JOURNAL OF PHYSICAL ORGANIC CHEMISTRY
J. Phys. Org. Chem. 2004; **17:** 978–982
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/poc.818

Blocker efflux through blocked pores†

Dawn Ronan, Nathalie Sordé and Stefan Matile*

Department of Organic Chemistry, University of Geneva, Geneva, Switzerland

Received 4 August 2003; revised 12 January 2004; accepted 14 January 2004

ABSTRACT: The objective of this paper is to clarify a frequent confusion that occurs with synthetic multifunctional pores: does molecular recognition by a synthetic pore exclude molecular translocation through the same pore? Evidence that this is not the case is provided with a set of competitive experiments using a rigid-rod β-barrel with internal histidine–arginine dyads as synthetic multifunctional pores (SMPs) as well as 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) as a fluorescent and 1,3,6,8-pyrenetetrasulfonate (PTS) as a non-fluorescent blocker. Direct evidence for the efflux of HPTS blockers through blocked SMPs is obtained by quenching of released HPTS with an externally added quencher. Blockage of blocker efflux through blocked pores was demonstrated by adding PTS as the opposing external blocker ($K_D = 3.3$ μM). A Hill coefficient of $n = 1.5$ may indicate that binding of more than one PTS blocker is necessary to inhibit the efflux of HPTS blockers. Supported by structural information on blockage and selectivity from biological potassium channels, blocker efflux through blocked pores is discussed as being implicated in selectivity. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: bioorganic chemistry; β-barrels; enzyme mimics; ion channels; molecular recognition; pores; rigid-rod molecules; supramolecular chemistry

INTRODUCTION

Synthetic multifunctional pores have been introduced as attractive systems to 'do' chemistry in confined and oriented 'nanospace'.[1] Studies on synthetic multifunctional pores (SMPs) as supramolecular hosts,[1] as enzyme sensors,[2] and as catalysts[3] became possible with the discovery of synthetic routes to rigid-rod β-barrels,[4] which in turn became accessible with the introduction of rigid-rod molecules in bioorganic chemistry.[5] Synthetic rigid-rod β-barrel pores are constructed using preorganization by non-planar *p*-octiphenyl 'staves' and functionalization by β-sheet 'hoops' (Plate 1). In most rigid-rod β-barrel pores prepared so far, leucine residues are positioned at the outer barrel surface to interact with the hydrophobic core of lipid bilayers. Amino-acid residues at the inner barrel surface serve to interact with molecules passing through the pore, i.e. to create multifunctionality. Rigid-rod β-barrel pores with internal histidines, lysines, aspartates and arginines have been prepared with the general objective of coupling molecular recognition and catalysis with molecular translocation across bilayer membranes.[1]

The synthetic multifunctional pore **1** used in this study comprises internal histidine–arginine dyads (Plate 1). In bilayer membranes composed of egg yolk phosphatidylcholine (EYPC), pore **1** forms very stable, ohmic ion channels.[6] Their pH-dependent anion/cation selectivity is modulated by inorganic phosphates bound to the guanidinium cations of internal arginines.[6] Molecular recognition by pore **1** was exemplified by, *inter alia*, α-helix recognition in polarized membranes.[7] In planar bilayer conductance experiments, 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) was an excellent blocker of pore **1** ($K_D \simeq 30$ μM at $V = 50$ mV)[6] and of homologous rigid-rod β-barrel pores with modified inner[8–10] and outer[11] surface. Molecular transformation by pore **1** was exemplified by hydrolysis of 8-acetoxypyrene-1,3,6-trisulfonate into HPTS. This esterolysis occurred with ground-state stabilization $\Delta G_{GS} = -30\ \mathrm{kJ\ mol^{-1}}$, corresponding to $K_M = 6$ μM, and transition-state stabilization $\Delta G_{TS} = -52\ \mathrm{kJ\ mol^{-1}}$, and it could be accelerated by supportive membrane polarization.[3] Esterolysis with a similar rigid-rod β-barrel pore could be inhibited competitively by 1,3,6,8-pyrenetetrasulfonate (PTS, $K_I = 500$ nM).[9]

Overall, there is ample experimental evidence on hand that pyrene-1,3,6-trisulfonates are recognized and converted by rigid-rod β-barrel pores such as SMP **1**. Pyrene-1,3,6- trisulfonates have even been used as cofactors to

