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A zinc(II) complex-conjugated polymer for selective recognition and separation of phosphates†

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ABSTRACT: A new polymer, Zn^{2+}–cyclen complex-conjugated polymer **4** (cyclen = 1,4,7,10-tetraazacyclododecane), was synthesized by copolymerization of a Zn^{2+}–(4-vinyl)benzylcyclen complex (**3**, ZnL^2) with ethylene glycol dimethacrylate (EGDMA). The contents of the zinc(II) ions in Zn^{2+}–cyclen polymer **4** were determined as 36–42 μmol g^{-1}, utilizing a Zn^{2+}-selective fluorophore, dansylamidoethylcyclen **10** (L^3). It was found that 31% of deoxyadenosine 5′-monophosphate (5′-dAMP) (50 μM) is adsorbed on **4** (corresponding to 50 μM) in 20 mM HEPES (pH 7.0) with $I = 0.1$ ($NaNO_3$) at 25 °C. The apparent complexation constants for the 1:1 complex of ZnL^2 on **4** with 5′-dAMP and 4-NPP, $\log K_{app}$(ZnL–S^{2-}) (S^{2-} denotes a phosphomonoester dianion), at pH 7.0 and 25 °C were determined as 4.1 ± 0.1, which was larger than that (3.5) for the 1:1 complex of **3** (ZnL^2) with 5′-dAMP in homogeneous aqueous solution at pH 7.0 and 25 °C. Deoxyadenosine (dA) and adenosine 3′,5′-cyclic-monophosphate (3′,5′-cAMP), a cyclic phosphodiester monoanion, were negligibly adsorbed on Zn^{2+}–cyclen polymer **4**, implying that **4** interacts selectively with phosphomonoester dianions. For reference, Zn^{2+}-free polymer **8** (prepared from **4**) and control polymer **9** (prepared by copolymerization of styrene with EGDMA) hardly adsorbed 5′-dAMP, dA and 3′,5′-cAMP. High-performance liquid chromatography (HPLC) of mononucleotides such as 5′-dAMP, cytidine 5′-monophosphate (5′-CMP), guanosine 5′-monophosphate (5′-GMP), and thymidine 5′-monophosphate (5′-dTMP) was achieved using **4** as a stationary phase and continuous gradient elution with 10:90 MeOH–1 mM HEPES (pH 7.0) and 10:90 MeOH–1 mM HEPES + 10 mM $(NH_4)_2HPO_4$ (pH 7.0). As expected, the retention times of mononucleotides were larger than those of the corresponding nucleosides, owing to the Zn^{2+}-phosphate interactions. These results indicated that Zn^{2+}–cyclen-conjugated polymer **4** affords a strong methodology to detect and separate biologically important phosphates. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: Zn^{2+} complexes; cyclen; synthetic polymer; phosphates; nucleotides; separation; high-performance liquid chromatography; Zn^{2+}-selective fluorophore

INTRODUCTION

Phosphate esters exist ubiquitously in nature in the form of nucleoside phosphates (nucleotides) as components of nucleic acids, sugar nucleotides for glycosylation of oligosaccharides or proteins, activated forms of proteins responding to extracellular signals and chemical mediators playing central roles in intracellular signals.[1] The development of artificial phosphate receptors would afford potent methodologies for the detection and separation of biologically important phosphates (for reviews of chemical receptors and sensors for phosphates, see Ref. 2). Most chemical receptors are soluble in solution and sometimes suffer from incapability of separation of specific compounds from a mixture. On the other hand, an artificial host molecule conjugated on an insoluble polymer affords a promising method to isolate only the desired molecules. Several strategies, such as molecularly imprinted polymers,[3] based on template-mediated synthesis and/or self-assembly systems utilizing metal-ligand coordination bondings[4] and hydrophobic interactions and hydrogen bondings, have been developed for the purpose of target-directed sensing and separation. However, examples of phosphate-targeted synthetic polymers are limited.[5]

It is well established that zinc(II) complexes of macrocyclic polyamines, such as Zn^{2+}–cyclen (**1**, ZnL^1) (cyclen = 1,4,7,10-tetraazacyclododecane), are good models for zinc(II) enzymes, including carbonic anhydrase and carboxypeptidase A (Scheme 1).[6] Based on the fact that phosphate anions act as substrates and inhibitors for zinc(II) enzymes,[6] the Zn^{2+}–cyclen complexes have been demonstrated to be good receptors for

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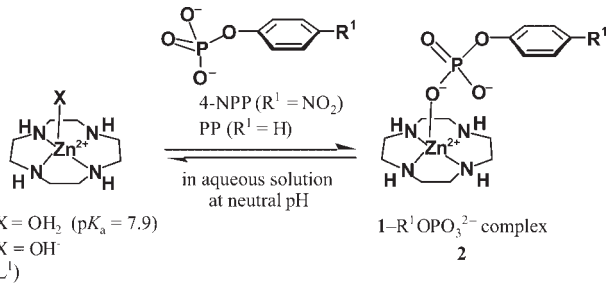


Scheme 1

phosphomonoester dianions including 4-nitrophenyl phosphate (4-NPP), phenyl phosphate (PP) and nucleotides,[7] and for other potential anions such as imides[8,9] and thiols[10] in aqueous solution. The affinities of carboxylates and sulfonates with Zn^{2+}–cyclen complexes are smaller than those of phosphomonoester dianions, imides, and thiols, because the basicities of the former functional groups are weaker than those of the latter.

In general, Zn^{2+}–cyclen complexes are thermodynamically and kinetically inert in aqueous solution at neutral pH (e.g., the dissociation constant (K_d) for **1** is ca 10^{-11} M). On the other hand, 1:1 ZnL^1–phosphate complex **2** is thermodynamically stable with dissociation constants, K_d { $= [\mathbf{1}]_{free}[phosphate]_{free}/[complex\ \mathbf{2}]$ (M)}, of submillimolar order and kinetically rather labile (Scheme 1).[6i,7] We therefore postulated that a synthetic solid-phase polymer conjugated with Zn^{2+}–cyclen complexes would be a promising tool for the detection and separation of phosphomonoester dianions under high-performance liquid chromatographic (HPLC) conditions. Here, we describe the synthesis of a Zn^{2+}–cyclen-conjugated polymer **4** by copolymerization of a Zn^{2+}–(4-vinylbenzyl)cyclen (**3**) with a cross-linker and separation of phosphates such as nucleoside phosphates (nucleotides) by HPLC using **4** as a stationary phase (Scheme 2).

