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**PAPER**

# Quantification of foscarnet with chromogenic and fluorogenic chemosensors: indicator displacement assays based on metal ion coordination with a catechol ligand moiety†

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The catechol moiety in a chromophore was used in an indicator displacement assay for the chemosensing of the antiviral drug foscarnet (trisodium phosphonoformate, abbreviated as PFA). Applications of two methods were investigated, namely UV-Vis absorption and fluorescence spectroscopy measuring coordination of a metal to a catechol-based indicator. $Yb^{3+}$ complexation with chromogenic pyrocatechol violet in 10 mM HEPES buffer at pH 7.0 yields a blue chemosensor that responds to the presence of PFA with the release of yellow pyrocatechol violet (PV). The YbPV coordination complex responds linearly to the PFA concentration with a 2 μM detection limit. Metal ion complexation of a range of metal ions (trivalent Al, Ga, In, Sc, La, Gd, Er, Yb, and Fe, and divalent Cu) to the fluorescent sensor 6,7-dihydroxy-4-methylcoumarin (also referred to as 4-methylesculetin and abbreviated ME) resulted in fluorescence quenching in 10 mM HEPES buffer at pH 7.0. Addition of foscarnet to the quenched coordination complex liberated the ligand fluorophore which could be observed by its fluorescence. The coordinating complex was optimized for determining foscarnet by varying the metal ion, resulting in increased sensitivity to the analyte and selectivity against phosphate. $Cu^{2+}$ was selected as the most effective ion and its performance in this assay was further investigated. The effect of the co-ligand in the ternary coordination complex, $Cu^{2+}$–6,7-dihydroxy-4-methylcoumarin–co-ligand, was examined, and 2-picolylamine was found to be the optimal co-ligand. This ternary complex improves the detection limit of PFA to 0.5 μM and is stable for at least 72 hours, rendering it a potential sensor for PFA in chromatographic analysis.

## Introduction

Analytical detection of biophosphates is a challenging problem, because the inherent spectroscopic signatures generally have limited sensitivities. Multiple approaches for microdetermination of biophosphates have been taken,[1] including molecular recognition events[2] and indicator displacement assays.[3] Although detection is an important problem, few methods compare to the traditional phosphate detection of the P–Mo complex.[1] Even less is known about the specific phosphate derivatives, such as phosphonates, bisphosphonates, and other biophosphates. The coordination chemistry of these systems have been reviewed,[4] and is used for a range of different applications.[5] Fluorogenic and chromogenic chemosensors and reagents have been used for sensing of phosphates,[1] pyrophosphates,[6] and other anions.[7] This report describes an investigation of solutions to this analytic problem based on metal complexation to chromophores observed by absorption and fluorescent spectroscopy. In particular $Cu^{2+}$-complexes in the presence of a range of ternary ligands are found to finetune the detection limit of the analyte.

Foscarnet (abbreviated PFA, also referred to as trisodium phosphonoformate hexahydrate or Foscavir) (Fig. 1) is a simple antiviral drug with a broad spectrum of activity on herpes viruses (HSV-1, HSV-2, VZV, CMV, and others) and on HIV[8] administered *via* an intravenous infusion. Adverse toxic effects require accurate analytical methods for monitoring of the drug both in formulations and biological samples.[9] The absence of a UV chromophore significantly limits the sensitivity

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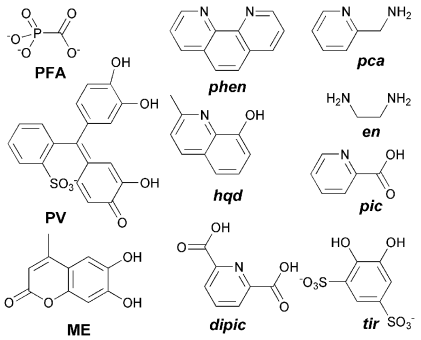


**Fig. 1** Phosphonoformate (PFA) was detected using the chromophore pyrocatechol violet (PV) or the fluorophore 6,7-dihydroxy-4-methylcoumarin (ME) in a ternary coordination complex with a range of co-ligands (1,10-phenanthroline (phen), 2-(aminomethyl)pyridine (pca), ethylenediamine (en), 8-hydroxyquinaldine (hqd), pyridine-2-carboxylic acid (pic), and 4,5-dihydroxybenzene-1,3-disulfonate (tir) and 2,6-pyridinedicarboxylic acid (dipic)).

