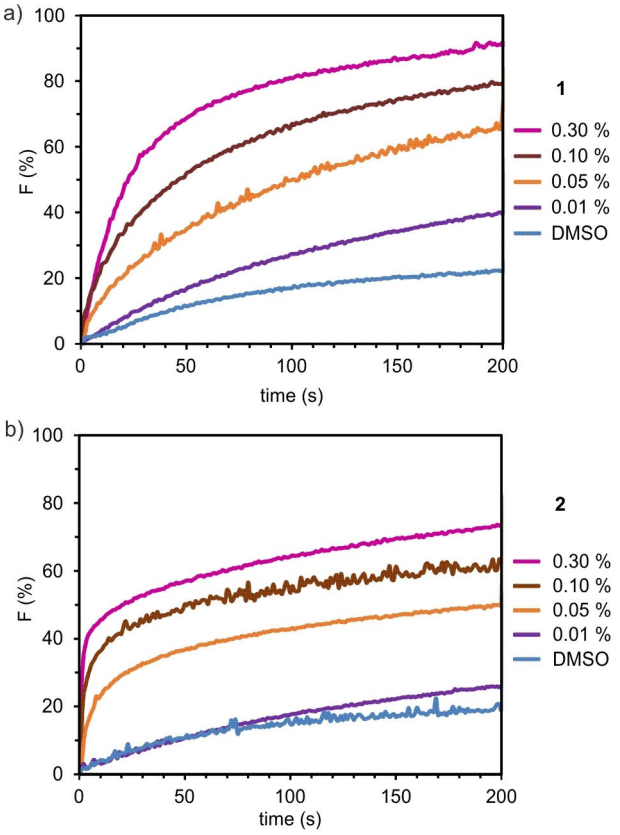

Figure 2. Dose-response experiments corresponding to a pH gradient equilibration of LUVs (mean diameter 100 nm) monitored using a single-excitation HPTS assay and carriers **1** (panel a) and **2** (panel b). Fluorescence emission at 510 nm ($\lambda_{ex}=$450 nm) was monitored during 200 s following the addition of the carrier as DMSO solution. All fluorescence curves are normalized considering the starting point as 0% *F*. The 100% *F* value corresponds to the emission value after addition of Gramicidin D (out of the transport window). Concentration of carriers are expressed in % carrier/EYPC molar ratio. To facilitate data comparison time values were also normalized: initial point of transporter addition ($t=$100 s normalized to $t=$0 s) and end-point of the experiment (just before addition of Gramicidin D, $t=$300 s normalized to $t=$200 s).

the dye's fluorescence in the time-course curves following the addition of high lipophilic anthracene bis-urea transporters even at low concentrations. Subsequently, the fluorescence intensity reached a plateau at high values.[14] The authors attributed this behaviour to a non-homogeneous distribution of the carriers into the membranes and/or to poor insertion when added as external solutions (post-insertion).[7]

We performed the Hill analysis of the dose-response chloride-transport curves obtained for carrier **2** and extracted a value of EC_{50} (**2**) $=0.124\pm0.004$%. This value is one order of magnitude larger than the one calculated for the "two-wall" counterpart **1** (EC_{50}(**1**) $=0.024\pm0.018$%), suggesting that the "four-wall" compound **2** features a significant reduction in chloride transport.

Next, we undertook chloride transport experiments using a higher concentration of the LUVs (≈120 μM EYPC) (Figure S7). In these conditions, the transporter concentrations must be also increased in order to cover the same range of relative concentrations (% carrier:EYPC molar ratio) used in the more dilut-

ed experiments explained above. At higher concentrations of carrier **1**, the time-course fluorescence curves showed analogous shapes (Figure S7a). We calculated a value of EC_{50} (**1**) = 0.020%, which is identical to the one obtained in more diluted conditions.

Also in the case of carrier **2**, the shape of the time-course fluorescence curves was not modified by the lipid's concentration increase (Figure S7b).

Nevertheless, the determined EC_{50} values for **2** turned out to be concentration dependent (2.5% for the concentrated conditions and 0.124%, under diluted conditions). In the concentrated conditions, a maximum increase of 60% of fluorescence was obtained at the end of the transport time at the highest concentration of the carrier **2** (2% mr) (Figure S7b).