**Correspondence to:* S. Matile, Department of Organic Chemistry, University of Geneva, Quai Ernest-Ansermet 30, CH-1211 Geneva 4, Switzerland.
E-mail: stefan.matile@chiorg.unige.ch
†Paper presented at the 9th European Symposium on Organic Reactivity, 12–17 July 2003, Oslo, Norway.
Contract/grant sponsor: Swiss NSF; *Contract/grant number:* 2000-064818.01.
Contract/grant sponsor: National Research Program 'Supramolecular Functional Materials', Swiss NSF; *Contract/grant number:* 4047-057496.

drag otherwise inaccessible substrates into supramolecular catalysts such as **1**.[12] There is equally rich experimental evidence available that molecular recognition of guests such as HPTS by SMP hosts in e.g. pore **1** causes pore blockage. Coincidence of molecular recognition by synthetic multifunctional pores and blockage of the same pore is an attractive characteristic of SMPs because it makes supramolecular host–guest chemistry detectable either at the single-molecule level or with the 'naked eye'.[1] The question of whether or not these blockers can move through the pore they are blocking is important for conception, comprehension and appreciation of molecular recognition and transformation by synthetic multifunctional pores.

Although this question may cause confusion when new chemistry with new SMPs is presented, the underlying concepts of physical organic chemistry are general and understood. In brief, every molecule moving through a pore (or an ion channel) interacts to some extent with the inner surface of the pore. In the case of weak interactions (i.e. high K_D values), the rate constant of entrance or association (i.e. k_{on}) and the rate constant of exit or dissociation (i.e. k_{off}) are of similar magnitude. This consideration, although oversimplifying, is sufficient to accept efflux as being possible under these conditions. There is also no problem to predict that, depending on the magnitude of k_{on}^{IN}, k_{off}^{OUT}, concentration gradients, number of binding sites, lifetime of open pores, and so on, efflux with weak interactions is not necessarily fast.

In the case of strong interactions (i.e. low K_D values), the entrance rate constant exceeds that of exit clearly (i.e. $k_{on} \gg k_{off}$). This does, however, indicate that association is neither irreversible nor that dissociation must be unidirectional.

Moreover, $k_{on} \gg k_{off}$ does not imply that efflux, influenced by the magnitude of k_{off}^{OUT}, concentration gradients and other parameters, is necessarily slow in the presence of strong interactions. This situation applies for blockers that are small enough to move through the pore but bind strongly to the pore and hinder the efflux of other compounds while they are bound. This situation has also been identified in the x-ray structures of biological potassium channels, revealing multiple, consecutive sites that bind potassium cations selectively.[13–17] One to two permanently bound potassium cations block the efflux of other ions, i.e. assure selectivity. Passive efflux of these potassium 'blockers' is, however, not a problem as long as a driving force such as a concentration gradient is present. Multiple, consecutive binding sites are thought to increase both selectivity and efflux rate substantially.

Blocker efflux through blocked channels is, therefore, the mechanism of selectivity employed by biological potassium channels. Translated from biological ion channels to synthetic multifunctional pores, efflux of the larger, organic blockers through blocked SMPs would demonstrate applicability of the same mechanism of selectivity. In the context of this paper, experimental evidence for HPTS efflux through blocked SMP **1** would confirm the existence of synthetic multifunctional pores with selectivity for HPTS, i.e. 'HPTS pores'. The objective of this study was to evaluate whether or not this is the case.

RESULTS AND DISCUSSION

8-Hydroxypyrene-1,3,6-trisulfonate (HPTS) is a superb fluorophore, particularly for ratiometric pH sensing at excitation wavelengths of 405 and 450 nm with emission at 510 nm.[3,18] Detection of HPTS efflux from spherical bilayers (i.e. vesicles or liposomes) seemed, therefore, not to be a problem. As routinely done with 5(6)-carboxyfluorescein,[2,18] vesicles could be loaded with HPTS at concentrations high enough to assure self-quenching. Efflux through pores would then lead to HPTS dilution that could be detected by an increase in fluorescence intensity. However, we failed to set up such an HPTS efflux assay based on self-quenching. We were also unable to find a helpful report on HPTS self-quenching assays in the literature.[18]