of direct UV detection,[10] thus more sensitive electrochemical methods such as coulometric[11] and amperometric[12] detection were previously investigated. Post-column derivatization in liquid chromatography by oxidation of the analyte to phosphate and reaction with molybdovanadate was used to improve the limit of detection of foscarnet.[13] Existing methods have quantification limits in the range of 30 μM[13] to 170 μM[10a] in pharmaceutical formulations and 15 μM[11a] to 33 μM[11b] in biological matrixes. The accurate determination of foscarnet at low concentration in biological samples is not trivial, since its high charge and instability in acidic solutions limits the range of available techniques for sample preconcentration.[9]

Foscarnet as a bidentate ligand readily forms both 1 : 1 and 1 : 2 complexes in aqueous solutions with a range of ions including $Mg^{2+}$, $Ca^{2+}$, $Sr^{2+}$, $Ba^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Mn^{2+}$, $Cu^{2+}$, $Cd^{2+}$, $Zn^{2+}$,[8a,14] $Fe^{3+}$,[8a] $VO^{2+}$,[15] and $Al^{3+}$.[16] Among divalent metals, $Cu^{2+}$ forms the most stable complex with PFA.[14a] In complexes, $Cu^{2+}$ has a strong preference for square-planar geometry.[17] The stability of copper–bidentate ligand complexes can be increased by ternary complex formation and finetuning of the co-ligand coordinated to the metal center.[17] PFA binding to the $Cu^{2+}$ center increases in the order: $[Cu(H_2O)_6]^{2+}$, $[Cu(bpy)]^{2+}$ (bpy—2,2′-bipiridyl), and $[Cu(phen)]^{2+}$ (phen—1,10-phenanthroline), as illustrated by the logarithm of the formation constant which ranges from 7.78 for aqueous $Cu^{2+}$ to 7.94 for $[Cu(bpy)]^{2+}$ and 7.99 for $[Cu(phen)]^{2+}$ ions.[14a]

An effective metal ion–indicator chemosensor forms a strong coordination complex M(ME) to quench the fluorescence, and an even stronger complex with the target anion, foscarnet also referred to as phosphonoformate [M(PFA)], to displace the indicator molecule and regain the fluorescence. In this work, we present studies of metal coordination with chromogenic (pyrocatechol violet abbreviated PV) and fluorogenic (6,7-dihydroxy-4-methylcoumarin also referred to as 4-methylesculetin and abbreviated ME) indicators for foscarnet detection in aqueous solution. Binding to a metal center to the catechol moiety in both indicator molecules results in bathochromic shifts in the UV-Vis[18] and fluorescence[19a] spectra or fluorescence quenching.[19b] Foscarnet added to the metal–indicator chemosensor solution displaces the indicator molecule with metal–foscarnet complex formation. The displacement can be readily detected by UV-Vis or fluorescence spectroscopy.

We describe here a UV-Vis foscarnet assay in 10 mM HEPES buffer at pH 7.0 using a $Yb^{3+}$–PV complex, previously used to detect phosphates, ATP[20a] and bisphosphonates.[20b] In addition, we have used coordination chemistry to develop a more sensitive and selective chemosensor with the fluorescent indicator 6,7-dihydroxy-4-methylcoumarin (also referred to as 4-methylesculetin and abbreviated ME).[19] Various metal ions were tested as quenchers at pH 7.0 in 10 mM HEPES buffer, with Cu(II) being the most effective. The fluorescence of the free indicator released by the addition of PFA was used for detection and quantification of PFA. The metal–ME chemosensor response to aqueous phosphate was also measured to evaluate the chemosensor selectivity towards PFA.

## Results and discussion

### UV-Vis foscarnet detection with a $Yb^{3+}$–pyrocatechol violet complex

The possibility of using YbPV to measure phosphonoformate (PFA) based on absorbance differences was examined. As we have shown previously,[20] $Yb^{3+}$ and the yellow ($\lambda_{max}$ = 444 nm) dye pyrocatechol violet (PV) forms a blue YbPV ($\lambda_{max}$ = 605 nm) 1 : 1 complex in HEPES buffer (10 mM, pH 7.0).

