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Fluorescent and Electrochemical Sensing of Polyphosphate Nucleotides by Ferrocene Functionalised with Two Zn^{II}(TACN)(pyrene) Complexes

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Abstract: The [Fc–bis{Zn^{II}(TACN)(Py)}] complex, comprising two Zn^{II}(TACN) ligands (Fc = ferrocene; Py = pyrene; TACN = 1,4,7-triazacyclononane) bearing fluorescent pyrene chromophores linked by an electrochemically active ferrocene molecule has been synthesised in high yield through a multistep procedure. In the absence of the polyphosphate guest molecules, very weak excimer emission was observed, indicating that the two pyrene-bearing Zn^{II}(TACN) units are arranged in a *trans*-like configuration with respect to the ferrocene bridging unit. Binding of a variety of polyphosphate anionic guests (PPi and nucleotides di- and triphosphate) promotes the interaction between pyrene units and results in an enhancement in excimer emission. Investigations of phosphate binding by ^{31}P NMR spectroscopy, fluorescence and electrochemical techniques confirmed a 1:1 stoichiometry for the binding of PPi and nucleotide polyphosphate anions to the bis(Zn^{II}(TACN)) moiety of [Fc–bis{Zn^{II}(TACN)(Py)}] and indicated that binding induces a *trans* to *cis* configuration rearrangement of the bis(Zn^{II}(TACN)) complexes that is responsible for the enhancement of the pyrene excimer emission. Pyrophosphate was concluded to have the strongest affinity to [Fc–bis{Zn^{II}(TACN)(Py)}] among the anions tested based on a six-fold fluorescence enhancement and 0.1 V negative shift in the potential of the ferrocene/ferrocenium couple. The binding constant for a variety of polyphosphate anions was determined from the change in the intensity of pyrene excimer emission with polyphosphate concentration, measured at 475 nm in CH_3CN/Tris-HCl (1:9) buffer solution (10.0 mM, pH 7.4). These measurements confirmed that pyrophosphate binds more strongly ($K_b = (4.45 \pm 0.41) \times 10^6\ M^{-1}$) than the other nucleotide di- and triphosphates ($K_b = 1–50 \times 10^5\ M^{-1}$) tested.

Keywords: bioinorganic chemistry · electrochemistry · ferrocene derivatives · fluorescence spectroscopy · polyphosphate anions

Introduction

The development of receptors and sensors for physiological anions represents a challenging and topical area of research.[1,2] Phosphate anions, such as the pyrophosphate anion (PPi) and DNA/RNA nucleotides, are an important class of anions that play a central role in physiologically important chemical and biological processes,[3,4] which include transport across membranes, DNA synthesis, cell signalling, energy or electron-transfer processes, kinase-catalysed protein phosphorylation and important cofactors of enzymatic reactions.[5] For example, adenosine triphosphate (ATP) and guanosine triphosphate (GTP) are sources of chemical energy.[6] In the case of ATP, the universal energy source available for cellular functions in living systems, PPi is released in DNA polymerisation to produce adenosine monophosphate (AMP), and also takes part in protein synthesis and DNA replication.[6] The development of receptor-based sensors for biologically important phosphate anions is essential to the understanding of their biological activity.

Given the biological importance of polyphosphate anion species, it is not surprising that a number of chemosensors and receptors for PPi and DNA/RNA nucleotides have been reported.[7] Several polyammomium-based receptors for PPi sensing utilise hydrogen bonding and electrostatic forces.[7b,c,8] Recently, conjugates bearing a fluorescent chro-

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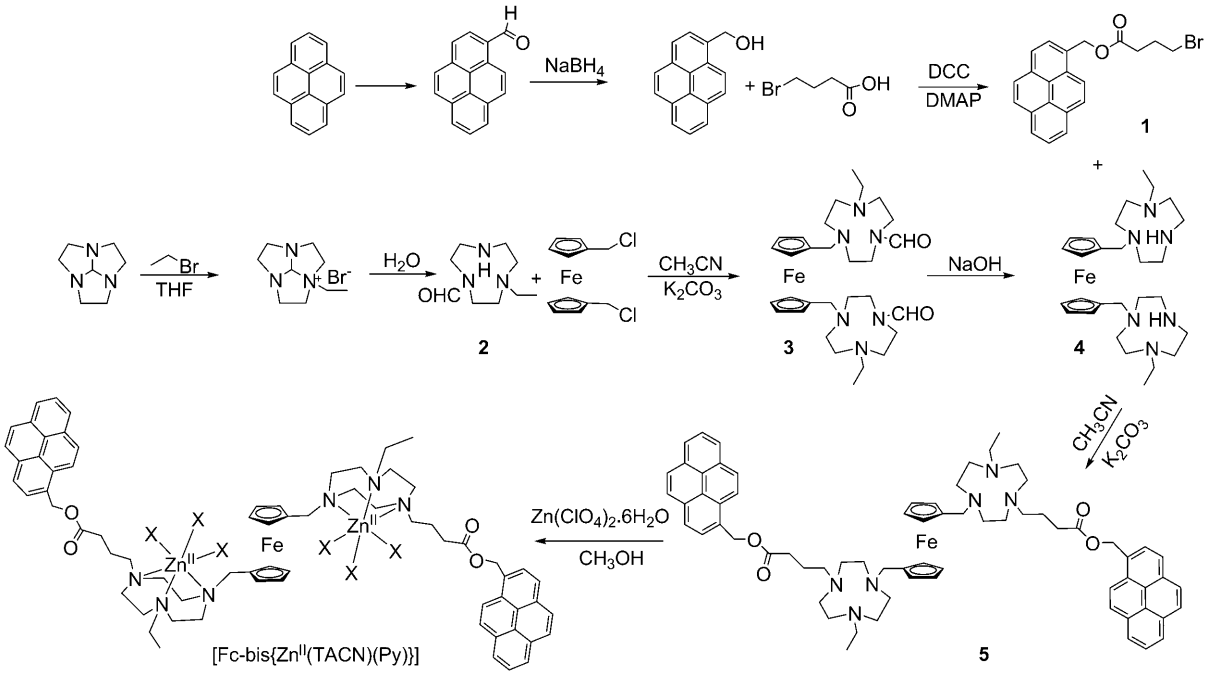
mophores and two di(2-picolyl)-amine (DPA) zinc(II) complexes have been reported, for which strong coordination of PPi and DNA/RNA nucleotides to the bis(Zn^{II}(DPA)) complex units induces fluorescence and UV spectral changes.[9] Yoon and co-workers also developed cationic imidazolium compounds as receptors for phosphate anions that exploit hydrogen bonding and electrostatic interactions between the imidazolium cations and phosphate anions in DNA/RNA nucleotides.[10] Tarraga and Molina reported a triazole-based receptor for PPi that incorporated ferrocene (Fc; electrochemical activity) and pyrene (Py; fluorescence activity). Binding of PPi was accompanied by significant electrochemical and fluorescence changes.[11] However, this sensor could only be used in organic solvents owing to poor solubility in aqueous media.