Quenching of HPTS emission by addition of quenchers such as *p*-xylenebispyridinium (DPX) is, however, not problematic. Extravesicular HPTS quenching by DPX has, for example, been used to detect the endovesiculation of vesicles.[19] Detectability of HPTS efflux from vesicles by external addition of DPX was, therefore, conceivable (Plate 2). Consequently, large unilamellar vesicles composed of egg yolk phosphatidylcholine (EYPC LUVs) were loaded with HPTS (Plate 2A). When exciting at the isosbestic point of $\lambda = 415$ nm, changes in the emission of EYPC-LUVs $\supset$ HPTS were then continuously monitored during the following operations. Firstly, pore **1** was added (Plate 2A and Fig. 1(a)). Incubation for one minute was allowed for eventual HPTS efflux [Plate 2(B) and Fig. 1(b)]. Then, DPX was added to quench extravesicular HPTS (Plate 2(C) and Fig. 1(c)]. At the end of each experiment, EYPC-LUVs $\supset$ HPTS were lysed with excess Triton X-100 to secure a constant emission value for calibration [Fig. 1(d)].

Addition of pore **1** at nanomolar concentrations sufficient to observe high activity in other assays[7,11] did not cause a significant change in the emission of EYPC-LUVs $\supset$ HPTS [Fig. 1(a)]. Subsequent addition of DPX, however, caused strong and instantaneous quenching [Fig. 1(c) solid trace]. Reduction of the concentration of pore **1** reduced the extent of this instantaneous HPTS quenching gradually down to a residual 'burst' [Fig. 1(e)] originating e.g. from membrane defects induced by pore addition (analogous to Fig. 1).

Whereas dependence on pore concentration already supports blocker efflux through blocked pore **1** as the origin of HPTS quenching by external DPX, comparison

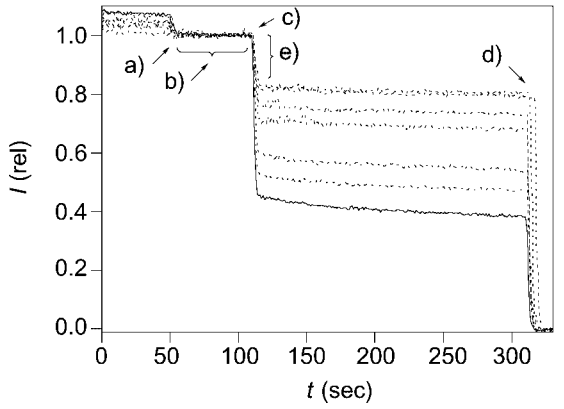

Figure 1. Fractional HPTS emission I (λ_{em} 510 nm, λ_{ex} 415 nm) as a function of time during (a) addition of pore **1** (150 nm), (b) incubation and addition of (c) DPX (7.5 mM) and (d) Triton X-100 to EYPC-LUVs ⊃ HPTS in presence of PTS [with increasing intensity after (c): 0 μM, 1 μM, 2.5 μM, 5 μM, 10 μM, 20 μM and 40 μM]. The traces were normalized to fractional emission $I = 1.0$ at (b) and $I = 0.0$ after lysis (d). (e) Quenching unrelated to efflux through pore **1**, see text

with other kinetic profiles was essential to secure the necessary corroborative evidence. Two sets of cross-comparisons confirmed validity of the direct experimental evidence that pore **1** blocked by HPTS can release HPTS in the presence of a concentration gradient (Fig. 2). DPX influx (rather than HPTS efflux) could be excluded as the origin of HPTS quenching by externally added DPX because, firstly, the instantaneous response to DPX addition to EYPC-LUVs ⊃ HPTS with pore **1** and to HPTS in buffer was identical (Fig. 2, solid versus dashed at $t = 60$ s) and, secondly, because right-angled profiles

