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Steroid Cyclophanes as Artificial Cell-surface Receptors. Molecular Recognition and its Consequence in Signal Transduction Behavior

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Abstract. Steroid cyclophanes, bearing four bile acid moieties covalently placed on a tetraazaparacyclophane skeleton, were designed and synthesized as artificial cell-surface receptors. Guest-binding behavior of the steroid cyclophanes embedded in a bilayer membrane formed with a synthetic peptide lipid was clarified by means of fluorescence and circular dichroism spectroscopy. We found that the steroid cyclophane effectively bound aromatic guests in both bilayer membranes and aqueous solution. In addition, copper(II) ions acted as a guest species for the steroid cyclophane and a competitive inhibitor toward a NADH-dependent lactate dehydrogenase (LDH). On these grounds, we constituted a supramolecular assembly as an artificial signaling system in combination with the steroid cyclophane, a cationic peptide lipid, and LDH. As a consequence, the steroid cyclophane acted as an effective artificial cell-surface receptor being capable of transmitting an external signal to the enzyme in collaboration with copper(II) ions as a signal transmitter.

Key words: artificial receptor, steroid cyclophane, molecular recognition, signal transduction.

1. Introduction

Many metabolic processes taking place in biological cells are triggered through recognition of external signals by specific receptor proteins. Such receptors are classified into two categories depending on their locations in cells: intracellular receptors in cytoplasms and cell-surface receptors in biomembranes. Furthermore, there are at least three known classes of cell-surface receptors – channel-linked, G-protein-linked, and catalytic [1]. However, receptor functions remain to be clarified at the molecular level with emphasis on molecular recognition of signaling ligands and the resulting responses. On these grounds, signal transduction based on molecular recognition by artificial receptors is one of the most attractive subjects in supramolecular chemistry [2]. While functional simulation of intracellular receptors in homogeneous solutions has been actively carried out with attention to various non-covalent interactions [3], signal transduction by artificial cell-surface

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receptors embedded in supramolecular assemblies has been scarcely studied up to the present time, except for channel-linked receptors [4–12].

We briefly review here our recent approaches to the development of artificial cell-surface receptors keeping attention to signaling mechanisms through G-protein-linked receptors; G-protein being a membrane-associated heterotrimeric protein composed of α, β, and γ subunits. The function of G-protein tends to couple with action of cell-surface receptors effective for hormones, neurotransmitters, and other bioactive molecules in their signaling pathways within a biological cell. The α-subunit of G-protein contains a guanine-binding site, which is occupied by guanosine diphosphate (GDP) in the inactive state. When the receptor binds a signaling ligand, the former undergoes a conformational change resulting in its association with G-protein. An interaction of G-protein with the activated receptor promotes the exchange of GDP for guanosine triphosphate (GTP) leading to dissociation of the β, γ-subunit complex from the α-subunit. The dissociated α-subunit is then capable of binding a specific effector enzyme such as adenylate cyclase and controlling the effector activity [13]. On these grounds, we can basically understand that G-proteins are signal transmitters operating between receptors and effectors. From a viewpoint of biomimetic chemistry, we need to design an artificial signaling system constituted with an artificial cell-surface receptor, an enzyme as an effector, and a bilayer membrane formed with a synthetic lipid. In such an artificial system, G-protein could be replaced by a simple signal transmitter which is capable of affecting both receptor and enzyme functions.

2. Design and Synthesis of Artificial Receptors

In the initial step of cell signaling, the recognition of an external signal by a receptor embedded in a biomembrane is a common and basic behavior. Thus, we first designed artificial receptor **1** capable of recognizing organic guests in bilayer membranes [14]. This host molecule is constituted in combination with two types of functional components, a cyclophane ring and four bile acid moieties, which are known to be individually effective in molecular recognition. We call it a "*steroid cyclophane*". Although simple cyclophane derivatives exhibit marked biomimetic functions when they constitute supramolecules with other molecular species, the supramolecular effect could be made more pronounced by modifications of their shallow cavities to afford three-dimensionally extended hydrophobic spaces [15, 16]. On the other hand, bile acids are well-known natural compounds, and many studies have been carried out on their characterization and their derivatives as hosts in various physical phases such as solid states, and aqueous and organic media [17–19]. In bilayer membranes, the bile acid moieties of the steroid cyclophane are expected to act as hydrophobic anchors as well as functional components for molecular recognition. The CPK and computer-aided molecular model studies reveal that the steroid cyclophane is capable of incorporating a hydrophobic guest

