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Review

Fluorescent probes for microdetermination of inorganic phosphates and biophosphates

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Abstract. This review covers the progress made in the development of fluorescent probes for inorganic and organic phosphates that are of significance in biosciences. Such probes need to work at physiological pH and at room temperature. The various modes of interactions between probe and phosphate species are discussed, not the least with the aim to assist in the design of more selective probes for which there is a substantial need.

Keywords: Phosphate probe; phosphate sensor; fluorescence; biophosphates

Phosphates are most ubiquitous in biological systems. In contrast to many other inorganic ions, they easily form esters (also referred to as organic phosphates) that play a most important role in a wide variety of processes that occur in biochemistry and cellular metabolism [1, 2]. Fluorescent probes have had a large success in the past 20 years as a result of the availability of probes for various ions and molecular species, for enzyme substrates and DNA detection. They also enable non-fluorescent (bio)molecules to be studied by fluorometric means. Fluorescence is sensitive (down to the single molecule level!), less prone to interferences by turbid samples such as cells, and comes in a variety of methods that range from measurement of intensity and decay time [3, 4] to the determination of the efficiency of quenching, FRET (fluorescence resonance energy transfer) [5, 6], anisotropy [7, 8], and combinations thereof. Detailed information on fluorescent techniques in general have been presented in the books by Lakowicz [9] and Valeur [10].

Numerous methods for optical determination of phosphate are known [11, 12] but only few fluorescent probes exist that can be used in water or electrolyte solutions [13–15]. Most methods often require experimental conditions that are not compatible with bioassays. The phosphomolybdate method, for example, is widely used but requires strongly acidic pH conditions. A selection of methods hardly applicable to bioassays is given in Table 1. Some require the use of organic solvents so that they are not applicable usually to cellular systems either.

pH effects on the charge of (bio)phosphates

Phosphoric acid and its esters undergo protolytic reaction often at near-neutral pH values, and this complicates the design and function of respective probes. As one result, pH also exerts an effect on the charge of the ion. Secondly, phosphate – unlike most other in-

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Table 1. Conventional methods for photometric or fluorometric determination of inorganic and organic phosphate that cannot be applied easily to probing phosphate in biological systems

Analyte	Reagent	Exptl. conditions; method	Comments	Refs.
Phosphate	molybdate	pH 1; photometry at 865 nm; 2 min incubation	LOD*: 0.5 μM	[12]
Phosphate	molybdate	pH 0.5, photometry at 880 nm; formation of reduced phosphomolybdate	LOD*: 80 nM	[12]
Phosphate	phenosafranine (Ph) and neutral red (NR)	pH 3; inhibition of Fe(III)-catalyzed photobleaching	LOD*: 10 μM	[16]
Phosphate	alizarin red sulfphonate (ARS)	pH 6.6, photometry at 478 nm	phosphate:ARS = 1:3	[17]
Phosphate	molybdate and rhodamine B or 6G	pH ~1; quenching of fluorescence by P-molybdate	LOD*: 20 nM (rhodamine 6G)	[18, 19]
Phosphate	thiamine	fluorescence	LOD*: 0.3 μM	[20]
Phosphate	Al(III)-morin complex	pH 3–4, fluorescence; 45 min incubation	complex decomposed (and quenched) by phosphate	[21]
ATP	anthracene-based open-chain polyamine	pH < 4; quenching of fluorescence	AMP and ADP show almost same stability constants	[22]
ATP	1,3,5-triarylpent-2-en-1,5-dione derivatve	pH 6; 1,4-dioxane/water (70/30); photometry at 550 nm	color change after transformation of diketonic to pyrylium form (cyclization)	[23]
2,3-Bisphospho-glycerate	Eu(III)-tetra-*N*-oxide bis-bipyridine complex	50% methanol/acetonitrile; quenching of fluorescence	complex not stable in aqueous solution	[24]

* Limit of detection.

organic acids – easily undergoes polycondensation to form species such as pyrophosphates, triphosphates, or even higher ones [25]. This is also true for inorganic phosphates. The following schemes overview the equilibria at near neutral pH's at 22 °C:

$H_2PO_4^-$ ⟷ HPO_4^{2-} (pK_a7.2)
prim. phosphate sec. phosphate

$H_2P_2O_7^{2-}$ ⟷ $HP_2O_7^{3-}$ (pK_a5.7)
sec. pyrophosphate tert. pyrophosphate

$HP_2O_7^{3-}$ ⟷ $P_2O_7^{4-}$ (pK_a8.2)
tert. pyrophosphate quart. pyrophosphate

It is mandatory to keep in mind that the different forms shown above bind with different affinity to the various probes, and that two forms are present simultaneously at certain pH's.

Organic phosphates also display acid-based equilibria that are governed by the respective pK_a values. Typical pK_a's are 6.5 for the last proton dissociation of ATP, and 3.6 for cAMP (at around 21 °C). Similar to inorganic phosphate, organic phosphates can form polyacids such as pyrophosphates (also referred to as ''diphosphates''; for example the well known ADP) and ''triphosphates'' such as ATP. Note that the term ''diphosphate'' is also used for species that carry two separated phospho groups, for example in glycerol-1,3-diphosphate which is not a pyrophosphate. A final group of organic phosphates consists in the group of cyclic esters such as cAMP. Typical species are given in Table 2.

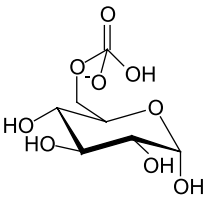
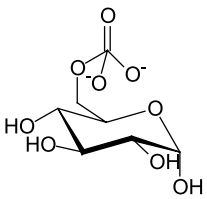
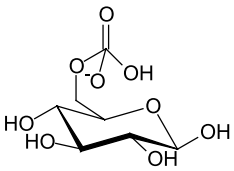
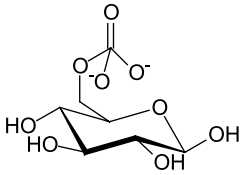
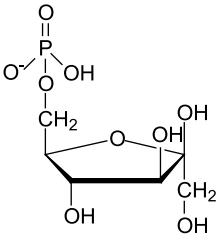
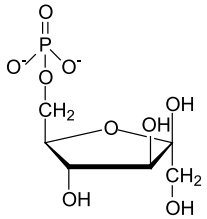
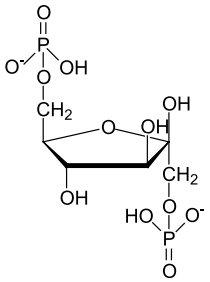
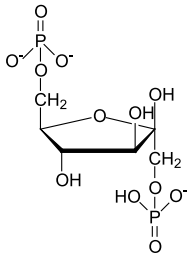
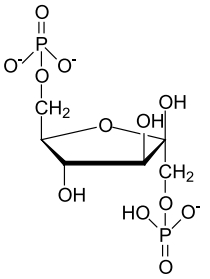
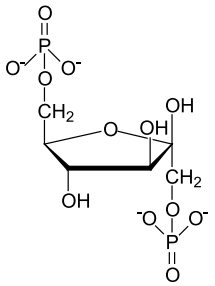
Numerous kinds of organic phosphates are essential for a functional metabolism. They range from inositol to NAD^+, NADH, FAD, vitamin B_{12}, thiamine, pyridoxal, choline, kephaline, farnesol, pentenyl alcohol, lecithine and other phospholipids, to mention a selection only. Polyalcohols also can carry two or more phospho groups. Hence, there is quite a variety of phosphates for which specific probes are sought. Optical probing of inorganic phosphates and of organophosphates [34, 35], of phosphoproteins [36], and of anions in general [37] has been reviewed recently. This, in turn, points to the significance of optical probing of phosphates in biological systems where numerous kinds of phosphate species can be present.

This review is confined to fluorescent probes that work at near-neutral pH and at room temperature. Its first section covers probes for primary and secondary inorganic phosphate (Pi) and inorganic pyrophosphate (PPi). The second part covers phosphoproteins, the third part phospho-nucleotides including DNA and RNA, and the final part presents probes for miscellaneous biophosphates.

Probes for inorganic phosphates

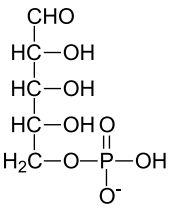
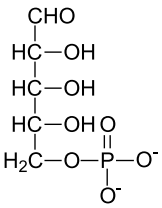
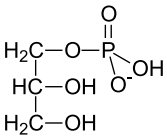
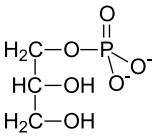
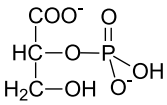
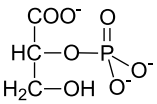
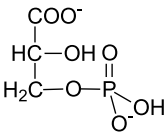
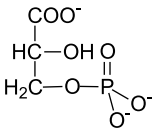
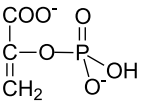
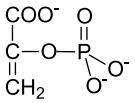
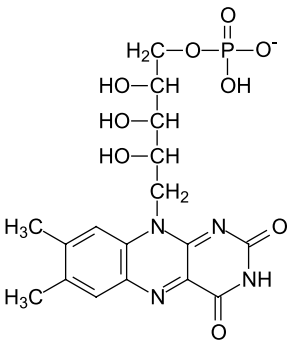
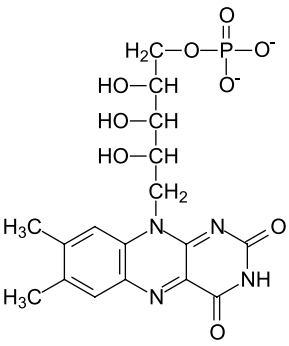
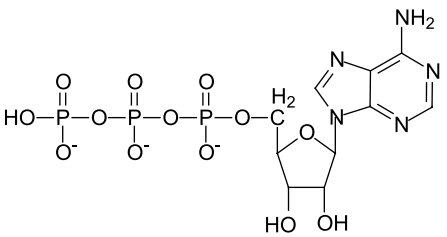
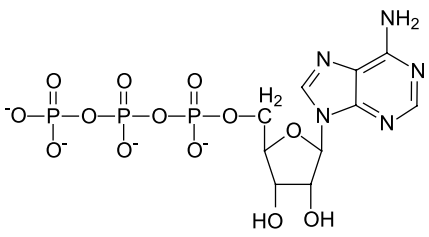
Phosphate has long been known to interfere in many optical (including fluorometric) assays. This, in turn,