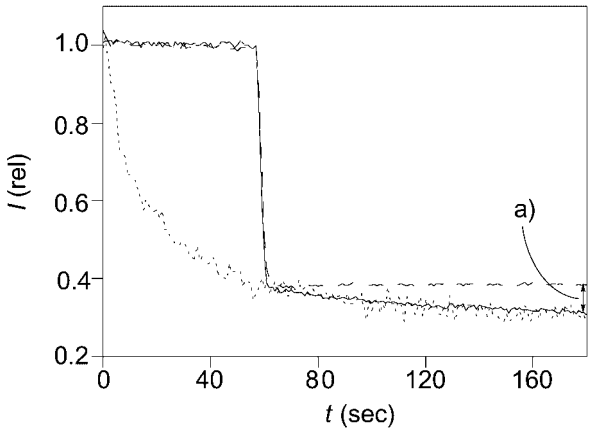

Figure 2. Overlay of kinetic profiles for the addition of DPX at $t = 60$ s to EYPC-LUVs ⊃ HPTS incubated with pore **1** (solid, from Fig. 1, solid), addition of DPX at $t = 60$ s to HPTS in buffer (dashed), addition of pore **1** to EYPC-LUVs ⊃ ANTS/DPX at $t = 0$ s (dotted, black) and at $t = 60$ s (dotted, gray; from Ref. 7). (a) HPTS emission is constant after addition of DPX to HPTS in buffer but not after addition to EYPC-LUVs ⊃ HPTS with **1**

were incompatible with molecular translocation across pores (Fig. 2, solid–dashed versus dotted–gray and extensive related evidence[2,7,8,11,20,21]). HPTS efflux through nonspecific 'leaks' (rather than through pore **1**) could be ruled out as the main origin of DPX quenching of released HPTS because, firstly, slow transport was still observable 1 min after pore addition [Fig. 2(a)], solid versus dashed) and, secondly, because the time course of this residual release was identical with that for ANTS/DPX efflux from EYPC-LUVs ⊃ ANTS/DPX through pore **1** (Fig. 2, solid versus dotted–black; ANTS = 8-aminonaphthalene-1,3,6-trisulfonate). Identity of the time course of dye efflux from EYPC-LUVs ⊃ ANTS/DPX and EYPC-LUVs ⊃ HPTS 1 min after addition of pore **1** was particularly interesting. It suggested that the 'invisible' first minute of HPTS efflux may resemble the first minute of ANTS/DPX efflux as well (Fig. 2, solid versus dotted–black). Identical kinetics for efflux of different dyes, in turn, implied that other processes such as pore formation contributed to the apparent rate of efflux.

With experimental evidence for blocker efflux through blocked pores established, we then considered that the efflux of HPTS blockers could be blocked by a second blocker added at the other side of the bilayer (Plate 3). 1,3,6,8-Pyrenetetrasulfonate (PTS) was selected as a second blocker because interference of this non-fluorescent chromophore with the detection of HPTS emission was unlikely. PTS has previously been used as a competitive inhibitor of the hydrolysis of 8-acetoxypyrene-1,3,6-trisulfonate by a rigid-rod β-barrel pore similar to pore **1**.[9]

To test this hypothesis, quenching experiments for HPTS release from EYPC-LUVs ⊃ HPTS were repeated in the presence of increasing concentrations of extravesicular PTS with a constant concentration of pore **1**. HPTS efflux decreased with increasing PTS concentration (Fig. 1, dotted). Dose response curves for PTS were established defining fractional pore activity $Y = 1.0$ in the absence of and $Y = 0.0$ at saturation with PTS (Fig. 3, circles). These dose response curves were analysed using the Hill equation

$$\log(Y/(1-Y)) = n \times \log[PTS] - n \times \log K_D \qquad (1)$$

where n is the Hill coefficient and K_D the global dissociation constant (Fig. 3, solid).[22] The obtained $K_D = 3.31 \pm 0.16$ μM was in the low micromolar range found repeatedly for the binding of pyrene-1,3,6-trisulfonates to pores such as **1**.[1] The Hill coefficient $n = 1.46 \pm 0.16$ may indicate that binding of one to two PTS blockers per pore **1** is necessary to block the efflux of HPTS blockers.