molecule into its three-dimensionally extended inner cavity created by the four steroid moieties and the macrocyclic skeleton.

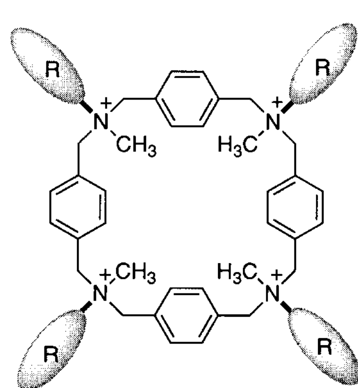
In order to achieve the functional simulation of signal transmission from a cell-surface receptor to an enzyme, the following requirements must be additionally fulfilled by artificial receptors. (1) The receptor is capable of recognizing both a first signaling ligand and a signal transmitter species. (2) The binding affinity of the receptor toward a signal transmitter varies as molecular recognition toward a signaling ligand changes. On these grounds, we structurally modified steroid cyclophane **1** by introduction of α-amino acid residues as connector units between a cyclophane skeleton and four bile acid moieties (Figure 1). Steroid cyclophanes **2–6** were prepared along this line [20]. For example, L-lysine residues covalently incorporated into the steroid cyclophane act as recognition sites for signal transmitters and as polar heads to solubilize the host in aqueous media (vide infra).

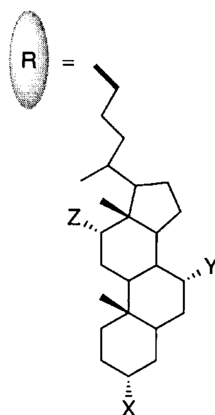
3. Molecular Recognition in Aqueous Solution

Prior to investigation of chemical functions of the steroid cyclophanes as cell-surface receptors, we clarified their fundamental capability in molecular recognition toward various organic guests in aqueous media [20–22].

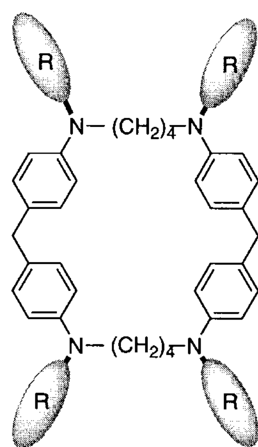
The steroid cyclophanes have an amphiphilic character capable of forming a molecular assembly in aqueous media. Critical aggregate concentration (cac) values, as evaluated by means of surface tension measurements based on the Wilhelmy principle, were 3.5×10^{-5} and 6.2×10^{-6} mol dm^{-3} for **1** and **2**, respectively. Although the aggregate structures of the steroid cyclophanes have not been clarified, the formation of relatively large aggregates was observed in a concentration range above the cac; hydrodynamic diameters for the aggregates of **1** and **2** were 140 and 650 nm, respectively, as evaluated by means of dynamic light-scattering measurements. In spite of such complexity in aggregation behavior, the steroid cyclophanes generally showed simple guest-binding behavior based on 1 : 1 host–guest complexation regardless of the concentration range, above or below the cac [22]. This indicates that the guest-binding affinity of the three-dimensional inner cavity provided by the steroid cyclophanes is much superior to that of the intermolecular hydrophobic space furnished by the aggregated host molecules. Other macrocyclic molecules having amphiphilic character were found to incorporate a guest molecule into their cavities in 1:1 stoichiometry and such binding behavior was maintained in a similar manner even under conditions that allow aggregation of the host [23–25].

