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Competition Assay of Thymidine Phosphates with a (Zn^{2+}-cyclen)-Lumazine Ensemble†

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Thymidine monophosphate (TMP) is synthesized in vivo from thymidine through phosphorylation catalyzed by thymidine kinase. TMP through further phosphorylations finally leads to thymidine triphosphate (TTP), which is used for DNA synthesis and repair. Imbalances in the cellular level of TTP have been implicated in the development of diseases such as Alpers syndrome, progressive external ophthalmoplegia, mitochondrial DNA depletion syndrome, and recessive mitochondrial neurogastrointestinal encephalomyopathy.[1] Because of its central role in growing cells, thymidine kinase (thymidylate synthase) has been a target in cancer therapy. Chemical assay methods that are selective for thymidine mono-, di- or triphosphates can be used for the assay of involved kinases; therefore, such assay methods would aid our efforts to diagnose or prognose those diseases as well as to elucidate related biological activities.

A few chemosensing systems for thymidine phosphates are known so far, whereas those for adenosine or guanosine phosphates are well documented.[2] Kimura and co-workers reported bis(Zn^{2+}-cyclen) **1** and its *meta* analogue that recognize thymidine phosphates (cyclen = 1,4,7,10-tetraazacyclododecane).[3] The Zn(cyclen) motif, which exists as aqua-zinc complex, strongly binds the thymine base through metal coordination to the deprotonated imide nitrogen coupled with complementary hydrogen bonding between two diagonal NH groups of the cyclen and two carbonyl oxygen atoms of the thymine base. The Zn(cyclen) motif also binds phosphate anion by metal coordination bonding. Therefore, receptor **1** can bind TMP to form the 1:1 complex (**2**) depicted in Figure 1.

Kimura and co-workers further utilized the binding mode in designing a $Ru(bpy)_3$-based bis(Zn^{2+}-cyclen) complex as a luminescent probe for inositol 1,3,5-trisphosphate (IP_3).[4] A chemosensing system for TMP or its analogues based on bis(Zn^{2+}-cyclen) **1** has not been further exploited. To develop luminescent probes based on bis(Zn^{2+}-cyclen) **1** through the traditional "binding site/signaling subunit" approach, we have to incorporate a signaling component into the structure. An alternative approach to avoid such a covalent incorporation of a signaling moiety to the recognition unit is the indicator-displacement assay (or displacement assay), which uses an ensemble of a receptor and an indicator (a supramolecularly interacting mixture). The indicator-displacement assay is now used as a standard sensing method since the Anslyn group has demonstrated its usefulness in the detection of various analytes.[5] Recently, we also reported indicator-displacement assays for phytate and IP_3 respectively, using tripodal receptors composed of Zn^{2+}-dipicolylamine binding groups.[6]

We have studied the chemical ensemble approach to sense TMP and its analogues using bis(Zn^{2+}-cyclen) **1** as the receptor component.[7] We have chosen lumazine as an indicator component of the ensemble based on the fact that the Zn(cyclen) binding motif forms a 1:1 adduct with lumazine (Figure 2).[8]

Two lumazine molecules are expected to coordinate bis(Zn^{2+}-cyclen) **1**. Therefore, an overall ensemble sensing scheme for TMP can be depicted as Scheme 1.

TMP is expected to completely replace the two lumazine molecules in sensing ensemble **3** because the association constant between bis(Zn^{2+}-cyclen) **1** and TMP ($\log K_{ass}$ = 6.4 at 25 °C)[3b] is much larger than that of lumazine and the Zn

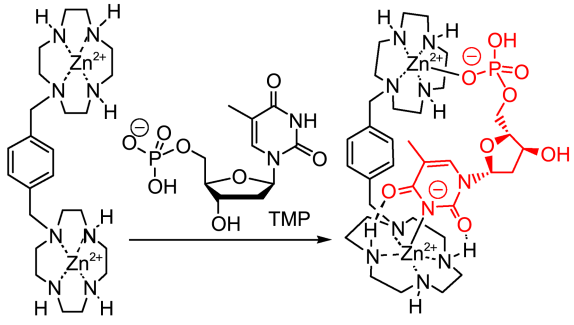


Figure 1. Structures of bis(Zn^{2+}-cyclen) **1** and its TMP complex **(2)**.

