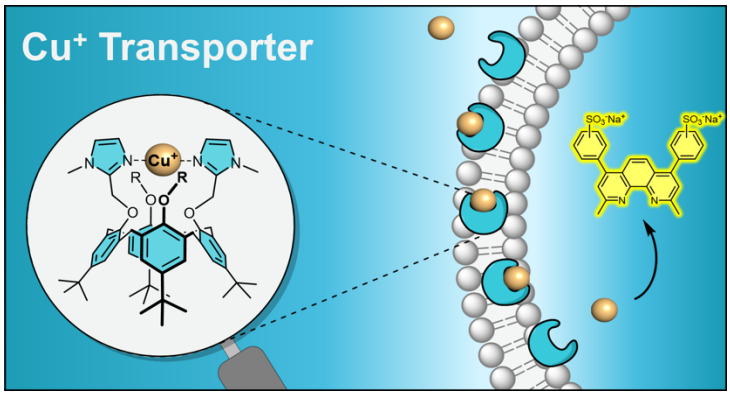
Transmembrane transport of copper(I) by imidazole-functionalised calix[4]arenes

Nathan Renier,[a] Olivia Reinaud,[b] Ivan Jabin,*[c] and Hennie Valkenier*[a]



Here we present the first synthetic transmembrane transporters for Cu^+. Calix[4]arenes with two imidazole groups have a linear coordination motif, which allows selective extraction of Cu^+ into chloroform. Transmembrane transport of Cu^+ into liposomes was investigated with a newly developed assay and the results open the way to biomedical applications of these Cu^+ ionophores.

Copper ions are involved in fundamental metabolic processes in living organisms, such as cellular respiration, and numerous enzymes use copper in their active site, taking advantage of its redox and catalytic properties.[1] Therefore, the intake of copper and its transport across cellular and organellar membranes are essential. Membrane proteins transport copper across lipid bilayers as Cu^+.[2] Dysfunctioning Cu^+ transport due to mutations in the genes encoding for such proteins leads to disruption of copper homeostasis and causes serious health problems. Examples are Menkes disease, characterised by neurological and muscular defects caused by copper deficiency, and Wilson's disease, characterised by the accumulation of copper in the liver due to inefficient secretion.[3]

A potential way to restore Cu^+ homeostasis could be by facilitating Cu^+ transmembrane transport with synthetic molecules acting as cationophores.[4,5] Transport of cations by small molecules has already been observed with naturally occurring cationophores, such as monensin and valinomycin. These cationophores bind cations, extracting them from the aqueous phase into the lipid bilayer, to then carry them across the membrane. Monensin is able to collapse Na^+ and H^+ gradients, leading to antibacterial effects and thus applications as antibiotic.[6] Similarly, valinomycin is able to complex and transport K^+ and also has antibacterial properties.[4]
These transport properties are not limited to naturally occurring molecules, as small synthetic molecules have been shown to transport cations, such as K^+, Na^+, Ag^+,[7] and also Zn^{2+} and Cu^{2+}.[8-10] While ionophores for many different cations have been described, ionophores able to transport Cu^+ across lipid bilayers have, to the best of our knowledge, not been reported.[11]

Reinaud and co-workers developed calixarene **1**, which is functionalised with two imidazole groups on its narrow rim, to act as an efficient Cu^+ receptor in organic solvents (Fig. 1a).[12] The X-ray crystal structure showed that the imidazole nitrogen atoms bind to Cu^+ in a linear geometry (N-Cu-N),[12] analogous to the structure observed in proteins that have two histidines coordinating to Cu^+.[13-15] A water soluble version of **1**, was shown to selectively complex Cu^+ in water in the presence of competing Na^+, K^+, Mg^{2+}, Ca^{2+}, Zn^{2+} and Cu^{2+} ions.[16] Here we describe a series of calix[4]arenes with two imidazole groups and their ability to bind Cu^+ and transport this cation across membranes.