Table 2. Selected organic phosphates and respective acid-base equilibria

Acidic form	Base form	Typical pK_a	Ref.
α-Glucose-6-phosphate$^-$	α-Glucose-6-phosphate^{2-}		
		6.2	[26]
β-Glucose-6-phosphate$^-$	β-Glucose-6-phosphate^{2-}		
		6.1	[26]
Fructose-6-phosphate$^-$	Fructose-6-phosphate^{2-}		
		6.4	[27]
Fructose-1,6-diphosphate^{2-}	Fructose-1,6-diphosphate^{3-}		
		6.1	[27]
Fructose-1,6-diphosphate^{3-}	Fructose-1,6-diphosphate^{4-}		
		6.9	[27]

(*continued*)

Table 2 (*continued*)

Acidic form	Base form	Typical pK_a	Ref.
Ribose-5-phosphate$^-$	Ribose-5-phosphate^{2-}		
		6.2	[28]
Glycerol-1-phosphate$^-$	Glycerol-1-phosphate^{2-}		
		6.2	[29]
2-Phosphoglycerate^{2-}	2-Phosphoglycerate^{3-}		
		7.0	[27]
3-Phosphoglycerate^{2-}	3-Phosphoglycerate^{3-}		
		6.8	[27]
Phosphoenolpyruvate^{2-}	Phosphoenolpyruvate^{3-}		
		6.4	[27]
FMN$^-$	FMN^{2-}		
		6.2	[29]
ATP^{3-}	ATP^{4-}		
		6.5	[30, 31]

(*continued*)

Table 2 (*continued*)

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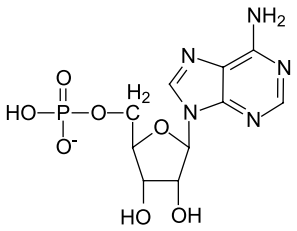
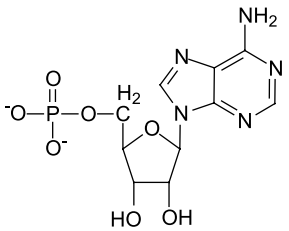
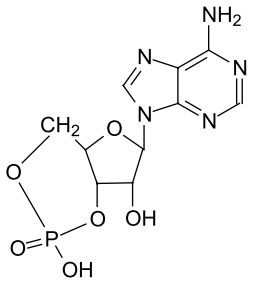
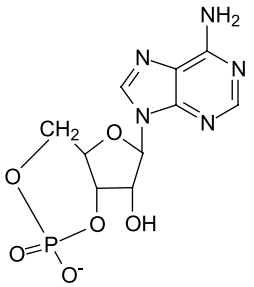
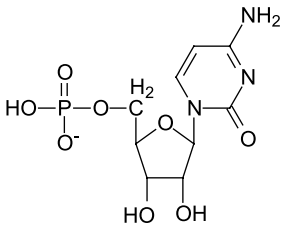
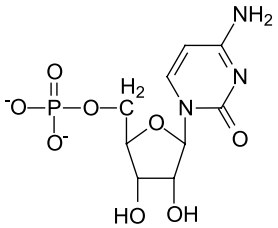
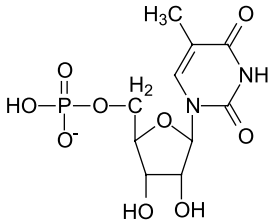
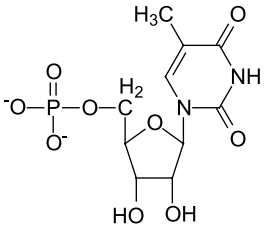
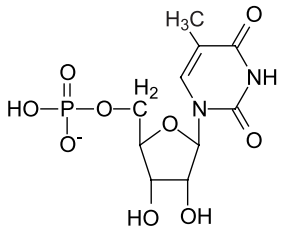
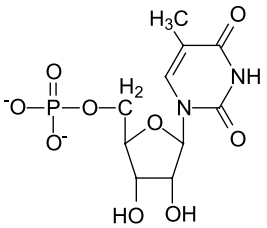
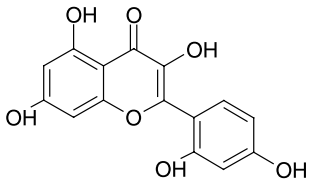
		6.2	[28]
cAMP	$cAMP^-$		
		3.6	[32]
CMP^-	CMP^{2-}		
		6.2	[33]
TMP^-	TMP^{2-}		
		6.4	[33]
UMP^-	UMP^{2-}		
		6.2	[33]

can be exploited to probe phosphate. Al(III) forms a fluorescent complex with morin (**1**) whose fluorescence is reduced by phosphate. This can be used to determine trace quantities of phosphate [21]. However, this probe was only tested in 50% ethanol mixtures since higher water contents lead to a reduced fluores-

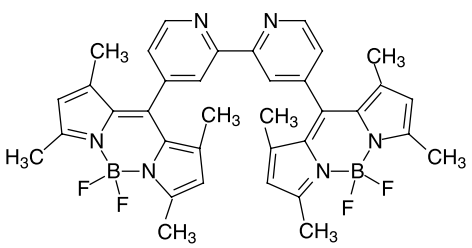
cence signal. Hence, the use of the Al(III)-morin reagent in biological matrices appears to be limited.



1

The Al(III)-morin complex was immobilized in a plasticized poly(vinyl chloride) matrix [38] to result in a sensor layer. Fluorescence at $\lambda_{ex}/\lambda_{em} = 424/510$ nm decreases in the presence of phosphate, when working at the optimum pH range between 4 and 6. This is the result of the decomposition of the complex and is accompanied by the release of morin. A linear response is found between 6 and 15 $\mu g\,mL^{-1}$ of phosphate, with a limit of detection of 0.02 $\mu g\,mL^{-1}$. Under these circumstances, the response is reversible when switching between phosphate and solutions of the Al(III)-morin complex which recovers the functionality of the sensor membrane.

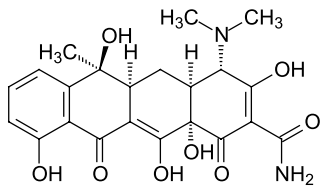
A substituted bipyridyl ligand **2** was introduced by Coskun et al. [39]. The ligand displays a high quantum yield in organic solvents. However, its fluorescence is quenched by metal chelation. Zn(II) was found to be the most effective quencher. The titration of the Zn(II)-**2** complex ($\lambda_{ex}/\lambda_{em} = 490/518$ nm) with phosphate revealed that phosphate strongly binds to the complex resulting in a 25-fold fluorescence enhancement whilst the absorption spectrum showed no changes. Other anions such as fluoride and acetate also bind to the Zn(II) complex. Hence it is likely that these anions interfere in phosphate determination although their binding affinity is smaller. Moreover, this probe was only applied in acetonitrile since it cannot be used under aqueous conditions.



2

Lanthanide ions display unique spectral properties such as line-like emission, large Stokes shifts, and long luminescence lifetimes. However, lanthanide ions themselves display only small absorptivity (and thus brightness) which requires laser excitation. Therefore, organic chromophores are often used as "antenna" ligands that have a lower energy gap between the lowest singlet state (S_1) and the triplet state (T_1). This enables energy transfer to occur from the ligand sphere to the central lanthanide ion. Appropriate ligands have broad-banded absorption characteristics. Following excitation, the ligands undergo intersystem crossing to T_1 which is followed by intramolecular energy transfer from the ligand to a 4*f* level within the central lanthanide ion to result in a sharp emission pattern typical of lanthanide ions.

The response of lanthanide emission to phosphates is assumed to be based on two mechanisms: (1) Phosphates can either replace water molecules coordinated to the 8th or 9th binding site and hence reduce quenching by water and/or reduce energy losses through O–H vibrations; (2) phosphates can bind to organic ligands, e.g. by electrostatic interaction, of the lanthanide complex and alter the effectivity of energy transfer from the ligand to the central lanthanide ion. One such antenna ligand, tetracycline (**3**), was used as a chelating agent for Eu(III) ion [40].