After the addition of phosphate, ATP, pyrophosphate, or a bisphosphonate drug (including etidronate, aledronate, and zolendronate), the PV is displaced from the YbPV chemosensor resulting in a change of the solution color from blue to yellow. Since PFA is also a phosphonate drug, we tested the YbPV chemosensor for the response to PFA. In Fig. 2a we show the absorbance spectra of 50 μM chemosensor YbPV (eqn (1)) in 10 mM HEPES buffer at pH 7.0 after the addition of increasing amounts of PFA (5 to 120 μM). Observed spectral changes show that PFA binds to $Yb^{3+}$ displacing the PV from the YbPV chemosensor (eqn (2)). Monitoring the absorbance increase at 443 nm and/or the decrease at 623 nm allows

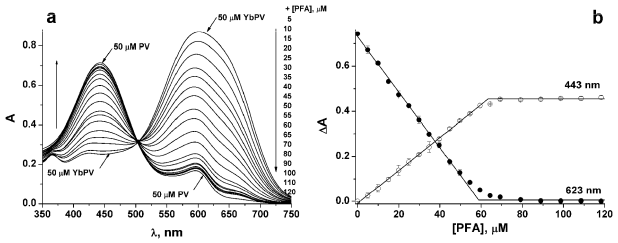


**Fig. 2** UV-Vis absorbance spectra for solutions of PV (50 μM) and YbPV (50 μM) complex in HEPES buffer (10 mM, pH 7.0). The spectra of the YbPV complex (50 μM) with varying amounts of PFA are also shown (a). The absorbance difference of the 50 μM YbPV complex at 443 and 623 nm is shown as a function of added foscarnet concentration (b). Error bars shown are three standard deviations from triplicate experiments.

 

quantitative determination of PFA in the 50 μM chemosensor solution (Fig. 2b). A linear response in the absorbance difference $\Delta A$ *versus* the PFA concentration is observed from 0 to 60 μM at 443 nm and from 0 to 50 μM at 623 nm with detection limits (defined as $3S/b$, where $S$—standard deviation of a PFA sample and $b$—the slope of the linear regression curve) of 3.7 μM and 2.0 μM at 443 and 623 nm, respectively.

$$Yb^{3+} + PV \rightleftharpoons YbPV \quad (1)$$

$$YbPV + PFA \rightleftharpoons [Yb(PFA)] + PV \quad (2)$$

Metal complexes with PFA generally are more stable than metal complexes with Pi[14a,21] because of the higher denticity of the PFA. Thus, strong PFA binding to $Yb^{3+}$ was expected. Sharp transitions in the absorbances at 443 and 623 nm in Fig. 2 support this conclusion. However, the $Yb^{3+}$ to PFA ratio obtained at the intersection of the two slopes is equal to 1 : 1.22 ($Yb^{3+}$ : PFA). The $Yb^{3+}$ ligand ratio obtained in this assay under identical conditions was equal to 1 : 1 for Pi and ATP,[20a] and 1 : 0.75 for pyrophosphate and bisphosphonates.[20b] The higher 1 : 1.22 ratio observed for the complex $Yb^{3+}$–PFA implies that either stoichiometry between Yb and PV is not 1 : 1, or that $Yb^{3+}$, PV, and PFA form a ternary complex with a stoichiometry 1 : 1 : 1. The $Yb^{3+}$ to ligand ratio was 4 : 3 with bidentate pyrophosphate and bisphosphonate drugs,[20b] showing that these ligands readily coordinate to the metal ion.

Although the YbPV chemosensor is very sensitive to PFA, its application to post-derivatization in liquid chromatography is complicated by oxidation in concentrated YbPV stock solutions. However, the desirable spectral changes with metal ion coordination to a catechol moiety and resulting potential chemosensing of PFA prompted us to explore the coordination chemistry of systems that are potentially more stable and more sensitive. We considered a range of commercially available fluorescent molecules with catechol moieties[22] and selected ME because it forms stable complexes with $Cu^{2+}$ and its absorbance (388 nm) is in a favorable spectral region.

### Fluorescent detection of foscarnet with metal–6,7-dihydroxy-4-methylcoumarin chemosensors: metal ion optimization

A useful metal ion–indicator chemosensor needs to form a strong M(ME) coordination complex to alter the fluorescence of the indicator (Fig. 1) and an even stronger complex with phosphonoformate to regain the fluorescence. The majority of d-metals are known as efficient fluorescence quenchers,[23] except $Zn^{2+}$, which induces chelation enhanced fluorescence.[19a,24] Seeking to increase the sensitivity of $Yb^{3+}$ sensor for PFA, we explored metal complexes of a coumarin derivative (6,7-dihydroxy-4-methylcoumarin, ME, Fig. 1) which as a fluorophore is potentially more sensitive to PFA detection than the Yb–PV complex. The aqueous fluorescence of ME is pH-sensitive, with a quenched neutral form $H_2$(ME), a fluorescent monoanion H(ME)$^-$, and a quenched dianion ME[19] ($pK_{a1}$ = 7.4 and $pK_{a2}$ = 11.7). The fluorescent spectra were recorded in 10 mM HEPES buffer at pH 7.0 where deprotonation takes place (eqn (1)). The fluorescence spectrum of 10 μM ME recorded with 375 nm excitation is shown in Fig. 3, curve 1. After addition of $Yb^{3+}$ to 10 μM, the fluorescence was quenched (Fig. 3, curve 2). The resulting ME–$Yb^{3+}$ mixture responded to the addition of