Next, we wanted to assess the chloride transport activity of carrier **3**. Compound **3** features an analogous binding affinity for chloride than **2**. However, **3** is more lipophilic than **2** (K_a and log*P* values in Table 1). Unfortunately, the analysis of the dose-response data obtained for carrier **3** using the Hill equation did not allow the calculation of a reliable EC_{50} value due to the poor fit of the experimental data to the theoretical curve (Table 1, Figure S7c).

Taken together, the chloride transport results derived from the HPTS assay indicated that, the "two-wall" calix[4]pyrrole **1** is a more effective carrier for chloride anions than the "four-wall" counterparts **2** and **3**.

On the one hand, we were aware that the binding affinities of the synthetic carriers for the targeted anions do not linearly correlate with their transport efficiencies. On the other hand, it has been established that differences in lipophilicity of the carriers and the lipid composition of liposomal membranes play an important role on their anion transport activities.[32] For example, Quesada, Gale and co-workers attributed the low transport activities shown by tambjamine carriers with high log*P* values either to their poor deliverability to the lipid bilayer and/or its low mobility within the membrane.[32,33]

Based on the above findings, we surmised that the higher transport activity displayed by the "two-wall" carrier **1** compared to the "four-wall" counterparts, **2** and **3**, might be produced by their poor delivery (insertion) into the membrane. Although the "four-wall" carriers (**2** and **3**, log*P* values in Table 1) are more lipophilic, they are possibly less water soluble, displaying a larger tendency to precipitation/aggregation under the used experimental conditions.

Influence of the pre-insertion method on the transport activities of the carriers

Aiming at supporting the previous hypothesis, we investigated the transport properties of the carriers by pre-inserting them in the membranes during the self-assembly of the LUVs. This methodology is different from the one employed in the standard HPTS assay. The EYPC-LUVs (ca. 100 nm) containing HPTS were self-assembled in the presence of the calix[4]pyrrole carrier (see experimental section for details). The resulting HPTS⊂EYPC-LUVs-**1**/**2**, containing the carrier already inserted in their membranes, were suspended in an aqueous NaCl (100 mM), HEPES (10 mM) solution at pH 7.0. Afterwards, a pulse of NaOH was added to the solution to initiate the ion transport.

Figure 3 depicts the fluorescence time-course curves obtained in chloride transport experiments for post- (dashed lines) and pre-inserted (continuous line) carriers, **1** (blue) and **2** (brown), at a 0.03% carrier:EYPC molar ratio. For a better comparison between the two insertion methods, an aliquot of DMSO was added in the pre-insertion experiments 50 seconds before initiating the chloride transport with the NaOH pulse.[34] In order to have comparable results, the relative transport activity was quantified as the initial transport rate of chloride efflux ($\Delta F/\Delta t$, $\%\,s^{-1}$). This value results from the linear fitting at the initial transport stage (0–10 s) of the fluorescence time–course curves representing the chloride efflux (Figures S11 and S12).

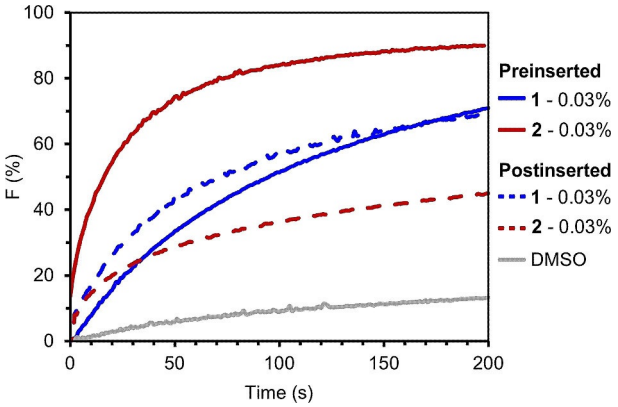

Figure 3. Fluorescence time-course curves of HTPS chloride transport experiments using post- and pre-inserted LUVs with carriers **1** and **2** at 0.03% carrier/EYPC molar ratio. Fluorescence emission at 510 nm (λ_{ex} = 450 nm) was monitored during 200 s after the addition of the base pulse. All fluorescence curves are normalized considering the starting point as 0% *F*. The 100% *F* value corresponds to the emission value after addition of Gramicidin D (out of the transport window). Concentration of receptors are expressed in % carrier/EYPC molar ratio. To facilitate data comparison, time values were also normalized: initial point of base pulse addition (t = 50 s normalized to t = 0 s) and end-point of the experiment (just before addition of Gramicidin D, t = 250 s normalized to t = 200 s).