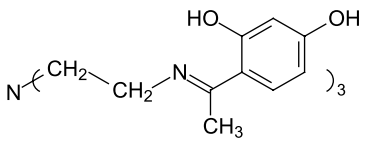


3

The luminescence of the europium-tetracycline (EuTC) complex in a 1:1 stoichiometry is sensitive to phosphate ions in that the emission of EuTC at 616 nm after excitation at 400 nm is quenched. It is assumed that phosphate anion coordinates electrostatically to EuTC which lowers the effectivity of energy transfer from the ligand to the central metal ion thus resulting in decreased luminescence. The determination of phosphate works best at pH 7, and this renders the probe suitable for applications to biochemical systems. The calibration plot is linear from 5 to 750 $\mu mol\,L^{-1}$ of phosphate with a limit of detection of 3 $\mu mol\,L^{-1}$, at a reagent concentration of 20.8 $\mu mol\,L^{-1}$. The results were obtained by taking advantage of the long luminescence lifetimes of EuTC using a lag time of 60 µsec and an integration time of 100 µsec. This resulted in a much steeper slope of the calibration plot compared to a lag time and integration time of 0 and 40 µsec, respectively. Among the many other ions tested, Mg(II), Al(III), Fe(III) and Tb(III) are tolerated up to concentrations of 20, 100, 15 and 33 $\mu mol\,L^{-1}$, respectively. However,

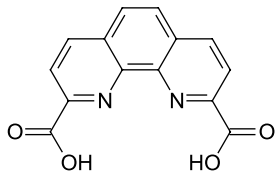
it was demonstrated that Cu(II) quenches the fluorescence of both the EuTC and the EuTC-H_2O_2 complex the latter displaying a 15-fold stronger luminescence intensity compared to EuTC [41]. Conceivably, anions containing oxygen may compete with phosphate for the binding to EuTC. However, only citric acid increases the emission intensity significantly at above 0.8 μmol L^{-1}. This probe offers the advantage of a relatively longwave excitation and a sharp emission band centered at 616 nm which allows its use in biological matrices where background fluorescence in the UV and near visible usually is strong. The sensitivity towards phosphate of the closely related Eu_3TC system (with its stoichiometry of 3:1) led to a time-resolved fluorescence-based assay for the determination of the acitivity of alkaline phosphatase [42], and to a screening scheme for its inhibitors.

A Eu(III) complex was reported [43] whose fluorescence is strongly increased in the presence of phosphate. Eu(III) is chelated by the Schiff base ligand **4**. The corresponding complex displays excitation and emission maxima at 365 and 615 nm, respectively. Fluorescence is enhanced 126-fold in presence of sodium phosphate solution and can be further increased about 12-fold after addition of the organic solvent dimethylsulfoxide (DMSO). Fluorescence reaches its maximum at 80% (v/v) of DMSO in the presence of 0.56 mmol L^{-1} of phosphate, but decreases at higher phosphate concentrations (0.87 mmol L^{-1}). The optimal pH for this system is 6.2. The fluorescence intensity decreases at pH values >7.0 owing to the quenching effect of hydroxide ions.



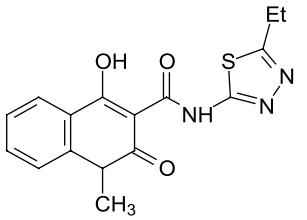
4

Coates et al. [44] reported on a Eu(III) complex with the ligand PDCA (1,10-phenanthroline-2,9-dicarboxylic acid) (**5**). It forms either a 1:1 or a 2:1 complex with the lanthanide ion. However, only the emission of the 1:1 PDCA:Eu complex is affected by phosphate. In HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) buffer of pH 6.5 the emission intensity at 615 nm (excitation at 290 nm) is enhanced by about 30% which is a rather small effect compared to the 126-fold fluorescence enhancement of probe **4**. The group of Sammes was the first to report on the effect of phosphate on the lifetime of a Eu(III) complex. A large increase in the fluorescence lifetime (from 230 μsec to 400 μsec) was observed. However, no phosphate assay based on fluorescence lifetime measurements of lanthanide ions has been established so far.



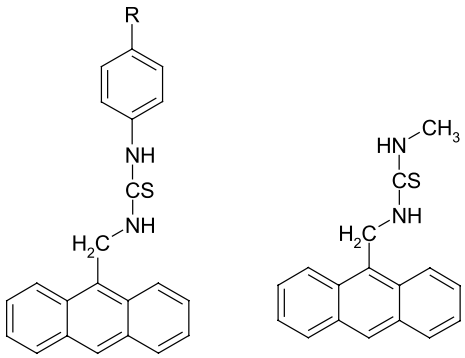
5

A Tb(III) complex with the ligand **6** (1:1 stoichiometry) was found to respond to phosphate [45]. The luminescence of the complex at 546 nm (λ_{exc} = 320 nm) is quenched in presence of phosphate. The probe works best at an optimum pH of 7.4. Quenching by phosphate follows a Stern-Volmer (SV) relationship over a concentration range of 0.5 to 100 μmol L^{-1} with a limit of detection of 0.25 μmol L^{-1}. The long lifetime of the Tb(III) complex (1.89 msec) makes it amenable to time-resolved fluorometry. ATP also induces quenching of the emission with a linear SV plot from 0.1 to 7.0 μmol L^{-1} and a limit of detection of 50 nmol L^{-1}.



6

The photoinduced electron transfer (PET) effect can be used to probe phosphate anions [46, 47]. This was established by de Silva 20 years ago. Gunnlaugsson et al. [48, 49] reported on the fluorescent anion PET probes **7**–**9** for phosphate.

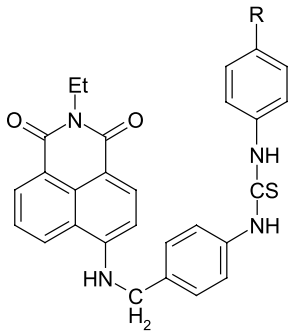


7 R = H
8 R = CF_3

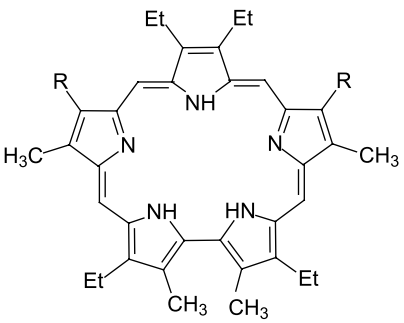
9

Probes **7**–**9** consist of an anthracene fluorophore connected to a thiourea anion receptor via a methylene

spacer. They are capable of binding phosphate via hydrogen bonding, thus resulting in an increased reduction potential of the receptor. Consequently, the emission of the anthracene fluorophore is quenched by approximately 50% in the case of phosphate. Compared to the 126-fold fluorescence enhancement of the Eu(III) complex with probe **4**, this effect is rather small. Receptor **8** has the highest affinity for phosphate. The intensity changes showed a sigmoidal dependency over two log units which signifies a 1:1 binding profile. Among other anions tested, acetate and fluoride display even larger binding affinities to the receptors which limits their selectivity. However, chloride and bromide do not interfere. Gunnlaugsson et al. also presented comparable PET probes in which a naphthalimide fluorophore was used instead of anthracene [50]. This makes the fluorophores **10** and **11** absorb in the visible region. Moreover, they have quantum yields of approximately 80–90%. The same fluorescent behavior was obtained for receptors **10** and **11** when exposed to phosphate anions as presented for receptors **7**–**9**. Unfortunately, receptors **7**–**11** have only been applied in DMSO. For applications in biological matrices, however, water compatibility is an essential prerequisite.

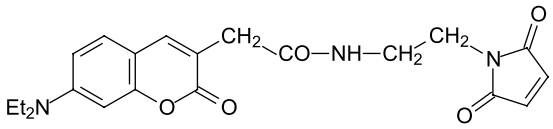


10 R = H
11 R = CF_3



13 : R = A
14 : R = B
15 : R = C

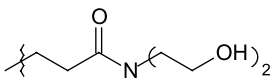
Phosphate recognition also can be accomplished via biosensors as reported by Salins et al. [51]. The phosphate binding protein (PBP), a member of the family of anion binding proteins, acts as the biorecognition element for inorganic phosphate. The protein consists of two domains which are linked by three peptide sequences. The phosphate binding region is located in the gap between the two domains. N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC) (**12**) was attached to the only available cystein residue which was introduced at the desired position in the PBP in advance. An increase in fluorescence at 470 nm after excitation at 425 nm was observed after addition of phosphate to the labeled PBP at pH 8. The calibration plot is linear over two orders of magnitude, and the limit of detection is 0.42 µmol L^{-1}.



12

This system was transferred to fiber optic sensing. PBP labeled with **12** was entrapped behind a dialysis membrane at the tip of a bifurcated optical fiber. A limit of detection of 1.8 µmol L^{-1} is observed for this system. Arsenate was found to be the only interfering ion among many others tested. Notably, this biosensor can discriminate clearly between phosphate and adenosine triphosphate which binds weaker by a factor of 3000 than phosphate. Apart from the required use of genetic engineering techniques for the introduction of one unique cysteine residue in PBP, the distinct discrimination between two phosphate-containing species makes PBP-**12** a very suitable probe for phosphate.

A =



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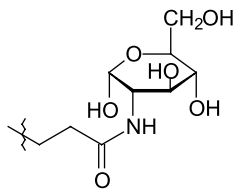
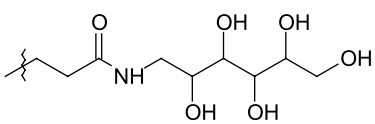


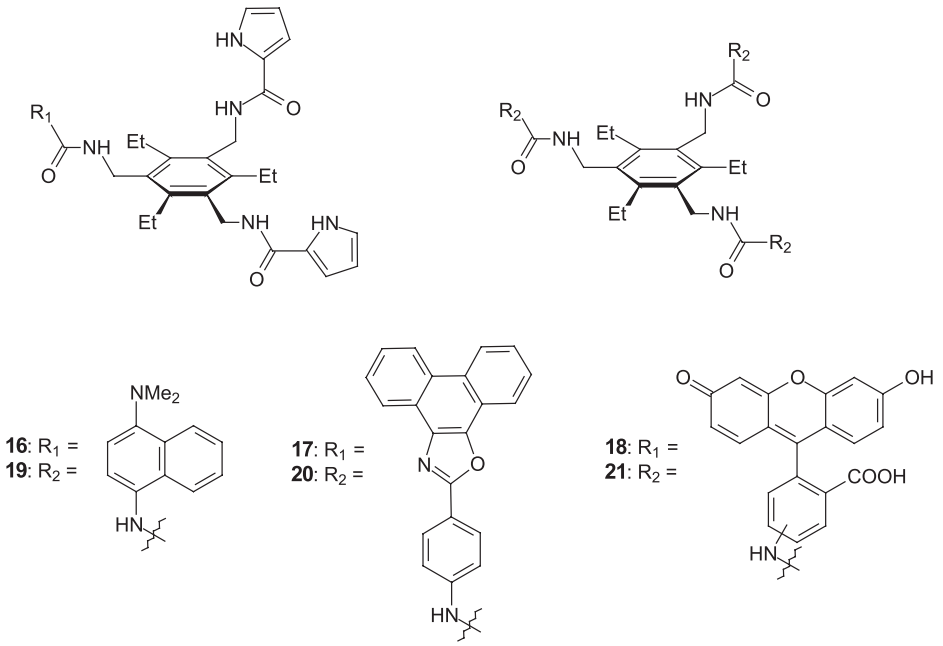
C =



Sapphyrins have been introduced by Sessler et al. [52] as fluorescent probes for phosphate. The water soluble sapphyrins **13**–**15** are highly aggregated and non-fluorescent at pH 7.0. The fluorescence of these probes is enhanced substantially after addition of phosphate. It has to be noted that water soluble sapphyrins are relatively non-toxic and can therefore be useful as biological phosphate probes. However, the influence of other anions has not been tested. Thus, predictions concerning selectivity are rather speculative.