This situation was comparable to the one to two potassium cations bound by biological potassium channels to assure selectivity. Given the presence of up to 64 cationic residues (i.e. up to 16 putative PTS binding sites)

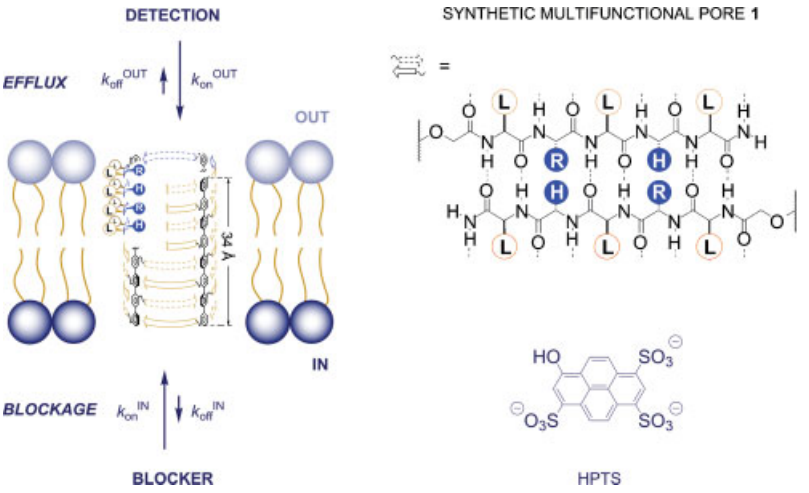

Plate 1. Blocker efflux through blocked synthetic multifunctional pores, using HPTS as blocker and barrel **1** as the pore. Pore blockage from the inside (IN) of a vesicle is defined by a low K_D, i.e. $k_{on}^{IN} \gg k_{off}^{IN} \approx k_{off}^{OUT}$, blocker efflux is influenced by the magnitude of k_{on}^{IN} and k_{off}^{OUT}. Pore **1** is a *p*-octiphenyl β-barrel, depicted as a cutaway suprastructure in bilayer membranes with β-sheets as arrows, external amino-acid residues in dark on bright, internal ones bright on dark (single-letter abbreviations). As in previous reports, we caution that the depicted structures may be viewed as, at worst, productive working hypothesis compatible with results from function. We further caution that molecular recognition between blocker and pore is depicted and described in an oversimplified manner, neglecting likely 'background' contributions from pores in the media, nonspecific leakage, and so on

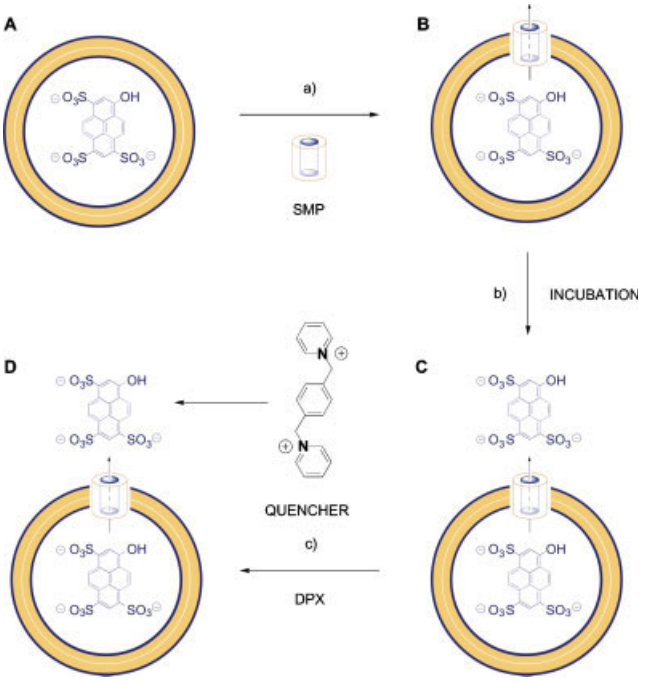

Plate 2. Assaying HPTS efflux from vesicles by external quenching with DPX, see text