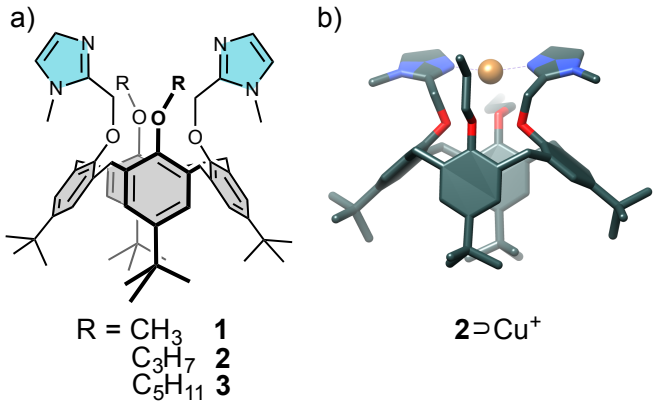


Fig. 1 a) Structure of the Cu^+ ionophores **1**, **2** and **3**. b) Molecular model of complex **2**⊃Cu^+ with Cu^+ bound by the imidazole groups.[17]

The binding abilities of ligand **1** and its sufficient lipophilicity (clogP 15)[18] make it a good candidate for the transmembrane transport of Cu^+. However, the methoxy-through-the-annulus rotation of the anisole units of **1** confers a high flexibility to this ligand. We thus synthesised the more rigid propyl and pentyl analogues **2** and **3** (clogP 17 and 19, Fig. 1a). While the ^1H NMR signals of the free receptor **1** in $CDCl_3$ were very broad, the signals of **2** and **3** are much sharper (see ESI), which is a consequence of locking of the macrocycle into a cone conformation, as any group larger than ethyl prevents the transannular rotation of the phenyl units.[19] The molecular model of complex **2**⊃Cu^+ (Fig. 1b) suggested that the binding of Cu^+ by the imidazole units was not affected by the replacement of methoxy by propoxy groups, as this model of **2**⊃Cu^+ strongly resembles the X-ray crystal structure of **1**⊃Cu^+.

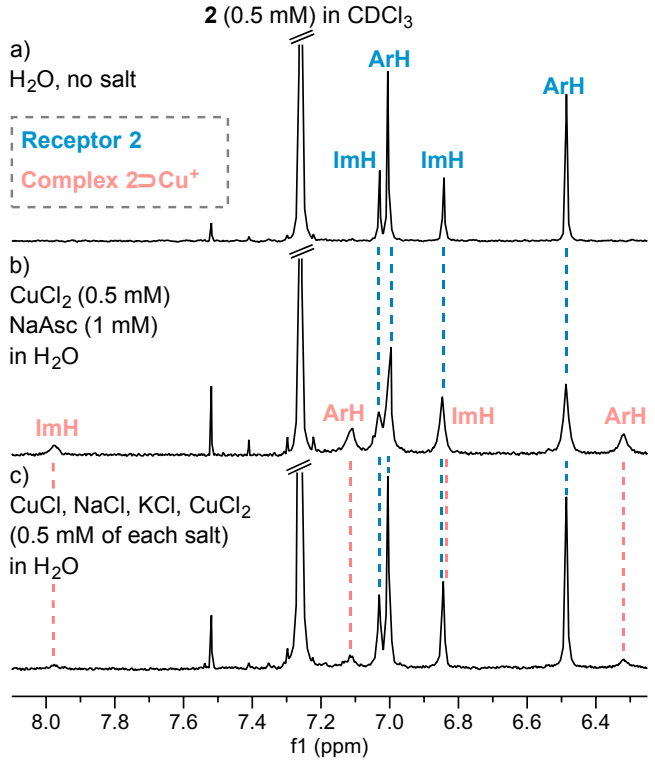


Fig. 2 Results from the extraction studies. Partial ^1H NMR spectra (298 K, 400 MHz) of **2** in $CDCl_3$ after addition of a) H_2O; b) $CuCl_2$ with sodium ascorbate in H_2O; c) NaCl, KCl, CuCl, and $CuCl_2$ in H_2O. "ImH" refers to the imidazole groups and "ArH" to the phenyl groups.

