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Synthetic Multifunctional Pores with External and Internal Active Sites for Ligand Gating and Noncompetitive Blockage

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Current research on the use of synthetic,[1−5] bioengineered,[6,7] and biological[8] pores as sensors focuses exclusively on pore blockage. In this report, we introduce the synthetic multifunctional pore **1** that opens rather than closes in response to chemical stimulation (Figure 1).[9]

This ligand gating was created as follows.[9] Directed by the nonplanarity of the *p*-octiphenyl staves, cylindrical self-assembly of rigid-rods **2** was designed to produce multifunctional β-barrel pore **1** with HH dyads at the inner surface and LRL triads at the outer surface. Blockage of rigid-rod β-barrel pores with internal HH dyads by guests that match their functionalized internal space has been demonstrated previously.[1] External LRL triads were introduced to exploit anion scavenging by oligoarginines[10] for ligand gating. Namely, the self-assembly of *p*-octiphenyl peptide conjugate **2** should afford water-soluble rigid-rod β-barrels with hydrophilic anions scavenged by the external oligoarginine arrays. Multifunctional pores **1** should then form in response to external ion exchange with amphiphilic anions **3** with high affinity for oligoarginine.[10,11]

p-Octiphenyl **2** was synthesized in 19 steps overall, following protocols reported previously for other sequences.[1] When added to EYPC-LUVs⊃CF, this new rigid-rod molecule **2** did not mediate substantial efflux of self-quenched intravesicular CF (Figure 2Aa, EYPC-LUVs⊃CF: large unilamellar vesicles composed of egg yolk phosphatidylcholine and loaded with 5(6)-carboxyfluorescein). Subsequent addition of ligands **3**, however, caused rapid, concentration-dependent CF efflux (e.g., Figure 2Ab−g). Hill analysis of the resulting dose response curve provided an effective concentration, $EC_{50} = 14.8\ \mu M$, for pyrenebutyrate **3c** to stimulate 50% pore activity. EC_{50}'s decreased from pyrene carboxylate **3c** to pyrene sulfate **3b**, calix[4]arene **3a** (Figure 2C(•)), and fullerene **3d**, with an $EC_{50} = 10.2 \pm 1.9$ nM (Table 1). Under experimental conditions, all ligands **3** were membrane inactive without pores (e.g., Figure 2Ah). Replacement of the external arginines by leucines in pores formed by **2a**[1] annihilated ligand gating (Figure 2Bc versus 2Bd). Pores with internal rather than external arginine arrays formed by rods **2b**[1] were partially blocked rather than opened by argininophile **3c**. Once bound to bilayer membranes, pores **1** were not able to permealize the newly added EYPC-LUVs⊃CF (Figure 2Be). This inability of intervesicular transfer contrasted sharply with conventional pores with external LLL triads,[12] suggesting that external ligands strengthened barrel−membrane interactions. This interpretation was supported by increasing the fluorescence resonance energy transfer (FRET) from the *p*-octiphenyl staves (i.e., the intrinsic fluorescent probe of rigid-rod β-barrels) to BODIPY lipids,[13] with increasing concentration of calixarene **3a** (Figure 2C).

Force-field geometry optimizations (MMFF94/MacroModel)[14] confirmed that the dimensions of the internal space of rigid-rod

