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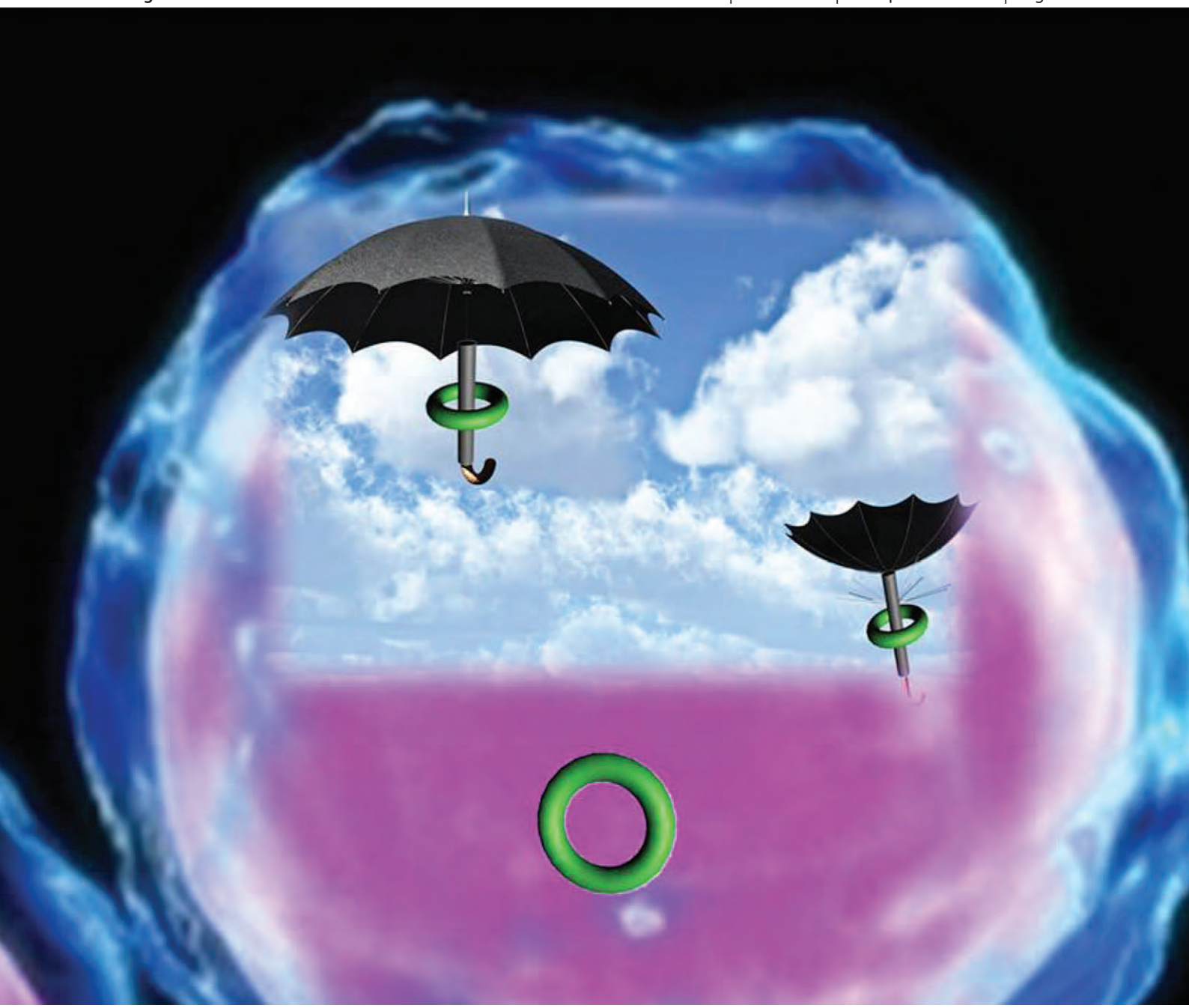

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Transport of macrocyclic compounds across phospholipid bilayers by umbrella-rotaxanes†

Christine Chhun, Josée Richard-Daniel, Julie Kempf and Andreea R. Schmitzer*

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We report the synthesis and assembly of umbrella-rotaxanes with transmembrane transport properties. We describe their amphomorphism and validate their ability to penetrate and cross phospholipid bilayers. Furthermore we present the strategy to release the macrocyclic compound by enzymatic cleavage inside egg yolk phosphatidylcholine (EYPC) liposomes.