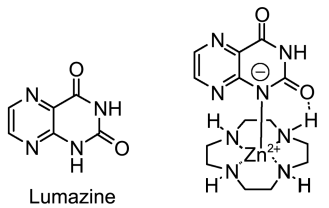
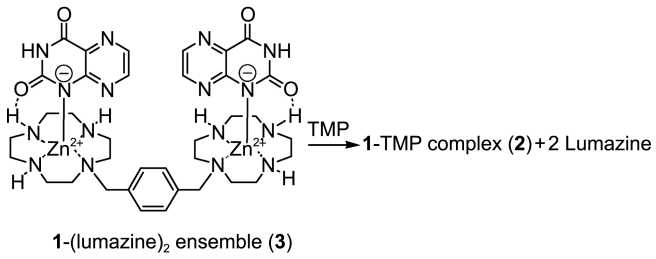


Figure 2. Lumazine and its Zn(cyclen) complex.

†This paper is dedicated to Professor Eun Lee on the occasion of his honourable retirement.



Scheme 1. Schematic representation for the competition assay of TMP using sensing ensemble **3** (a 1:2 complex between **1** and lumazine).

(cyclen) unit (K_{ass} = 6500 M^{-1} at 30 °C).[9] Lumazine shows enhanced fluorescence in the deprotonated state (quantum yield, Φ = 0.24) from its neutral state (Φ = 0.03).[10] Therefore, the displacement of lumazine is expected to result in fluorescence changes.

To find an optimum ratio of bis(Zn^{2+}-cyclen) **1** to lumazine in the sensing ensemble, we determined the UV absorbance changes of the solutions of lumazine with changing molar ratios of **1** in HEPES buffer at pH 7 (HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid). As the molar ratio of bis(Zn^{2+}-cyclen) **1** increases, the absorbance of lumazine itself (λ_{max} = 325 nm) decreases along with the absorbance maximum (λ_{max}) being shifted to longer wavelengths (λ_{max} = 357 nm). A Job plot, the absorbance changes (which is a function of the molar concentration of the lumazine-**1** complex) depending on the mole fraction of **1**, shows a maximum at 0.7 (close to 2/3), suggesting a 1:2 stoichiometry for the major complex between **1** and lumazine (Figure 3).

Next, the fluorescence change of lumazine upon binding with bis(Zn^{2+}-cyclen) **1** were measured. As expected, the addition of lumazine to an aqueous solution of **1** (50 μM) buffered at pH 7 (HEPES, 10 mM) resulted in enhancement in the fluorescence intensity along with a shift in the maximum wavelength from 458 to 480 nm (Figure 4a); with two equivalents of lumazine, about 95% of the maximum fluorescence intensity was attained. Such fluorescence changes are due to the deprotonation of the imide proton of lumazine by forming **1**·(lumazine)$_2$ complex **3**. Also it was determined that the addition of TMP to lumazine did not result in any fluorescence change. On the basis of these results, we have chosen an aqueous solution of bis(Zn^{2+}-cyclen) **1** (50 μM)

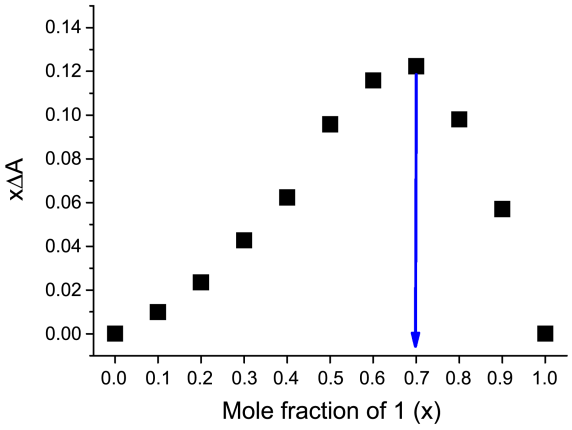


Figure 3. A Job plot for the complex formation between lumazine and bis(Zn^{2+}-cyclen) **1** (X = mole fraction of **1**; ΔA = A_x − $A_{x=0}$, where A is the absorbance of lumazine observed in HEPES buffer at pH 7).