1,4,7-Triazacyclononane (TACN) is a cyclic organic compound, which coordinates strongly to transition-metal ions through three amine nitrogen donor atoms.[12] Binding of one TACN macrocycle to a metal ion generally leaves one or more coordination sites occupied by weakly binding ligands (e.g., solvent molecules, halides) that can be displaced by ligands with a higher affinity for that particular metal ion. For example, in the case of Cu^{II}(TACN) and Zn^{II}-(TACN) complexes, the phosphate anions in DNA/RNA sequences have been shown to displace such weakly binding ligands.[13] Exploiting these properties and recognising that the application of ferrocene in the electrochemical sensing of anions and DNA is well established,[14] we have developed a new Zn^{II}(TACN) complex, [Fc–bis{Zn^{II}(TACN)-(Py)}] shown in Scheme 1, as a receptor or chemosensor for biological phosphate anions. This receptor is comprised of two pyrene-bearing Zn^{II}(TACN) complexes linked by ferrocene, which can be applied in the two channels (or dual) sensing of biologically important phosphate anions. Detection of PPi and nucleotide di- and triphosphates has been achieved through electrochemistry (ferrocene) and fluorescence emission spectroscopy (pyrene) in aqueous or partially aqueous media.

Results and Discussion

Synthesis of [Fc–bis{Zn^{II}(TACN)(Py)}]: The synthetic route for receptor [Fc–bis{Zn^{II}(TACN)(Py)}] is summarised in Scheme 1. Reaction of tacnorthoamide[15] with bromoethane gave ethyl–TACN–orthoamidinium bromide, which was hydrolysed to give **2**. Compound **3** was obtained by the reaction of **2** with 1,1′-bis(dichloromethyl)ferrocene[16] followed by hydrolysis in base to give **4**. In parallel, 1-pyrenecarboxaldehyde was converted to 1-pyrenemethanol, which was reacted with 4-bromobutyric acid in the presence of dicyclohexylcarbodiimide (DCC) and a catalytic amount dimethylaminopyridine (DMAP) to afford **1**. Reaction of compounds **4** and **1** gave the free ligand **5**. Finally, reaction of **5** with zinc(II) perchlorate gave the zinc(II) complex, [Fc–bis{Zn^{II}-(TACN)(Py)}]. Details of the characterisation of these compounds are provided in the Experimental Section.

Fluorescence study: The complexation of the free ligand, **5**, with Zn^{2+} to form the [Fc–bis{Zn^{II}(TACN)(Py)}] complex was investigated by measuring changes in the fluorescence emission of the pyrene units present in **5** on addition of in-



Scheme 1. Synthetic route used to prepare receptor [Fc–bis{Zn^{II}(TACN)(Py)}].

creasing concentrations of Zn^{2+} (Figure S1 in the Supporting Information). Application of the continuous variation method determined the **5** to Zn^{2+} binding stoichiometry to be 1:2 (Figure S2 in the Supporting Information). Fitting of the variation in pyrene excimer emission with concentration of Zn^{2+} to Equation (1) yielded values of $K_{11}=7.6\times10^{8}\,M^{-1}$ and $\beta_{21}=3.2\times10^{16}\,M^{-2}$ for the apparent stability constants for the formation of the 1:1 and 1:2 **5**:Zn^{II} complexes, respectively (Figure S3 in the Supporting Information). This established that the receptor [Fc–bis{Zn^{II}(TACN)(Py)}] is stable under the conditions chosen for the study of the binding of phosphate anions.

$$F=\{F_0+c_M bK_{11}[M]+F_b\beta_{21}[M]^2\}/\{1+K_{11}[M]+\beta_{21}[M]^2\} \quad (1)$$

In Equation (1), F_0 and F are the fluorescence intensities in the absence and presence of Zn^{2+}, respectively, [M] is the concentration of Zn^{2+}, F_b is the maximum fluorescence intensity, K_{11} and K_{21} are the first and second binding constant, respectively, and $\beta_{21}=K_{11}K_{21}$.

The interaction between the receptor [Fc–bis{Zn^{II}-(TACN)(Py)}] and phosphate anions was investigated by measuring changes in the fluorescence emission of the pyrene units in the absence and presence of various guests. When PPi was added to a CH_3CN/Tris-HCl (1:9) buffer solution (10.0 mM, pH 7.4) containing 1.0 μM [Fc–bis{Zn^{II}-(TACN)(Py)}] (Figure 1), a large fluorescence enhancement

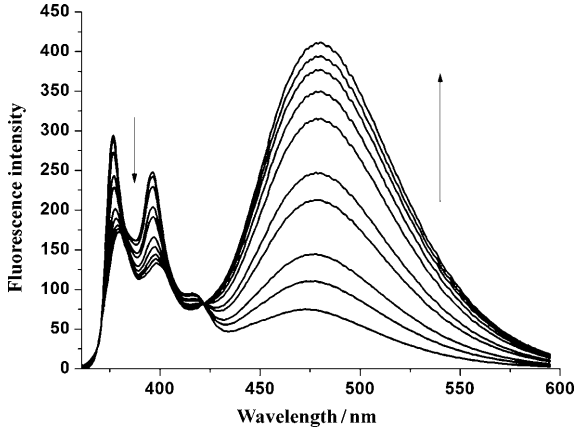


Figure 1. Fluorescence emission spectra of [Fc–bis{Zn^{II}(TACN)(Py)}] (1.0 μM, excitation at 350 nm) recorded upon addition of PPi (0, 0.1, 0.2, 0.4, 0.5, 0.8, 1.0, 1.5, 2.5 and 4.0 equiv) in CH_3CN/Tris-HCl (1:9) buffer solution (10.0 mM; pH 7.4; $T=(20\pm1)$°C).

was observed at 475 nm together with a gradual decrease in the intensity of the emission at 375 nm. This result indicates that binding of PPi forces the two pyrene units in the receptor [Fc–bis{Zn^{II}(TACN)(Py)}] into closer proximity such that interaction between the aromatic rings is promoted and the static pyrene excimer emission at 475 nm is strongly enhanced. As in previous studies applying this fluorophore, we conclude that the fluorescence bands at 475 nm and at 375 nm arise from excimer and monomer emission,[17] respectively. Titration of the receptor with up to 1.0 equivalent of PPi results in a six-fold increase in the intensity of excimer emission together with a 40% decrease in the monomer emission intensity. The continuous variation method was used to determine that the [Fc–bis{Zn^{II}(TACN)(Py)}] to PPi binding stoichiometry was 1:1 (Figure S4 in the Supporting Information). Fitting of the variation in pyrene excimer emission with concentration of PPi (Figure 1) to Equation (2) gave a PPi binding constant (K_b) value of $(4.45\pm0.41)\times10^{6}\,M^{-1}$ (Figure S5 in the Supporting Information).

$$(F-F_0)/F_0=F_bK_b[PPi]/F_0\{1+K_b[PPi]\} \quad (2)$$

In Equation (2), F_0 and F are the fluorescence intensities in the absence and presence of the guest, respectively, and F_b is the maximum fluorescence intensity.