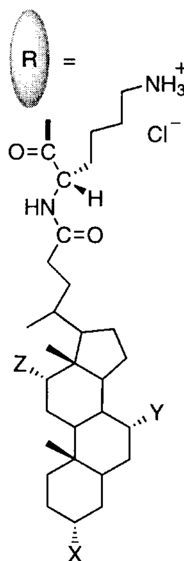
Binding constants (K) for several naphthalene derivatives with the steroid cyclophanes in aqueous solution, as evaluated by means of fluorescence spectroscopy, are summarized in Table I. The cationic steroid cyclophanes bind anionic and nonionic guests with large K values. Among these guest molecules, the hosts prefer to take up slenderer molecules **9** and **11** relative to **8** and **10**. On the other hand, these cyclophanes show no capacity to bind a guest with a positive charge. This

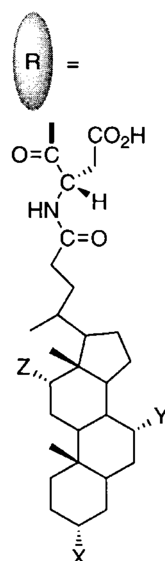




1 (X = Y = Z = OH)







2 (X = Y = Z = OH)
3 (X = Z = OH, Y = H)
4 (X = OH, Y = Z = H)
5 (X = Y = Z = H)

6 (X = Y = Z = OH)

Chart 1.

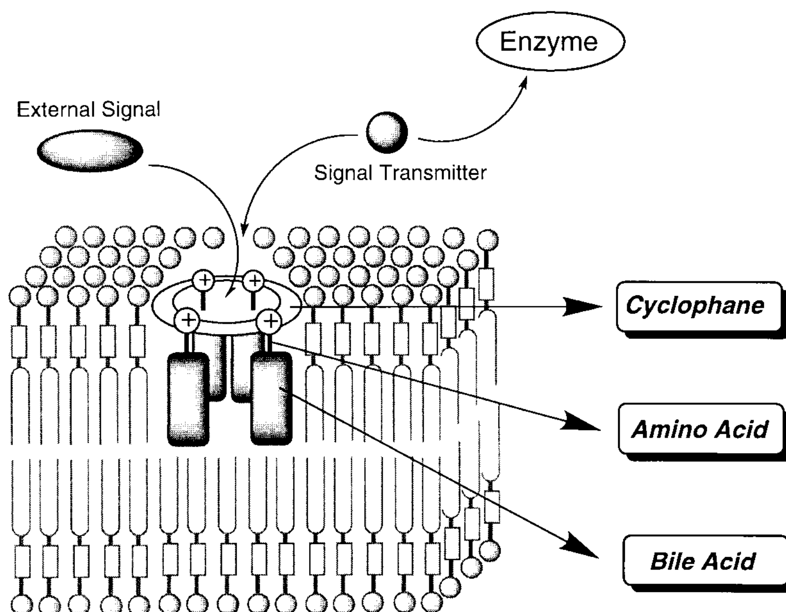


Figure 1. Schematic representation of a steroid cyclophane as an artificial cell-surface receptor embedded in a bilayer membrane and collaboration modes with an enzyme and an external signal.

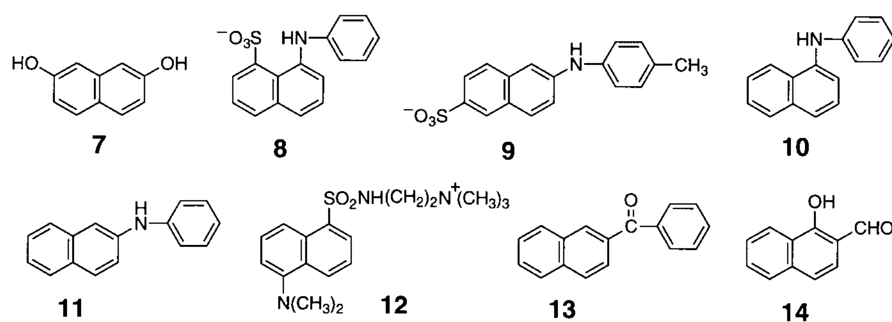


Chart 2.