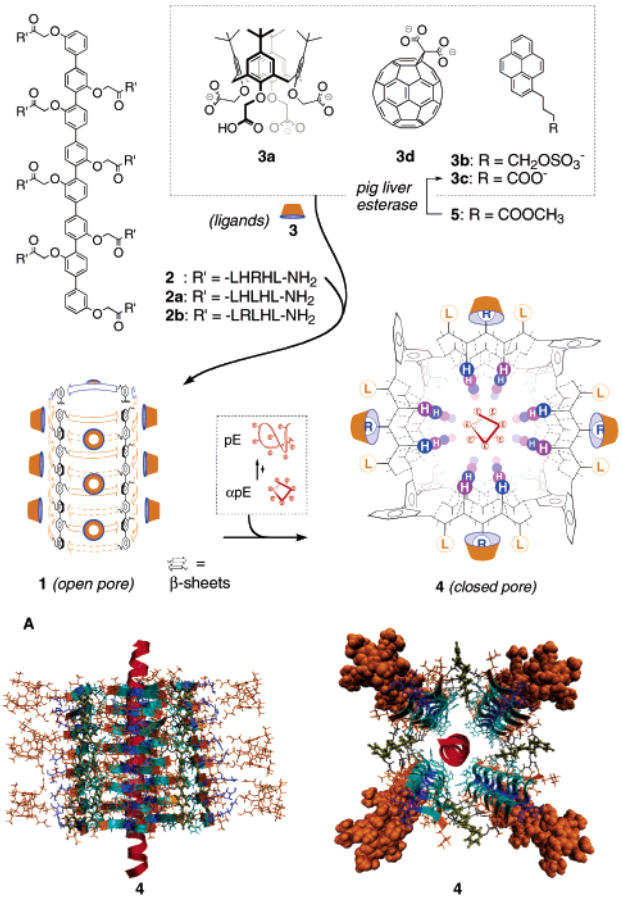

Figure 1. Ligand-gated self-assembly of rod **2** into pore **1** and noncompetitive blockage of pore **1** with poly-L-glutamate (pE, random coil; α-pE, α-helix) to give complex **4**. Rigid-rod β-barrels are depicted in side (1) and axial (4) views, with β-sheets as arrows (N → C, **1**) or solid (backbone) and dotted lines (H bonds, **4**; external amino acid residues, dark on white and internal ones, white on dark, single-letter abbreviations). (A) Molecular mechanics simulations of complex **4** with **3a** as the ligand in side (left) and axial (right) view. Arginines are in blue, histidines in cyan, leucines and calixarenes in gold, β-sheets and α-helix as ribbons, calixarenes space-filling (right), and *p*-octiphenyls, in ball-and-stick, in tan color. We caution that all shown suprastructures are, in part, speculative simplifications that are, however, consistent with molecular models (A) and experimental data.

β-barrel pores are ideal for α-helix recognition (Figure 1A).[1] It was, therefore, not surprising that poly-L-glutamate (pE) blocked pores **1** with IC_{50}'s, which were independent of the EC_{50}'s of the external ligands (Table 1 and Figure 2D(•),Bf). The IC_{50}'s for blockage by heparin, in contrast, varied with the EC_{50}'s of ligands **3a**−**c** (Table 1). This difference supported the view that oligoargininophile[10] heparin acted, at least in part, competitively on the outer barrel

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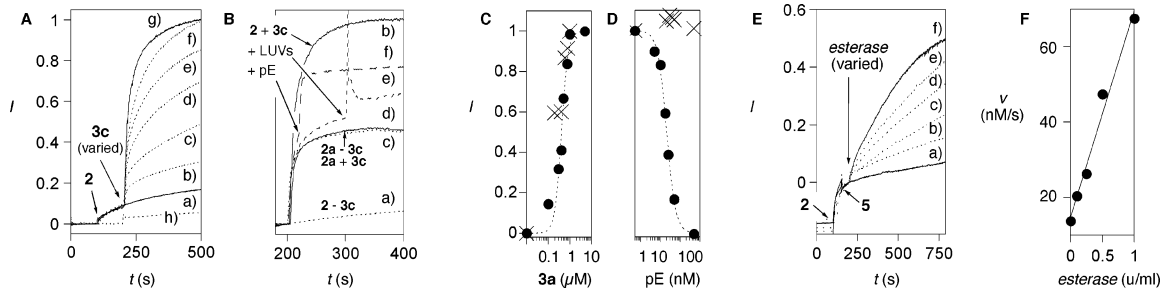