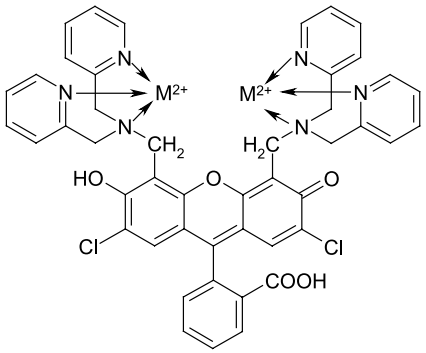
The recognition of phosphates by a specific probe often is accomplished via formation of hydrogen bonds. Each of the receptors **16**–**21** bears six hydrogen bond donors which enable the recognition of various anions such as phosphate, pyrophosphate, acetate, chloride, and bromide [53]. The binding of phosphate, pyrophosphate, and other anions in an organic solvent (not specified any closer) induces an up to 200-fold increase in fluorescence. The strong fluorescence enhancement was used for the development of differential probing of anions in arrays despite the relatively low selectivity of the probes. The specificity results from the response pattern which is unique for each analyte.

Probes **16**–**21** were incorporated into thin polyurethane films in the wells of a micro titer plate. The addition of various analytes (fluoride, chloride, acetate, benzoate, phosphate, pyrophosphate, AMP, and ATP) results in a dramatic increase of fluorescence of the doped polyurethane films which can be observed by naked eye. The degree of fluorescence enhancement is specific for each particular analyte. The identification of anions was carried out in pure water and HEPES buffer solution of pH 7.4. The array also has been applied to the analysis of phosphate-anions in blood serum.



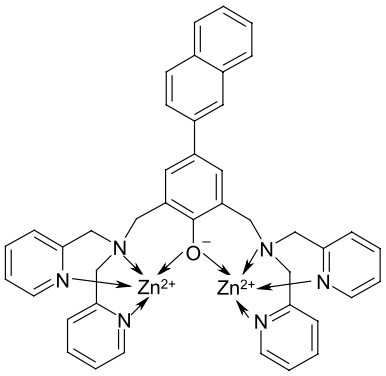
Probes for pyrophosphate

Jang et al. reported on the fluorescein-derived probes **22** and **23** for pyrophosphate (PPi). They are based on binding of PPi by metal cations coordinated by dipicolylamine (DPA) ligands [54]. The emission maximum of **22** is shifted from 523 to 534 nm at an excitation wavelength of 517 nm after addition of PPi. Moreover, a chelation enhanced fluorescence (CHEF) effect of around 150% is observed. The ratio of the signals at the two wavelengths was used to record a calibration curve. The same red shift in emission occurred when **23** was excited in presence of pyrophosphate, and the CHEF effect was around 25%. Both PPi probes have been used under aqueous conditions at pH 7.4 at a reagent concentration of 1 μmol L^{-1}. A series of other anions was found not to interfere, including phosphate. Therefore, **22** and **23** can serve as highly selective fluorescent probes for the determination of PPi under physiological conditions with the advantage of longwave excitation and emission.

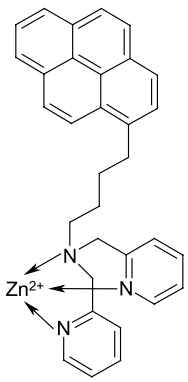


22 M = Zn
23 M = Cu

The group of Hong et al. [55] developed another probe (**24**) for pyrophosphate. It is based on the DPA recognition unit of **22** and **23** and is connected to a fluorophore via a linker. Addition of one equivalent of PPi to **24** resulted in a 9.5-fold enhancement of fluorescence. A bathochromic shift of the emission maximum from 436 to 456 nm is observed in water of pH 7.4. In the same course, a new absorbance band occurs at 316 nm besides the absorbance maximum at 305 nm. ATP and ADP display a twofold and 1.5-fold fluorescence enhancement after addition of a 4-fold and 50-fold excess, respectively. AMP and phosphate did not lead to any intensification of the emission intensity of **24** even in presence of 100 equivalents, thus rendering this probe a highly selective detection tool for PPi. This selectivity can be explained by the binding of PPi to the binuclear zinc complex by bridging the two metal ions leading to two hexacoordinated zinc(II) ions.



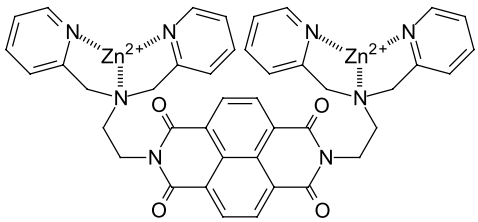
24



25

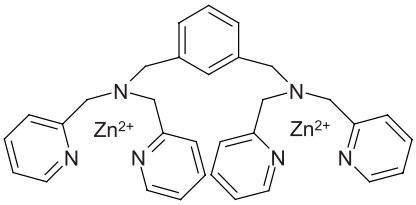
Cho et al. [56] also developed probe **25**, again based on the DPA recognition unit that benefits from a possible bridging effect. In contrast to probes **22**–**24**, compound **25** only bears one DPA unit connected to a pyrene fluorophore. Anion binding was supposed to bridge two probe molecules via their zinc(II) ions to result in excimer formation between two pyrene residues. Indeed, the effect of PPi binding on the pyrene emission confirmed the assumption of excimer formation due to the presence of a broad excimer emission band with a maximum at 475 nm (excitation at 343 nm) whereas the monomer emission was quenched. Among other phosphates such as ATP, AMP and Pi, only ATP induced a similar but less strong response. **24** as well as **25** can detect PPi selectively over other anions and can be used under physiological conditions. However, both probes suffer from shortwave excitation in the UV which limits their application in biological matrices, and the fact that the fluorescence of pyrene is strongly quenched by oxygen.

Lee et al. [57] have introduced probe **26** which also displays excimer formation exhibiting an emission peak at 490 nm upon addition of PPi. A weak enhancement of fluorescence at 490 nm at an excitation wavelength of 383 nm was observed in presence of ATP, whereas ADP, AMP or Pi did not induce any change in emission intensity. This accounts for a high selectivity of **26** for PPi. The complexation of PPi is again based on the DPA recognition unit. Compared to **25**, **26** can be excited at a longer wavelength close to the visible region.

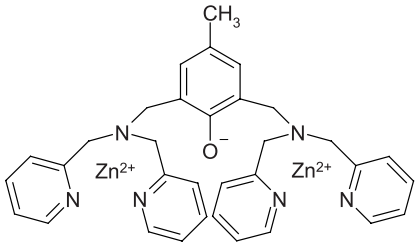


26

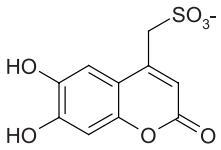
The zinc(II)-DPA recognition units also were applied in an indicator displacement mechanism scheme for fluorescent probing of PPi [58]. Receptors **27** and **28** were each attached to the soluble coumarin **29** via electrostatic interactions. In the resulting ion pair complexes, the fluorescence emission of **29** at 480 nm after excitation at 347 nm is quenched and can be restored after addition of PPi which displaces the indicator. Hydrogen phosphate also induces the displacement of **29** from **27** and **28**. However, **27** and **28** have an affinity that is almost two orders of magnitude higher for PPi than for hydrogen phosphate. Thus, the receptor-indicator complexes can selectively probe PPi under physiological conditions but again suffer from shortwave excitation at ∼340 nm where background luminescence can be quite strong.



27

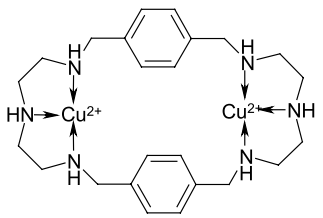


28

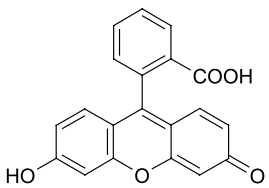


29

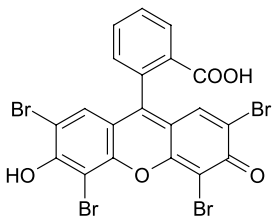
Receptor-indicator complexes based on the above displacement mechanism have been reported by Fabbrizzi et al. [59]. These can better discriminate between PPi and phosphate. The copper(II) complex **30** was used as the receptor which completely quenched the fluorescence of both indicators **31** and **32** after the formation of the corresponding receptor-indicator complex. Fluorescence is restored after binding of PPi by **30**, thus releasing the indicators from the complex. No interference was observed from a series of other anions including phosphate. This selectivity is ascribed to the bridging capability of PPi which coordinates the two copper ions in the receptor. Moreover, both the excitation and emission wavelength are in the visible, and the probes can be used at pH 7.