At 0.03% molar ratio of transporter, the transport curves show similar initial transport rates for carrier **1** used in both insertion methods ($\Delta F/\Delta t$ = 1.06% s^{-1} and 0.90% s^{-1} for post- and pre-insertion of the transporter, respectively, Table 2). Moreover, identical fluorescence values, *F* at 200 s, are obtained at the end-points (≈70%). These observations indicate that the transport activity of **1** assessed using HTPS assays is not influenced by the method employed for its insertion.

In striking contrast, we observed a significant difference in the initial transport rates of carrier **2** depending on the method used for its insertion in the LUVs membranes. Indeed, the initial transport rate calculated for the chloride transport experiment using LUVs with pre-inserted carrier is three-fold larger than the one calculated using the post-insertion methodology (2.78% s^{-1} and 0.86% s^{-1} for pre- and post- inserted transporter, respectively, Table 2).

Table 2. Initial rates and fluorescence % at 200 s derived from the chloride transport data of carriers **1** and **2** at 0.03% mr using post- and pre-insertion methods in 30 μM EYPC LUVs.

Carrier	Insertion method	$\Delta F/\Delta t$ [% s^{-1}][a]	F_{200} [%][b]
1	Post-insertion[c]	1.06	70
	Pre-insertion[d]	0.90	71
2	Post-insertion[c]	0.86	45
	Pre-insertion[d]	2.78	90

[a] Initial rates calculated from the transport fluorescence curves from 0 to 10 s (Figures S11 and S12). [b] Fluorescence values at 200 s (Figure S11). [c] The carrier was added as DMSO stock solution. [d] 1% of DMSO was added for a better comparison between the two insertion methods.

Table 3. Initial rates and fluorescence % at 200 s derived from the chloride transport data of carriers **1** and **2** using the pre-insertion method of the transporter in 30 μM EYPC LUVs.

Carrier	[carrier]/[EYPC]	$\Delta F/\Delta t$ [% s^{-1}][a]	F_{200} [%][b]
1	0.01%	0.52	47
	0.03%	0.85	69
2	0.01%	1.76	79
	0.03%	3.61	92

[a] Initial rates calculated from the transport fluorescence curves from 0 to 10 s (Figures S9 and S10). Most likely, the slight differences observed in initial rates at 0.03% mr between Table 2 and 3 are due to the addition of 1% of DMSO in the experimental conditions used in the transport assays reported in Table 2. [b] Fluorescence values at 200 s (Figure S9).

In addition, the end-point fluorescence value, *F* at 200 s, is two-fold larger in the case of the LUVs with pre-inserted carrier (F_{200} = 90% and 45%, for pre- and post-inserted transporter, respectively, Table 2).

We performed control experiments using analogous experimental conditions (0.03% **2**/EYPC molar ratio, 30 μM EYPC) for the mixing of the LUVs components but changing the hydration medium (HEPES 10 mM, pH 7.0 and 100 mM Na_2SO_4) for liposomes' formation. In doing so, the extra- and intra-vesicular media of the self-assembled LUVs contain SO_4^{2-} anions instead of chloride. These LUVs are suitable to assess the transport activity of the synthetic carriers towards SO_4^{2-}. Sulphate is typically poorly transported through the lipophilic membrane of LUVs using synthetic carriers owing to its higher hydrophilicity compared to chloride (i.e. double negative charge).[35]

Using the above prepared LUVs, we did not observe significant changes of fluorescence following the NaOH pulse (i.e. no significant SO_4^{2-} transport) (Figure S13). This result supported that the pre-insertion of carrier **2** in the LUVs do not disrupt their membranes allowing a non-specific transport of ions.

On the other hand, we tested the transport activity of carrier **2** towards other anions such as NO_3^- and Br^- (Figure S14). Taking into account the passive diffusion of each studied anion, we conclude that the transport efficiency of the pre-inserted carrier **2** is slightly reduced for NO_3^- and Br^- with respect to chloride.