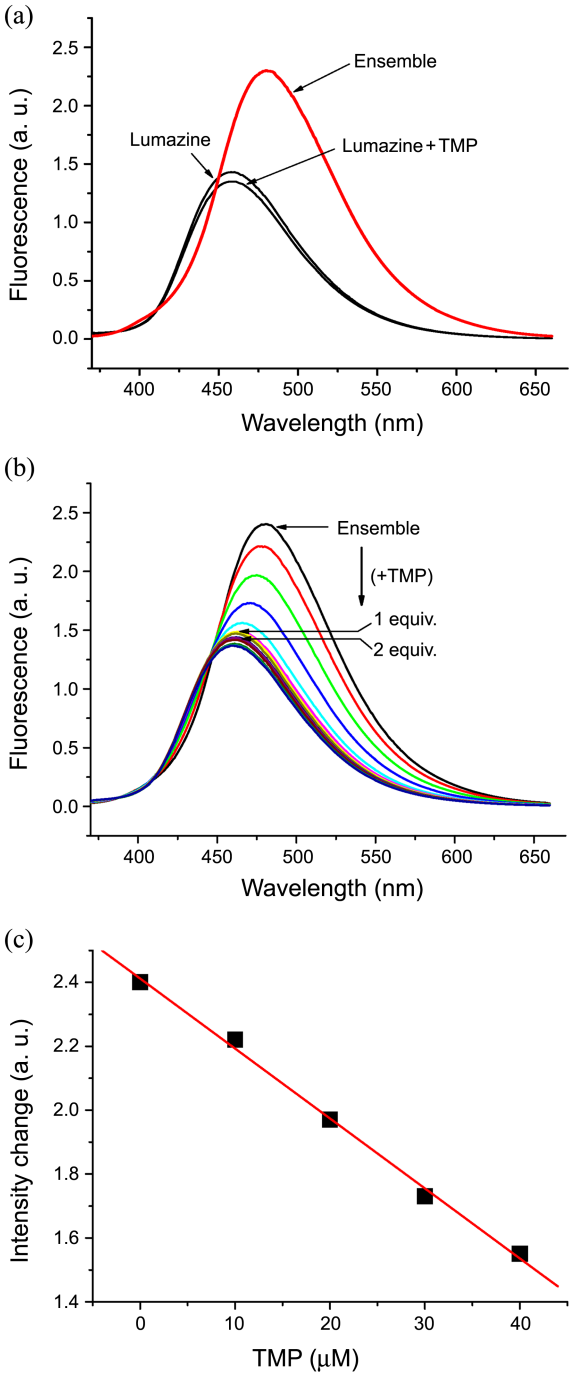


Figure 4. a) Fluorescence changes of lumazine (100 μM) upon addition of TMP (100 μM) and bis(Zn^{2+}-cyclen) **1** (50 μM), respectively; b) Fluorescence titration of the ensemble (100 μM **1** + 50 μM lumazine) with TMP (0 – 160 μM) in a HEPES buffer solution at pH 7.0. Excitation wavelength = 350 nm; c) A plot of fluorescence intensity change depending on [TMP] (0 – 40 μM).

and lumazine (100 μM) buffered at pH 7 (HEPES 10 mM) as the ensemble solution for sensing thymidine phosphates.

When an equimolar amount of TMP was added to sensing ensemble **3**, the emission spectrum of the ensemble (λ_{max} = 480 nm) returned to that of lumazine (λ_{max} = 458 nm); the result indicates that the lumazine bound to the zinc complex undergoes replacement with TMP, as expected (Figure 4a). Indeed, the fluorescence titration of ensemble **3** against increasing concentration of TMP (as monosodium salt, 0 - 160 μM) in the buffer solution resulted in a gradual decrease in the fluorescence intensity up to one equivalent of TMP (Figure 4c); after that point, the decrease slowed down.

On the basis of the titration results of TMP, we evaluated the selectivity of the sensing ensemble towards other monophosphates of adenosine-, cytidine-, and guanosine (TMP, AMP, CMP, and GMP) as their monosodium salts (Figure 5).

Under the same titration conditions (50 μM **1** + 100 μM lumazine; 10 mM pH 7 HEPES buffer; room temperature), the sensing ensemble responded to the nucleotides in the decreasing order of TMP > GMP > AMP > CMP (Figure 6a). Fluorescence decrease was largest in the case of TMP, followed by GMP. Little change in the fluorescence intensity was observed in the case of CMP. These results can be explained by evoking the binding mode in Scheme 1, where the imide functionality of thymine (T) base in TMP binds the Zn(cyclen) moiety through the metal coordination and the diagonal (two) hydrogen bonds. In the case of GMP, the guanine (G) base can have a similar metal coordination bond but only one hydrogen bond through the amide carbonyl oxygen, resulting in the smaller response than the case of TMP. Whereas both AMP and CMP show slight or negligible fluorescence changes because their bases, adenine (A) and cytosine (C) respectively, are unable to provide strong metal coordination bonds as in the cases of the T and G base moieties in TMP and GMP, respectively.