The binding of cations by calixarene **2** was investigated with extraction studies from aqueous solutions into chloroform (Fig. 2), as a model for the first step of transmembrane transport by cationophores. Multiple aqueous salt and acid solutions (NaCl, KCl, CuCl, HCl, $CuCl_2$, and $CuCl_2$ with sodium ascorbate to generate Cu^+ in situ) were mixed with **2** in $CDCl_3$ and the resulting samples were analysed by ^1H NMR spectroscopy (Fig. S14). The ^1H NMR spectra showed a new set of signals only for the samples where Cu^+ was present (Fig. 2b *vs* 2a). The appearance of the same signals was observed when multiple salts were added simultaneously (Fig. 2c). These new signals were unambiguously assigned to the **2**⊃Cu^+ complex (Fig. S15). Binding studies with Cu^+ salt in organic solvents showed the formation of the complex in a pinched cone conformation due to the binding of Cu^+ by the imidazole groups (Fig. S17b), in agreement with Fig. 1b. In contrast, the binding of K^+ and Na^+ salts involves the oxygen atoms of the small ring, resulting in a straight cone conformation of the complexes (Fig. S17c,d).[19] The difference in the binding mode of Cu^+ compared to Na^+ and K^+ can explain why **2** is able to selectively extract Cu^+ from an aqueous phase into chloroform, retaining the selectivity that was observed for the water soluble version of **1** in an aqueous environment.[16]

The ability of calixarenes **1**-**3** to transport Cu^+ was studied by fluorescence spectroscopy in a newly developed assay, using bathocuproine disulphonate (BCS). BCS is a fluorescent water soluble phenantroline derivative that can be used as a fluorescent dye for the sensing of Cu^+ through the formation of a non-fluorescent 2:1 complex (Fig. 3a).[20] Calixarenes **1**-**3** were pre-incorporated into the membrane of POPC/cholesterol (7:3) large unilamellar vesicles (LUVs, diameter ~0.2 μm) at 1:500 receptor:lipids ratio (Fig. 3b). 0.5 mM BCS was encapsulated and the liposomes were suspended in sodium phosphate buffer (50 mM, pH 7). A Cu^+ solution prepared from $CuCl_2$ (0.5 mM) and sodium ascorbate (1 mM) was added to the liposomes to create a 0.03 mM Cu^+ gradient, while the fluorescence intensity of BCS at 393 nm (excitation at 278 nm) was monitored. After addition of the Cu^+, a decrease of the fluorescence intensity was observed for the samples containing calixarenes **1**-**3**, but not for the liposomes without receptor (Fig. 4a), which implies transport of Cu^+ into the liposomes.

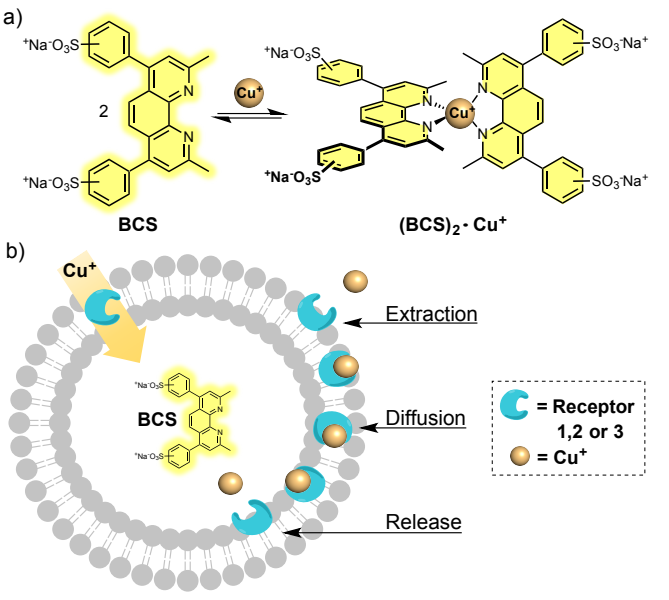


Fig. 3 a) Formation of the non-fluorescent 2:1 BCS•Cu^+ complex.[20] b) The transmembrane transport assay for Cu^+ into liposomes and proposed mechanism for the transport.