Introduction

Many efforts have been made to deliver biologically active molecules inside cells. The size and lack of liposolubility of many bioactive hydrophilic drugs require synthetic transporters because of their poor cell membrane permeability. The main role of these synthetic transporters is to shield the polarity of the drug and ease its insertion inside the phospholipid bilayer. Different strategies have been developed, such as encapsulation by cyclodextrin derivatives[1] or dendrimers,[2] formation of nanotubes,[3] cell penetrating peptides,[4] *etc.*[5] The molecular umbrellas developed by Regen *et al.* were extensively studied for the transport of hydrophilic molecules,[6] where the facial amphiphilic bile acids are the key feature for their transport properties. By changing their conformation depending on the polarity of the media, these molecular umbrellas were able to minimize disfavored interactions between the hydrophilic agent and the hydrophobic interior of the lipid bilayer. The covalent attachment of molecular umbrellas to hydrophilic compounds was successfully used for the transmembrane transport of oligopeptides (glutathione,[7] DADLE[8]), ATP and AMP[9] inside 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) vesicles, where the hydrophilic compounds were released by an exchange reaction with intravesicular glutathione.

Only a few examples of pseudorotaxanes[10] or rotaxanes used as transmembrane transporters have been reported,[11] all involving covalent attachment of the biologically active molecule to one of the pseudo- or rotaxane components. We recently designed and reported an umbrella thread presenting chloride transmembrane transport properties and membrane interactions.[12] The umbrella thread possessing also the capacity to complex crown ether-like macrocycles on the ammonium recognition site, was used in this previous study to assemble umbrella-pseudorotaxanes. We report here, the synthesis and assembly of umbrella-rotaxanes, as well as evidence of their amphomorphism when used to transport a fluorescent macrocycle inside liposomes. The umbrella-rotaxane concept and design offer the strength of both molecular umbrellas and interlocked molecules. While the membrane insertion is insured by the umbrella moiety, the ammonium recognition site allows complexation of a hydrophilic cyclic wheel without any derivation and assembly of a rotaxane. The umbrella-rotaxane can be used as a new vehicle for drug transmembrane transport and the macrocycle can be released after internalization into the cell.