Next, we have evaluated the sensing ensemble toward nucleoside diphosphates (TDP, GDP, ADP and CDP) under the same conditions; the results are shown in Figure 6b. Fluorescence changes decrease in the order of TDP > GDP > CDP > ADP, showing significant decreases in the cases of TDP and GDP. This time little change was observed in the case of ADP, rather than CDP as in the cases of the monophosphate series. These opposite behaviors between the mono- and dinucleotides of A and C bases suggest that A/C base moieties interact with the Zn(cyclen) unit, albeit it is weak; these weak interactions seem to be also dependent on the distance between the metal-coordinating phosphate anion to the A/C base site.

Finally, we have evaluated the sensing ensemble toward nucleoside triphosphates (TTP, GTP, ATP and CTP) under the

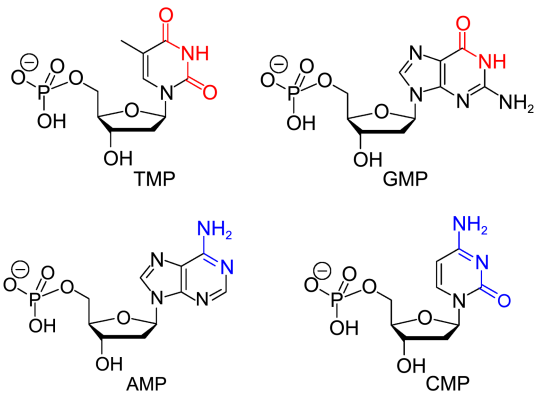


Figure 5. Structures of nucleotide monophosphates examined.