Table I. Binding constants (K) for aromatic guests with steroid cyclophanes in aqueous solution at 30.0 °C

Steroid cyclophane	pH	$K/10^3$ dm^3 mol^{-1}				
		8	**9**	**10**	**11**	**12**
1	8.0	330	2000			–[a]
2	5.0	110	1200	360	1100	–[a]
5	5.0	42	380	54	120	–[a]
6	8.0	23	130	89	250	11

[a] Complex formation was not detected.

means that the hosts recognize guests mainly through hydrophobic and electrostatic interactions. In addition, larger K values with **2** relative to those with **5** for all the guests imply that the hydroxyl groups placed in the steroid skeleton of **2** give out a significant effect on the guest recognition ability through hydrogen-bonding interactions. In contrast, the anionic steroid cyclophane (**6**) demonstrates an efficient binding affinity toward hydrophobic guests regardless of their charged states; cationic, anionic, and non-ionic. Thus, the hydrophobic interaction overcomes the electrostatic ones in the course of guest-binding by the present anionic host having L-aspartate residues.

Upon complexation of the fluorescent guests with the steroid cyclophanes, the emission maximum of each guest shows a large blue shift in a manner similar to the corresponding fluorescence behavior observed in nonaqueous organic solvents [20,21]. The results indicate that the guest-binding sites of the steroid cyclophanes in aqueous solution are well desolvated due to formation of a three-dimensionally extended hydrophobic cavity constructed with a cyclophane skeleton and four steroid moieties.

Odashima et al. have reported that 2,7-dihydroxynaphthalene (**7**) incorporated into 1,6,20,25-tetraaza[6.1.6.1]paracyclophane (CP44), the basic skeleton of steroid cyclophanes **2**–**6**, assumes a pseudo-axial geometry with the long axis of its naphthalene ring penetrating the cavity obliquely, as clarified by ^{1}H NMR spectroscopy in D_2O at pD 1.2 [26]. Upon complexation of **7** with steroid cyclophane **2** at pD 5.0, the spectral behavior is analogous to that reported for a complex of CP44 and **7**, except for the following aspects. The aromatic proton signals of the steroid cyclophane are broader than those of CP44 both in the presence and in the absence of the guest molecule, reflecting restricted molecular motion of the benzene rings in the former host due to the presence of the hydrophobic branches. The extents of upfield shifts for the guest signals are 1-H $\approx$ 4-H $>$ 3-H with the steroid cyclophane and 1-H $>$ 4-H $>$ 3-H with CP44. Judging from the complexation induced shifts (CIS), the shifts of NMR signals for the guest upon 100% complexation, the guest is incorporated into the macrocyclic cavity of **2** with the long axis of the naphthalene ring parallel to the molecular axis of the steroid cyclophane, i.e. axial geometry [22]. Figure 2 shows the CIS values and schematic representation for the geometry of 2-naphthyl phenyl ketone (**13**) bound to **2** in D_2O–CD_3OD (8:2 v/v) at pD 5.0 and 40.0 °C. The steroid cyclophane induces marked upfield shifts of the proton signals of the guest, especially for 1-H, 4-H, 5-H, and 8-H on the naphthalene ring, upon complexation. It is noteworthy that the phenyl ring of the guest molecule is presumably surrounded by four steroid moieties in order to minimize hydrophobic hydration in aqueous media. Thus, hydrogen-bonding between the carbonyl oxygen of the guest and the 12-OH on a steroid moiety of the host becomes plausible when the complex is formed. Such a hydrogen-bonding interaction in the host–guest complex is supported on the basis of experimental results; induced CD bands for the guest molecule can be observed upon complexation with **2** or **3** but not with **4** or **5**. The K value for the complex of the guest with **2** increases to 1.2×10^6 dm^3

Figure 2. CIS values (ppm) (a) and schematic representation of geometry (b) of **13** bound to steroid cyclophane **2**.