The affinity of the receptor for a variety of other biological inorganic anions and DNA/RNA nucleotides was also investigated by measuring their effect on the excimer emission intensity. Thus, fluorescence titrations were carried out for thymine (T); adenine (A); cytosine (C); guanine (G); thymidine mono-, di- and triphosphate (TMP, TDP, TTP); adenosine mono-, di- and triphosphate (AMP, ADP, ATP); cytidine mono-, di- and triphosphate (CMP, CDP, CTP); and guanosine mono-, di- and triphosphate (GMP, GDP, GTP) and inorganic salts (phosphate (Pi), CH_3COO^- and F^-). For comparison, the fluorescence emission of solutions of [Fc–bis{Zn^{II}(TACN)(Py)}] (1.0 μM) containing 1.0 equivalent of PPi, ATP, ADP, AMP, Pi, CH_3COO^- and F^- is shown in Figure 2. The binding constants were determined by measuring the change in fluorescence with guest concentration and fitting this titration data to Equation (1). The data are summarised in Table 1. Titration data for ATP and ADP, as examples of di-, and triphosphate nucleotides, are shown in Figure S6 in the Supporting Information. In all cases, the

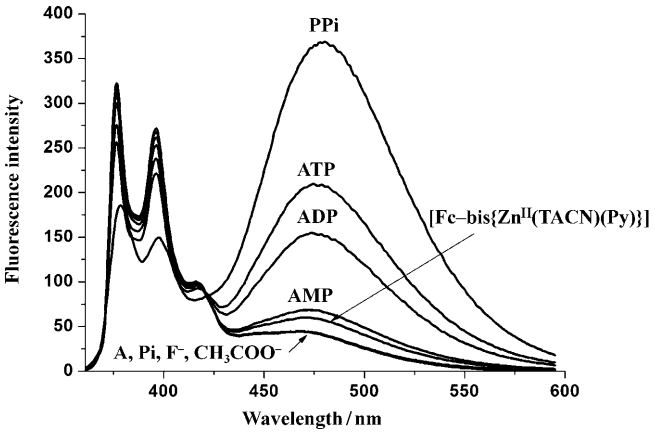


Figure 2. Fluorescence emission spectra of [Fc–bis{Zn^{II}(TACN)(Py)}] (1.0 μM, excitation at 350 nm) measured in a 1:9 mixture of CH_3CN/Tris-HCl buffer (10.0 mM; pH 7.4; $T=(20\pm1)$°C) for solutions containing 1.0 μM of PPi, ATP and ADP, and 100 μM of A, AMP, Pi, CH_3COO^- and F^-.

Table 1. Summary of the binding constants between [Fc–bis{Zn^{II}-(TACN)(Py)}] and various anions.

Anion	K_b [M^{-1}]	Anion	K_b [M^{-1}]
PPi	$(4.45\pm0.41)\times10^6$	TDP	$(2.03\pm0.18)\times10^4$
ATP	$(9.31\pm0.84)\times10^4$	AMP	ND[a]
CTP	$(2.32\pm0.19)\times10^5$	CMP	ND[a]
GTP	$(2.13\pm0.29)\times10^5$	GMP	ND[a]
TTP	$(5.05\pm0.46)\times10^5$	TMP	ND[a]
ADP	$(9.63\pm0.93)\times10^3$	Pi	ND[a]
CDP	$(1.55\pm0.14)\times10^4$	F^-	ND[a]
GDP	$(1.41\pm0.12)\times10^4$	CH_3COO^-	ND[a]

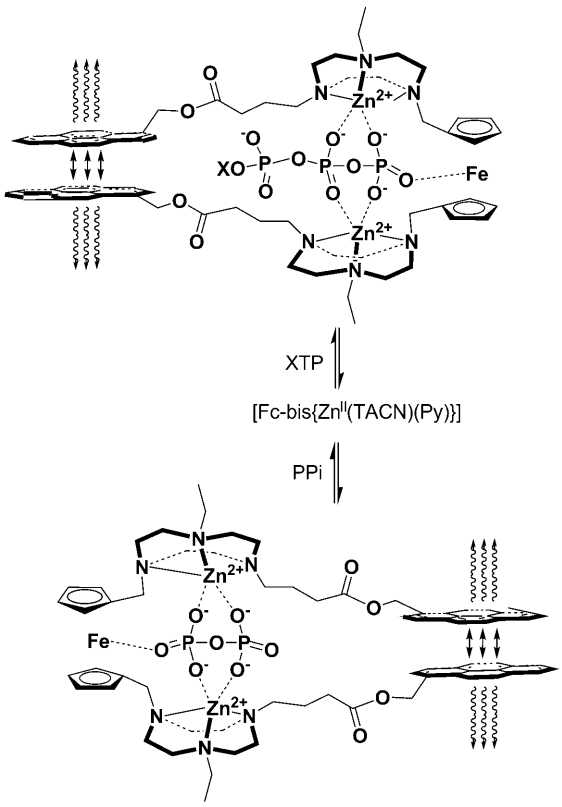
[a] Not determined (ND) owing to the small changes in fluorescence emission upon addition of the analyte.

binding stoichiometry was found to be 1:1 (Figure S4 in the Supporting Information).

The receptor [Fc–bis{Zn^{II}(TACN)(Py)}] shows stronger affinity to triphosphate nucleotides (e.g., $K_b=(9.31\pm0.84)\times10^4\,M^{-1}$ for ATP) than diphosphate nucleotides ($K_b=(9.63\pm0.93)\times10^3\,M^{-1}$ for ADP). The fluorescence change was negligible for the addition of monophosphate nucleotides, the nucleotide bases, Pi, CH_3COO^- and F^-, even when present at a much higher concentrations (100 equiv). The fluorescence results indicate that [Fc–bis{Zn^{II}(TACN)(Py)}] can selectively bind to polyphosphate anions. The largest binding constant was obtained for PPi ($K_b=(4.45\pm0.41)\times10^6\,M^{-1}$), which probably arises from the higher negative charge per phosphorous centre of PPi when compared with di- and triphosphate nucleotides, and is reflected in stronger binding to the 4^+ charged [Fc–bis{Zn^{II}(TACN)(Py)}] receptor. Hong and co-workers also concluded that bis(Zn^{II}(DPA)) complexes exhibit selective binding to PPi over ATP owing to the more negatively charged phosphorous in PPi.[9a,b,d,g]

To account for the enhanced excimer emission in the presence of phosphate anions, it is proposed that the two positively charged bis(Zn^{II}(TACN)) complexes bearing pyrene are arranged in a *trans*-like configuration with respect to the ferrocene bridging unit, to minimise electrostatic repulsion and energy, as was concluded in previous studies that employed a receptor with a pairs of Zn^{II}(cyclen) units to detect TpT.[16b] Thus, in the absence of the polyphosphate the dominant band in the fluorescence spectrum of [Fc–bis{Zn^{II}-(TACN)(Py)}] measured in CH_3CN/Tris-HCl (1:9) buffer solution is from monomer rather than excimer emission. On introduction of PPi, triphosphate or diphosphate nucleotides, the interaction between these polyphosphate anions and bis(Zn^{II}(TACN)) complexes induces a rearrangement of the two Zn^{II}(TACN) complexes bearing two pyrene units from a *trans* conformation (which minimises electrostatic repulsion), to a *cis* conformation. This rearrangement brings the pyrene units into much closer proximity, leading to strong excimer emission (static) and quenching of monomer emission, on the basis of the modified metal–anion coordination interaction (Scheme 2).

After it was established that [Fc–bis(Zn^{II}(TACN)(Py))] binds more strongly to PPi than to mono-, di-, or triphosphate nucleotides and other anions, competition experiments were carried out to quantify the selectivity level of the receptor for PPi. As shown in Figure 3, the addition of 100 equivalents of inorganic anions and monophosphate nucleotides and 1.0 equivalent of di- and triphosphate nucleotides does not interfere with PPi binding to the receptor [Fc–bis{Zn^{II}(TACN)(Py)}]. In contrast, addition of 10 equiv-



Scheme 2. Proposed mode of binding between [Fc–bis{Zn^{II}(TACN)(Py)}] and polyphosphate anions (PPi and XTP (X = A, T, C or G)).