30

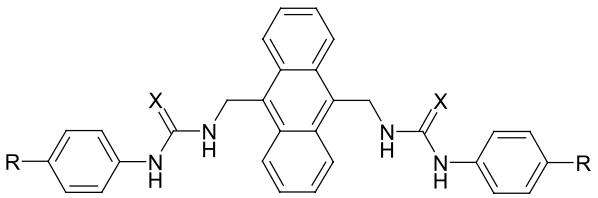


31



32

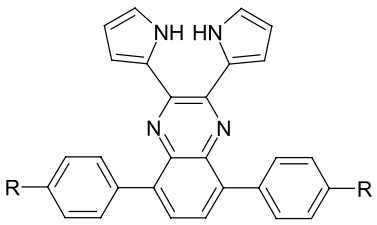
Gunnlaugsson et al. [60, 61] synthesized the metal-free probes **33**–**35** which represent an extension of the earlier PET probes. **33**–**35** are based on the *receptor-spacer-fluorophore-spacer-receptor* motif and are the first examples of uncharged fluorescent PET probes for PPi. The receptors are excited at 378 nm and show emission bands at 409, 431 and 454 nm whose intensities are quenched on interaction of PPi with the thiourea or urea groups present in the receptors. The binding between receptors **33**–**35** and PPi was found to have 1:1 stoichiometry indicating that PPi bridges the fluorophore moiety. Phosphate strongly interferes with PPi since it shows the same quenching effect. Moreover, probes **33**–**35** have only been used in DMSO since fluorescence is restored when measurements are made in hydrogen bonding solvents such as ethanol or water which compete for the recognition moieties. Other aprotic solvents have not been tested.



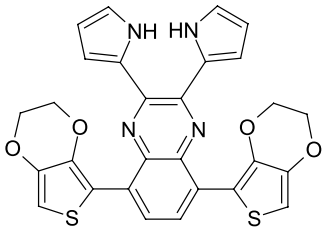
33: R = CF_3, X = S
34: R = H, X = S
35: R = CF_3, X = O

Aldakov and Anzenbacher [62] reported fluorescent probes having two pyrrole moieties connected to different fluorophores. Receptors **36**–**39** are capable of detecting PPi in aqueous solution. Fluorescence is

quenched by as much as 95% after addition of PPi. Phosphate, in contrast, does not change intensity, and this allows selective determination of PPi in presence of phosphate. The emission maxima for probes **36**–**39** are located at 500, 502, 610 and 590 nm, respectively, with excitation maxima found in the visible throughout.

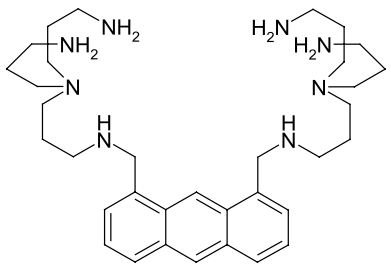


36: R = H
37: R = OMe
38: R = $N(Me)_2$



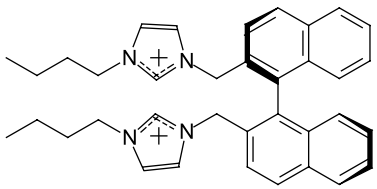
39

The PET effect is suppressed in another type of fluorescent probe for PPi detection [63]. The recognition unit in **40** consists of two tris(3-aminopropyl)amine residues which can bind both ends of PPi simultaneously. On binding of PPi by the nonfluorescent receptor **40**, fluorescence is strongly enhanced at 414 nm after excitation at 368 nm. The enhancement is due to protonation of the amine which reduces the PET effect. Additionally, **40** can discriminate between PPi and phosphate since the former is bound 2200 times more tightly. All measurements were carried out at pH 7. Probe **40** has been applied in an assay for inorganic pyrophosphatase. Fluorescence intensity decreased as a result of PPi hydrolysis and subsequent release of phosphate.



40

Kim et al. [64] reported another synthetic receptor (**41**) based on the PET principle. The UV emission of **41** is quenched by PPi. The emission maxima are located at 330 and 342 nm at an excitation wavelength of 310 nm. Phosphate induces a negligible decrease only in emission intensity and therefore does not significantly interfere in the detection of PPi, except if Pi > PPi. The application of **41** in biological matrices can only be realized when working under aqueous conditions. Unfortunately, **41** has not been tested in other solvents than acetonitrile.



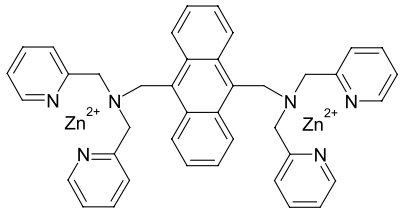
41

Probes for phosphopeptides and phosphoproteins

This section covers fluorescent probes for proteins that carry a phospho group on serine, threonine, or tyrosine, but also for phosphorylated amino acids. It does not cover probes that can recognize phosphoproteins by interaction with a conceivable saccharide moiety on such proteins, for example via a boronic acid group. It also does not cover methods of detection of phosphoproteins via immunological methods.

Ojida et al. [65–67] introduced the Zn(II)-dipicolylamine (DPA) dependent anthracene fluorophores **42** and **43** which can be applied to probing phosphorylated peptides. The probes are based on suppression of the photoinduced electron transfer (PET) process. The fluorescence intensity of **42** and **43** is increased four to five fold after recognition of a tyrosine-phosphorylated peptide having an overall negative charge. This, in turn, was not the case for the corresponding non-phosphorylated peptide. The emission was detected at 420 nm after excitation at 380 nm and at pH 7.2. It was assumed that the phosphate group on the surface of the peptide is bound to

the two zinc-DPA units due to electrostatic interactions. The probing of two other phosphopeptides, however, revealed that the overall charge of the peptide is also essential for phosphopeptide recognition. Positively charged phosphopeptides are not detected at all, whereas neutral ones only show minor responses.

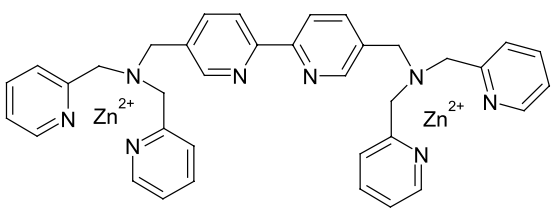


42

Moreover, receptors **42** and **43** can be used for probing phospho-tyrosine itself which induced an enhancement in emission intensities by a factor of three and 1.2, respectively. However, only **42** is capable of discriminating between phosphotyrosine and phosphate whereas in **43** phosphate and phosphotyrosine cause a similar fluorescence enhancement.

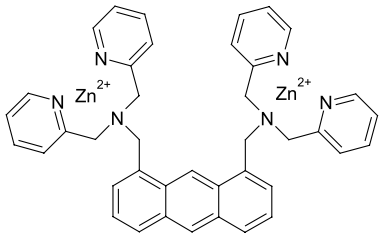
42 also has been utilized as a staining agent in SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) experiments [66, 68]. It can selectively stain phosphorylated proteins such as phosphoovalbumin and phospho-α-casein in a mixture of various different proteins present on the gel. A UV-lamp is adequate to visualize the phosphoprotein bands.

Ojida et al. [69, 70] also reported on a receptor based on the zinc(II)-DPA recognition principle for the detection of a multi-phosphorylated peptide in aqueous solution. Receptor **44** displays a decrease in fluorescence intensity at 389 nm after addition of a diphosphorylated peptide. A corresponding monophosphorylated peptide also induces a decrease in emission intensity but to a much lesser extent. The nonphosphorylated peptide does not affect the emission at all. **44** works best under aqueous conditions at pH 8.0. The receptor suffers from UV excitation and shortwave emission.



44

Anai et al. [71] subsequently developed a bioprobe based on the Zn(II)-DPA recognition unit. The so-called Pin1 protein selectively binds the multiphosphorylated C-terminal domain (CTD) repeat sequence of RNA polymerase II through the phosphoprotein binding domain (WW domain). It was shown by



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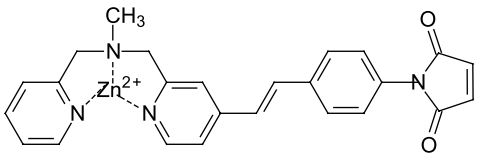
X-ray crystallographic analysis that the Pin1 WW domain only interacts with one (pS5) of two phospho groups on a serine phosphorylated pS2,5-CTD peptide.



45

It was intended to improve the probing capability of the Pin1 WW domain for the CTD peptide by introducing the artificial probe **45** (see Fig. 1). The maleimide group of **45** was used to attach the probe to a cysteine residue that was mutationally introduced into the WW domain. After addition of the bisphosphorylated pS6,9-CTD peptide (an analogue of the pS2,5-CTD peptide) the fluorescence intensity at 440 nm ($\lambda_{exc} = 340$ nm) increased by about 60%. The fluorescence enhancement is said to be due to the rigidification of the stilbazole fluorophore. The developed probing system also was used to establish a kinase assay in which the phosphorylation of the pS9-CTD peptide at Ser6 by a kinase was monitored.