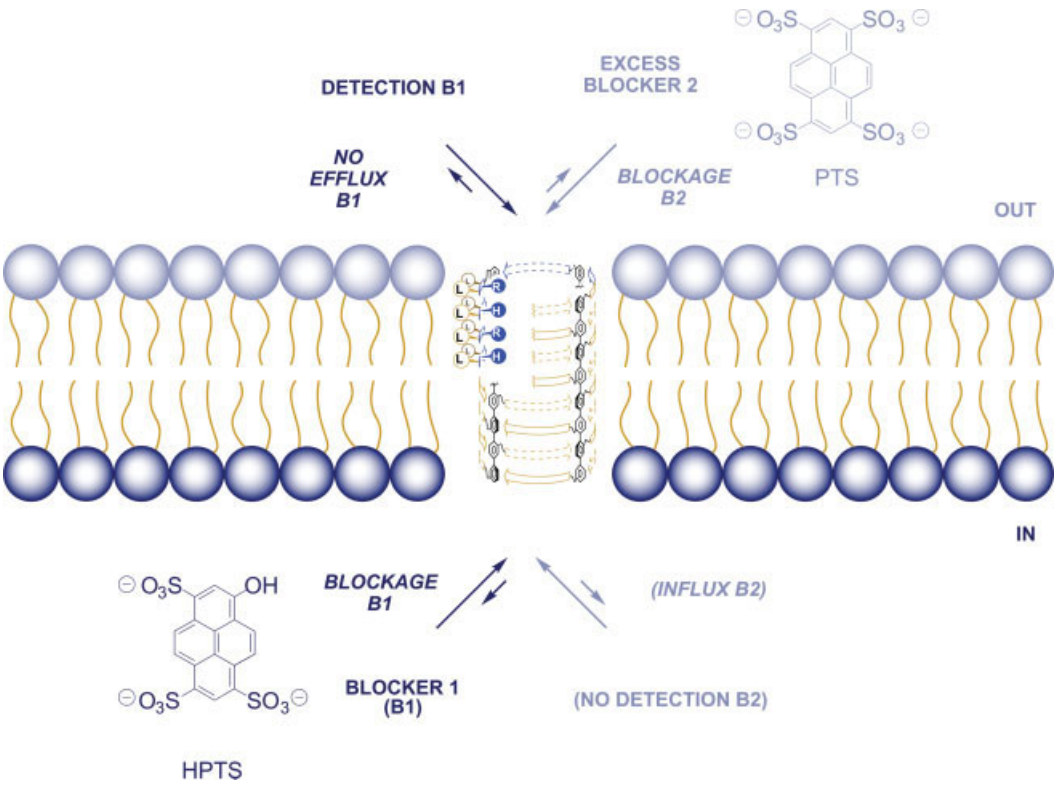

Plate 3. Blocking blocker efflux through blocked synthetic multifunctional pores, using HPTS as fluorescent blocker 1 (B1), PTS as non-fluorescent blocker 2 (B2), and barrel **1** as pore. See Plate 1 for details

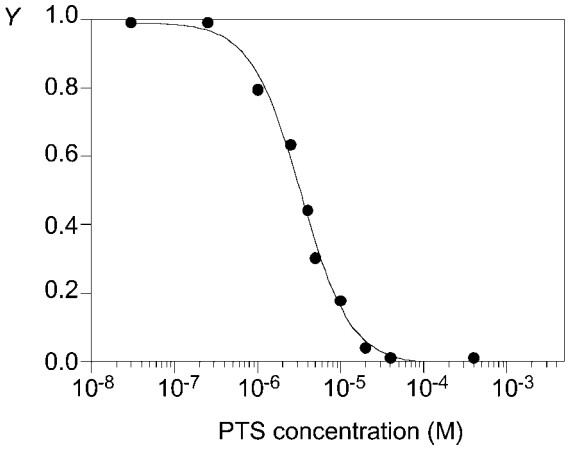

Figure 3. Fractional activity *Y* of pore **1** in EYPC-LUVs ⊃ HPTS as a function of the concentration of PTS with curve fit to the Hill Eqn (1)

along the ion conducting pathway of tetramer **7**, the suspected binding of two tetraanionic PTS blockers (length ≤ 1 nm) per pore (length 3.4 nm) is possible from a structural point of view. In view of their not always clear meanings, such interpretations of Hill coefficients warrant, however, appropriate reservations.[22] Further caution toward over-interpretation of PTS blockage is indicated considering that several blockage mechanisms, reaching from the competitive PTS binding to active pores as outlined in Plate 3 to the less likely[3,7,9,10,23–25] non-competitive PTS binding to aqueous prepores or monomers, remain valid possibilities. The finding, however, that blocker efflux through blocked pores is not problematic and can be blocked by external blockers is unambiguous. It confirms that expectations on selectivity from biological ion channels apply to synthetic multifunctional pores.