Chart 3.

mol^{-1} in aqueous acetate buffer containing 1%(v/v) ethanol at pH 5.0 and 303 K, as evaluated by means of CD spectroscopy.

4. Molecular Recognition in Bilayer Membranes

We have recently developed synthetic lipids bearing α-amino acid residue(s), interposed between a polar head moiety and a hydrophobic double-chain segment through peptide bonds [27]. In comparison with conventional phospholipids, such peptide lipids generally afford morphologically stable bilayer aggregates in aqueous media and are useful as molecular elements for the simulation of biomembrane-mediated processes. When the steroid cyclophane is embedded in the bilayer membrane formed with the peptide lipid, the host behaves as an artificial cell-surface receptor exhibiting marked discrimination toward guest molecules [14, 20, 21, 28].

For example, **9** is effectively bound to the hydrophobic binding site of **2** embedded in the bilayer vesicle formed with **15** through hydrophobic and electrostatic interactions (Figure 3). We evaluated the stoichiometry and formation constant for the host–guest complexation by fluorescence spectroscopy under conditions that allow a composition of the steroid cyclophane vs. the peptide lipid to be maintained

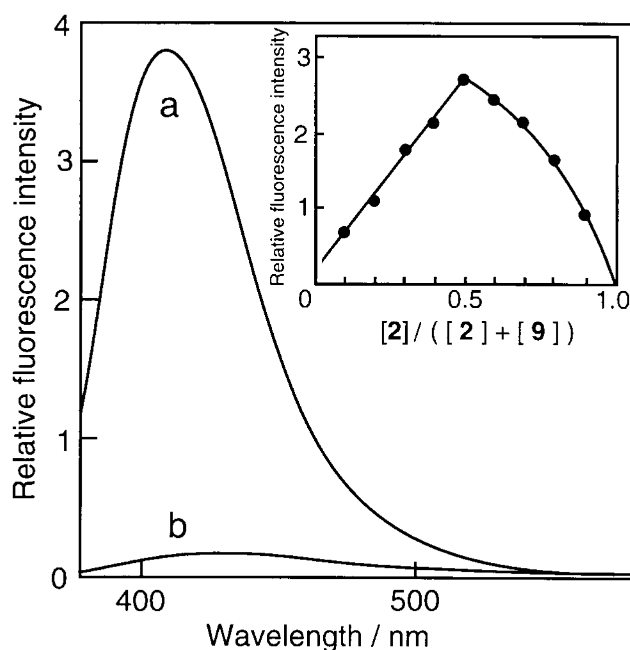


Figure 3. Fluorescence spectra of **9** (0.001 mmol dm^{-3}) in the presence of a bilayer membrane composed of peptide lipid **15** (n = 16) (0.4 mmol dm^{-3}) (a) with and (b) without steroid cyclophane **2** (0.01 mmol dm^{-3}) in aqueous acetate buffer (10 mmol dm^{-3}, pH 5.0) at 30.0 °C. Inset shows a continuous variation plot for complexation of **9** with **2** embedded in the bilayer vesicle: total concentration of **2** and **9**, 0.01 mmol dm^{-3}; molar ratio of **2** to **15**, 0.025.

constant in a molar ratio of 1 : 40. The continuous variation method applied to the present system clearly indicates that **2** and **9** forms a 1 : 1 complex in the bilayer vesicle as well as in aqueous solution. The *K* value determined on the basis of a Benesi-Hildebrand plot was 5.1×10^4 dm^3 mol^{-1}. The corresponding *K* value for the complex of **9** with **5** was 1.8×10^4 dm^3 mol^{-1}. Although the binding constants for the steroid cyclophanes embedded in the bilayer membrane are somewhat lower than those in aqueous solution, the superiority of **2** over **5** for molecular recognition of **9** is also retained in the bilayer membrane.