We have used the Tb(III) complex with esculetin (see **46**) for the determination of phosphorylated amino acids and phosphopeptides. The Tb(III):**46** 1:1 complex displays an emission maximum at 465 nm (excitation at 405 nm) which is ascribed to the ligand, while no Tb^{3+} emission is observed. On addition of phosphoserine, -threonine, or -tyrosine at pH 7.0, the fluorescence intensity at 465 nm is increased by around 50% whereas the nonphosphorylated analogues only had negligible effects. Corresponding limits of

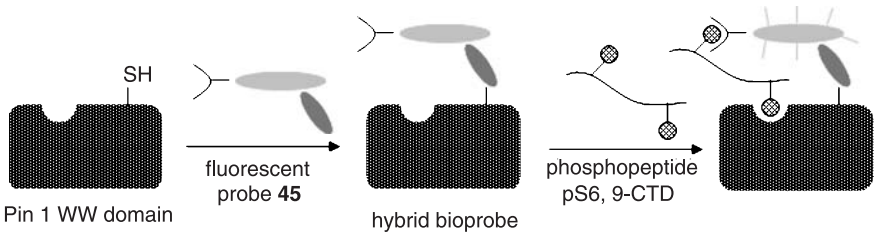
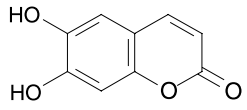


Fig. 1. Principle of probing a doubly phosphorylated peptide. The fluorescent probe **45** is covalently attached to the Pin1 phospoprotein binding domain (*Pin1 WW domain*) by thiol-maleimide coupling. The doubly phosphorylated peptide pS6,9-CTD is bound by both the Pin1 WW domain and **45**. Fluorescence increases due to rigidification of the stilbazole fluorophore in **45**

detection of 21.8, 8.5, and 5.1 μmol L^{-1} for phosphoserine, -threonine, and -tyrosine, respectively have been determined.



46

We also have tested the effect of the peptide GAEEKEYHAEGGK whose Y in position 7 was phosphorylated. The fluorescence intensity of the Tb(III):**46** probe was doubled in presence of the phosphopeptide (see Fig. 2) whereas the nonphosphorylated control hardly had any effect. This allows the selective determination of phosphorylated peptides. The LOD (limit of detection) in MOPS buffer [3-(N-morpholino)propanesulfonic acid] is 11.4 μmol L^{-1}.

Moreover, we have tested the effect of phosphoserine, -threonine, and -tyrosine and the respective nonphosphorylated controls on the fluorescence of the Al-morin complex. An increase in fluorescence at 535 nm after excitation at 405 nm was observed for the phosphorylated amino acids. The emission intensity was enhanced by around 50, 70, and 120% after addition of phosphoserine, -threonine, and -tyrosine, respectively. The control samples displayed very small (1–2%) quenching effects, in contrast. Limits of detection for phosphoserine, -threonine, and -tyrosine were found at 108.8, 103.8, and 239.8 μmol L^{-1}, respectively.

The effect of a phosphotyrosine containing peptide (see above) and its nonphosphorylated analogue on the emission intensity of the Al(III) complex is shown in Fig. 3. A 2.5-fold fluorescence enhancement in presence of the phosphorylated peptide is observed. The control sample also induces a 1.5-fold increase in fluorescence. However, the probe can clearly discriminate between the two peptides and can be applied at aqueous conditions of pH 7.0.

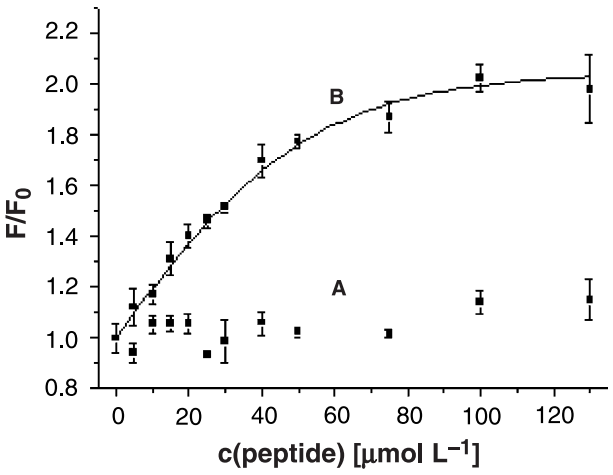


Fig. 2. Fluorescence response of the Tb(III):**46** complex (c = 20 μmol L^{-1}) at 465 nm to a nonphosphorylated (*A*) and phosphorylated peptide (*B*) in 10 mmol L^{-1} MOPS buffer of pH 7.0 at 25 °C

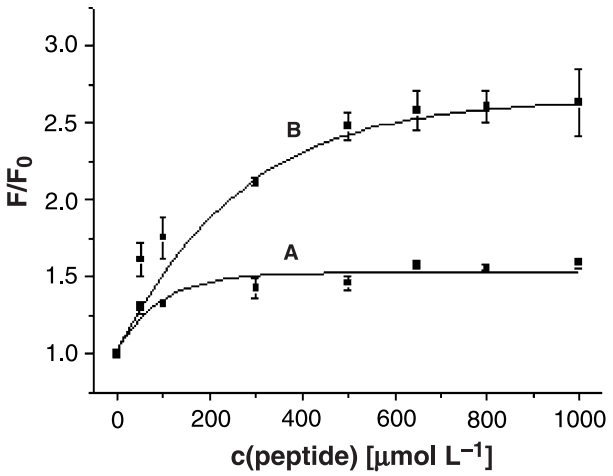


Fig. 3. Fluorescence intensity of the Al(III)-morin complex (c = 10 μmol L^{-1}) at 535 nm in presence of various concentrations of the non-phosphorylated peptide GAEEKEYHAEGGK (*A*) and of its Y-phosphorylated analog (*B*) in 10 mmol L^{-1} MOPS buffer of pH 7.0 at 25 °C

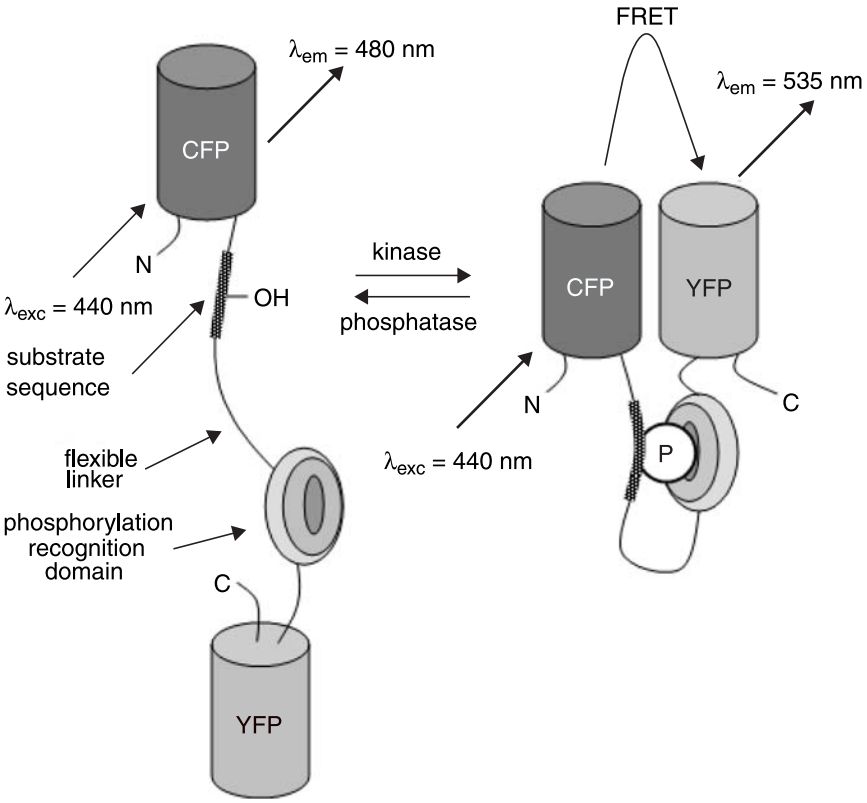


Fig. 4. Protein phosphorylation system (''PHOCUS'') [72]. The phosphorylation recognition domain binds to the phosphate group, after phosphorylation of the substrate sequence by a kinase, and a FRET signal is thereby induced. The reverse reaction can be effected by a phosphatase which diminishes FRET efficiency. *CFP* Cyan fluorescent protein; *YFP* yellow fluorescent protein; *C* C-terminus; *N* N-terminus

An imaging method for protein phosphorylation in single living cells was presented by Sato and Umezawa [72]. They have visualized the signal transduction induced by protein phosphorylation steps by developing a new indicator system (Fig. 4). A substrate domain for a certain kinase was connected, via a flexible linker, to a phosphorylation recognition domain. The two ends of this unit were labeled with both cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). These serve as donor and acceptor, respectively, in a fluorescence resonance energy transfer (FRET) system.

After phosphorylation has occurred at the substrate by the kinase of interest, the phosphorylation recognition unit interacts with the phosphate moiety introduced. As a result, FRET is induced between the two fluorescent proteins, and the degree of phosphorylation can be determined from the changes in fluorescence intensities of the donor and acceptor, respectively. This system, termed ''PHOCUS'', was applied for visualizing the signal transduction pathways for the insulin receptor, for a serine/threonine kinase that regulates a series of cellular processes, and for a non-receptor tyrosine kinase responsible for cell regulation and transformation.

The same concept was used to visualize protein phosphorylation by an extracellular signal-regulated kinase (ERK) [73]. The indicator used was named Erkus and consists of CFP and YFP sandwiching a substrate domain and a phosphorylation recognition domain which are connected via a flexible linker. An ERK-docking domain is fused to YFP. The substrate domain binds to the phosphorylation recognition domain upon phosphorylation by ERK resulting in decreased FRET efficiency between CFP and YFP due to a larger distance between the fluorophores.