CONCLUSION

Coming back to the frequent confusion with synthetic multifunctional pores: does molecular recognition by a synthetic pore exclude molecular translocation through the same pore? Based on results from a novel assay using external quenching of released fluorescent blocker HPTS by quencher DPX, the straightforward answer is 'no'. Imagining a blocked pore such as a corked wine bottle is, therefore, not always correct, particularly not with symmetrical synthetic pores.

This conclusion does not come as a surprise. According to the principles of physical organic chemistry, it simply confirms the obvious. Experimental support for the possibility of blocking blocker efflux through blocked pores by an opposing external blocker corroborates similarly obvious expectations. The conclusion that blocker efflux through blocked pores demonstrates selectivity, however, is crucial to conceive, comprehend and appreciate practical applications of synthetic supramolecular pores (e.g. remote control of catalysis within synthetic pores by electrostatic steering).[3] Although agreement with previous studies[1–11,23–25] and present controls confirm validity of these important conclusions as such, we reiterate that the system of interest is more complex in reality than it may appear in this report, and that many less relevant considerations have been neglected or simplified with the only intention being to preserve clarity and focus.

EXPERIMENTAL

General

EYPC was from Avanti, HPTS and DPX from Molecular Probes, PTS, buffers, salts and Sephadex G-50 from Sigma and Fluka-Aldrich. A Mini-Extruder with two stacked polycarbonate membranes, pore size 100 nm was used for LUV preparation (Avanti Polar Lipids). UV–visible spectra were recorded on a Varian Cary 1 Bio spectrophotometer, fluorescence spectra and kinetics on a FluoroMax-2, Jobin Yvon-Spex) equipped with a stirrer, a temperature controller and an injector port. Data analysis was made with a KaleidaGraph 3.5.

Synthesis

Monomeric $1^3,2^3,3^2,4^3,5^2,6^3,7^2,8^3$-octakis(*Gla*-Leu-Arg-Leu-His-Leu-NH_2)-*p*-octiphenyl for self-assembly of barrels **1** was prepared in 19 steps as described previously.[7]

EYPC-LUVs ⊃ HPTS

25 µl of EYPC (1 g ml^{-1} in EtOH) was diluted in 1 ml $CHCl_3$ and 1 ml MeOH. The solvents were slowly evaporated on rotavapor to produce a thin, transparent film. After drying *in vacuo* (>2 h), the lipid film was rehydrated with 1 ml HPTS-containing buffer {1.0 mM HPTS, 5.0 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonate (TES), 96.8 mM KCl, pH 7.0} for more than 30 min, subjected to 5 freeze–thaw cycles and more than 10 extrusions. External HPTS was removed by gel filtration (Sephadex G-50) with HPTS-free buffer (5 mM TES, 100 mM KCl, pH 7.0). In the HPTS-free buffer, the KCl concentration was increased from 96.8 to 100 mM to compensate for the absence of 1.0 mM HPTS with an osmolarity $\approx 3.2 \times$ KCl.[3] The LUV fractions were unified and diluted to 6 ml with the same buffer to give EYPC-LUVs ⊃ HPTS stock solutions with the following characteristics: 2.1 mM EYPC (phosphate analysis); inside: 1.0 mM HPTS, 5.0 mM TES, 96.8 mM KCl, pH 7.0; outside: 5.0 mM TES, 100 mM KCl, pH 7.0.

HPTS efflux

EYPC-LUVs ⊃ HPTS (100 μl) were added to gently stirred, thermostated buffer [1.9 ml, 10 mM 2-morpholinoethanesulfonic acid monohydrate (MES), 100 mM KCl, pH 5.5) in a fluorescence cuvette (2 ml). Fluorescence emission intensity I_t ($\lambda_{em} = 510$ nm, $\lambda_{ex} = 415$ nm) was recorded as a function of time during addition of pore **1** (20 μl of solutions in DMSO; 0–150 nM final tetramer concentration) at time (a) (Fig. 1). After incubation for about 1 min, DPX (20 μl of 750 mM in water; 7.5 mM final concentration) was added at time (c) followed by 40 μl 1.2% aqueous Triton X-100 at time (d) for lysis. Fluorescence kinetics were normalized to fractional emission I using the equation

$$I = (I_t - I_0)/(I_\infty - I_0) \quad (2)$$

where $I_0 = I_t$ at pore addition (b) and $I_\infty = I_t$ at saturation after lysis (d).