Results and discussion

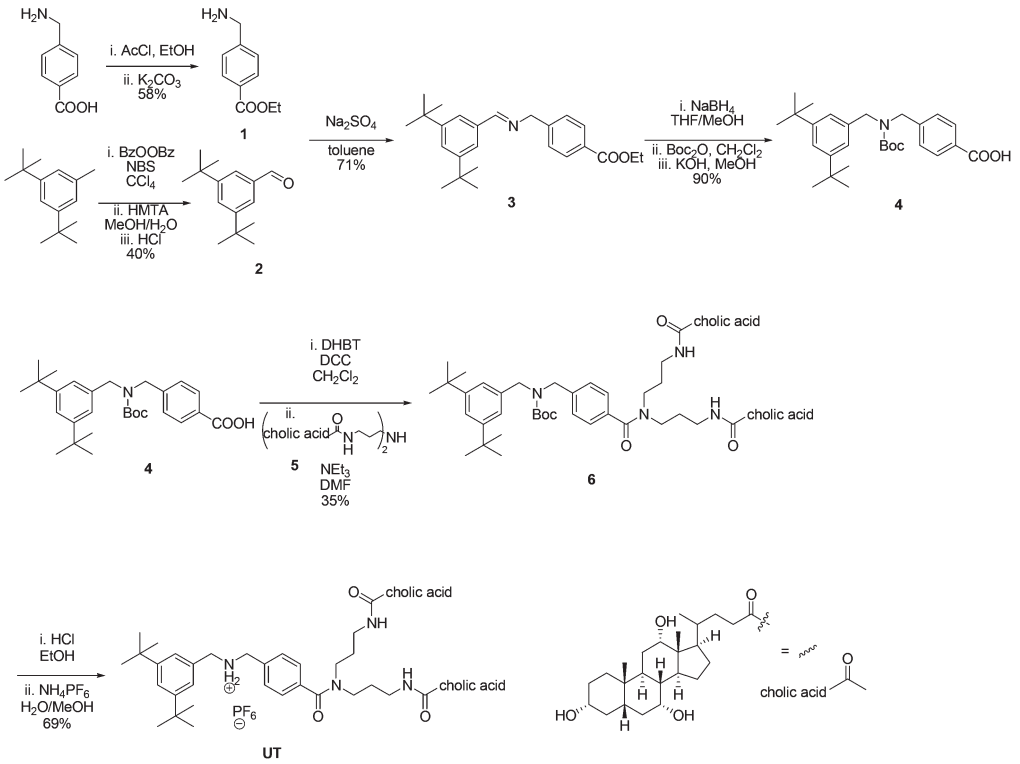
Synthesis

An analogue of the reported umbrella thread was synthesized.[12] One of the stoppers was directly integrated into the thread to assemble the rotaxane. The umbrella thread **UT** was obtained in ten steps with 6% overall yield. The thread moiety was first prepared by condensation of di-*tert*-butylbenzaldehyde **2** with the ethyl 4-aminomethylbenzoate **1**. After reduction of the imine bond by sodium borohydride, the resulting secondary amine was protected with a Boc group. The ester was hydrolyzed in basic conditions to prepare the coupling reaction with the umbrella **5**, which was previously reported.[12] The Boc-protected umbrella thread **6** was deprotected and the counter-anion exchanged by metathesis into hexafluorophosphate resulted in the umbrella thread **UT** (Scheme 1).

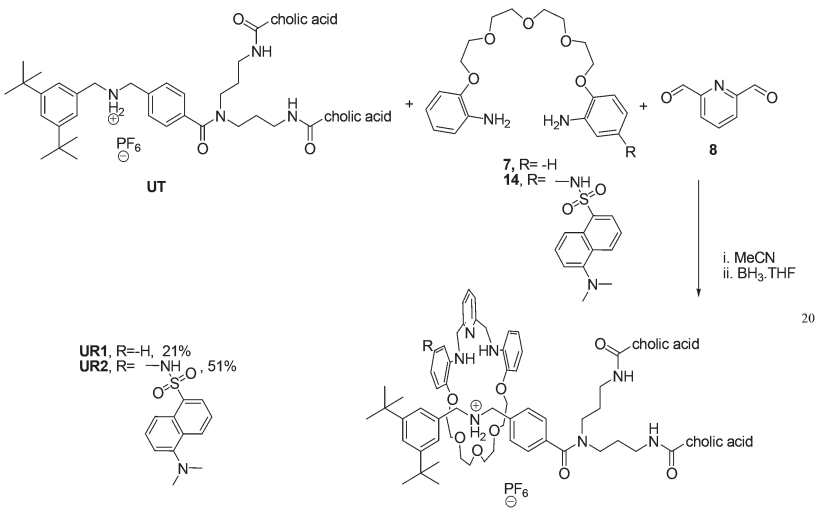
The umbrella-rotaxanes **UR1** was assembled by condensation of 2,6-pyridinedicarboxaldehyde and tetraethyleneglycol bis(2-aminophenyl)ether[12] to form a crown-ether-like

Department of Chemistry-Université de Montréal, 2900 Edouard Montpetit, CP 6128 Succursalle Centre ville, Montréal, QC, Canada H3C3J7.
E-mail: ar.schmitzer@umontreal.ca; Fax: (+1) 514 343 7586

† Electronic supplementary information (ESI) available: Synthesis and characterization of the compounds, preparation of liposomes, enzymatic cleavage. See DOI: 10.1039/c3ob41209a



Scheme 1 Multistep synthesis of the umbrella thread **UT**.