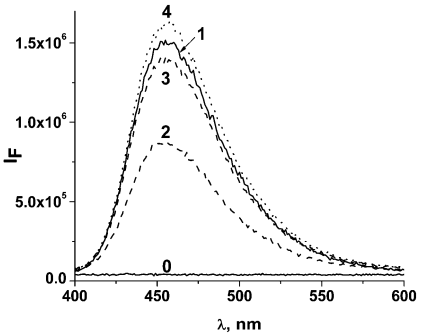


**Fig. 3** Fluorescence of ME. Background subtracted fluorescence at 455 nm is shown after the addition of 10 μM of aqueous metal ion to 10 μM of ME (Q, solid bars) in HEPES buffer (10 mM, pH 7.0). The fluorescence after the addition of PFA (to 10 μM) to the 10 μM 1 : 1 metal–ME chemosensor is shown (PFA, open bars). The fluorescence after the addition of 0.9 mM $P_i$ (Pi, shaded bars) is shown for comparison. Metal ions were added from 0.10 mM aqueous stock solutions of $LaCl_3$, $ErCl_3$, $GdCl_3$, $YbCl_3$, $Ga_2(SO_4)_3$, $InCl_3$, $Fe(NO_3)_3$, $ScCl_3$, $Al(NO_3)_3$, and $Cu(NO_3)_2$. Excitation wavelength is 375 nm.

PFA to 10 μM (Fig. 3, curve 3) or 0.9 mM aqueous phosphate (Fig. 3, curve 4) with partial fluorescence recovery. These observations suggest that ME forms a quenched $Yb^{3+}$–ME chemosensor (eqn (3)), and that ME fluorescence is restored after the addition of PFA (eqn (4)). Excess phosphate also produced strong fluorescence due to the release of free ME with $YbP_i$ formation.[20a] However, relatively low $Yb^{3+}$–ME chemosensor sensitivity and its insufficient selectivity suggested the need to explore complexation of other metal ions with ME.

$$ME + Yb^{3+} \rightleftharpoons YbME \quad (3)$$

$$YbME + PFA \rightleftharpoons ME + Yb(PFA) \quad (4)$$

Chemosensors in the presence of aqueous $Al^{3+}$, $Ga^{3+}$, $In^{3+}$, $Sc^{3+}$, $La^{3+}$, $Gd^{3+}$, $Er^{3+}$, $Yb^{3+}$, $Fe^{3+}$, and $Cu^{2+}$ were tested for PFA detection and selectivity against $P_i$ using identical concentrations. In Fig. 4, we show ME fluorescence quenching by these metal ions at pH 7.0 in 10 mM HEPES buffer (see Experimental for details). The fluorescence recovery

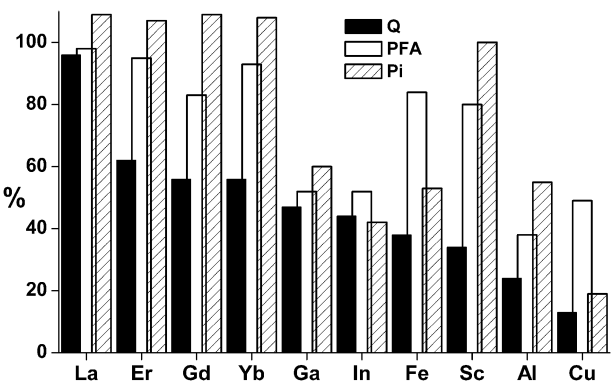


**Fig. 4** Fluorescence spectra of 10 μM 4-methylesculetin (1) in 10 mM HEPES buffer (pH 7.0) upon subsequent addition of 10 μM of $Yb^{3+}$ (2), 10 μM of $Yb^{3+}$ and 10 μM of PFA (3), and 10 μM of $Yb^{3+}$ and 0.9 mM of Pi (4). Background fluorescence spectrum is shown in the buffer solution (0). Excitation wavelength is 375 nm.

after the addition of PFA or $P_i$, respectively, is also shown in Fig. 4.