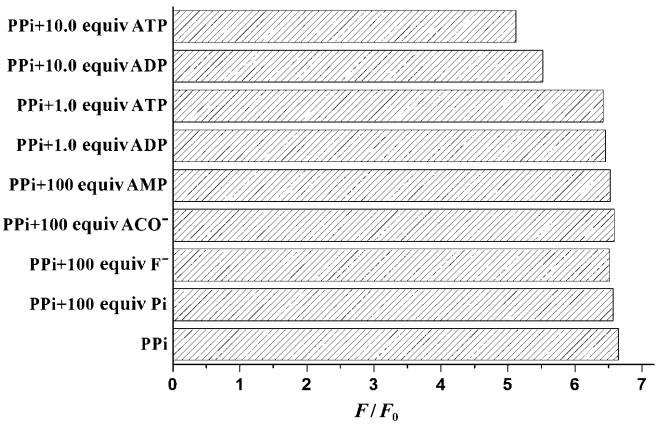


Figure 3. Fluorescence change (F/F_0) at 475 nm for [Fc–bis{Zn^{II}-(TACN)(Py)}] (1.0 μM, excitation at 350 nm) on addition of 1.0 μM of PPi and mixtures containing 1.0 μM of PPi and 100.0 μM of either Pi, F^-, AcO^- or AMP, 1.0 μM of either ADP or ATP; and 10.0 μM of either ADP or ATP in CH_3CN/Tris-HCl (1:9) buffer solution (10.0 mM; pH 7.4; $T=(20\pm1)$ °C). F_0 and F are the fluorescence intensities in the absence and presence of the guest/guest mixture, respectively.

alents of ATP and ADP caused a decrease in the fluorescence by 20 and 15%, respectively, indicating that these polyphosphates can compete effectively with PPi for the binding site on [Fc–bis{Zn^{II}(TACN)(Py)}].

^{31}P NMR study: To gain further insight into the mechanism of binding of the phosphate anions to [Fc–bis{Zn^{II}-(TACN)(Py)}], ^{31}P NMR spectroscopy was used to investigate the interaction between bis(Zn^{II}(TACN)) complexes and phosphate anions in CD_3CN/Tris-HCl (1:9) buffer solution (10.0 mM; pD = 7.6; $T = (20 \pm 1)$ °C) with the addition of 85% H_3PO_4 as a reference.[18] In these experiments, changes in the ^{31}P NMR spectrum of PPi, ATP, ADP and AMP were recorded on addition of the [Fc–bis{Zn^{II}(TACN)(Py)}] receptor (from 0.1 to 4.0 equiv) as shown in Figure S7 in the Supporting Information. The ^{31}P NMR chemical shift change for PPi became 2.5 ppm upon addition of 1.0 equivalent of [Fc–bis{Zn^{II}(TACN)(Py)}], whereas for ATP the chemical shift changes for the P_γ, P_β, and P_α centres were found to be 1.4, 1.1 and 0.18 ppm, respectively. For the P_β, and P_α centres in ADP, the respective changes were 0.9 and 0.37 ppm in the presence of same amount receptor and, interestingly, the change was negligible for the signal of the P centre in AMP (Figure 4d). Thus, the ^{31}P NMR chemical shift changes indicate the receptor [Fc–bis{Zn^{II}(TACN)-(Py)}] shows the highest affinity to PPi among the phosphate anions tested, which is consistent with the fluorescence experiment. Analysis of the change in ^{31}P NMR chemical shift as a function of added receptor confirmed 1:1 binding stoichiometry (Figure 4), which was proposed from the fluorescence emission studies.

In the case of ATP, the chemical shift change in the inner P_α is very small (0.2 ppm) compared with P_γ and P_β (1.4 and 1.1 ppm, respectively). This implies that the two Zn^{II}-(TACN) units mainly bind to the outer and middle phosphorous, P_γ and P_β, and lends support to our proposed binding mode between [Fc–bis{Zn^{II}(TACN)(Py)}] and ATP, shown in Scheme 2. Hong and co-workers proposed a similar binding mode for the bis(Zn^{II}(DPA)) complexes and ATP.[9a,b,d,g] The bigger change in the chemical shift for the outer phosphorous than for the middle (ATP) and inner (ADP), indicates that bis(Zn^{II}(TACN)) complexes bind more strongly to the phosphate with the greater negative charge (2^- when fully deprotonated) than the inner phosphate (1^-). Given

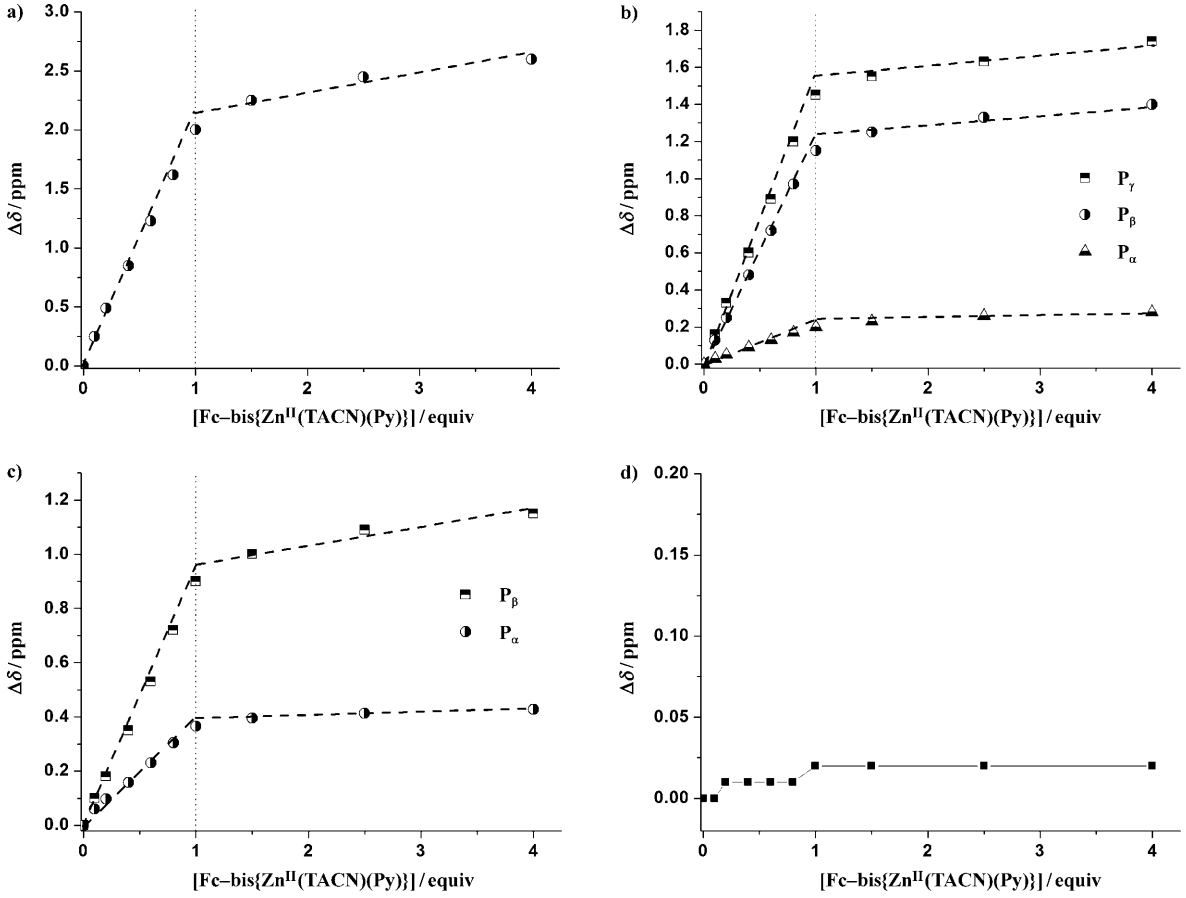
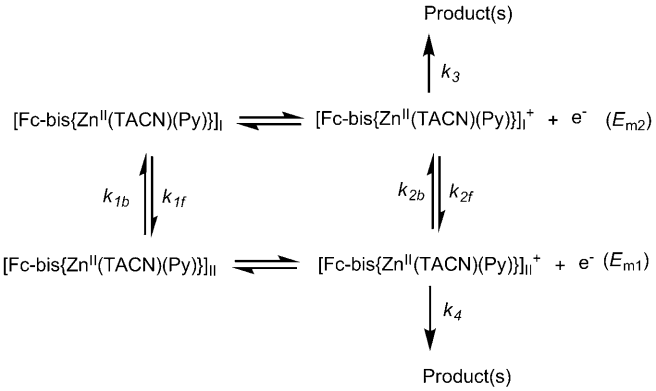