Scheme 2 Assembly of umbrella rotaxanes **UR1** and **UR2** using the clipping approach.

macrocycle.[13] The fluorescent derivative **UR2** was likewise prepared using a novel crown-ether-like clip tagged by a dansyl group **14** (Scheme 2), designed to follow the change of conformation of the umbrella depending on the polarity of the media.

Amphomorphism

The variation of the dansyl group fluorescence depending on the environment polarity[14,15] is a good probe to monitor the umbrella folding. A more polar environment led to the stabilization of the excited state of the dansyl group and in the case of the unthreaded fluorescent macrocycle, where a maximum emission was obtained in water (see ESI†).[16] In the first series of experiments, fluorescence variation of **UR2** was monitored in mixtures of solvents, covering a large range of solvent polarity (Fig. 2). In MeOH–CCl_4 solutions (Fig. 2a), the emission maximum λ_{max} shifts to longer wavelength, from 425 nm to 530 nm, when increasing the methanol ratio. This red shift

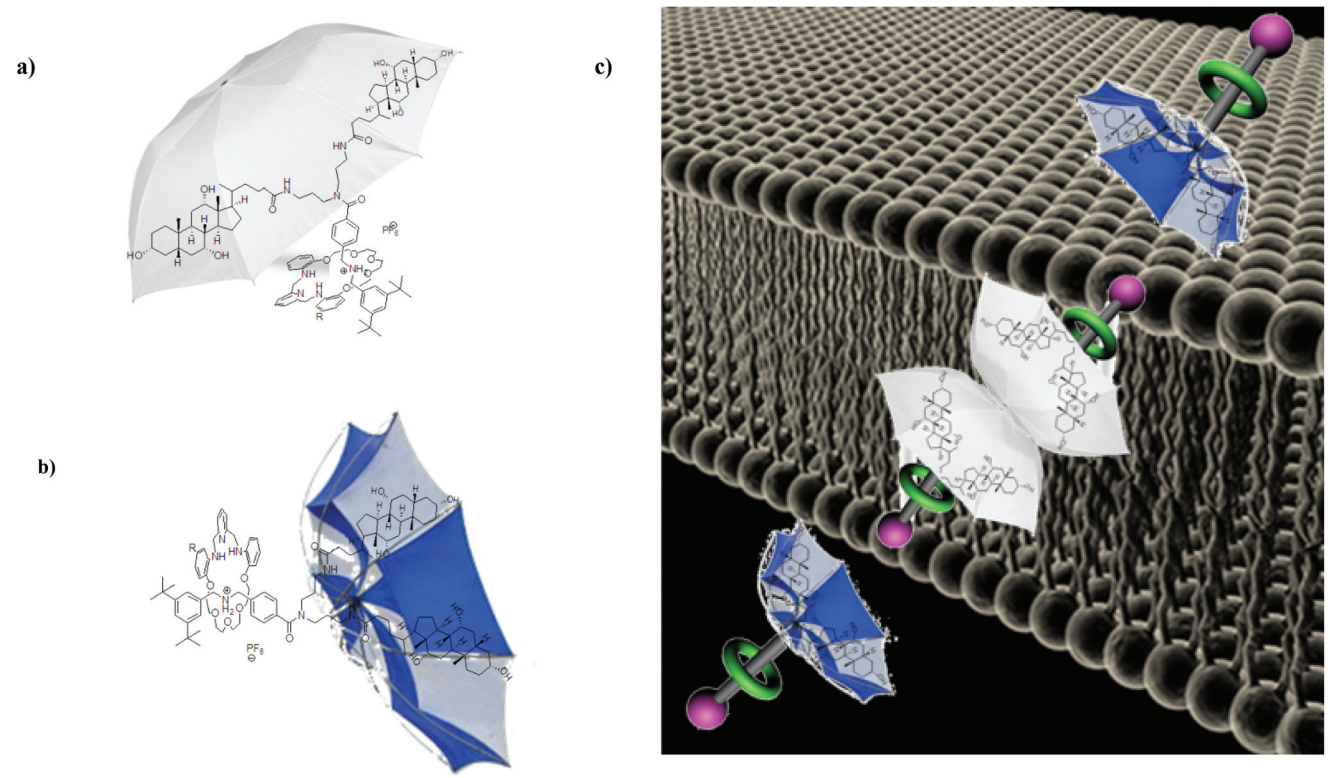


Fig. 1 Proposed different conformations of the umbrella rotaxane: (a) shielded; (b) exposed; (c) schematic transmembrane transport process of the umbrella-rotaxane.

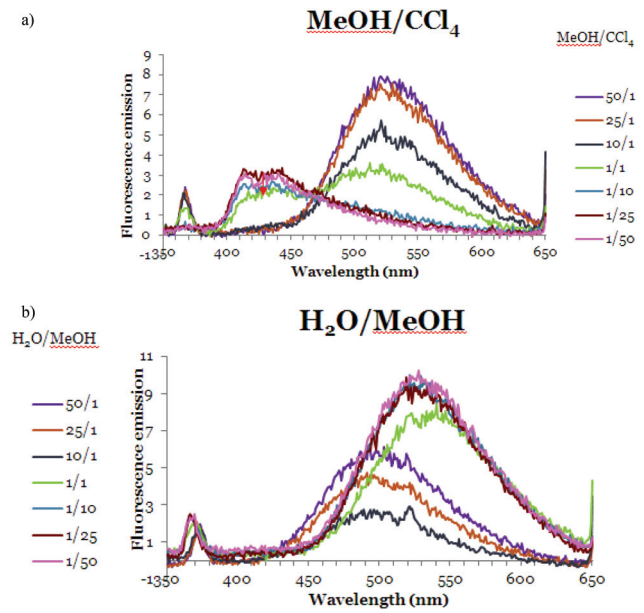


Fig. 2 Fluorescence emission spectra of **UR2** at 2 μM in different (a) MeOH–CCl_4 and (b) MeOH–H_2O solutions. Excitation at 330 nm.

is coherent with the variation of solvent polarity and its effect on the fluorescence emission. The umbrella may adopt an exposed conformation in relatively polar media (see Fig. 1b), with the dansyl group exposed to the solvent. The same procedure was followed in MeOH–H_2O solutions, by progressively increasing the water content. In mixtures with high MeOH content, the fluorescence curves remained similar. When 50% of H_2O in MeOH was reached, another red shift was observed (from 530 nm to 540 nm). Again, the phenomenon was expected with the increase of polarity of the media. However, a blue shift took place when the water ratio continued to increase, leading to a λ_{max} at 500 nm. This blue shift suggests a more hydrophobic environment around the fluorophore, which can only be explained by a conformation change, towards a shielded conformation of the umbrella (see Fig. 1a), where the two hydrophobic faces of the cholic acids face the macrocycle. Thus, the umbrella rotaxane shows a shielded conformation in more hydrophobic media and an exposed conformation in more hydrophilic media. The umbrella rotaxane showed its ability to change its conformation depending on the polarity of the media (similar to an umbrella motion). This property is a *sine qua non* condition for the transmembrane transport of polar drugs.