Fluorescence quenching by the aqueous metal ions in HEPES buffer (10 mM, pH 7.0) increased in the following order: La ≫ Er ≈ Gd ≈ Yb > Ga ≈ In > Fe > Sc > Al > Cu. The first six metal ions in this sequence follow the metal complex stability order observed for metal catechol complexes.[25] La(catechol)$^+$ is the weakest (log $K_f$ = 1.52 at pH 7.0) and Fe(catechol)$^+$ (log $K_f$ = 11.8 at pH 7.0) the most stable complex. The quenching efficiency of Fe, Sc, Al and Cu does not follow metal–catechol complex stability, suggesting that specific speciation of an aqueous metal ion[26] and stronger acidic properties of ME (p$K_{a1}$ = 7.4 and p$K_{a2}$ = 11.7[19b]) compared to the catechol (p$K_{a1}$ = 13.1[25]) are affecting this order.

Aqueous $La^{3+}$ is a very weak quencher and not suitable for application as a chemosensor. $Er^{3+}$, $Gd^{3+}$ and $Yb^{3+}$ show limited quenching efficiency. $Ga^{3+}$ and $In^{3+}$ binding to ME is too strong, preventing displacement by the PFA and phosphate. Both $Fe^{3+}$ and $Sc^{3+}$ quench the ME fluorescence and recover it after addition of PFA, however, the $Sc^{3+}$–ME chemosensor is also sensitive to phosphate. $Al^{3+}$ is known to bind strongly to phosphates,[27] and its strong binding to ME quenches the fluorescence, however, PFA does not displace the indicator from the complex. The $Cu^{2+}$ ion quenches ME fluorescence and the resulting $Cu^{2+}$–ME coordination complex dissociates in the presence of PFA.

$Cu^{2+}$ forms the strongest complex with PFA among (2+) metal ions[14a] as depicted in Fig. S3, ESI.† As a result, Cu was the only divalent metal ion studied here. We tested the affinities of various trivalent metal ions because formation constants for M(PFA) made with (3+) metal ions are not available in the literature (Fig. 4). Although most of the (3+) metal ions form stable complexes with catechol,[28] all trivalent metal ions were less efficient fluorescence quenchers than divalent Cu (Fig. 4).

We attribute this observation to aqueous metal ion speciation. The hydrolysis of metal ion and/or interaction of metal ion with the buffer reduces the amount of free metal available for quenching. Metal coordination to the catechol indicator moiety gives a quenched chemosensor, M(ME), which regains fluorescence when PFA is added as a competitive ligand.

In summary, we have shown that chemosensors for the antiviral drug foscarnet can be assembled from certain aqueous metal ions and a fluorescent indicator (6,7-dihydroxy-4-methylcoumarin). The metal center was optimized to increase the detection sensitivity and selectivity *versus* phosphate and we conclude that the $Cu^{2+}$–ME chemosensor is the most suitable one for this application among the divalent and trivalent metal ions tested for this assay.

### Fluorescent detection of foscarnet (PFA) with a 1 : 1 $Cu^{2+}$–4-methylesculetin mixture: the fine tuning of a co-ligand

Ternary coordination complexes can effectively be used to modify and fine tune the properties of a desirable chemosensor in a displacement assay. Since one $Cu^{2+}$ center can bind two ME ligands in solution,[19a] we explored the effect of a co-ligand to prevent the binding of two ME molecules to one metal center which minimized the possibility of ternary $Cu^{2+}$–ME–PFA complex formation. Furthermore, a bidentate co-ligand bound to four-coordinate $Cu^{2+}$ affects the stability and solubility of the $Cu^{2+}$ complex with PFA[14a] and possibly the $Cu^{2+}$ complex stability and solubility with ME (Fig. 5). Although the 0.1–0.2 log unit increase in the stability constant may seem small, it can substantially improve assay sensitivity. We assembled a $Cu^{2+}$–co-ligand center with two or three binding sites occupied by a co-ligand and tested its efficacy in the quenching of ME (eqn (5)), and fluorescence recovery after the addition of PFA (eqn (6)). Fluorescence intensity was measured at 455 nm in HEPES (10 mM, pH 7.0) buffer solution. In Fig. 5 we show the fluorescence ratio for $Cu^{2+}$–co-ligand systems studied, and the ratio after the addition of (to 10 μM) PFA and 0.9 mM of $P_i$ to the 10 μM $Cu^{2+}$–co-ligand–ME chemosensor is also shown for comparison.