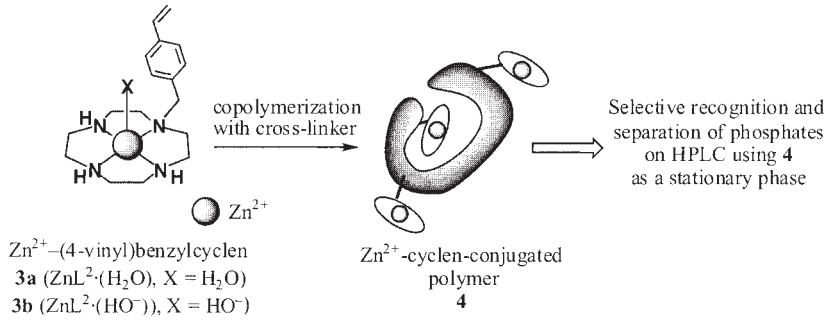
RESULTS AND DISCUSSION

Synthesis of Zn^{2+}–(4-vinyl)benzylcyclen complex (ZnL^2) (3) and Zn^{2+}–cyclen-conjugated polymer (4)

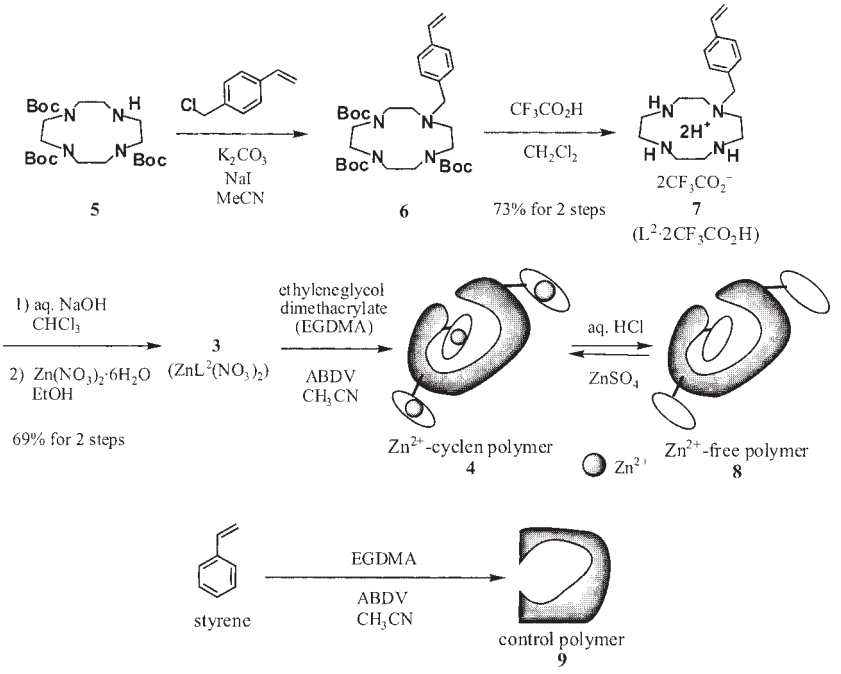
3Boc-cyclen **5**[7f] was reacted with 4-vinylbenzyl chloride to yield **6**, whose three Boc groups were deprotected with TFA to afford L^2 (**7**) as a TFA salt (Scheme 3). After $L^2 \cdot (TFA)_2$ had been neutralized with aqueous NaOH, the free ligand L^2 was treated with $Zn(NO_3)_2 \cdot 6H_2O$ in EtOH to give Zn^{2+}–(4-vinyl)benzylcyclen complex **3** (ZnL^2). Copolymerization of **3** with ethylene glycol dimethacrylate (EGDMA)[3a] in the presence of azobis(4-methoxy-2,4-dimethylvaleronitrile) (ABDV) yielded Zn^{2+}–cyclen-conjugated polymer **4**. The Zn^{2+} ions in **4** were removed in 0.1 M HCl to give Zn^{2+}-free polymer **8**, to which $ZnSO_4$ was reloaded to give Zn^{2+}–cyclen-conjugated polymer **4**. Control polymer **9** was similarly synthesized by copolymerization of styrene with EGDMA in the presence of ABDV.

Complexation constants for the 1:1 complex of 3 with 4-nitrophenyl phosphate (4-NPP) and deoxyadenosine 5′-monophosphate (5′-dAMP) in aqueous solution at neutral pH

A typical potentiometric pH titration curve of 1 mM ZnL^2 (**3**) against 0.1 M NaOH with $I = 0.1$ ($NaNO_3$) at 25 °C is shown in Fig. 1(a) and the titration data were analyzed for according to Eqn (1), where a_{H^+} is the activity of H^+, by using the program BEST.[11] The deprotonation constant of the Zn^{2+}-bound water of ZnL^2 (**3**) (for **3a** $\rightleftharpoons$ **3b** + H^+), pK_a, defined by Eqn (1) was determined as 7.62 ± 0.05. From the titration curves of 1 mM 4-NPP^{2-} + 2 mM HNO_3 [Fig. 1(b)]. The pK_{a1} and pK_{a2} values for 4-NPP, defined by Eqns (2) and (3), are <2 and 5.09 ± 0.05, respectively, at 25 °C with $I = 0.1$ ($NaNO_3$)[7b] and a mixture of 1 mM ZnL^2 (3) and 1 mM NPP^{2-} [Fig. 1(c)] with $I = 0.1$ ($NaNO_3$) at 25 °C, the 1:1 complexation constant of ZnL^2 and 4-NPP^{2-}, $\log K_s(ZnL\text{–}S^{2-})$, defined by Eqns (1)–(4), was determined as 3.7 ± 0.1, from which the 1:1 apparent complexation constant, $\log K_{app}(ZnL\text{–}S^{2-})$, at pH 7.0 and 25 °C defined by Eqn (5)–(7) was calculated as 3.6 ± 0.1 [$K_d(ZnL\text{–}S^{2-}) = 0.25 \pm 0.07$ mM]. These values are considerably larger than the $\log K_s(ZnL\text{–}S^{2-})$ of 3.0 ± 0.1 and $\log K_{app}(ZnL\text{–}S^{2-})$ of 2.9 ± 0.1 at pH 7.0 for the 1:1 ZnL^1–(4-NPP^{2-}) complex,[7b,e,f] which might be attributable to hydrophobic and/or π–π interactions between styryl moiety of ZnL^2 and 4-nitrophenyl group of 4-NPP.[8b,i] The distribution diagram for the five species



Scheme 2



Scheme 3

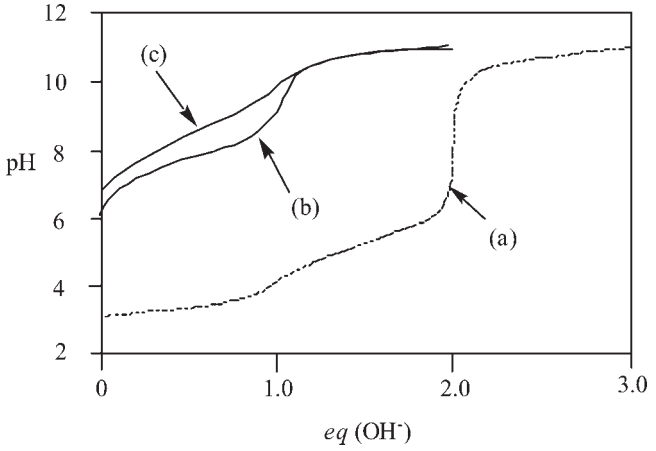


Figure 1. Typical titration curves for (a) 1 mM 4-NPP + 2 mM HNO_3, (b) 1 mM **3** and (c) a mixture of 1 mM **3** + 1 mM 4-NPP at 25 °C with $I = 0.1$ ($NaNO_3$), where *eq*(OH^-) indicates the number of equivalents of base added