Figure 4. Variation in the ^{31}P NMR chemical shift of PPi, ATP, ADP and AMP (1.0 mM) upon addition of [Fc–bis{Zn^{II}(TACN)(Py)}] (from 0.1 to 4.0 equiv) in a mixture of CD_3CN and Tris-HCl D_2O buffer (1:9) solution (10.0 mM; pD = 7.6; $T = (20 \pm 1)$ °C). a) PPi; b) ATP; c) ADP; d) AMP measured with H_3PO_4 (85% in D_2O) as the reference.

that PPi has a negative charge of 4^- when fully deprotonated, it would be anticipated to bind more strongly to the [Fc–bis{Zn^{II}(TACN)(Py)}] receptor. On addition of the receptor to PPi, broadening of the ^{31}P NMR resonance is observed (see Figure S7 in the Supporting Information), whereas on addition of ATP, the ^{31}P NMR signal of the outer phosphorous atom broadens. This is likely to be a consequence of slow exchange between free and bound polyphosphate and/or slow structural rearrangement between different conformations of the receptor:polyphosphate adduct (slow dynamic equilibrium on the NMR timescale).

Electrochemistry of [Fc–bis{Zn^{II}(TACN)(Py)}]: Because a redox-active molecule, Fc, is present as the linker, it is possible to probe the electrochemical behaviour of ferrocene/ferrocenium couple in [Fc–bis{Zn^{II}(TACN)(Py)}] and to explore the influence of the bis(Zn^{II}(TACN)) binding agent. Typically, a Fc centre undergoes a chemically and electrochemically reversible one-electron oxidation process to give a ferrocenium (Fc^+) centre. As Figure 5 shows, the addition of Zn^{II}(TACN)(Py) to Fc to give [Fc–bis{Zn^{II}(TACN)(Py)}] decreases the stability of the Fc^+ centre formed on oxidation and leads to a substantial increase in the complexity of the voltammetry. Examination of cyclic voltammograms obtained as a function of scan rate over the range 0.02 to 1.00 $V s^{-1}$ in both CH_3CN/Tris-HCl (1:9) and CH_3CN/HEPES (1:9) buffer solutions (50.0 mM; pH 7.4) at (20±1) °C, revealed a clear dependence on scan rate. Thus, a single nearly chemically reversible diffusion-controlled redox process, with a mid-point potential (E_m) of 0.45 V versus Ag/AgCl (in NaCl, 3 M) is observed at low scan rates. However, a new process emerges at potentials that are almost 0.10 V more positive when the scan rate is increased up to 1.0 $V s^{-1}$. These observations imply the existence of a square-type mechanism outlined in Scheme 3, which interrelates four chemical forms ([Fc–bis{Zn^{II}(TACN)(Py)}]$_I$/[Fc–bis{Zn^{II}(TACN)(Py)}]$_{II}$ and [Fc–bis{Zn^{II}(TACN)(Py)}]$_I^+$/[Fc–bis{Zn^{II}(TACN)(Py)}]$_{II}^+$, two closely related conformers or other structural forms in two oxidation states). The scan rate data also indicate that chemical reactions are linked to the electrochemically generated [Fc–bis{Zn^{II}(TACN)(Py)}]$_I^+$ and [Fc–bis{Zn^{II}(TACN)(Py)}]$_{II}^+$ species, also as shown in Scheme 3.



Scheme 3.

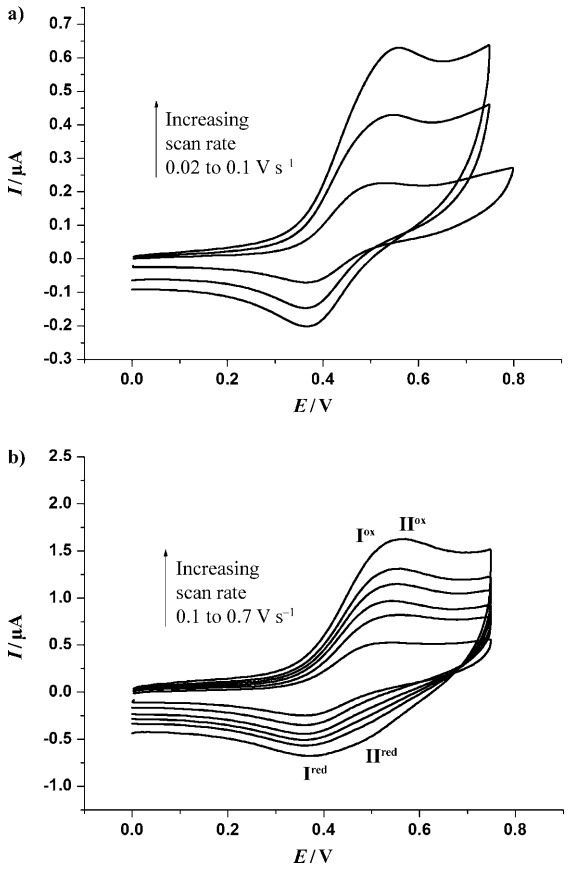


Figure 5. Cyclic voltammograms obtained at a glassy carbon electrode (1 mm diameter) for oxidation of 1.00 mM of [Fc–bis{Zn^{II}(TACN)(Py)}] in CH_3CN/Tris-HCl (1:9) buffer solution (50.0 mM; pH 7.4; T=(20±1) °C) at scan rates of a) 0.02, 0.05, 0.10 and 0.20 $V s^{-1}$; and b) 0.1, 0.2, 0.3, 0.4, 0.5 and 0.7 $V s^{-1}$. Potentials are versus Ag/AgCl (NaCl, 3 M).