$$\mathrm{ME} + [\mathrm{Cu(LL)}] \rightleftharpoons [\mathrm{Cu(LL)(ME)}] \quad (5)$$

$$[\mathrm{Cu(LL)(ME)}] + \mathrm{PFA} \rightleftharpoons \mathrm{ME}^- + [\mathrm{Cu(LL)(PFA)}] \quad (6)$$

The quenching efficacy of the $Cu^{2+}$–co-ligand complex decreased in the following co-ligand order: *phen* > $H_2O$ ≈ *pca* ≈ *hqd* > *en* > *pic* ≫ *tir* ≈ *dipic*, suggesting that coordination of the aromatic nitrogen ligands provides an

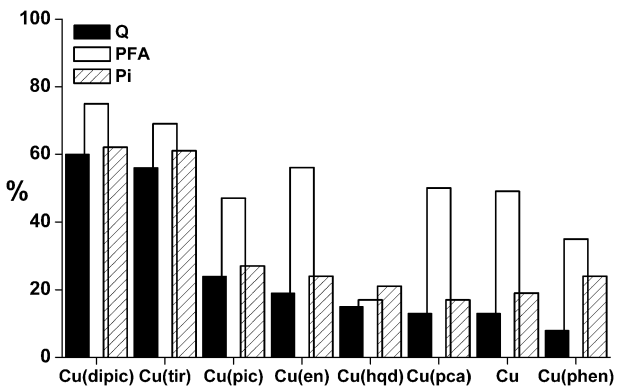


**Fig. 5** Residual fluorescence ratio after the addition of 10 μM of a $Cu^{2+}$–co-ligand quencher (solid bars, Q) to the 10 μM of 4-methylesculetin, and after subsequent addition of 10 μM of PFA (open bars, PFA) or 0.9 mM of Pi (shaded bars, Pi) in 10 mM HEPES buffer (pH 7.0). Excitation wavelength is 375 nm, fluorescence measured at 455 nm. Ligand structures are shown in Fig. 1.

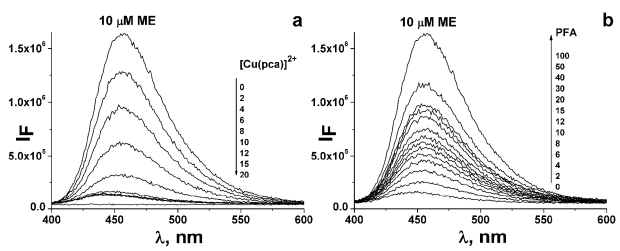


**Fig. 6** Fluorescence spectra of ME (10 μM) in HEPES buffer (10 mM, pH 7.0) upon successive addition of $[Cu(pca)]^{2+}$ (a). The μM $[Cu(pca)]^{2+}$ concentrations are shown next to the spectra. Fluorescence spectra of Cu–pca–ME (10 μM) with successive additions of PFA in HEPES buffer (10 mM, pH 7.0) (b). PFA concentrations (μM) are shown next to the spectra. The excitation wavelength is 375 nm.

 

increase in the metal center binding affinity to ME. In contrast, saturated nitrogen and oxygen ligands decrease the metal center binding affinity compared to the aqueous $Cu^{2+}$ ion. The $Cu^{2+}$–*dipic* complex is the least efficient quencher. PFA addition to the coordination complex, [Cu(LL)(ME)], results in fluorescence recovery due to $H(ME)^{-}$ release. Fluorescence recovery when quenching the $Cu^{2+}$–co-ligand–ME complex decreases in the following order: *en* ≈ *pca* > $H_2O$ ≫ *phen* > *pic* ≫ *dipic* > *tir*, with *en* and *pca* being equally effective co-ligands. Since these systems are governed by equilibria, the fact that a different response is observed upon PFA addition to the aqueous $Cu^{2+}$–ME and $Cu^{2+}$–co-ligand–ME chemosensors implies that the co-ligands remain coordinated to the metal after ME displacement with PFA. This provides indirect support for ternary complex formation of [Cu(LL)(PFA)] (eqn (6)).