(4-NPP^-, 4-NPP^{2-}, $ZnL^2(H_2O)$ (**3a**), $ZnL^2(HO^-)$ (**3b**), and the 1:1 ZnL^2–(4-NPP^{2-}) complex) for a mixture of 1 mM 3 and 1 mM 4-NPP is shown in Fig. 2, in which the 1:1 ZnL^2–(4-NPP^{2-}) complex is formed in 60–63% yield at pH 6.0–7.0. Similarly, $\log K_s(ZnL\text{–}S^{2-})$ for the 1:1 ZnL^1–($5'\text{-dAMP}^{2-}$) complex was determined as 3.5 ± 0.1 and hence $K_d(ZnL\text{–}S^{2-})$ at pH 7.0 was 3.4 ± 0.1 [$K_d(ZnL\text{–}S^{2-}) = 0.40 \pm 0.10$ mM] (for the structure of $5'\text{-dAMP}^{2-}$, see Scheme 4). The pK_{a1} and pK_{a2} values for 5′-dAMP were determined as 3.96 ± 0.05 and 6.25 ± 0.05, respectively, at 25 °C with $I = 0.1$ ($NaNO_3$).

$$\mathbf{3a} \rightleftharpoons \mathbf{3b} + H^+$$
$$K_a = [\mathbf{3b}]a_{H^+}/[\mathbf{3a}] \quad (1)$$

$$SH_2 \rightleftharpoons (SH)^- + H^+ (SH_2 = ROPO_3H_2)$$
$$K_{a1} = [(SH)^-]a_{H^+}/[SH_2] \quad (2)$$

$$(SH)^- \rightleftharpoons S^{2-} + H^+ [(SH)^- = ROPO_3H^-]$$
$$K_{a2} = [S^{2-}]a_{H^+}/[SH] \quad (3)$$

$$ZnL + S^{2-} \rightleftharpoons ZnL - S^{2-}\text{complex}$$
$$K_s[ZnL - S^{2-}] = [ZnL - S^{2-}]/[ZnL][S^{2-}](M^{-1}) \quad (4)$$

$$K_{app}(ZnL - S^{2-}) = [ZnL - S^{2-}]/([ZnL]_{free} \times [\text{uncomplexed S}]_{free}) = 1/K_d(ZnL - S^{2-}) \quad (M^{-1}) \quad (5)$$

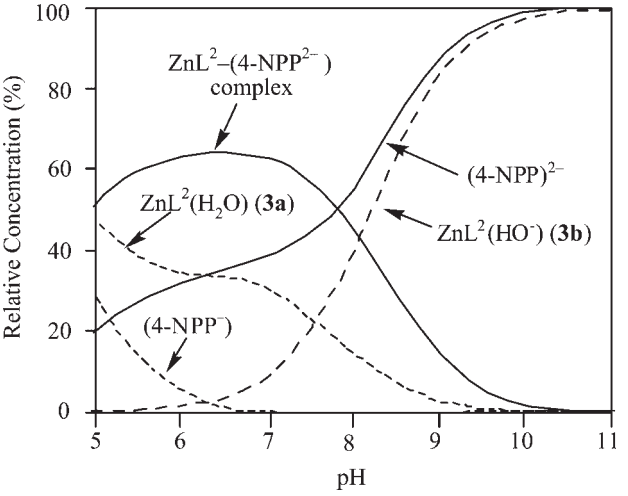
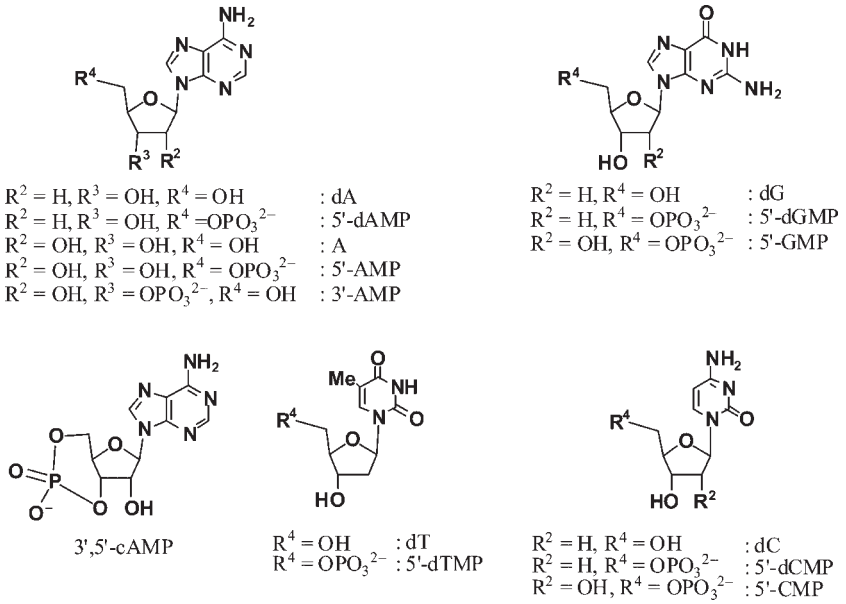


Figure 2. Speciation diagrams for a mixture of 1 mM **3** + 1 mM 4-NPP as a function of pH at 25 °C with $I = 0.1$ ($NaNO_3$). Other species which exist at <5% are omitted



Scheme 4

$$[\mathrm{ZnL}]_{\mathrm{free}} = [\mathrm{ZnL(H_2O)}] + [\mathrm{ZnL(HO^-)}] \qquad (6)$$

$$[\text{uncomplexed S}]_{\mathrm{free}} = [\mathrm{S}] + [\mathrm{S^-}] + [\mathrm{S^{2-}}] \qquad (7)$$

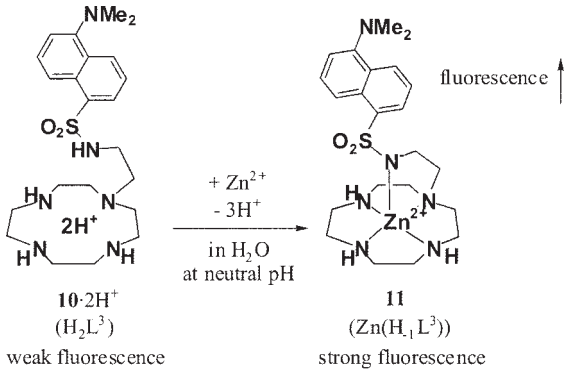
Determination of Zn^{2+} contents in Zn^{2+}–cyclen-conjugated polymer 4 by a Zn^{2+}-selective fluorophore, dansylamidoethylcyclen 10

The contents of Zn^{2+} in synthesized Zn^{2+}–cyclen-conjugated polymer **4** were determined. We previously reported that dansylamidoethylcyclen **10** is a Zn^{2+}-selective fluorophore (Scheme 5).[12,13] The dansylamide deprotonation in Zn^{2+}-complex **11** at neutral pH increased the emission intensity 4.8- fold at 540 nm, while the fluorescence emission intensity of the non-metalated dansylamide deprotonation of L^3 to $H_{-1}L^3$ in the absence of Zn^{2+} at high pH (>12) increased only 1.2-fold. The $K_d[Zn(H_{-1}L^3)]$ value for the 1:1 Zn^{2+}–**10** complex, **11** $[Zn(H_{-1}L^3)]$ is 0.14 nM at pH 7.0,[12a–c] implying that the fluorescence emission of **10** increases linearly upon quantitative complexation with Zn^{2+} at micromolar order concentration in aqueous solution at neutral pH.