At very slow scan rates (Figure 5a) equilibrium conditions are almost maintained for the [Fc–bis{Zn^{II}(TACN)-(Py)}]$_I$/[Fc–bis{Zn^{II}(TACN)(Py)}]$_{II}$ and [Fc–bis{Zn^{II}(TACN)-(Py)}]$_I^+$/[Fc–bis{Zn^{II}(TACN)(Py)}]$_{II}^+$ systems, because only a single process is observed. At high scan rates (Figure 5b), in which two oxidation and two reduction processes are observable, approximate E_{m1} and E_{m2} values can be obtained, whereas at low scan rates an average E_m value is found. This process, which is split in to two processes under short timescales, was also found under conditions of square wave voltammetry (SWV, Figure S8 in the Supporting Information). The proposal of a square scheme is based on an analogy with previously studied systems that encompass redox-dependent isomerisation, ligand exchange and protonation.[19]

The oxidation of [Fc–bis{Zn^{II}(TACN)(Py)}] at a GC rotating-disc electrode (RDE) at a scan rate of 0.02 $V s^{-1}$ and a rotation speed (f) of 100 rpm produced sigmoidal-shaped I/E curves. By using the Levich equation,[20] a diffusion coef-

ficient of $4.22\times10^{-6}\,cm^2\,s^{-1}$ was estimated for [Fc–bis{Zn^{II}(TACN)(Py)}]. However, a plot of $\log[I/(I_{lim}-I)]$ versus potential did not yield the straight lines predicted for a single-electron transfer step, again consistent with the presence of a complex reaction of the kind given in Scheme 3.

A titration experiment with PPi was conducted by the higher resolution SWV method (Figures 6 and S9 in the Supporting Information). A significant shift in the oxidation

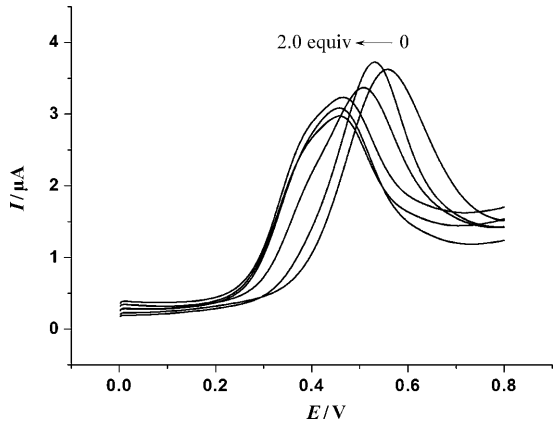


Figure 6. Square-wave voltammograms of 0.5 mM [Fc–bis{Zn^{II}(TACN)(Py)}] in the presence of 0.0, 0.25, 0.50, 0.80, 1.0, and 2.0 equivalents of PPi in CH_3CN/Tris-HCl (1:9) buffer solution (50.0 mM; pH 7.4; $T=(20\pm1)$ °C). Potentials are versus Ag/AgCl (NaCl, 3 M). The working electrode is a 1 mm diameter GC-disc electrode. SWV parameters: amplitude 25 mV, frequency = 100 Hz, $\Delta E=2$ mV.

peak potential (ΔE_p) of [Fc–bis{Zn^{II}(TACN)(Py)}] of about 60 and 100 mV towards less positive values was found at frequencies of 15 and 100 Hz, respectively, as the concentration of PPi increases from zero to 1.0 equivalent. The dependence of the ΔE_p values on frequency is related to differences in the time scale, and therefore to the level of interaction of the [Fc–bis{Zn^{II}(TACN)(Py)}] and [Fc–bis{Zn^{II}(TACN)(Py)}]$^+$ systems with PPi, in an analogous manner to that discussed in related work.[16b] Importantly, changes in ΔE_p when additional PPi is added beyond the 1.0 equivalence are negligible. This infers the binding stoichiometries between [Fc–bis{Zn^{II}(TACN)(Py)}] and the oxidised form with PPi are both 1:1 (Figure S9 in the Supporting Information). Hence, this result provides data for the oxidised form, not available with fluorescence results in which only [Fc–bis{Zn^{II}(TACN)(Py)}] in the reduced form can interact with PPi.

Consistent with fluorescence titration data, the change of peak potential is smaller (<20 mV) when single bases and monophosphate nucleotides are added to [Fc–bis{Zn^{II}(TACN)(Py)}]. However, titration with di- and triphosphate nucleotides induces a ≥0.05 V shift to less positive values. The maximum negative shift found for PPi indicates that [Fc–bis{Zn^{II}(TACN)(Py)}] has a stronger affinity for PPi relative to other phosphate anions (Figure 7). As mentioned

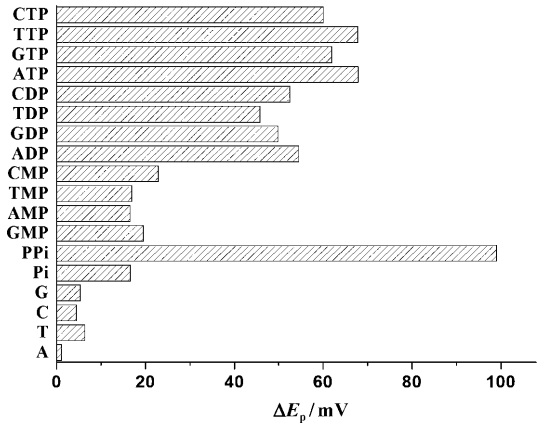


Figure 7. Peak potential shifts obtained by SWV for the oxidation of 0.5 mM of [Fc–bis{Zn^{II}(TACN)(Py)}] in CH_3CN/Tris-HCl (1:9) buffer solution (50.0 mM; pH 7.4; $T=(20\pm1)$ °C) in the absence ($E_p=0$ mV) and presence of 0.5 mM designated bases, nucleotides, Pi and PPi. SWV parameters: amplitude 25 mV, frequency = 100 Hz, $\Delta E=2$ mV.

above, PPi is negatively charged, and shows the strongest binding properties to the positively charged receptor [Fc–bis{Zn^{II}(TACN)(Py)}] in its oxidised or reduced forms. As the adduct is formed, the charge on the receptor is lowered and the configuration changes from *trans* to *cis*, which alters the electron density on the Fc/Fc^+ linker.[16b] As indicated above, the NMR spectra show evidence of line broadening, which is consistent with the existence of a dynamic equilibrium. As can be seen in Figure 6, this equilibrium is also reflected in the electrochemistry, in which concomitantly with the potential shift, a second process is detected at about 0.07 V more negative, the peak current value of which increases as the ratio between [Fc–bis{Zn^{II}(TACN)(Py)}] and PPi approaches 1:1. This complexity could be associated with a different conformational form of the same adduct. Nucleotide polyphosphates can also induce configuration changes, but presumably not as readily because a smaller potential shift for oxidation of [Fc–bis{Zn^{II}(TACN)(Py)}] is observed relative to that induced by PPi.