The decrease of fluorescence was faster for **2** and **3** than for **1**, indicating that **2** and **3** transport Cu^+ at higher rates than **1**. The transport rates were quantified by fitting the curves to single and double exponential decay functions to determine the half-

lives and the initial rates of transport (see ESI for details). The initial rate of Cu^+ transport was more than two times higher for **2** (0.0015 s^{-1}) and **3** (0.0018 s^{-1}) than for **1** (0.006 s^{-1}) and the half-life was almost two times lower for **2** (70 s) and **3** (75 s) than for **1** (120 s) (Fig. S24). These higher transport rates by **2** and **3** compared to **1** can be attributed to the locked cone conformation of those compounds, while the similarity in rates between **2** and **3** indicates that the increased lipophilicity has a negligible effect on the rates of transport by these calixarenes.

A fragment of **2** with a single imidazole group (**4**) and a calix[4]arene without imidazole groups (**5**)[21] (Chart 1) did not show any transport (Fig. 4a), showing that coordinating imidazole groups and their preorganisation on the calix[4]arene-based platform are necessary for Cu^+ transport activity.

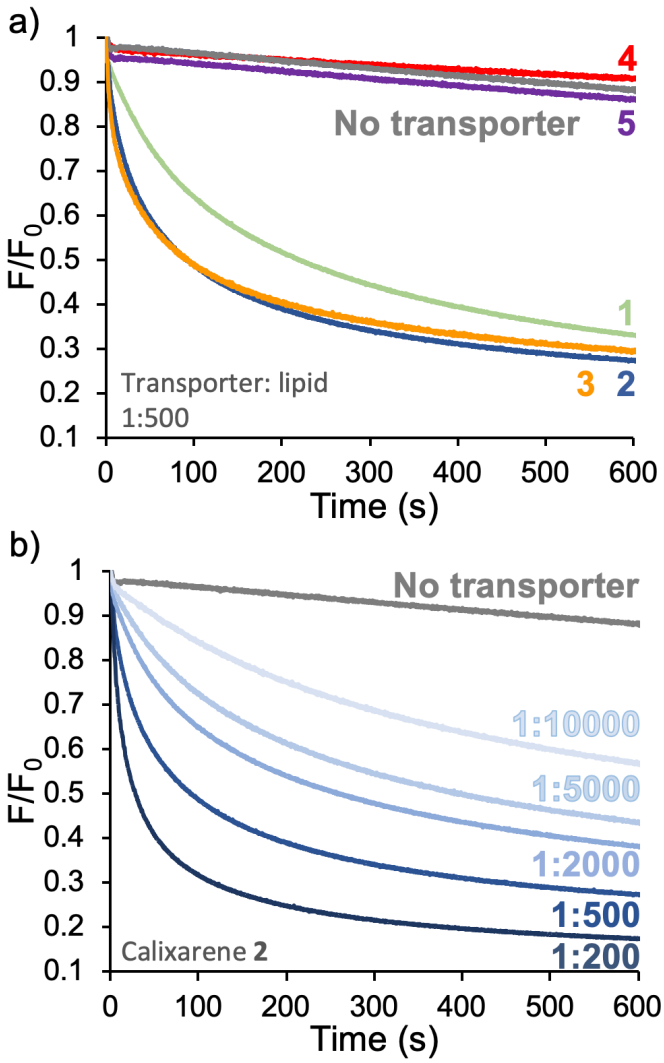


Fig. 4 Transport measurements of Cu^+ by following the fluorescence of BCS encapsulated inside LUVs in phosphate buffer (50 mM, pH 7) upon addition of $CuCl_2$ and sodium ascorbate to create a Cu^+ gradient. a) Comparison of Cu^+ receptors **1**-**3**, and control compounds **4** and **5**. b) Transport of Cu^+ by different concentrations of **2**.