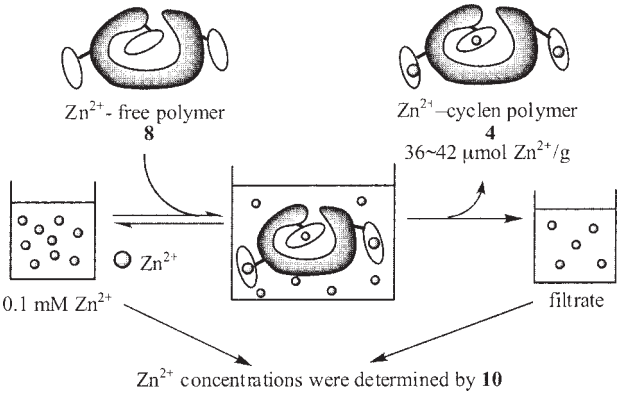
Zn^{2+}-free polymer **8** was added to a solution of 0.3 mM Zn^{2+} in 20 mM HEPES [pH 7.4 with $I = 0.1$ ($NaNO_3$)]. The mixture was stirred for 1 h at 25 °C and an insoluble polymer was filtered off. The concentrations of Zn^{2+} in an aqueous solution before and after the addition of **4** were determined by using dansylamidoethylcyclen **10** (Scheme 6). From the decrease in Zn^{2+} concentrations, the content of Zn^{2+}–cyclen in **4** was determined as 36–42 μmol g^{-1}. For reference, the adsorption of Zn^{2+} in control polymer **9** was negligible.

Complexation properties of Zn^{2+}–cyclen polymer 4 with phosphates in aqueous solution

The adsorption of phosphates, such as 5′-dAMP and 4-NPP, adenosine 3′,5′-cyclic-monophosphate (3′,5′-cAMP), and the corresponding dephosphorylated compounds, deoxyadenosine (dA) and 4-nitrophenol (4-NP),



Scheme 5



Scheme 6

on Zn^{2+}–cyclen polymer **4** was examined (for the structures of these nucleotides, see Scheme 4). A given amount of a Zn^{2+}–cyclen polymer [corresponding to 50–100 μM Zn^{2+} (= 1–2 equiv. against a guest, in a sample solution] was added to a 50 μM phosphate solution in 20 mM HEPES (pH 7.0 with $I = 0.1$ ($NaNO_3$)). After the mixture had been stirred for 1 h at 25 °C, polymer was filtered off. The efficiency of adsorption of a guest on **4** was determined by measuring the decrease in guest concentrations by UV spectrophotometry. As shown in Fig. 3, 5′-dAMP was adsorbed on **4** (1 equiv. Zn^{2+}–cyclen against phosphates) to the extent of 31%, whereas dA was hardly adsorbed (<1%), implying that **4** has a selective affinity with phosphomonoester dianions. From these adsorption efficiencies, the $\log K_{app}(ZnL–S^{2-})$ values for ZnL^2 on **4** with 5′-dAMP was calculated as 4.1 ± 0.1 [$K_d(ZnL–S^{2-}) = 1/K_{app}(ZnL–S^{2-}) = 79 \pm 20$ μM], which is larger than the $\log K_{app}(ZnL–S^{2-})$ value for the 1:1 ZnL^2–(5′-$dAMP^{2-}$) complex of 3.5 [$K_d(ZnL–S^{2-}) = 0.32$ mM) in homogeneous aqueous solution obtained by potentiometric pH titrations. These facts mean that the ZnL–phosphate interaction on the polymer surface is 4–5 times stronger than that in homogeneous aqueous solution, which may be explained by the hydrophobic environments at the solid–liquid interface. The concentration of 5′-dAMP and 4-NPP did not decrease on addition of control polymer **9** and Zn^{2+}-free polymer **8**. The fact that 36% of 4-NP was adsorbed not only on **4** but also on **9** and **8** indicated that less polar compounds tend to be adsorbed non-specifically on a styrene-based copolymer.

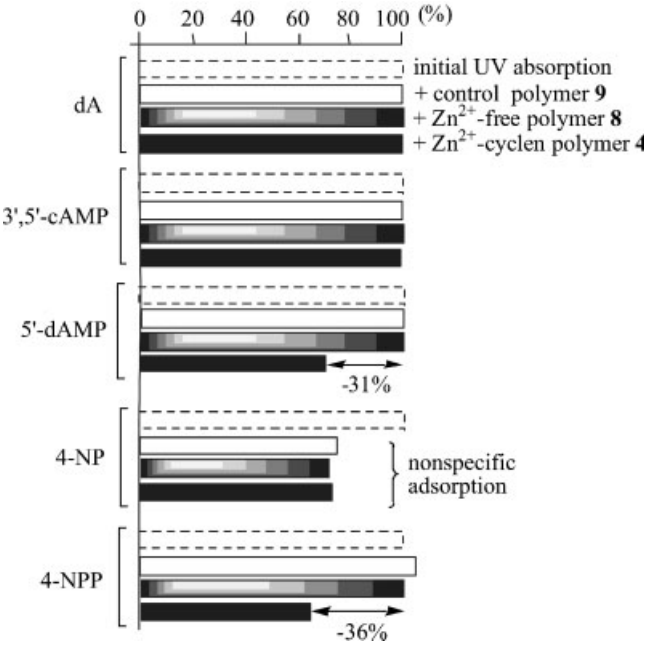


Figure 3. Concentration of a guest (dA, 3′,5′-cAMP, 5′-dAMP, 4-NP and 4-NPP) before addition of a polymer (dashed rectangles), after addition of control polymer **9** (white rectangles), after addition of Zn^{2+}-free polymer **8** (shaded rectangles) and after addition of Zn^{2+}–cyclen polymer **4** (filled rectangles) at pH 7.0 [20 mM HEPES with $I = 0.1$ ($NaNO_3$)] and 25 °C

Figure 2 indicates that the ZnL^2–phosphate complex dissociates at pH > 10. Indeed, adsorbed 4-NPP and 5′-dAMP were recovered almost quantitatively on addition of 20 mM CAPS [pH 10.5 with $I = 0.1$ ($NaNO_3$)] to phosphate-bound **4**. It was confirmed that no decomposition of 4-NPP or 5′-dAMP occurred in the presence of polymers **4**, **8** and **9**.

Separation of nucleotides by HPLC using a Zn^{2+}–cyclen polymer as a stationary phase

HPLC of dA, 5′-dAMP and 3′,5′-cAMP was achieved using Zn^{2+}–cyclen polymer **4**, Zn^{2+}-free polymer **8** and the control polymer **9** as stationary phases. Typical chromatographic separations are displayed in Fig. 4 and retention times for the guests are summarized in Table 1. Continuous gradient elution with 10:90 MeOH–1 mM HEPES (pH 7.0) and 10:90 MeOH–1 mM HEPES + 10 mM $(NH_4)_2HPO_4$ (pH 7.0) was utilized (flow-rate: 2.0 ml^{-1} min, UV detection at 254 nm). Inorganic phosphate is necessary to obtain sharp elution of nucleotides. As expected, the retention time for 5′-AMP was larger than those for dA and 3′,5′-cAMP. The retention times for adenosine 5′-monophosphate (5′-AMP), adenosine 3′-monophosphate (3′-AMP), cytidine 5′-monophosphate (5′-CMP), guanosine 5′-monophosphate (5′-GMP) and thymidine 5′-monophosphate (5′-dTMP) were found to be larger than those for the corresponding nucleosides, dA, A, cytidine (C), guanosine (G), deoxyadenosine (dA) and 3′,5′-cAMP, whose retention times were less than 2–3 min (Table 1). Thymidine (dT) has the largest retention time (11 min) among nucleosides, possibly owing to the formation of ZnL–(dT^-) complex **12** on **4** (Scheme 7).[8] For reference, HPLC using the control polymer **9** [Fig. 4(b)] and the Zn^{2+}-free polymer **8** exhibited immediate (< 4 min) elution of dA, 5′-dAMP and 3′,5′-cAMP [Fig. 4(c)].