Conclusion

A receptor consisting of two pyrene-bearing Zn^{II}(TACN) complexes linked by ferrocene, [Fc–bis{Zn^{II}(TACN)(Py)}], has been developed, which combines an electrochemically active group with an off–on fluorescent chromophore, and shows selectivity for biological polyphosphate anions. Fluorescence, electrochemistry and ^{31}P NMR spectroscopy established that PPi binds most strongly to [Fc–bis{Zn^{II}(TACN)(Py)}] amongst the polyphosphate anions examined, due to a higher negative charge density when compared with the nucleotide di- or triphosphate anions. The binding of the polyphosphate anions to the [Fc–bis{Zn^{II}(TACN)(Py)}] receptor is proposed to force the bis(Zn^{II}(TACN)) units to same side of the ferrocene unit, giving rise to significant

changes in both electrochemical behaviour of the Fc/Fc^+ centre (≈ 0.1 V), due to major disruption in the structure, and off–on fluorescence enhancement of pyrene excimer emission induced by $\pi\cdots\pi$ stacking of the two aromatic pyrenes.

Experimental Section

Materials: Unless otherwise specified, reagents and chemicals were purchased from commercial sources and were used without further purification. In the synthetic work, the solvents were used as received or dried over 4 Å molecular sieves. Deionised water was distilled prior to use. Tetrabutylammonium hexafluorophosphate (Bu_4NPF_6, Fluka) was recrystallised from ethanol prior to use as the electrolyte in electrochemical studies. Tris-HCl buffer solutions (pH 7.4; 10.0 and 50.0 mM) were prepared by dissolving tris(hydroxymethyl)aminomethane (Tris base) in distilled water (500 mL) followed by titration to pH 7.4 with HCl (1.0 M) solution, measured by using a pH meter (HANNA instrument pH 211) at (20 ± 1) °C, and adjustment of the total solution volume to 1000 mL in a volumetric flask. ^{31}P NMR titration experiments were carried out in Tris-HCl D_2O buffer solution (10.0 mM, pD = 7.6, (20 ± 1) °C), in which the pD value was determined to pH meter value plus 0.4. The HEPES buffer solution (pH 7.4; 50.0 mM) was prepared by dissolution of HEPES in distilled water (500 mL) and titrated to pH 7.4, by using NaOH (0.1 M) solution at (20 ± 1) °C, and then diluted with distilled water to 1000 mL in a volumetric flask.

Instrumentation and methods: ^1H NMR and ^{13}C NMR spectra were recorded in deuterated solvents by using an Avance DRX400 Bruker spectrometer at 30 °C. The chemical shifts are reported in ppm (parts per million). Tetramethylsilane (TMS) or the residual solvent peaks have been used as an internal reference. The abbreviations for the peak multiplicities are as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet) and br (broad). Infrared spectra were recorded a Perkin–Elmer 1600 series FTIR spectrophotometer at 4 cm^{-1} resolution on samples doped in KBr pellets. CHN analyses were performed by the Campbell Microanalytical Services, University of Otago, Dunedin (New Zealand). Low-resolution electrospray mass spectra were recorded with a Micromass Platform II Quadrupole mass spectrometer fitted with an electrospray source. Samples were introduced by a syringe pump at a rate of 1 L min^{-1} and the capillary voltage was at 200 V. Thin layer chromatography (TLC) was performed by using silica gel 60 F-254 (Merck) plates and basic alumina followed by preparative column chromatography on silica gel and alumina. Fluorescence titration experiments were carried out in Tris-HCl buffer solution (10.0 mM, pH 7.4, (20 ± 1) °C). Fluorescence spectra were recorded with a Varian Cary Eclipse fluorescence spectrophotometer, with excitation and emission slit widths of 2.5 mm.

Electrochemical Studies: Electrochemical experiments were carried out at (20 ± 1) °C in a standard three-electrode cell arrangement with a BAS 100B electrochemical workstation (Bioanalytical Systems, West Lafayette, IN, USA). A platinum wire auxiliary electrode, and an aqueous Ag/AgCl (NaCl, 3 M) reference electrode were used for studies in the mixed solvent CH_3CN/Tris-HCl (50.0 mM; 1:9) and CH_3CN/HEPES (50.0 mM; 1:9) buffer solutions (pH 7.4). A glassy carbon (GC) working electrode with a diameter of 1.0 mm and an area of 0.72 mm^2 was used for the cyclic voltammetric experiments. Rotating-disc experiments employed a GC working electrode (3.0 mm diameter, 7.10 mm^2 effective area) and were performed with a BAS RDE-2 accessory. The area or radii of the voltammetric working electrodes was determined from cyclic voltammograms by using the peak current from the oxidation of a 1.0 mM Fc solution (diffusion coefficient of 2.3×10^{-5} cm^2s^{-1}) in CH_3CN (Bu_4NPF_6, 0.1 M) and with the application of the Randles–Sevcik equation. The area of the rotating-disc electrode was similarly determined by using the Levich equation. Prior to each voltammetric experiment, the working electrode was polished with 0.3 μm alumina (Buehler, Lake Bluff, IL) on a clean polishing cloth (Buehler), sequentially rinsed with distilled water and acetone, and then dried with lint-free tissue paper. The solutions used for voltammetric measurement were purged with solvent-saturated nitrogen gas before each experiment and were blanketed with nitrogen gas during the experiment.

Synthesis

1-Pyrenecarboxaldehyde: Pyrene (2.1 g, 10 mmol) was added to a mixture of $POCl_3$ and *N*-methylformanilide at room temperature. The solution was heated to 100 °C under an atmosphere of nitrogen for 6 h. A yellow precipitate was obtained after the reaction mixture was poured into an ice-water mixture. The precipitate was collected through vacuum filtration. The solid was recrystallised from methanol to give yellow needles of 1-pyrenecarboxaldehyde. Yield: 1.5 g (70 %); ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.92$–8.34 (m, 9H), 10.2 ppm (s, 1H); ESIMS: *m*/*z*: 231.1 [M^++H].

1-Pyrenemethanol: 1-pyrenecarboxaldehyde (1.15 g, 5.0 mmol) was dissolved in THF (30 mL) and a solution of $NaBH_4$ (210 mg, 5.5 mmol) dissolved in methanol (10 mL) was added dropwise into the 1-pyrenecarboxaldehyde solution at room temperature. After stirring overnight, a few drops of acetic acid were added to the reaction mixture to quench the excess $NaBH_4$. After the organic solvent was removed on a rotary evaporator, the solid was extracted into chloroform twice and washed twice with an aqueous solution. The collected organic solution was dried with sodium sulfate and was concentrated to give yellow solid 1-pyrenemethanol. Yield: 1.0 g (90 %); ^1H NMR (400 MHz, $CDCl_3$): $\delta = 5.07$ (s, 2H), 7.75–8.14 ppm (m, 9H); ESIMS: *m*/*z*: 233.2 [M^++H].

Compound ***1***: Dicyclohexylcarbodiimide (460 mg, 2.2 mmol) and a catalytic amount of 4-dimethylaminopyridine were added to a solution prepared by dissolving 1-pyrenemethanol (464 mg, 2.0 mmol) and 4-bromobutyric acid (335 mg, 2.0 mmol) in CH_2Cl_2 (20 mL), and the mixture was stirred overnight at room temperature. The mixture was filtered and the solvent was removed from the filtrate to yield a yellow oil, which was purified by silica-gel column chromatography by using ethyl acetate and hexane (1:2) as the eluent to afford a yellow solid **1**. Yield: 565 mg (75 %); ^1H NMR (400 MHz, $CDCl_3$): $\delta = 2.15$–2.36 (m, 4H), 3.49 (t, 2H), 5.68(s, 2H), 7.72–8.16 ppm (m, 9H); ESIMS: *m*/*z*: 383.2, 381.2 [M^++H].