The detection of protein phosphorylation is also possible by using only one fluorescent indicator connected to a substrate domain (where the phosphorylation takes place) and a phosphorylation recognition domain [74]. The so-called sinphos (single color florescent indicator for protein phosphorylation) can be phosphorylated by intracellular protein kinases. The fluorescence intensity of the attached fluorophore (mutants of green fluorescent protein) is changed after phosphorylation of the substrate domain by a protein kinase. Conformational alterations of the fluorophore are induced originating in binding of the phosphorylated substrate domain to the phosphorylation recognition domain.

A commercially available kit for the determination of phosphoproteins is being offered by Molecular Probes [75]. The so called Pro-Q Diamond gel stain can be used to selectively detect phosphoproteins in

gel electrophoresis. The stain, whose chemical nature is not revealed, is applicable to proteins bearing phosphate groups on serine, threonine and tyrosine residues. The limits of detection (LODs) range from 1 to 16 ng of phosphoprotein per band depending on the phosphorylation state of the protein. The response is linear over three orders of magnitude, and the fluorescent signal correlates well with the number of phospho groups. The excitation and emission maxima are located at 555 and 580 nm, respectively. Pro-Q Diamond also has been applied to quantifiy protein phosphorylation on microarrays. The detection limit for phosphoproteins was found to be 3–6 fg, depending on the number of phosphor groups [76].

Molecular Devices [77] has developed a reagent named IMAP for phosphopeptide detection via measurement of fluorescence polarization (Fig. 5). This technology is based on the use of nanoparticles containing trivalent metal ions which undergo a specific, high-affinity interaction with phospho groups. These can be free, or linked to serines, threonines or tyrosines, or to other molecules important to assess kinase activity. Fluorescently labelled peptides first are furnished with phospho groups in a kinase-assisted reaction. After addition of the IMAP reagent, the kinase reaction is stopped by the reagent and the phosphopeptides formed are bound by it. Hence, the autocorrelation time of the comparably small phosphopeptide

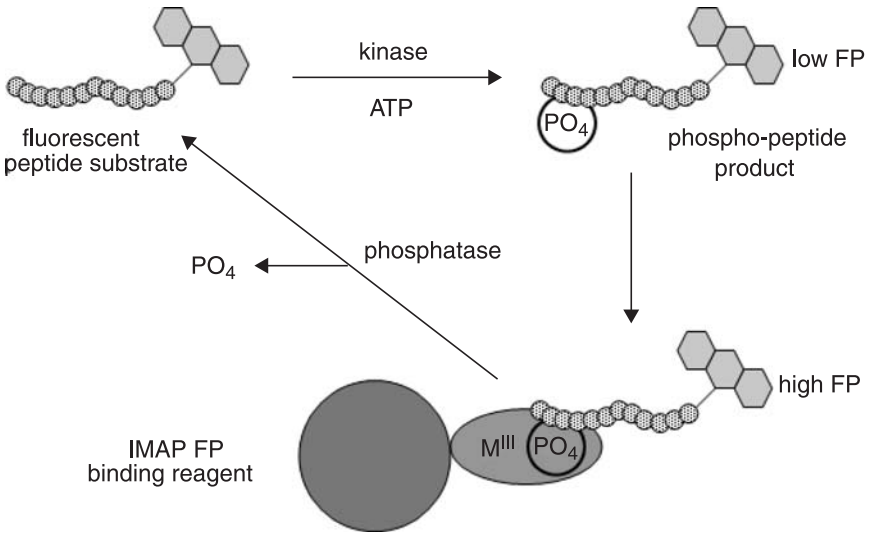


Fig. 5. Determination of phosphopeptides using the IMAP reagent. A fluorescently labeled peptide is phosphorylated in a kinase reaction. Addition of the IMAP reagent stops the kinase reaction and the phosphorylated peptide binds to IMAP. The reduced autocorrelation time of the phosphopeptide increases its fluorescence polarization (*FP*). M^{III} Trivalent metal ion

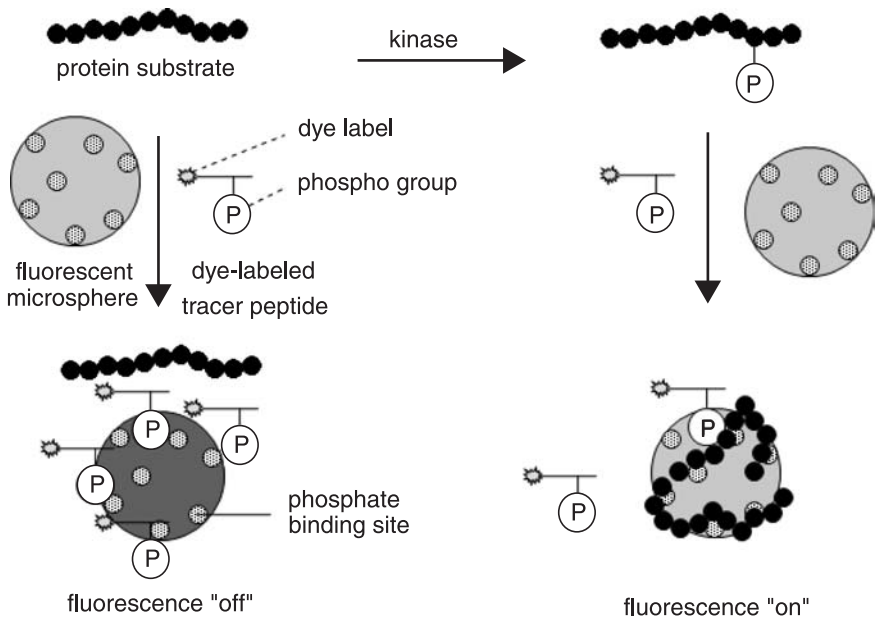
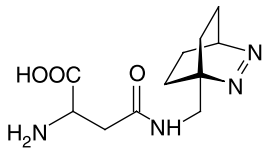


Fig. 6. Principle of monitoring kinase activity using the QTL Lightspeed assay. Phosphoproteins (product of kinase reaction) compete with phosphorylated dye-labeled tracer peptides for phosphate binding sites on the fluorescent microspheres. Displacement of tracer peptides (that cause quenching of polymer fluorescence) by phosphoproteins restores fluorescence which correlates with the extent of protein phosphorylation

is reduced so that an increase in fluorescence polarization can be measured.

A kinase assay has been developed which directly can detect phosphoproteins [78]. The so-called QTL Lightspeed assay consists of metal-ion coordinating groups which are co-located with a fluorescent polymer deposited onto microspheres. The phosphorylation of proteins inhibits the binding of phosphorylated dye-labeled tracer peptides to the phosphate binding sites on the microspheres due to competition with the phosphoproteins (see Fig. 6). Hence, the quenching of polymer fluorescence by the dye-labeled tracer peptides is inhibited generating a fluorescent signal. This system was applied to the determination of the activity of protein kinase Cα (PKCα) and of interleukin-I receptor-associated kinase 4 (IRAK4). Myelin basic protein (MBP), histone HI and phosphorylated heat- and acid-stable protein (PHAS-I) were used as substrates for the kinase reactions.

Phosphorylation of peptides can be monitored by the attachment of an artificial amino acid Dbo (**47**) to one end of the peptides with subsequent detection of FRET efficiency from the intrinsic fluorescent amino acid tryptophan (W) in the peptide chain to **47** [79]. It has been shown [80] that the W/**47** (donor/acceptor) FRET pair has a small transfer distance of about 10 Å which allows the determination of distances in small peptides.



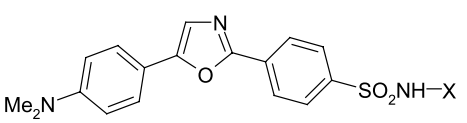
47

Two model peptides **I** and **II** (see Table 3) have been designed according to the recognition motifs of protein kinase A and C, respectively. The sequences of the peptides and their phosphorylated analogues are presented in Table 3. The FRET efficiency for **I** is decreased after phosphorylation of the serine residue to give **pI**. Contrastingly, the FRET efficiency for **II** increases upon phosphorylation (**pII**). This is said to be due to differing conformational changes after the introduction of the phospho group. Phosphorylation of **I** converts the serine residue into a sterically more demanding group leading to a larger donor-acceptor distance. The introduction of the phospho group in **II** favors hydrogen-bonding interactions between the phospho group and neighboring amino acid residues such as arginine.

Table 3. Sequences of model peptides for the determination of FRET efficiency between tryptophan (W) and **47**

Peptide	Amino acid sequence
I	LRRWSLG-**47**
pI	LRRWpSLG-**47**
II	WKRTLRR-**47**
pII	WKRpTLRR-**47**

Phosphorylated products of a kinase reaction can be determined by a method introduced by Wang and Lawrence [81]. Peptides containing phosphotyrosines are bound by protein-binding domains such as SH2 [82]. A tyrosine-containing peptide consisting of 11 amino acids first was labelled with either of the fluorophores **48**–**50** at various positions, and then phosphorylated by Src protein kinase. The kinase reaction product was subsequently bound by the SH2 domain and this results in enhanced fluorescence intensity, probably because of an altered microenvironment around the fluorophores. The increase in fluorescence was dependent on the amount of SH2 domain present. Hence, it is possible to determine the kinase activity at a fixed SH2 domain concentration.