Steroid cyclophane **2** is capable of providing a chiral binding site for **9** in a bilayer membrane as well as in aqueous solution. The bilayer vesicle formed with **15** showed a CD band at 231 nm. No induced CD was observed upon addition of **9** to this vesicular solution. A CD spectrum for the hybrid bilayer assembly composed of **2** and **15** was nearly identical to a sum of the individual spectra, strongly suggesting that the steroid cyclophane was incorporated into the membrane without meaningful conformational change. In the present hybrid assembly, however, we observed induced CD bands at 323 and 371 nm upon addition of **9**, reflecting the fact that the guest is incorporated into the cyclophane cavity. On the other hand,

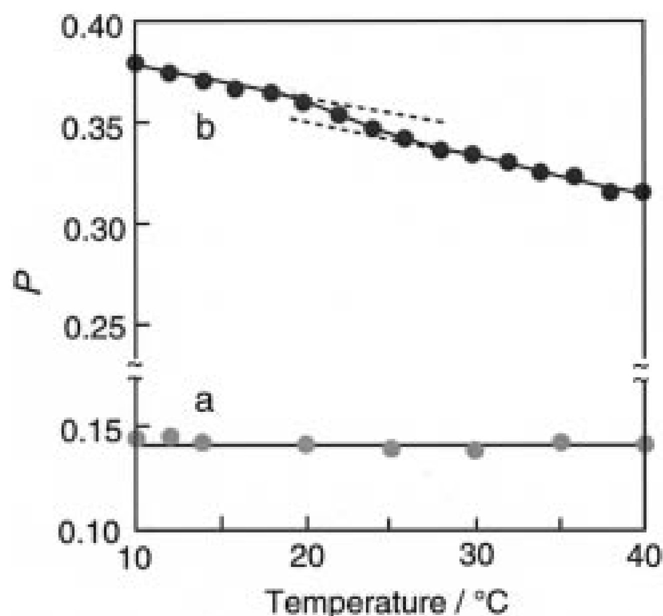


Figure 4. Correlations of temperature with steady-state fluorescence polarization of **8** (0.001 mmol dm^{-3}) bound to steroid cyclophane **1** (0.01 mmol dm^{-3}) in the (a) presence and (b) absence of bilayer membrane formed with peptide lipid **15** (n = 16) (0.4 mmol dm^{-3}) in aqueous phosphate buffer (100 mmol dm^{-3}, pH 8.0).

such an induced CD band was not detected in the hybrid assembly composed of **5** and **15**.

A microscopic environment around the guest-binding site of the steroid cyclophane embedded in the bilayer membrane can be evaluated from steady-state fluorescence polarization values (P) for the fluorescent guests bound to the host. Figure 4 shows an example of such measurements performed for guest **8** bound to receptor **2** in the presence and absence of the bilayer membrane formed with **15**, in a temperature range above and below its phase transition from gel to liquid-crystalline state; the phase transition temperature (T_m) being 24.4 °C. The P value in aqueous solution remains nearly constant at 0.14 in the range of 10—40 °C. In the case of the corresponding hybrid assembly formed with the peptide lipid, however, the P value decreased significantly as temperature was raised along with a slight inflection in the phase transition temperature range. Hence, the guest molecule is obviously incorporated into the hydrophobic aggregate domain, in which molecular motion of the guest is affected by the phase transition.

Since the P value is subject to change by the fluorescence lifetime (τ) and the rotational correlation time (θ), these values for **8** bound to receptor **2** were evaluated at 20 °C in the presence and absence of the bilayer membrane formed with **15**. The fluorescence lifetimes (τ = 15.4 and 15.0 ns in the presence and absence of the bilayer membrane, respectively) are large relative to that in water without