The assay must be pH-controlled because ME fluorescence is very sensitive to solution pH with only the deprotonated form being fluorescent.[19] Since the p$K_a$'s of ME (7.4[19b]) and PFA (7.3–7.6[14a,15,29]) are near the pH 7.0 used in the assay (7.0), $Cu^{2+}$ binding to ME, and to PFA are likely to be sensitive to the solution pH. To optimize the assay pH, we explored several coordination complexes in the pH range of 6.0 to 8.5. PFA detection of four $Cu^{2+}$–co-ligand–ME complexes, with *pca*, *en*, *phen*, and *tir* as co-ligands, was tested at pH values of 7.0, 7.5, 8.0, and 8.5. With increasing pH, the response towards PFA decreases, showing that the optimal pH for these chemosensors is 7.0 (Fig. S1, ESI†). We attribute this observation to increasing $Cu^{2+}$ binding to ME with increasing pH compared to $Cu^{2+}$ binding to PFA. Solutions below pH 7.0 were limited by abrupt ME fluorescence quenching at pH 6.5 upon protonation (Fig. S2, ESI†), which is in agreement with previous reports.[19]

The binary $Cu^{2+}$ complex with *pca* was selected for further quantitative study of $Cu(pca)^{2+}$ response towards PFA. ME fluorescence quenching upon successive $Cu(pca)^{2+}$ additions is shown in Fig. 6a.

Further studies of the ternary system $Cu^{2+}$–pca–ME towards PFA were conducted in an equimolar mixture: in Fig. 6b, we show the ME fluorescence reappearance after successive additions of PFA to the 10 μM solution. The linear response to low (0–10 μM) PFA concentrations (Fig. 7) suggests that this ternary system can be successfully used for micromolar PFA detection at neutral pH with a 0.5 μM detection limit. Furthermore, in contrast to the YbPV chemosensor, the mixture of $Cu^{2+}$, *pca*, and ME is stable at 0.1 mM concentrations with no sensitivity loss to PFA even after 72 hours. Therefore, this ternary chemosensor can be readily applied to fluorescent PFA detection in a post-derivative step in liquid chromatography. Commercial availability of the chemosensor components, assay sensitivity and selectivity against phosphate are three major advantages of this assay.

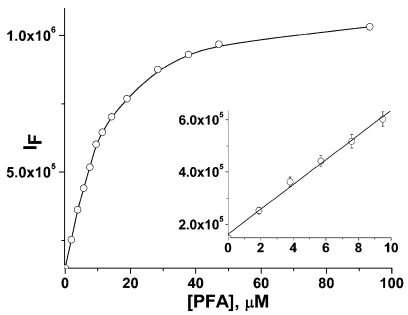


**Fig. 7** Fluorescence intensity at 475 nm as a function of PFA concentration in 10 μM $[Cu(pca)]^{2+}$ in 10 mM HEPES buffer at pH 7.0. The figure inset shows a linear fluorescence increase upon the addition of PFA from 0 to 10 μM. Error bars shown are 3× the standard deviation for three independent measurements.

## Conclusions

The coordination chemistry between metal ions and the catechol moiety in colorimetric (pyrocatechol violet) and fluorescent (4-methylesculetin) chromophores was examined with the objective of using the spectroscopic signature as a chemosensor for the antiviral drug foscarnet (also referred to as phosphonoformate) in aqueous solutions. The coordination complex (YbPV) allows determination of foscarnet *in situ* with a detection limit of 2.0 μM. However, this chemosensor degrades over time and loses sensitivity towards other $P_i$-containing ions [20] which limits its usefulness.

In contrast, the copper(II) coordination complex of fluorescent 6,7-dihydroxy-4-methylcoumarin also referred to as 4-methylesculetin has more potential as a chemosensor due to its greater sensitivity to PFA (detection limit 0.5 μM). Furthermore, it is more selective as evidenced by the fact that millimolar concentrations of $P_i$ do not cause interference.

Although large formation constants with 4-methylesculetin are reported for a range of trivalent metal cations, none of them were as effective as divalent $Cu^{2+}$ in 4-methylesculetin fluorescence quenching. Fine-tuning the coordination chemistry at the $Cu^{2+}$ center yielded the most sensitive, selective, and stable ternary complex suitable for PFA quantification. In comparison to YbPV, our fluorescent chemosensor [Cu(*pca*)(ME)] can be prepared in a concentrated stock solution from an equimolar mixture of $Cu^{2+}$, 2-picolylamine, and 6,7-dihydroxy-4-methylcoumarin and is stable over time.

In conclusion, two systems based on coordination complexes and indicator displacement assays were evaluated. Although both systems extend the detection range of current methods for detection of PFA, the fluorescent methods were not only more sensitive but also more stable showing no change in PFA detection for at least 72 hours.