We also compared the fluorescence time-course curves of pre-inserted LUVs with carriers **1** and **2** at a lower molar ratio concentration (0.01% carrier/EYPC molar ratio, Figures S9 and S10).

The fluorescence-time curves evidenced a higher transport activity for the "four-wall" calix[4]pyrrole compared to the "two-wall" analogue also under these diluted conditions. The initial transport rate ($\Delta F/\Delta t$) and the end-point fluorescence value at 200 s (F_{200}) at 0.01% mr are larger for the "four-wall" carrier even when compared with the ones derived for the "two-wall" carrier at 0.03% mr (Table 3).

These findings contrast with our observations derived from the post-insertion HPTS assay, which assigned superior transport activities to the "two-wall" carrier **1**.

Most likely, the superior chloride transport activity displayed by the "four-wall" calix[4]pyrrole carrier **2** in the pre-insertion methodology of the HTPS assay, which overcomes insertion related problems, is produced by the presence of two extra aromatic walls in comparison to the "two-wall" carrier **1**. The cone conformation adopted by **2** upon chloride binding defines a deep aromatic polar cavity with only one open end (Figure 1b). The binding-site of **2** improves the isolation of the chloride anion during transport across the lipophilic membrane of the liposome. This hypothesis is also supported by the fact that *meso*-octamethylcalix[4]pyrrole shows a reduced chloride transport efficiency compared to the aryl-extended carriers **1** and **2**.[23] We claim that the pre-insertion method of the HTPS assay allows a suitable delivery of carrier **2** into the liposomal membrane making the comparison of the transport activity with the "two-wall" carrier **1** more reliable and accurate.

Finally, we tested the chloride transport efficiency of carriers **1** and **2** using an assay based on lucigenin, a halide sensitive fluorescent probe.[36] The addition of a pulse of concentrated sodium chloride (final concentration of 25 mM) to a 200 nm mean diameter LUVs suspension with carrier **2** pre-inserted in their membranes provoked the incremental quenching of the lucigenin fluorescence along time. We observed the increase of the chloride transport mediation in response to higher carrier's loadings (control, 0.03% and 0.1% **2**/POPC molar ratio, Figure S15). Nevertheless, it is worth noting that the transport activity shown by carrier **2** using the lucigenin assay is low when compared to that of other transporters evaluated using this strategy.[14]

We also investigated the transport efficiency of the "two-wall" carrier **1** using the same assay. Surprisingly to us, the "two-wall" carrier **1** displayed similar transport efficiency than the "four-wall" counterpart **2** (0.1% carrier/POPC molar ratio, see Figure S16). This finding is in striking contrast with the results obtained using the pre-insertion method of the HPTS assay. A sensible explanation for this discrepancy might be related to the different experimental conditions of the two assays and the dissimilar transport mechanism of the carrier associated with them.[37]

Conclusion

We showed that the use of the standard HPTS assay, involving the post-incorporation of the carrier into the liposomes' mem-

brane as DMSO solution, assigned a superior chloride transport activity to the "two-wall" calix[4]pyrrole carrier **1** compared to the "four-wall" counterpart **2**. Remarkably, when the carriers were incorporated into the membranes during the liposomes' formation (pre-insertion methodology) the HTPS assay revealed that the chloride transport activity of the "four-wall" calix[4]-pyrrole **2** over-performed the "two-wall" carrier **1**. Most likely, the reduced chloride transport activity displayed by the "four-wall" calix[4]pyrrole carrier **2** in the post-insertion method of the HTPS assay was caused by its poor deliverability into the lipid bilayer when used as a DMSO solution. We concluded that the pre-insertion method of the HPTS assay allowed a more accurate and reliable comparison of the transport activities of these two carriers. The superior chloride transport activity observed for the "four-wall" carrier **2** in the pre-insertion HTPS assay was assigned to the presence of two additional aromatic walls in the carrier. The polar aromatic cavity of carrier **2** in cone conformation is open only at one end providing a better shielding of the chloride anion during its transport across the lipophilic membrane.

In contrast, when lucigenin assay was used, carriers **1** and **2** displayed similar transport efficiencies. This is probably due to the differences in the experimental conditions and the transport mechanism that are involved in the two transport assays.