any coexisting component (τ = 0.55 ns), and indicate that the guest molecule is placed in a hydrophobic microenvironment well isolated from the bulk aqueous phase. It is noteworthy that the rotational correlation time in the bilayer assembly (θ = 66.7 ns) is much larger than that in aqueous solution (θ = 6.4 ns). The θ values for **8** bound to liposomal membranes formed with lecithin and bound to various proteins are in ranges of 3—6 and 9—63 ns, respectively. Thus, the θ value for the guest incorporated into the hybrid assembly formed with the steroid cyclophane and the peptide lipid in its gel state seems to be the largest value for **8** bound to hosts. It is interesting to note that such marvelous restriction of molecular motion can be achieved by combination of the steroid cyclophane having rigid steroid moieties, being incapable of tight guest-binding by itself, with the anionic peptide lipid, which cannot perform effective binding of an anionic guest in the individual aggregate state. On the other hand, the molecular motion of **8** bound to octopus cyclophanes having flexible hydrocarbon branches does not undergo significant change upon formation of a hybrid assembly with the peptide lipid [21].

1-Hydroxy-2-naphthaldehyde (**14**) was effectively recognized by the steroid cyclophanes (**2–5**) embedded in a bilayer vesicle composed of a peptide lipid (**16** or **17**) to form an imine bond between the formyl group of the guest and the amino group of the cyclophane, as confirmed by electronic absorption spectroscopy; the K value being about 10^6 dm^3 mol^{-1} in aqueous HEPES buffer (100 mmol dm^{-3}) at pH 7.0 and 30.0 °C. The complexation is reversible and much enhanced upon addition of copper(II) ions due to the formation of the corresponding metal complex.

4.1. Signal Transduction as Artificial Cell-Surface Receptors

A NADH-dependent lactate dehydrogenase (LDH) catalyzes transformation between pyruvate and lactate and its activity is inhibited by metal ions [29]. We have clarified that the inactivation of LDH by copper(II) ions is a reversible process and that the enzyme is bound to bilayer vesicles of cationic peptide lipids mainly through electrostatic interactions [30]. On these grounds, we constituted a supramolecular assembly as an artificial signaling system in combination with a steroid cyclophane, a cationic peptide lipid, and pig heart LDH, as schematically shown in Figure 5. The catalytic activity of LDH in the reduction of pyruvate to L-lactate was evaluated spectrophotometrically by following the consumption rate of NADH in the presence and absence of signaling species such as **14** and copper(II) ions.

Each block representation in Figure 6 represents a magnitude of LDH activity in the bilayer system formed with **17** in the presence of copper(II) ions, relative to that of the corresponding metal-free system [31]. In the absence of an artificial receptor and a signaling ligand, 67% of LDH was inactivated by coordination of copper(II) ions to the enzyme active site under the conditions employed here. Although micelle-forming single-chain amphiphiles such as hexadecyltrimethylammonium

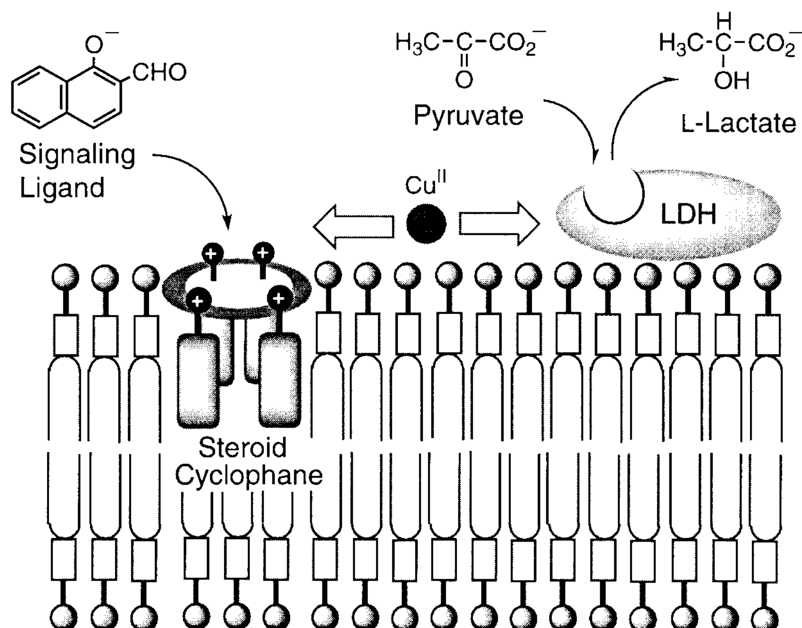


Figure 5. Schematic representation of a signal transduction system mediated by a steroid cyclophane as an artificial cell-surface receptor.