Interaction with the phospholipid bilayers and chloride transport properties

To gain insights into the behavior of our umbrella-rotaxanes in the presence of lipid bilayers, **UR2** was injected into EYPC liposomes and its fluorescence emission monitored (Fig. 3). In this case, a λ_{max} shift from 500 nm to 510 nm accompanies the increase of fluorescence intensity, showing the interaction of **UR2** with the phospholipid bilayer. However, no direct

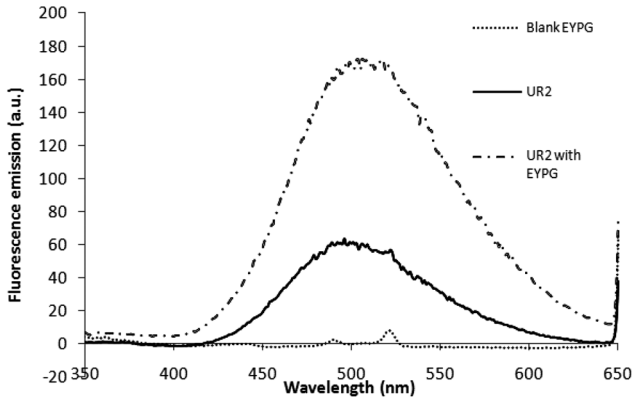


Fig. 3 Fluorescence emission spectra of **UR2** in phosphate buffer (pH = 7.8) at 20 μM in presence and absence of EYPC liposomes.

evidence of conformation changes was obtained in this study, whereas the inclusion into the membrane is supported by the ability of our umbrella-rotaxanes to transport Cl^-.[12] We compared the ion transport properties of **UR1** and **UR2** in EYPC liposomes using the lucigenin assay, a standard protocol in the evaluation of membrane active transporters.[12,22] Chloride efflux out of the vesicles was measured by following the fluorescence changes in emission when an aliquot of **UR1** and **UR2** was added to lucigenin-containing vesicles, as a function of time and rotaxane content. By using similar procedures to those we previously described,[17] the mole percentages of **UR1** and **UR2** were varied from 0.1 μM to 0.1 mM. The effectiveness of **UR1** and **UR2** was characterized in the chloride transport process across the bilayer by their EC_{50} values, using dose–response analysis (see ESI†) based on data shown in Fig. 4 and 5. **UR1** and **UR2** present comparable EC_{50} values (Table 1) suggesting similar transport mechanism and kinetic parameters. The biphasic nature of the kinetic profile is probably the consequence of the use of substoichiometric amounts of **UR1** and **UR2**, which are expected to be distributed according binomial statistics in the liposomes. The fast phase can be attributed to those liposomes that contain one or more

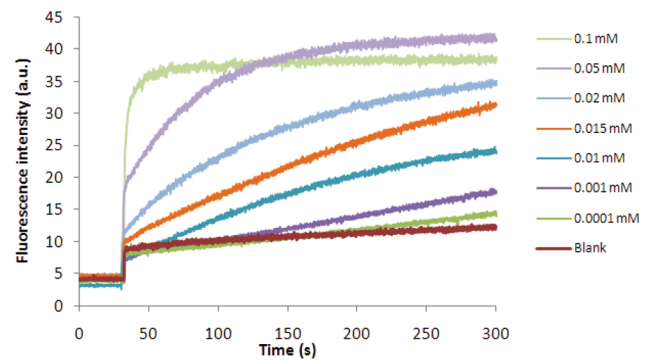


Fig. 4 Relative activity of **UR1** in the lucigenin-based Cl^- transport assay. Intravesicular conditions: 100 mM NaCl, 10 mM phosphate buffer, 2 mM lucigenin; extravesicular conditions: 100 mM $NaNO_3$, 10 mM phosphate buffer (pH 6.4). 50 μl of methanol (blank) or of **UR1** at different concentrations were injected at t = 30 s.