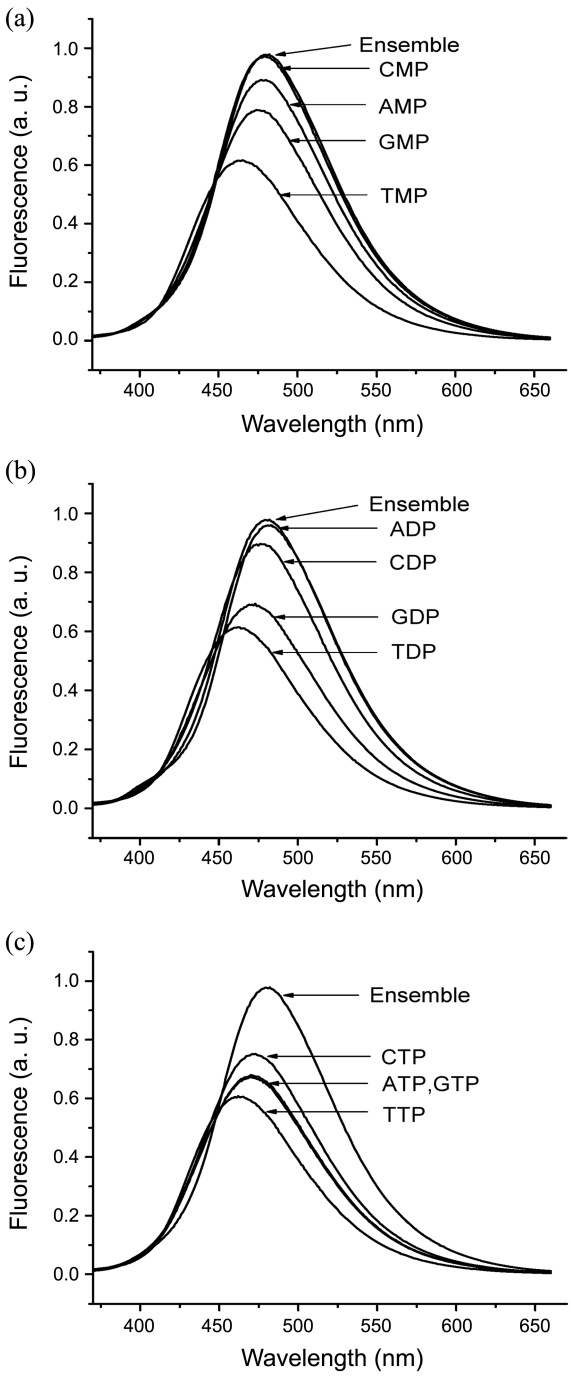


Figure 6. Collective fluorescence data obtained from titrations of the ensemble (50 μM **1** + 100 μM lumazine) with nucleoside mono- di- and triphosphates (50 μM) in water buffered at pH 7.0 (HEPES 10 mM). Excitation wavelength = 350 nm.

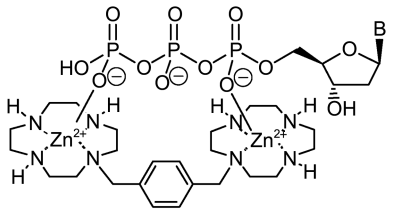


Figure 7. An alternative binding mode in the cases of nucleotide triphosphates (B represents the T, G, A, or C base).

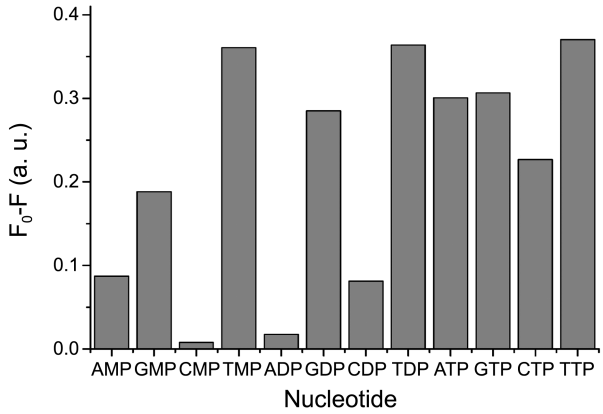


Figure 8. Changes in the fluorescence intensity of sensing ensemble **3** (50 μM **1** + 100 μM lumazine) in the presence of nucleotides (50 μM) in a buffer solution at pH 7 (HEPES, 10 mM), expressed as $F_0 - F$ (F_0 = intensity of the ensemble alone, F = intensity of the ensemble in the presence of an equimolar amount of the nucleotide; the intensity was estimated as the maximum peak height).

same conditions; the results are shown in Figure 6c. Interestingly, all the nucleoside triphosphates show sizeable decreases in the fluorescence intensity in the order of TTP > GTP ~ ATP > CTP. Although an overall selectivity trend is not far from the cases of the mono- and diphosphates, ATP shows almost the same response as GTP does. In these triphosphate cases, it is likely that the two Zn(cyclen) moieties in **1** can coordinate the triphosphate group alone, plausibly to the 1,3-phophate moieties (Figure 7). This alternative binding mode seems to compete with the binding mode that involves one phosphate group and one thymine or guanine base at the same time, as the suggested one in Scheme 1. In the cases of the nucleoside di- and triphosphates, the phosphate group involved in such a binding mode seems to be that of 5-site, taking into account of the allowed distance between the two zinc ions in bis(Zn^{2+}-cyclen) **1**.

The fluorescence intensity changes depending on the nucleotides are collectively compared as bar graphs in Figure 8, which shows an overall selectivity trend of the ensemble over the nucleoside mono-, di-, and triphosphates.

In summary, an indicator-displacement approach to sense thymidine phosphates has been investigated using a sensing ensemble composed of *para*-xylyl-bis(Zn^{2+}-cyclen) and lumazine in a 1:2 molar ratio. The ensemble senses thymidine monophosphate, as the latter displaces lumazine bound to the zinc complex by causing fluorescence changes. Among the nucleoside mono- and diphosphates of thymine, guanine, adenine, and cytosine bases examined, the sensing ensemble shows the largest fluorescence changes toward the thymine derivatives, followed by the guanine derivatives. The results are explained by strong metal coordination bonds between the thymine/guanine base and the Zn(cyclen) moiety. In the cases of the corresponding triphosphate derivatives, the sensing ensemble responds with greater extents to all the nucleotides; the results suggest that the bis(Zn^{2+}-cyclen) complex also binds the triphosphate moiety alone, in competition with the known binding mode involving the base groups.

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