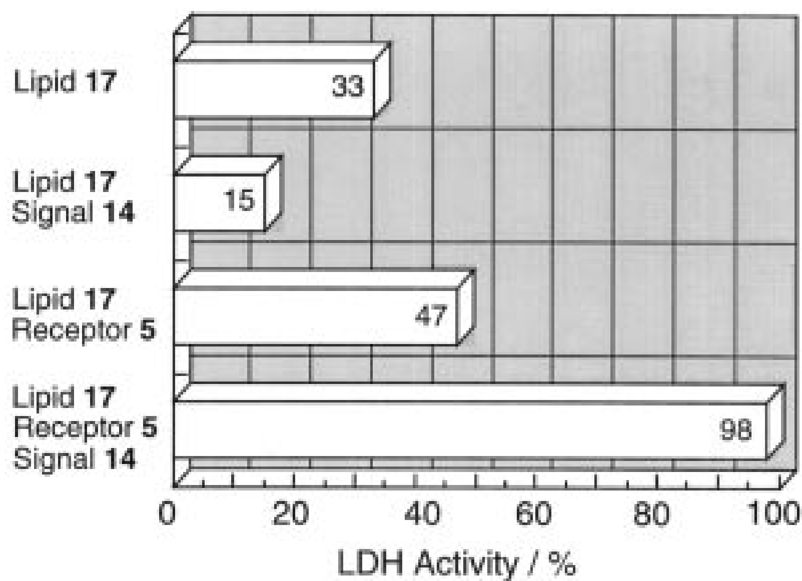


Figure 6. LDH activities in the presence of supramolecular bilayer assemblies in aqueous HEPES buffer (0.1 mol dm^{-3}, pH 7.0) at 30.0 °C. Concentrations in mmol dm^{-3}: pyruvate, 0.5; NADH, 0.25; $Cu(ClO_4)_2$, 0.008; **17**, 1.6; **14**, 0.04, **5**, 0.04. LDH, 170 μg dm^{-3}.

bromide inactivate the enzyme, the peptide lipid scarcely affects the LDH activity when they form a stable bilayer membrane. Upon addition of **14** as a signaling ligand, the LDH activity decreased to 15%. Since **14** does not act as an inhibitor to LDH directly, a lower enzyme activity is presumably due to destabilization of an aggregate state of the peptide lipid caused by perturbation of a copper(II) chelate of **14**. On the other hand, the LDH activity is recovered to 47% in the presence of steroid cyclophane **5**, reflecting that copper(II) ions bound to the enzyme are partly transferred to the receptor. It is noteworthy that the LDH activity is nearly completely recovered upon addition of **14** to the hybrid assembly composed of **5** and **17** in a molar ratio of 1 : 40. Such recovery of the enzyme activity would come from metal binding by the receptor embedded in the membrane, which is much enhanced due to recognition of the signaling ligand by the receptor. The result clearly indicates that an artificial signaling system depicted in Figure 5 has now turned into reality.

The present signaling system shows marked signal selectivity. For example, **14** is much a better signal than salicylaldehyde and 2-naphthaldehyde. When we replaced **5** with **2** in the present signaling system, signal transduction efficiency was somewhat lowered.

5. Conclusions

We demonstrated here the first example of supramolecular assemblies in which an artificial cell-surface receptor transmits an external signal to an enzyme in collaboration with a signal transmitter. Although we employed a bilayer membrane without discrimination between the inner and the outer surfaces at present, we believe more intelligent supramolecular systems may be developed by further modifications of an artificial receptor from the viewpoints of structural feature and chemical function.

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