PTS blockage

EYPC-LUVs ⊃ HPTS (100 μl) and PTS (0–40 μM final concentration) were added to gently stirred, thermostated buffer (1.9 ml, 10 mM MES, 100 mM KCl, pH 5.5) in a fluorescence cuvette (2 ml). Fluorescence emission intensity I_t ($\lambda_{em} = 510$ nm, $\lambda_{ex} = 415$ nm) was recorded as a function of time during addition of pore **1** (150 nM final tetramer concentration) at time (a). After incubation for about 1 min, (20 μl of 750 mM in water; 7.5 mM final) was added at time (c) followed by 40 μl 1.2% aqueous Triton X-100 at time (d) for lysis. Fluorescence kinetics were normalized to fractional emission I using Eqn (2).

REFERENCES

1. Sakai N, Matile S. *Chem. Commun.* 2003; 2514–2523.
2. Das G, Talukdar P, Matile S. *Science* 2002; **298**: 1600–1602.
3. Sakai N, Sordé N, Matile S. *J. Am. Chem. Soc.* 2003; **125**: 7776–7777.
4. Matile S. *Chem. Soc. Rev.* 2001; **30**: 158–167.
5. Matile S. *Chem. Rec.* 2001; **1**: 162–172.
6. Sakai N, Sordé N, Das G, Perrottet P, Gerard D, Matile S. *Org. Biomol. Chem.* 2003; **1**: 1226–1231.
7. Sordé N, Matile S. *Supramol. Chem.* 2002; **2**: 191–199.
8. Baumeister B, Som A, Das G, Sakai N, Vilbois F, Gerard D, Shahi SP, Matile S. *Helv. Chim. Acta* 2002; **85**: 2740–2753.
9. Baumeister B, Sakai N, Matile S. *Org. Lett.* 2001; **3**: 4229–4232.
10. Som A, Sakai N, Matile S. *Bioorg. Med. Chem.* 2003; **11**: 1363–1369.
11. Talukdar P, Sakai N, Sordé N, Gerard D, Cardona VMF, Matile S. *Bioorg. Med. Chem.* 2004; **12**: 1325–1336.
12. Som A, Matile S. *Eur. J. Org. Chem.* 2002; 3874–3883.
13. Doyle DA, Cabral JM, Pfuetzner RA, Kuo A, Gulbis JM, Cohen SL, Chait BT, MacKinnon R. *Science* 1998; **280**: 69–77.
14. MacKinnon R, Cohen SL, Kuo A, Lee A, Chait BT. *Science* 1998; **280**: 106–109.
15. Roux B, MacKinnon R. *Science* 1999; **285**: 100–102.
16. Jiang Y, Ruta V, Chen J, Lee A, MacKinnon R. *Nature* 2003; **423**: 42–48.
17. Kuo A, Gulbis JM, Antcliff JF, Rahman T, Lowe ED, Zimmer J, Cuthbertson J, Ashcroft FM, Ezaki T, Doyle DA. *Science* 2003; **300**: 1922–1926.
18. Haugland RP. *Handbook of Fluorescent Probes and Research Chemicals* (9th edn). Molecular Probes: Eugene, OR; 2002.
19. Tedesco MM, Matile S. *Bioorg. Med. Chem.* 1999; **7**: 1373–1379.
20. Ladokhin AS, Wimley WC, White SH. *Biophys. J.* 1995; **69**: 1964–1971.
21. Ladokhin AS, Wimley WC, Hristova K, White SH. *Methods Enzymol.* 1997; **278**: 474–486.
22. Connors KA. *Binding Constants*. John Wiley & Sons: New York; 1987.
23. Sakai N, Baumeister B, Matile S. *Chem. Bio. Chem.* 2000; **1**: 123–125.
24. Das G, Onouchi H, Yashima E, Sakai N, Matile S. *Chem. Bio. Chem.* 2002; **3**: 1089–1096.
25. Das G, Matile S. *Proc. Natl. Acad. Sci. USA* 2002; **99**: 5183–5188.