The elution profile for 5′-AMP, 5′-GMP, 5′-CMP and 5′-dTMP on a column packed with **4** is depicted in Fig. 4(d). It should be noted that 5′-GMP and 5′-CMP are separated from 5′-AMP, whereas the dissociation constants for ZnL^2–(5′-GMP^{2-}), ZnL^2–(5′-CMP^{2-}) and ZnL^2–(5′-AMP^{2-}) are almost identical (0.5–0.6 mM) at pH 7.0, as confirmed by potentiometric pH titrations. Therefore, the interaction of nucleobase parts with a polymer also affects the retention times of nucleotides. Separation of adenosine-5′-diphosphate (5′-ADP) and adenosine-5′-triphosphate (5′-ATP) was also attempted. However, these two nucleotides were eluted with extreme broadening under the same conditions for the elution of 5′-dAMP or 5′-AMP. This is possibly due to the kinetic inertness of Zn^{2+}–di(tri)phosphate interaction under HPLC conditions. Thymidine-5′-monophosphate (5′-dTMP) has the largest retention time (20.3 min) among

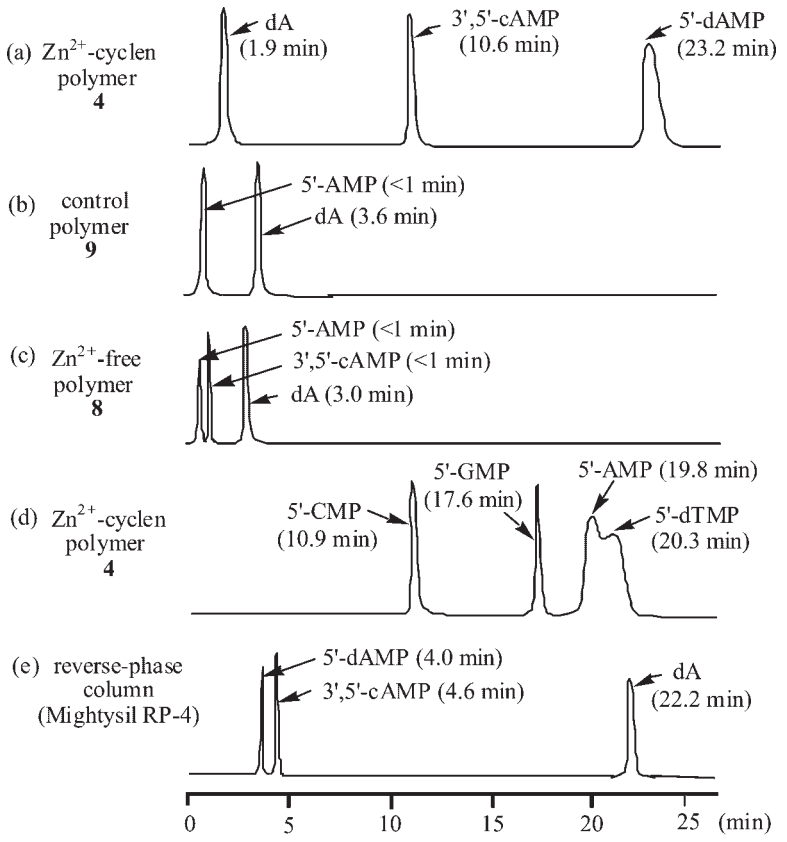


Figure 4. Typical chromatographic separations of mononucleotides on a column packed with (a, d) Zn^{2+}--cyclen polymer **4**, (b) control polymer **9** and (c) Zn^{2+}-free polymer **8** and (e) a commercially available reversed-phase column, Mightysil RP-4. Elution was achieved with continuous gradient elution (0–80% solvent B, 5–25 min, linear) with solvent A [10:90 MeOH–1 mM HEPES (pH 7.0)] and solvent B [10:90 MeOH–1 mM HEPES + 10 mM $(NH_4)_2HPO_4$ (pH 7.0)] (flow-rate 2.0 ml min^{-1}, UV detection at 254 nm) for (a)–(d). For (e), elution was carried out with continuous gradient elution (0–80% solvent C, 5–25 min, linear) with solvent C [1 mM HEPES buffer (pH 7.0)] and solvent D [1:10 MeOH–1 mM HEPES buffer (pH 7.0)] (flow-rate 0.5 ml min^{-1}, UV detection at 254 nm)

Table 1. Retention times (min) of nucleosides and nucleotides in HPLC using control polymer **9**, Zn^{2+}-free polymer **8** and Zn^{2+}–cyclen polymer **4**[a,b]

Analyte	Polymer **9**	Polymer **8**	Polymer **4**
dA	3.6	3.0	1.9
A	2.6	n.d[c]	1.9
5′-dAMP	<1	<1	23.2
5′-AMP	<1	<1	19.8
3′-AMP	<1	<1	18.5
3′,5′-cAMP	<1	<1	10.6
G	1.4	1.1	1.5
5′-GMP	<1	<1	17.6
C	1.0	n.d[c]	<1
5′-CMP	<1	<1	10.9
dT	~1	n.d[c]	11.7
5′-dTMP	<1	<1	20.3

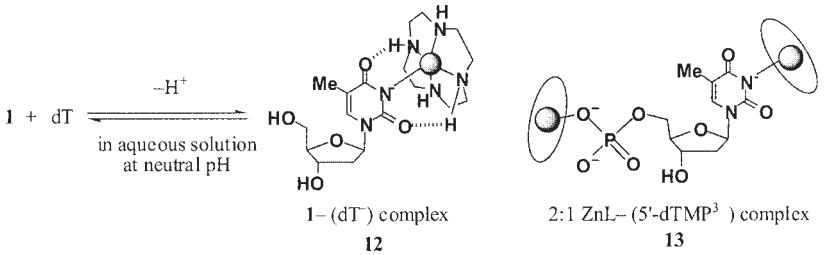
[a] Elution was achieved with continuous gradient elution (0–80% solvent B, 5–25 min, linear) with solvent A [10:90 MeOH–1 mM HEPES (pH 7.0)] and solvent B [10:90 MeOH–1 mM HEPES buffer with 10 mM $(NH_4)_2HPO_4$ (pH 7.0)] (flow-rate 2.0 ml min^{-1}, UV detection at 254 nm).
[b] Errors in retention times are within ± 0.2 min.
[c] Not determined.

the mononucleotides tested, which is attributable to the Zn^{2+}-$ROPO_3^{2-}$ and the Zn^{2+}-(dT^-) interactions, as shown in **13** (Scheme 7).[7g]

For further evaluation of Zn^{2+}–cyclen polymer **4**, HPLC experiments using a commercially available reversed-phase column, Mightysil RP-4, were carried out. Continuous gradient elution with 1 mM HEPES buffer (pH 7.0) and 1:10 MeOH–1 mM HEPES buffer (pH 7.0) was utilized (flow rate: 0.5 ml min^{-1}, UV detection at 254 nm). As displayed in Fig. 4(e), more polar 5′-dAMP was eluted earlier than 3′,5′-dAMP and dA. Hence it is concluded that the mechanism for phosphate separation by **4** is completely different from that for conventional reversed-phase column chromatography.