Figure 2. Changes in activity and membrane affinity of **2** and **2a** (Bc,d) in response to ligands **3c** (A and B) and **3a** (C), blockers (pE, Bf and D), and enzymes (PLE, E and F) in lipid bilayers. (A) Fractional change in CF emission I (λ_{ex} 492 nm, λ_{em} 517 nm) as a function of time during addition of rod **2** (100 nM (a−g) and 0 nM (h)) and ligand **3c** (0 (a), 5 (b), 10 (c), 20 (d), 22 (e), 30 (f), and 50 μM (g and h)) to EYPC-LUVs⊃CF (250 μM EYPC) in buffer (10 mM HEPES, 107 mM NaCl, pH 7.0), calibrated by final lysis (excess Triton X-100). (B) Same for addition sequences (a) **2** (100 nM), (b) **2** (100 nM) → **3c** (50 μM), (c) **3c** (50 μM) → **2a** (1 μM), (d) **2a** (1 μM), (e) **2** (100 nM) → **3c** (50 μM) → EYPC-LUVs⊃CF (250 μM EYPC), and (f) **3b** (6 μM) → **2** (500 nM) → pE (1 μM). (C) Fractional activity of pore **2** (500 nM) as a function of the concentration of ligand **3a** (●, fit to Hill eq) and BODIPY emission under identical conditions (×). (D) Fractional activity of pore **2** (500 nM) with ligand **3a** (1 μM) as a function of the concentration of blocker pE (●, fit to Hill eq) and BODIPY emission under identical conditions (×). (E) Fractional change in CF emission I as a function of time during addition of rod **2** (100 nM), proligand **5** (600 μM), and pig liver esterase (0 (a), 0.10 (b), 0.25 (c), 0.50 (d), 1.00 (e), and 10.0 units/ml (f)) to EYPC-LUVs⊃CF (250 μM EYPC). (F) Initial velocity of formation of ligand **3c** as a function of esterase concentration (summary E).

Table 1. Data on Ligand Gating and Blockage of Pore **1**[a]

ligand[a]	EC_{50} (μM)[b]	IC_{50} (nM)[c] polyglutamate	IC_{50} (nM)[c] heparin
3a	0.44 ± 0.04	42.0 ± 2.2	24.6 ± 3.3
3b	3.30 ± 0.50	47.0 ± 4.0	19.0 ± 1.2
3c	14.80 ± 2.40	41.0 ± 7.1	4.2 ± 0.5

[a] See Figure 1 for structures. [b] Concentration of ligand **3** required for 50% pore activation. [c] Concentration of blocker required for 50% pore blockage.

surface, whereas pE was recognized by spatially separated internal active sites to give complex **4** (Figure 1). The functional evidence for noncompetitive pore blockage was corroborated on the structural level by unchanged FRET from fluorescent pore **1** to BODIPY lipids during blockage by pE (Figure 2D(×)).

We have previously shown that synthetic multifunctional pores are of practical use as adaptable detectors of enzyme activity.[15,16] The continuous detection of enzyme activity was, however, problematic with pores that operate on blockage because substrates bound within pores seem to be less accessible to enzymes, whereas continuous detection of pore closing upon production of blockers is incompatible with routine fluorescence detection. This challenge was therefore ideal for probing the practical usefulness of ligand-gated pore sensors. Pyrenebutyrate methylester **5** was considered as a substrate of pig liver esterase (PLE) that would yield ligand **3c** as a product (Figure 1). Addition of PLE to a mixture of substrate **5**, rod **2**, and CF vesicles caused the CF efflux indicative for "enzyme gating" (Figure 2E). Linear dependence on enzyme concentration suggested that the observed initial velocity of CF efflux reflected the initial velocity of ligand formation (i.e., enzyme kinetics (Figure 2F)). We summarize that the rational design of synthetic multifunctional pores that can be opened[9] and closed noncompetitively by external ligands[9] and internal blockers is possible, and that such pores can be of practical use for, namely, the continuous fluorometric detection of chemical processes.

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Supporting Information Available: Experimental details and brief discussion of FRET experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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