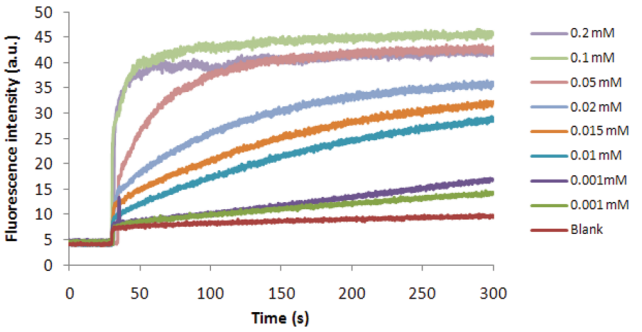


Fig. 5 Relative activity of **UR2** in the lucigenin-based Cl^- transport assay. Intravesicular conditions: 100 mM NaCl, 10 mM phosphate buffer, 2 mM lucigenin; extravesicular conditions: 100 mM $NaNO_3$, 10 mM phosphate buffer (pH 6.4). 50 μl of methanol (blank) or **UR2** solutions at different concentrations were injected at t = 30 s.

Table 1 Determination of EC_{50}

	EC_{50} [a] (M)	EC_{50} mol% (transporter/lipid)
UR1	1.56×10^{-5}	1.56
UR2	1.67×10^{-5}	1.67

[a] The concentration of rotaxane needed to achieve a 50% release of encapsulated chloride.

rotaxane molecules and the slow phase to a redistribution of these rotaxanes among the liposomes.

α-Chymotrypsin cleavage *in vitro*

In order to develop an enzymatic method to release the macrocyclic wheel, the enzymatic cleavage of **UT** was tested in the presence of wide-spectrum enzymes able to hydrolyze amide bonds (α-chymotrypsin and trypsin). α-Chymotrypsin proved to be more efficient in the cleavage of the umbrella-thread **UT**.[23] The optimal conditions for enzymatic digestion of **UT** were first determined by UV-Vis spectroscopy (Fig. 6) and the

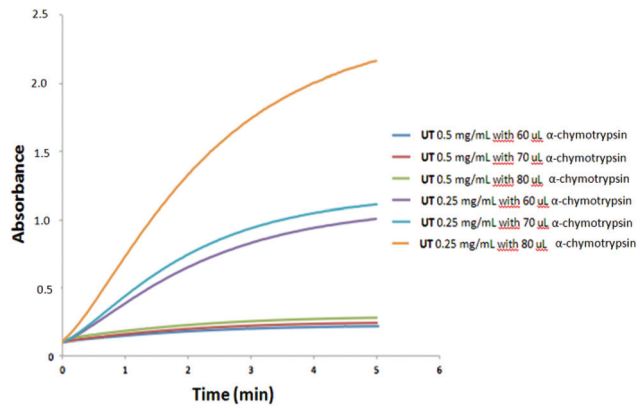


Fig. 6 Enzymatic activity of α-chymotrypsin in the presence of **UR2**. 0.25 or 0.5 mg mL^{-1} solutions of **UR2** in a 1 : 1 MeOH–Tris-HCl buffer 80 mM (pH 7.6), 80 μl of $CaCl_2$ 2 M and different volumes of α-chymotrypsin (60 units per mL) at 37 °C. Absorbance variation was followed at 280 nm.

Table 2 Determination of the α-chymotrypsin cleavage sites in UT by MS

	$[M]^+_{calc.}$	$[M]^+_{found}$	Present before digestion	Present after digestion
UT	1246,9285	1247,929 ($M + NH_4^+$)	Yes	Yes
Cholic acid	408,2876	426,3208 ($M + NH_4^+$)	No	Yes
5	911,6963	912,7051 ($M + H^+$)	No	Yes
N-(3-Amino propyl)-1,3 propane diamine	131,1422	149.1754 ($M + NH_4^+$)	No	Yes

cleavage sites were determined by MS spectrometry (Table 2). As shown in Fig. 6, the enzymatic digestion is a fast process and would not be the rate-limiting step for a delivery strategy where the release of the macrocycle is assured by the enzymatic cleavage of the thread. From the fragments detected by MS, we were able to show that all the peptide bonds in **UT** structure are cleavage sites for α-chymotrypsin.