CONCLUSION

We have developed Zn^{2+}–cyclen-conjugated polymer **4** as a new tool for the detection and separation of mononucleotides. The Zn^{2+} contents in **4** were determined by



Scheme 7

our Zn^{2+} fluorophore **10**. The interactions of **4** with nucleotides were found to be stronger than ZnL^2–nucleotides interactions in homogeneous aqueous solution, suggesting additional hydrophobic interactions on the polymer surface. In HPLC using **4** as a stationary phase, the retention times of mononucleotides were larger than those of the corresponding nucleosides, as expected. This work is a reasonable extension of our Zn^{2+} chemistry in homogeneous aqueous solution to a new chemistry at the solid–liquid interface.

Recently, much attention has been paid to the phosphorylation of proteins and sugars as an intracellular signaling.[1b,1c] Further development of artificial polymers, which discriminate phosphate moieties and other functional groups, will contribute to the development of analytical chemistry, bio-organic chemistry, biochemistry, and medicinal chemistry.

EXPERIMENTAL

General. All reagents and solvents were purchased at the highest commercial quality and used without further purification. Anhydrous acetonitrile (CH_3CN) was obtained by distillation from calcium hydride. All aqueous solutions were prepared using deionized, distilled water. Good's buffer reagents (Dojindo) were commercially available: HEPES [2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid, $pK_a = 7.5$] and CAPS [3-(cyclohexylamino)propanesulfonic acid, $pK_a = 10.4$]. Melting-points were measured on a Yanaco melting-point apparatus and are listed without correlation. UV spectra were recorded on a Hitachi U-3500 spectrophotometer and a Hitachi F-4500 spectrofluorimeter at 25 ± 0.1 °C. IR spectra were recorded on a Horiba FTIR-710 spectrophotometer at room temperature. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra at 35 ± 0.1 °C were recorded on a JEOL Alpha 400 spectrometer. 3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP) sodium salt in D_2O and tetramethylsilane in $CHCl_3$ and CD_3CN were used as internal references for ^1H and ^{13}C NMR measurements. Elemental analyses were performed on a Perkin-Elmer CHN 2400 analyzer. Thin-layer (TLC) and silica gel column chromatography were performed using a Merck 5554 (silica gel) TLC plate and a Fuji Silysia Chemical FL-100D column, respectively. HPLC experiments were performed on a JASCO Gulliver PU-480 system.

*1-(4-Vinylbenzyl)-1,4,7,10-tetraazacyclododecane 2TFA salt (***7**·2TFA). A mixture of 3Boc-cyclen (**5**)[7f] (1.5 g, 3.2 mmol) and 4-vinylbenzyl chloride (0.72 g, 4.7 mmol) in CH_3CN (50 mL) was stirred at 70 °C for 3 h in the presence of K_2CO_3 (0.66 g, 4.78 mmol) and NaI (0.48 g, 3.1 mmol). Insoluble inorganic salts were filtered off and the filtrate was concentrated under reduced pressure. The remaining residue was purified by silica gel chromatography to afford 1-(4-vinylbenzyl)-4,7,10-tris(*tert*-butyloxycarbonyl)-1,4,7,10-tetraazacyclododecane (**6**) as a colorless amorphous solid (1.4 g). Trifluoroacetic acid (10 ml, 0.13 mol) was added dropwise to a solution of **6** (1.4 g, 2.4 mmol) in CH_2Cl_2 (90 ml) at 0 °C and the mixture was stirred overnight at room temperature, then concentrated under reduced pressure. Toluene (10 ml) was added to the remaining residue and evaporated under reduced pressure. The remaining powders were recrystallized from Et_2O–EtOH to give **7**·2TFA as a colorless powder (1.2 g, 73%). M.p. >200 °C; IR (KBr), 3292, 3019, 2854, 2828, 1689, 1556, 1454 1415, 1201, 1124, 825, 798, 717 cm^{-1}; ^1H NMR (D_2O–TSP): δ 2.9–3.0 (m, 8H, CH_2 of cyclen), 3.1–3.4 (m, 8H, CH_2 of cyclen), 3.88 (s, 2H, $ArCH_2$), 5.38 (d, 1H, $J = 11.2$ Hz, $ArCH{=}CH_2$), 5.94 (d, 1H, $J = 20$ Hz, $ArCH{=}CH_2$), 6.85 (dd, 1H, $J = 11.2$, 20 Hz, $ArCH{=}CH_2$), 7.40 (d, 1H, $J = 10.5$ Hz, Ar*H*), 7.59 (d, 1H, $J = 10.5$, 20 Hz, Ar*H*); ^{13}C NMR (CD_3CN–D_2O), δ 42.61, 42.73, 45.02, 48.62, 57.10, 115.75, 127.50, 131.12, 135.64, 136.89, 138.34, 163.59. Anal. Calcd for $C_{21}H_{30}F_6N_4O_4$: C, 48.84; H, 5.85; N, 10.85. Found: C, 48.69; H, 5.80; N, 10.87%.

1-(4-Vinylbenzyl)-1,4,7,10-tetraazacyclododecane Zn-$(NO_3)_2$ salt, [$ZnL^2(NO_3)_2$] (**3**). An aqueous solution of **7**·2TFA (934 mg, 1.8 mmol) was added to 1 M NaOH solution and the solution was extracted with $CHCl_3$ (50 ml, × 5). After the combined organic layers had been dried over anhydrous Na_2SO_4, the solvent was concentrated under reduced pressure to obtain the free ligand **7** as a colorless oil. A solution of $Zn(NO_3)_2 \cdot 6H_2O$ (592 mg, 2.0 mmol) in EtOH (10 ml) was added to an EtOH (10 ml) solution of acid-free **7** at 60 °C and the mixture was stirred for 1 h. After the solvent had been cooled and evaporated, the remaining solid was

crystallized from EtOH–H_2O to obtain $ZnL^2\cdot(NO_3)_2$ (**3**) (602 mg, 69% yield) as colorless powder. M.p. > 200 °C; IR (KBr), 3430, 3256, 3239, 2928, 1628, 1510, 1385, 1296, 1092, 976, 855, 826, 750 cm^{-1}. ^1H NMR (CD_3CN), δ 2.67–2.73 (m, 2H, CH_2 of cyclen), 2.77–2.90 (m, 8H, CH_2 of cyclen), 2.97–3.03 (m, 4H, CH_2 of cyclen), 3.19–3.27 (m, 2H, CH_2 of cyclen), 4.00 (s, 2H, $ArCH_2$), 5.38 (d, 1H, $J = 11.0$ Hz, $ArCH{=}CH_2$), 5.90 (d, 1H, $J = 17.7$ Hz, $ArCH{=}CH_2$), 6.83 (dd, 1H, $J = 11.0$, 17.7 Hz, $ArCH{=}CH_2$), 7.37 (d, 1H, $J = 8.2$ Hz, Ar*H*), 7.56 (d, 1H, $J = 8.2$ Hz, Ar*H*); ^{13}C NMR (D_2O–CD_3CN), δ 44.93, 45.05, 46.47, 46.49, 47.29, 51.98, 118.02, 129.11, 133.83, 134.39, 138.87, 140.74. Anal. Calcd for $C_{17}H_{28}N_6O_6Zn$: C, 42.73; H, 5.91; N, 17.59. Found: C, 42.39; H, 5.92; N, 17.55%.