## Experimental

### Materials and methods

High purity pyrocatechol violet, ytterbium(III) chloride hexahydrate, ethylenediamine dihydrochloride, 8-hydroxyquinaldine, picolinic acid, 2,6-pyridinedicarboxylic acid (Sigma Aldrich), 6,7-dihydroxy-4-methylcoumarin, trisodium phosphonoformate hexahydrate, 2-picolylamine (AlfaAesar), copper(II) nitrate 2.5-hydrate, HEPES (Fisher Scientific), 1,10-phenanthroline monohydrate (TCI), and disodium 4,5-dihydroxybenzene-1, 3-disulfonate (tiron) (Fluka) were used as received. The UV-Vis spectra were obtained at ambient temperature on a Perkin

Elmer Lambda 25 spectrophotometer in dual beam mode using a buffer solution blank.

### Fluorescence spectra

Fluorescence spectra were acquired in the steady state mode with a FluoroLog spectrofluorometer (HORIBA Jobin Yvon). The excitation wavelength was set at 375 nm unless specified otherwise. Water purified using the E-pure Barnstead system (specific resistivity $\geq 17.8$ MΩ $cm^{-1}$) was used throughout.

The fluorescence maximum was measured at 455 nm for the 10 μM ME solution ($I_{max}$) in 10 mM HEPES (pH 7.0) and compared to the quenched fluorescence after the addition of 10 μM of metal ion ($I_F$). The fluorescence ratio was calculated as $(I_F - I_{bg})/(I_{max} - I_{bg}) \times 100\%$, where $I_{bg}$ is the background fluorescence of blank buffer solution.

### Stock solutions and standardization procedures

Except for 6,7-dihydroxy-4-methylcoumarin, 10–50 mM stock solutions were prepared for all compounds by dissolving them in deionized water. Pyrocatechol violet, 6,7-dihydroxy-4-methylcoumarin and 2-picolylamine stock solutions were standardized in 10 mM acetate buffer (pH 5.3, $\varepsilon_{445}$ = 14 000 $M^{-1}$ $cm^{-1}$),[30] potassium hydrogen phthalate buffer (10 mM, pH 4.0, $\varepsilon_{340} = 1.16 \times 10^4$ $M^{-1}$ $cm^{-1}$),[19b] and in NaOH solution (0.1 M, $\varepsilon_{260}$ = 3020 $M^{-1}$ $cm^{-1}$),[31] respectively. The $Cu(NO_3)_2$ stock solution was standardized by complexometric titration with 10 mM $Na_2H_2EDTA$ in ammonia buffer (0.5 M, pH 8.1) using murexide as an indicator.

To prepare the 0.20 mM 4-methylesculetin stock solution, the solid was added to deionized water and sonicated with dropwise addition of 0.05 M NaOH until complete dissolution. The prepared stock solution (pH 7.0) was stored in the dark. The solutions of 1 : 1 $Cu^{2+}$ and a co-ligand complex were prepared by mixing $Cu(NO_3)_2$ and co-ligand stock solutions freshly each day. Studies that varied the pH of a ternary system $Cu^{2+}$–co-ligand–ME were carried out in MES (10 mM, pH 6.0 and 6.5), HEPES (10 mM, pH 7.0, 7.5, and 8.0) and CHES (10 mM, pH 8.5) buffers.

### Chemosensor sample preparation

The YbPV complex was prepared in HEPES buffer (10 mM, pH 7.0) *in situ* as described previously.[20] The $Cu^{2+}$ and ME fluorescent chemosensor was prepared as follows. Equal volumes of 10 mM stock solutions of $Cu^{2+}$ and a co-ligand were premixed and combined. 250 μL of 0.2 mM ME stock solution was added to 5.00 mL of HEPES solution (10 mM, pH 7.0) followed by the 10 μL addition of premixed $Cu^{2+}$–co-ligand stock solution. The 5 mM PFA stock solution was added subsequently and fluorescence spectra were recorded.

The stoichiometric 1 : 1 : 1 mixtures of $Cu^{2+}$, co-ligand, and ME were prepared as 0.10 mM stock solutions. However, only the mixtures with ethylenediamine and 2-picolylamine as co-ligands were stable. No time-dependent decrease in sensitivity towards PFA detection was observed for at least 72 h after preparation of solutions. Other ternary mixtures, such as $Cu^{2+}$, phen, and ME sensor, were effective only when prepared *in situ* immediately prior to PFA addition and with significant loss of sensitivity with time.



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