Translocation into liposomes and *in situ* release of the macrocycle

The transmembrane translocation of **UR2**, followed by the *in situ* release of the macrocycle was studied in intravesicular α-chymotrypsin/lucigenin containing EYPC liposomes. The lucigenin incorporation in the liposomes permitted monitoring of the liposomes during the extrusion steps, their stability during injection of the transporter and during the incubation time. After different times of incubation after the injection of the transporter, the liposomes were removed from solution by low speed centrifugation, the α-chymotrypsin was inactivated, the liposomes were lysed and the amount free macrocycle was monitored by LC-MS. After 4 hours of incubation after the injection of the transporter, up to 45% of the free macrocycle was present in the liposomes, even if no intact **UR2** was detected in the liposomes. This suggests that at least 45% of **UR2** had been crossed the lipid bilayer and were digested by the enzyme in 4 h. For comparison glance, the same experiments were performed with the unthreaded macrocycle. As shown in Fig. 7, the macrocycle itself can penetrate and cross the bilayer, as we previously demonstrated.[12] However, the amount of unthreaded macrocycle inside liposomes never exceeded 10%. The ability of translocation of the macrocycle outside the liposomes once internally released, could explain the fact that only 45% of free macrocycle was found inside the liposomes. However, the slow efflux process results in sufficient time for accumulation of the free macrocycle in the liposomes, demonstrating the proof of concept of a drug delivery strategy. These results demonstrate the transport and release of the macrocycle inside a vesicle by using an umbrella-rotaxane. Nonactin[18] and valinomycin[19] are cyclic antibiotic ionophores specific to alkali cations[20,21] able to complex ammonium cations and could be used to assemble interlocked systems.[22] The assembly of rotaxane with these ionophores as wheel could be the solution to their cell transmembrane transport. Keeping in mind that these cyclic antibiotic ionophores are more hydrophilic than the model macrocycle we used in this study, the concept of umbrella thread is a promising vehicle for their transmembrane transport.

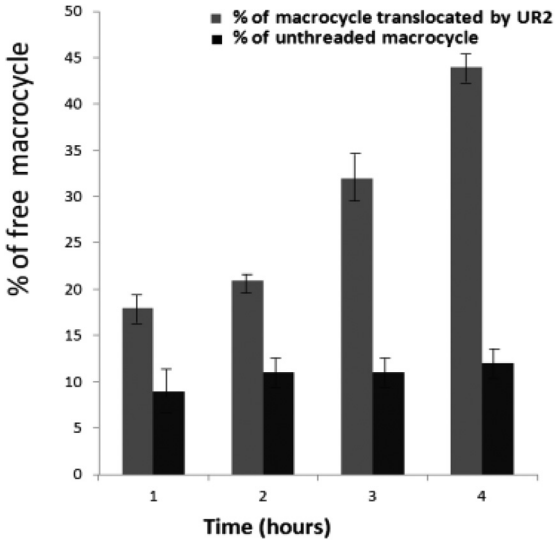


Fig. 7 Amount of free macrocycle present in the α-chymotrypsin-loaded liposomes after different incubation times of 20 μM solutions of **UR2** and unthreaded macrocycle.

Conclusions

We presented here the synthesis of a novel umbrella-thread that allows the assembly of an umbrella-rotaxane by clipping of a crown-ether-like wheel on the ammonium recognition site. The umbrella-rotaxane showed amphomorphic properties in solvents with different polarities supporting the hypothesis of umbrella motion required for the bilayer transport mechanism. Finally, we demonstrated transmembrane transport and release of the free macrocycle by digestion with intravesicular α-chymotrypsin. This study validates our concept of umbrella-rotaxane and opens the door to the development of umbrella-rotaxanes as a vehicle for macrocyclic biologically active molecules. Work is under progress in our group to assemble umbrella-rotaxanes with these biologically active macrocycles.

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