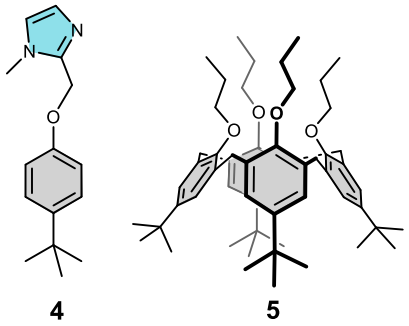


Chart 1. Control compounds **4** and **5**.

The Cu^+ transport process by calixarene **2** was investigated in more detail (see ESI for details). Firstly, the transporter:lipid ratio was varied (Fig. 4b) and the initial rates (I_0) when plotted against the transporter concentration showed a linear trend (R^2 = 0.99, Fig. S24). This implies that transporters function independently from each other and transport only one Cu^+ ion at a time.

Changing the counter anions of the copper salt (SO_4^{2-} was used instead Cl^-) did not have a significant impact on the rate of Cu^+ transport (see ESI, Section 4.6), implying that Cu^+ is not transported by a symport mechanism. Changing the counter cations of the phosphate buffer (K^+ instead of Na^+) did not give any significant changes either. The ability of calixarene **2** to transport H^+, Na^+, and K^+ was further evaluated with HPTS, a pH sensitive dye (see ESI, Section 4.7). In presence of a pH gradient, **2** was unable to transport H^+, neither by uniport nor by antiport with Na^+ or K^+. Furthermore, **2** did not show any Na^+ or K^+ uniport activity in presence of the protonophore carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP). These results indicate that transport of Cu^+ could not occur by an antiport mechanism with H^+, Na^+ or K^+ and that calixarene **2** is a highly selective transporter for Cu^+.

Having ruled out symport and antiport mechanisms, the Cu^+ transport as observed by **2** in the BCS assay appears thus to occur by a uniport mechanism. This electrogenic transport process should result in the build-up of a potential gradient, which is however limited by the low Cu^+ concentration used (30 µM) and therefore not visible with the membrane potential probe Safranin O.

No transport of Cu^+ by **2** was observed in DPPC liposomes at 25°C (gel phase), while transport was retrieved at 45°C (fluid phase, Fig. S26), in agreement with a mobile carrier mechanism (Fig. 3b). To further demonstrate the ability of **2** to function as a carrier for Cu^+, U-tube experiments were conducted. An aqueous phase was placed at either end of a $CHCl_3$ solution containing **2** (1 mM). One of the aqueous phases contained $CuCl_2$ and sodium ascorbate (0.5 mM and 1 mM, donor phase), the other was water without any salt (receiving phase). After 7 days, Cu^+ was detected in the receiving phase as a decrease of the fluorescence of added BCS (Fig. S32). The amount of Cu^+ transported was estimated to be 22 µM, which represents about 4% of the Cu^+ concentration initially present in the donor phase, in agreement with U-tube results reported in the literature.[22,23] These U-tube experiments corroborate that Cu^+

can be transported with imidazole-functionalised calixarenes via a carrier mechanism.

We have demonstrated that a calixarene with two preorganised imidazole groups is capable of selectively extracting Cu^+ from an aqueous into an organic phase. The bidentate linear coordination of Cu^+ is at the basis of the observed selectivity for Cu^+ over other cations, including Cu^{2+} and Na^+. These calixarenes do not only bind Cu^+, but are also able to transport Cu^+ across lipid bilayers, as shown using a newly developed assay with a Cu^+ sensitive fluorescent dye encapsulated in liposomes. Additional transport studies showed that calixarene **2** is highly selective for transport of Cu^+ compared to other monovalent cations (Na^+, K^+, H^+) and that transport of Cu^+ occurs via a uniport mechanism. This is the first time that transmembrane transport of Cu^+ by synthetic molecules was demonstrated and this study opens a way to further develop Cu^+ ionophores. These could find applications in the study of Cu^+ homeostasis and in the development of treatments for channelopathies linked to deficient transmembrane transport of Cu^+, such as Menkes and Wilson's diseases. Current treatments for those diseases focus on the symptoms,[24] while Cu^+ cationophores could target their cause.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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