Zn^{2+}–cyclen polymer (**4**), Zn^{2+}-free polymer (**8**), and control polymer (**9**). A solution of $ZnL^2\cdot(NO_3)_2$ (**3**) (176 mg, 0.37 mmol), EGDMA (6.4 g, 32 mmol, distilled under reduced pressure immediately before use) and ABDV (0.24 g, 0.96 mmol) in CH_3CN was heated at 50 °C for 12 h in a test-tube, which was put in a shaker bath. The resulting colorless polymer mass was released by breaking the test-tube, ground to a fine powder, passed through a testing sieve (Nonaka Rikaki, Japan) collecting particles of 36–63 μm in diameter, washed with CH_3CN and dried at room temperature *in vacuo*. The obtained polymers were treated with 0.1 M HCl to remove Zn^{2+}, filtered, washed with water and dried under reduced pressure. Zn^{2+}-free polymer **8** was reloaded with 10 mM $ZnSO_4$ (20 ml) for 1 day, washed with water, and dried at room temperature *in vacuo*. The obtained powder of Zn^{2+}–cyclen polymer **4** was suspended in CH_3CN and packed in a stainless-steel column (100 × 4.6 mm i.d.) (purchased from GL Science, Japan). The **4**-loaded column was washed with CH_3CN and used for HPLC experiments. Control polymer **9** was obtained by copolymerization of styrene (19 mg, 0.19 mmol), EGDMA (3.2 g, 16 mmol), and ABDV (120 mg, 0.48 mmol) under identical conditions.

Potentiometric pH titrations. The preparation of the test solutions and the calibration method for the electrode system (Potentiometric Automatic Titrator AT-400 and Auto Piston Buret APB-410, Kyoto Electronics Manufacturing, with Orion Research Ross Combination pH Electrode 8102BN) were described earlier.[7] All the test solutions (50 ml) were kept under an argon (> 99.999% purity) atmosphere. The potentiometric pH titrations were carried out with $I = 0.10$ ($NaNO_3$) at 25.0 ± 0.1 °C and at least two independent titrations were performed (0.1 M NaOH was used as the base). The deprotonation constants of Zn^{2+}-bound water K'_2 (= [HO^--bound species][H^+]/[H_2O-bound species]) were determined by means of the program BEST.[11] All the sigma fit values defined in the program were < 0.05. The K_W ($= a_{H^+}\cdot a_{OH^-}$), K'_W ($= [H^+][OH^-]$) and f_{H^+} values used at 25 °C were $10^{-14.00}$, $10^{-13.79}$ and 0.825 respectively. The corresponding mixed constants, K_2 (= [HO^--bound species]a_{H^+}/[H_2O-bound species]), were derived using $[H^+] = a_{H^+}/f_{H^+}$. The species distribution values (%) against pH ($= -\log[H^+] + 0.084$) were obtained using the program SPE.[11]

Determination of Zn^{2+} contents of Zn^{2+}–cyclen-conjugated polymer **4** by fluorescent Zn^{2+} sensor **10**. Fluorescence spectra were recorded on a JASCO FP-6500 spectrofluorimeter at 25.0 ± 0.1 °C. A given aliquot of Zn^{2+}-free polymer **8** was added to 0.3 mM $ZnSO_4$ in 50 mM HEPES [pH 7.4 with $I = 0.1$ ($NaNO_3$)]. The reaction mixture was stirred at 35 °C overnight and insoluble polymer was filtered off. A 50 μl volume of the filtrate was added to 2.5 ml of 10 μM **10**[12a,c] in 20 mM HEPES [pH 7.4 with $I = 0.1$ ($NaNO_3$)] in a cuvette and fluorescent emission spectra were recorded (excitation at 330 nm). Based on the linear calibration curves for the [Zn^{2+}] fluorescence (emission at 540 nm) profile, the contents of the Zn^{2+} ions per gram of **4** were determined.

UV spectra. UV spectra were recorded on a Hitachi U-3500 spectrophotometer at 25.0 ± 0.1 °C. The molar absorption coefficients (ε) ($M^{-1}\cdot cm^{-1}$) of the guest molecules in aqueous HEPES buffer solutions at pH 7.4 with $I = 0.1$ ($NaNO_3$) were as follows: 4-NP, (7.3×10^3 at 310 nm; 4-NPP, 1.0×10^4 at 310 nm; dA, 1.46×10^4 at 258 nm; 5′-dAMP, 1.2×10^4 at 258 nm; and 3′,5′-cAMP, 1.3×10^4 at 258 nm.

HPLC. The HPLC system consisted of two PU-980 intelligent HPLC pumps (JASCO, Japan), a UV-970 intelligent UV–visible detector (JASCO), a Rheodine injector (Model No. 7125) and a Chromatopak C-R6A (Shimadzu, Japan). The separations were carried out at room temperature using a flow-rate of 0.5 ml min. A 2.5–5 μl volume from each sample (5 mg ml^{-1}) was injected into the HPLC system for analysis. The eluent was monitored with a UV-970 UV–visible detector at 254 nm. The column packed with Zn^{2+}–cyclen polymer **4** can be reused after washing with 10 mM HEPES (pH 7.0). For reference, a reversed-phase packed column, Mightysil RP-4 GP 250-4.6 (5 μm) (250 × 4.6 mm i.d.) (Kanto Chemical, Japan), was used.