To the best of our knowledge, this work reports for the first-time comparative studies of synthetic chloride transporters using post- and pre-incorporation methodologies of the HPTS assay. We are convinced that the described methodology could be useful in other comparative studies of anion transport activity using synthetic carriers. We highly recommend performing anion transport experiments with the pre-insertion method in order to assess the reliability of the standard post-insertion methodology of the HPTS assay, whenever possible.

Experimental Section

Vesicles' preparation. ***HPTS⊂EYPC-LUVs***: 25 mg of egg yolk L-α-phosphatidylcholine (EYPC) were dissolved in 1 mL of degassed chloroform. The solvent was removed under reduced pressure at room temperature with continuous rotation to form a thin lipid film. The resulting film was kept in high vacuum overnight. The lipid film was hydrated for 1 hour with 1 mL of a buffered HPTS solution (100 mM NaCl, 10 mM HEPES, 1 mM HPTS, pH 7.0) under continuous stirring. The lipids' suspension was then subjected to eight freeze-thaw cycles and extruded twenty-one times using a polycarbonate membrane of 100 nm pore diameter. Finally, the extravesicular HPTS dye was removed by Sephadex-G50 size exclusion chromatography using buffer (100 mM NaCl, 10 mM HEPES, pH 7.0) as eluent. The lipid concentration of the final solution was estimated considering the initial lipid concentration and the final volume resulting from the column chromatography (≈3.7 mM). The resulting LUVs were characterized by DLS and TEM (Supporting Information).

HPTS⊂EYPC-LUVs-1 or 2 (pre-insertion of the carrier): 12.5 mg of egg yolk L-α-phosphatidylcholine (EYPC) were dissolved in 0.5 mL of degassed chloroform. Carrier **1** or **2** were then added as chloroform solutions (97 μL and 32 μL of a 50 μM solution for 0.03 and 0.01% carrier:EYPC molar ratio, respectively). The solvent was removed under reduced pressure at room temperature with continuous rotation to form a thin lipid film. The resulting film was kept in high vacuum overnight. The rest of the procedure was exactly the same described above for the HPTS⊂EYPC-LUVs. (Final estimated [EYPC]≈3.4–4.5 mM).

Transport experiments. ***Standard HPTS assay***: In a typical experiment, freshly prepared HPTS⊂EYPC-LUVs (16 μL or 64 μL for final lipid concentration ≈30 μM and ≈120 μM, respectively) were added to gently stirred buffer (2 mL; 100 mM NaCl, 10 mM HEPES, pH 7.0) in a polystyrene fluorescence cuvette. Fluorescence emission was monitored at $\lambda_{em}=510$ nm ($\lambda_{ex}=450$ nm) during the addition of first 0.5 M NaOH (20 μL, at 50 s), second the carrier (20 μL stock solution in DMSO, at 100 s) and finally excess of Gramicidin D (20 μL stock solution in DMSO, at 300 s). Continuous stirring and constant temperature was maintained (25.0±0.1 °C with a Peltier device) throughout the transport experiment. Analysis of the experimental data by the Hill equation is accurately described in the Supporting Information.

Transport evaluation with LUVs prepared using the pre-insertion method: In a typical transport experiment, freshly prepared HPTS⊂EYPC-LUVs-**1** or **2** (13–18 μL for final lipid concentration ≈30 μM) were added to gently stirred buffer (2 mL; 100 mM NaCl, 10 mM HEPES, pH 7.0) in a polystyrene fluorescence cuvette. Fluorescence emission was monitored at $\lambda_{em}=510$ nm ($\lambda_{ex}=450$ nm) during the addition of first 0.5 M NaOH (20 μL, at 50 s), and finally excess of Gramicidin D (20 μL stock solution in DMSO, at 250 s). Continuous stirring and constant temperature was maintained (25.0±0.1 °C with a Peltier device) throughout the transport experiment.

Accurate comparison of anion transport efficiency using post- and pre-insertion methods required slightly modified procedures.

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Conflict of interest

The authors declare no conflict of interest.

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[37] The experimental conditions used to evaluate the transport efficiency using the lucigenin assay are completely different than the used for the HPTS assay (membrane lipid composition, liposome's size, mechanism).

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