Compound ***2***: Tacnorthoamide[15] (700 mg, 5.0 mmol) was dissolved in THF (3 mL) and a solution of bromoethane (600 mg, 5.5 mmol) in THF (3 mL) was added to this solution over a 10 min period. The mixture was then stirred overnight and the precipitate that resulted was collected by filtration and washed several times with THF. The precipitate was dissolved in water (20 mL) and the aqueous solution was heated at reflux for 4 h. After the solution was cooled to room temperature, NaOH solution (10 M) was carefully added dropwise to the reaction solution to adjust the pH to 12. The product was extracted with $CHCl_3$ (3 × 30 mL). Removal of the organic solvent on a rotary evaporator gave a colourless oil. Yield: 625 mg (67 %); ^1H NMR (400 MHz, $CDCl_3$): $\delta = 2.15$–2.36 (m, 4H), 3.49 (t, 2H), 5.68(s, 2H), 7.72–8.16 ppm (m, 9H); ESIMS: *m*/*z*: 771.2 [M^++H].

Compound ***3***: 1,1′-bis(dichloromethyl)ferrocene (270 mg, 1.0 mmol), which was obtained by a literature method,[16] and **2** (405 mg, 2.2 mmol) were dissolved in acetonitrile (40 mL), and potassium carbonate (550 mg, 4 mmol) was added. The mixture was stirred for 1 hour at room temperature and was then heated to 80 °C overnight. The mixture was filtered and the solvent removed from the filtrate in vacuo to yield a solid residue which was dissolved in chloroform and was extracted twice with distilled water. After removing the organic solvent, the crude product was isolated as a brown crude solid. Further purification was carried out by chromatography on a silica-column with $MeOH/CH_2Cl_2$ in a 1:22 ratio as the eluent. The product was collected as a pure yellow oil after the solvent was removed on a rotary evaporator. Yield: 290 mg (50 %); ^1H NMR (400 MHz, $CDCl_3$): $\delta = 1.28$ (t, $J = 7.8$ Hz, 6H), 2.39–2.46 (m, 4H), 2.55–2.64 (m, 8H), 2.80–2.88 (m, 4H), 3.00–3.06 (m, 8H), 3.30–3.38 (m, 4H), 3.45 (s, 4H), 4.05–4.12 (m, 8H), 8.02 ppm (d, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 12.5$, 47.2, 48.2, 51.7, 52.1, 52,8 53.6, 58.3, 68.3, 70.4, 84.4, 164.6 ppm; ESIMS: *m*/*z*: 291.2 [M^++2H]/2, 581.2 [M^++H].

Compound ***4***: Compound **3** (290 mg, 0.5 mmol) was dissolved in methanol (5 mL) and sodium hydroxide solution (20 mL, 5 M) was added. The mixture was heated to reflux overnight, cooled to room temperature and

extracted three times with $CHCl_3$. This solution was washed three times with distilled water. After removing the organic solvent, a yellow oil was obtained, which was purified by chromatography on a silica column with MeOH/diethylamine in a 10:3 ratio as the eluent. Removal of the eluent gave **4** as yellow oil. Yield: 220 mg (84%); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.28$ (t, $J = 8.2$ Hz, 6H), 2.42–2.86 (m, 28H), 3.42 (s, 4H), 4.04–4.10 ppm (m, 8H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 12.9$, 46.4, 47.1, 51.1, 51.3, 52, 52.4, 56.1, 68.3, 70.4, 84.4 ppm; ESIMS: 264.2 [M^++2H]/2, 525.3 [M^++H].

Compound ***5***: Compound **4** (131 mg, 0.25 mmol) and **1** (230 mg, 2.2 mmol) were dissolved in a mixture of acetonitrile and THF (25 mL, 10:1), and potassium carbonate (275 mg, 2.0 mmol) was added. The mixture was stirred for 1 hour at room temperature and was then heated to 50°C overnight. The precipitate was filtered and washed three times with acetone. The collected solvent was removed in vacuo to give a solid residue that was dissolved in chloroform and extracted twice with distilled water. After removing the organic solvent a crude yellow solid was obtained, which was purified by chromatography on a basic alumina column by using MeOH/CH_2Cl_2 in a 1:15 ratio as the eluent. A pure yellow oil was collected after the solvent was removed on a rotary evaporator. Yield of **5**: 81 mg (29%); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.26$ (t, $J = 7.3$ Hz, 6H), 1.85–1.97 (m, 4H), 2.15–2.26 (m, 4H), 2.46–2.81 (m, 32H), 3.44 (s, 4H), 4.02–4.11 (m, 8H), 5.61 (s, 4H), 7.70–8.08 ppm (m, 18H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 12.9$, 24.6, 32.5, 45.2, 47.5, 50.3, 51.7, 51.9, 52.6, 56.5, 57.9, 68.2, 69.1, 71.6, 85.2, 123.9, 124.9, 125.5, 125.9, 126.3, 126.9, 127.5, 128.2, 127.6, 128.2, 128.4, 131.3, 131.5, 131.8, 156.1, 168.4 ppm; ESIMS: *m/z*: 563.3 [M^++2H]/2, 1125.7 [M^++H].

Caution: Although no problems were encountered in this work, metal perchlorate complexes are potentially explosive. They should be prepared in small quantities and handled with care.

[Fc–bis{Zn^{II}(TACN)(Py)}] receptor: Compound **5** (57 mg, 0.05 mmol) and $Zn(ClO_4)_2{\cdot}6H_2O$ (23 mg, 0.06 mmol) were dissolved in methanol (10 mL) and the solution was heated at reflux for 30 min. After cooling to room temperature, diethyl ether was added to precipitate the complex, which was filtered, washed three times with a 1:1 mixture of diethyl ether and ethanol and dried in vacuo. Yield: 61 mg (72%); ^{1}H NMR (400 MHz, CD_3CN): $\delta = 1.35$ (t, $J = 7.5$ Hz, 6H), 1.88–1.98 (m, 4H), 2.20–2.28 (m, 4H), 2.56–3.03 (m, 32H), 3.52 (s, 4H), 4.12–4.20 (m, 8H), 5.63 (s, 4H), 7.68–8.24 ppm (m, 18H); ^{13}C NMR (100 MHz, CD_3CN): $\delta = 13.6$, 24.8, 32.2, 46.8, 48.8, 51.8, 53.8, 54.1, 55.2, 57.8, 59.2, 68.4, 69.5, 71.9, 85.5, 123.2, 124.5, 124.8, 125.6, 126.6, 126.8, 127.1, 127.5, 127.8, 128.1, 128.6, 131.1, 131.4, 131.6, 155.3, 169.1 ppm; Selected FTIR bands (KBr): $\tilde{\nu} =$ 3235 (b), 3102 (b), 2763 (b), 1724 (s), 1640 (s), 1419 (s), 1434 (b), 1225 (b), 1095 (b), 1027 (s), 894 (b), 751 cm^{-1} (s); ESIMS: *m/z*: 888.2 [M^+]/2; elemental analysis calcd (%) for $C_{74}H_{108}Cl_4FeN_6O_{26}Zn_2$ [Fc–bis{Zn^{II}(TACN)(Py)}]·6CH_3OH: C 49.6, H 5.4, N 4.5; found: C 50.6, H 5.0, N 4.1.

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