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REFERENCES

1. (a) Fraústo da Silva JJR, Williams RJP. *The Biological Chemistry of the Elements*. Clarendon Press: Oxford, 1991; (b) Sefton BM, Hunter T. *Protein Phosphorylation*. Academic Press: New York, 1998; (c) *Chem. Rev.* 2001; **101**: issue 8 (Protein Phosphorylation and Signaling).
2. (a) Bianchi A, Bowman-James K, Garcia-Espana E (eds). *Supramolecular Chemistry of Anions*. Wiley-VCH: New York, 1996; (b) Prasanna de Silva A, Gunaratne HQN, Gunnlaugsson T, Huxley AJM, McCoy CP, Rademacher JT, Rice TE. *Chem. Rev.* 1997; **97**: 1515–1566; (c) Seel C, Galan A, de Mendoza J. *Top. Cur. Chem*. 1995; **175**: 101–132; (d) Scheerder J, Engbersen JFJ, Reinhoudt DN. *Recl. Trav. Chim. Pays-Bas* 1996; **115**: 307–320; (e) Sessler JL, Andrievsky A, Genge JW. In *Advances in Supramolecular Chemistry*, vol. 4, Gokel GW (ed). JAI Press: Greenwich, CT, 1997; 97–142; (f) Sessler JL, Gale PA. In *The Porphyrin Handbook*, Kadish KM, Smith KM, Guilard R (eds). Academic Press: San Diego, CA, 2000; (g) Beer PD. *Acc. Chem. Res.* 1998; **31**: 71–80; (h) Beer PD. *J. Chem. Soc., Chem. Commun.* 1996; 689–696; (i) Beer PD, Gale PA. *Angew. Chem., Int. Ed. Engl.* 2001; **40**: 487–516; (j) Wiskur SL, Ait-Haddou H, Anslyn EV, Lavigne JJ. *Acc. Chem. Res.* 2001; **34**: 963–972.
3. (a) Sellergren B (ed). *Molecularly Imprinted Polymers: Man-made Mimics of Antibodies and Their Applications in Analytical Chemistry*. Elsevier: Amsterdam, 2001; (b) Mosbach K. *Trends Biochem. Sci*. 1994; **19**: 9–14; (c) Shea KJ. *Trends Polym. Sci*. 1994; **2**: 166–173; (d) Wulff G. *Angew. Chem., Int. Ed. Engl.* 1995; **34**: 1812–1832; (e) Vidyasanker S, Arnold FH. *Curr. Biol*. 1995; **6**: 218–224; (f) Mosbach K, Ramström O. *Biotechnology* 1996; **14**: 163–171; (g) Takeuchi T, Haginaka J. *J. Chromatogr. B* 1999; **728**: 1–20; (h) Haupt K, Mosbach K. *Chem. Rev.* 2000; **100**: 2495–2504; (i) Asanuma H, Hishiya T, Komiyama M. *Adv. Mater.* 2000; **12**: 1019–1030.
4. (a) Dhal PK, Arnold FH. *Macromolecules* 1992; **25**: 7051–7059; (b) Dhal PK, Arnold FH. *New J. Chem.* 1996; **20**: 695–698; (c) Severin K. *Curr. Opin. Chem. Biol*. 2000; **4**: 710–714.
5. (a) Bünemann H, Dattagupta N, Schuetz HJ, Müller W. *Biochemistry* 1981; **20**: 2864–2874; (b) Shea KJ, Spivak DA, Sellergren B. *J. Am. Chem. Soc*. 1993; **115**: 3368–3369; (c) Iverson BL, Thomas RE, Kral V, Sessler JL. *J. Am. Chem. Soc*. 1994; **116**: 2663–2664; (d) Turkewitsch P, Wandelt B, Darling GD, Powell WS. *Anal. Chem*. 1998; **70**: 2025–2030; (e) Gale PA, Sessler JL, Král V. *J. Chem. Soc., Chem. Commun.* 1998; 1–8.
6. (a) Kimura E. *Prog. Inorg. Chem.* 1994; **41**: 443–491; (b) Kimura E, Koike T. *Adv. Inorg. Chem.* 1997; **44**: 229–261; (c) Kimura E, Koike T. In *Comprehensive Supramolecular Chemistry*, vol. 10, Reinhoudt DN (ed). Pergamon Press: Tokyo, 1996; 429–444; (d) Kimura E, Koike T. *J. Chem. Soc., Chem. Commun*. 1998; 1495–1500; (e) Kimura E, Koike T. In *Bioinorganic Catalysis* Reedijk J, Bouwman E (eds). Marcel Dekker: New York, 1999; 33–54; (f) Kimura E, Kikuta E. *J. Biol. Inorg. Chem.* 2000; **5**: 139–155; (g) Kimura E. *Curr. Opin. Chem. Biol.* 2000; **4**: 207–213; (h) Kimura E. *Acc. Chem. Res.* 2001; **34**: 171–179; (i) Aoki S, Kimura E. *Rev. Mol. Biotechnol.* 2002; **90**: 129–155; (j) Aoki S, Kimura E. In *Comprehensive Coordination Chemistry II*, vol. 8, McCleverty J, Meyer TJ (eds-in-chief). Elsevier Ltd: Oxford, 2004; 601–640.
7. (a) Kimura E, Shiota T, Koike T, Shiro M, Kodama M. *J. Am. Chem. Soc*. 1990; **112**: 5805–5811; (b) Koike T, Kimura E. *J. Am. Chem. Soc*. 1991; **113**: 8935–8941; (c) Koike T, Takamura M, Kimura E. *J. Am. Chem. Soc*. 1994; **116**: 8443–8449; (d) Koike T, Kajitani S, Nakamura I, Kimura E, Shiro M. *J. Am. Chem. Soc*. 1995; **117**: 1210–1219; (e) Fujioka H, Koike T, Yamada N, Kimura E. *Heterocycles* 1996; **42**: 775–787; (f) Kimura E, Aoki S, Koike T, Shiro M. *J. Am. Chem. Soc.* 1997; **119**: 3068–3076; (g) Aoki S, Kimura E. *J. Am. Chem. Soc.* 2000; **122**: 4542–4548; (h) Aoki S, Iwaida K, Hanamoto N, Shiro M, Kimura E. *J. Am. Chem. Soc.* 2002; **124**: 5256–5257.
8. (a) Shionoya M, Kimura E, Shiro M. *J. Am. Chem. Soc.* 1993; **115**: 6730–6737; (b) Shionoya M, Ikeda T, Kimura E, Shiro M. *J. Am. Chem. Soc.* 1994; **116**: 3848–3859; (c) Kimura E, Shionoya M. In *Transition Metals in Supramolecular Chemistry*, Fabbrizzi L, Poggi A (eds). Kluwer: Dordrecht, 245–259; (d) Kimura E, Shionoya M. In *Metal Ions in Biological Systems*, vol. 33, Sigel A, Sigel H (eds). Marcel Dekker: New York, 1996; 29–52; (e) Kimura E, Kikuta E. *J. Biol. Inorg. Chem*. 2000; **5**: 139–155; (f) Kimura E, Kikuta E. *Prog. React. Kinet. Mech.* 2000; **25**: 1–64; (g) Kikuta E, Aoki S, Kimura E. *J. Am. Chem. Soc.* 2001; **123**: 7911–7913; (h) Kikuta E, Aoki S, Kimura E. *J. Biol. Inorg. Chem*. 2002; **7**: 473–482; (i) Kimura E, Katsube N, Koike T, Shiro M, Aoki S. *Supramol. Chem*. 2002; **14**: 95–102.
9. Aoki S, Shiro M, Koike T, Kimura E. *J. Am. Chem. Soc*. 2000; **122**: 576–584.
10. (a) Aoki S, Shiro M, Kimura E. *Chem. Eur. J.* 2002; **8**: 929–939; (b) Aoki S, Zulkefeli M, Shiro M, Kimura E. *Proc. Natl. Acad. Sci. USA* 2002; **99**: 4894–4899.
11. Martell AE, Motekaitis RJ. *Determination and Use of Stability Constants* (2nd edn). VCH: New York, 1992.
12. (a) Koike T, Watanabe T, Aoki S, Kimura E, Shiro M. *J. Am. Chem. Soc*. 1996; **118**: 12696–12703; (b) Kimura E. *S. Afr. J. Chem.* 1997; **50**: 240–248; (c) Kimura E, Aoki S, Kikuta E, Koike T. *Proc. Natl. Acad. Sci. USA* 2003; **100**: 3731–3736; (d) Aoki S, Kaido S, Fujioka H, Kimura E. *Inorg. Chem.* 2003; **42**: 1023–1030; (e) Aoki S, Kawatani H, Goto T, Kimura E, Shiro M. *J. Am. Chem. Soc.* 2001; **123**: 1123–1132.
13. (a) Kimura E, Koike T. *Chem. Soc. Rev.* 1998; **27**: 179–184; (b) Kimura E, Aoki S. *BioMetals* 2001; **14**: 191–204; (c) Kimura E, Takasawa R, Tanuma S, Aoki S. *Science STKE* 2004; pl7.