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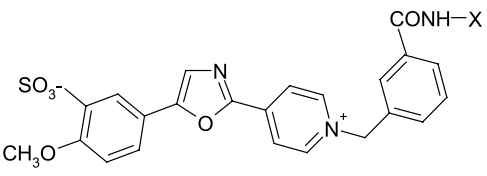

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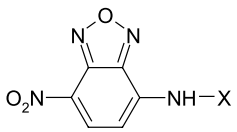


50

X = tyrosine containing peptide

Probes for phosphonucleotides

This section covers fluorescent probes for nucleotides, notably the monomers and their oligomers and polymers, both of the DNA and RNA type, of the respective di- and triphosphates, and of cyclic phosphoesters. It does not cover fluorescent probes that signal the presence of nucleosides by virtue of the interaction of the probe with the ribose unit of a nucleoside such as those based on organic boronic acids [83]. It also does not cover intercalators and other probes that do not interact with phospho groups. The issue here is selectivity since many probes have been reported to respond to phosphonucleotides which also are likely to detect other biophosphates.

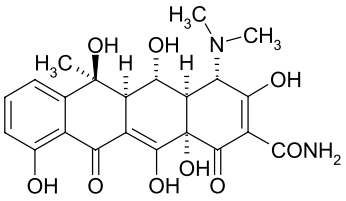
Probes for adenosine nucleotides

Phosphate is known to bind to Eu(III) complexes and thus can alter the spectrofluorometric properties of those complexes as presented earlier in this review. Hou et al. [84, 85] recently reported that the Eu(III) complexes with oxytetracycline **51** and doxycycline **52** can be used to probe ATP. Both complexes show enhanced fluorescence at 612 nm after excitation at 385 nm. While Eu(III)-**51** works best at pH 7.5, Eu(III)-**52** has its strongest fluorescence at pH 10. The limits of detection for ATP using Eu(III)-**51** and Eu(III)-**52** are 2.7 nmol L^{-1} (linear range from 0.08 to 1.5 μmol L^{-1}) and 41 nmol L^{-1} (linear range from 0.1 to 2.0 μmol L^{-1}), respectively. Fluorescence increases maximally fourfold for both probes. The coordination number of Eu(III) in its complexes with **51** and **52** is eight according to the authors. However, the coordination number cannot be reached due to ligand:Eu(III) ratios of 1.4:4 and 3:5 for Eu(III)-**51** and Eu(III)-**52**, respectively. ATP can replace water molecules bound to the free coordination sites and therefore compensates the energy loss through the O-H vibration of water. Hence, energy transfer from **51** and **52** to the central Eu(III) ion is facilitated resulting in increased fluorescence intensity. Regarding the pH conditions and limit of detections for ATP, Eu(III)-**51** is the probe of choice for ATP determination. Eu(III)-**51** and Eu(III)-**52** both show fluorescence enhancement after addition of ATP while the emission intensity of the closely related Eu(III) complex with tetracycline (**3**) is quenched in presence of phosphate. It should be reminded here that the limits of detection depend on the concentrations of probes employed.

It was shown that the luminescence of the EuTC complex also is influenced by ATP [86]. Seventy five percent of the initial fluorescence is quenched in presence of ATP whilst, rather surprisingly, ADP has no significant effect. A limit of detection for ATP of 1.5 μmol L^{-1} was determined. Protein kinases transfer the terminal phospho group of ATP to serine or tyrosine residues of the kinase substrate thereby releasing ADP. Hence, EuTC was applied to the determination of the activity of a creatin kinase since no free phosphate anions (that could interfere due to their quenching properties) are present in solution. The enzyme assay was carried out at 30 °C at pH 7.4. These results clearly point to the potential of Eu(III) complexes for the determination of phosphate-containing analytes even in association with enzymatic assays.

Another Eu(III) based complex was used by Shtykov et al. [87] for the selective determination of ATP. The fluorescence of the Eu(III)-trifluoro-(2-thenoyl)acetone (TTA) (**53**) complex displays a maximum at 615 nm after excitation at 330 nm. The fluorescence of this complex is strongly enhanced if it is solubilized in Brij-35 micelles. Addition of ATP to the Eu(III)-TTA-Brij-35 system leads to a reduction in emission intensity by two orders of magnitude. The calibration graph revealed a linear concentration range of 100 to 1.0 μmol L^{-1} with a detection limit of 0.6 μmol L^{-1} for ATP. AMP does not interfere, however, the effect of other adenosine nucleotides was not tested. The probe was also applied to the determination of ATP in fruit juices showing that sucrose, fructose and glucose do not interfere.

Li et al. [88] introduced the Eu(III) complex with ligand **54**. Its fluorescence intensity ($\lambda_{ex}/\lambda_{em} = 575/615$ nm) is extremely amplified in presence of ATP due to the replacement of **54** by ATP. Phosphate, ADP and AMP also induce a fluorescence enhancement which restricts the use of the Eu(III)-**54** complex for in situ tracking of single-molecule reactions. Furthermore, Li et al. [89] also reported a Eu(III) iron-sulfur hybrid cluster (**55**) deduced from the structure of **54**. Again, adenosine mono-, di- and triphosphate cause an increase in fluorescence intensity. The emission band is centered at 440 nm after excitation at 300 nm. The non-selectivity of both Eu(III) complexes is partially compensated for by the option of applying them at pH 7.4.



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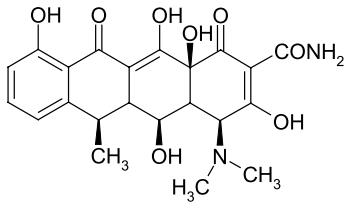

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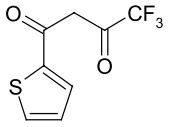

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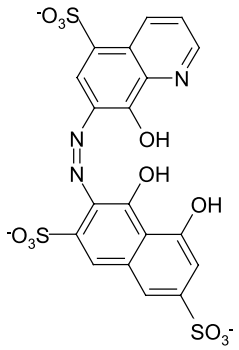

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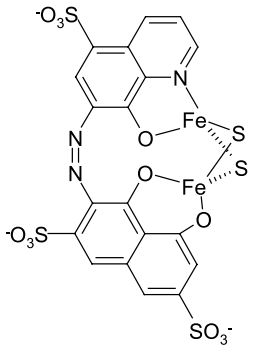

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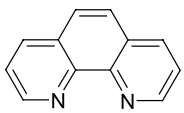
Certain Tb(III) based complexes also have been described as fluorescent phosphonucleotide probes [90, 91]. 1,10-phenanthroline (**56**) was reported to form a complex with Tb(III) that displays an emission maximum at 543 nm at an excitation wavelength of 298 nm. The fluorescence intensity increases in the presence of ATP at pH 7.0. Regrettably, ADP and AMP also induce a fluorescence amplification which is slightly smaller but cannot be ignored. The limits of detection for pure solutions of ATP, ADP and AMP were found to be 1.0, 0.6, and 0.2 $\mu mol\,L^{-1}$, respectively.



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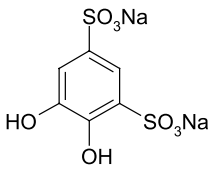

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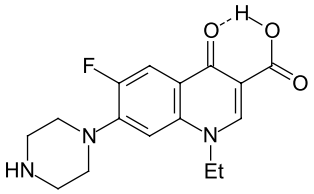
Sensitive determination of nucleotides was demonstrated [91] by making use of the Tb(III) complex with tiron (**57**) whose fluorescence is quenched by nucleotides. The emission intensity at 546 nm decreased significantly after addition of ATP, ADP and AMP when excited at 317 nm. The extent of quenching is dependent on the nucleotide added: ATP > ADP > AMP. This is assigned to the number of phosphate residues available for binding to Tb(III). The experimentally determined number of binding sites of ATP, ADP and AMP (3.09:1.96:1.00) was equal to the number of phospho groups in these nucleotides. The quenching mechanism was explained by competition between the phospho anions and **57** for the Tb(III) ion. Under the optimal conditions (at pH 6.9), the nucleotides form a non-fluorescent complex with Tb(III). However, Al(III), Cu(II) and phosphate severely interfere.

Norfloxacin (**58**) forms a fluorescent complex with Tb(III) that displays the typical narrow emission of Tb(III) ion at 545 nm after excitation at 335 nm. ATP was reported to enhance the emission at a pH of 7.4 [92]. A linear relation between fluorescence enhancement and ATP concentration was found between 1 and 16 $\mu mol\,L^{-1}$ with a detection limit of 41 $nmol\,L^{-1}$. The influence of a series of metal ions on the emission intensity of the complex was tested. Ions such as Co(II) and Ca(II) alter fluorescence intensity by 9.4 and 7.8%, respectively, while other ions had negligible effects. We have tested the selectivity of the fluorescence response towards other nucleotides and phosphates. ADP causes a similar fluorescence enhancement when compared to ATP, whereas cAMP does not interfere. Thus, we were able to establish a fluorescent assay for the determination of adenylyl cyclase activity using Tb-norfloxacin [93, 94].

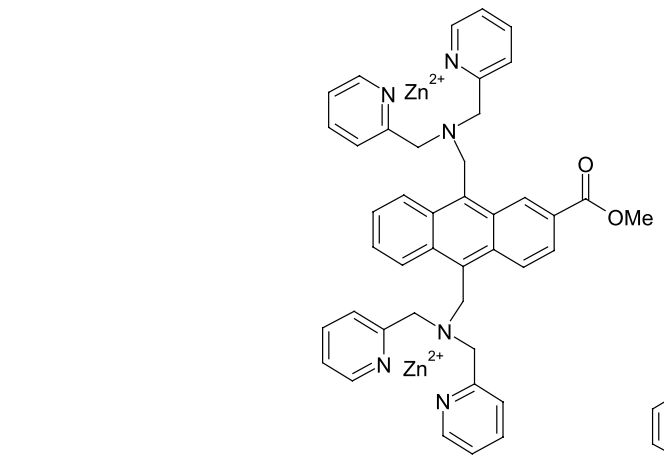


58

The group of Hamachi [95–97] has developed Zn(II)-dipicolylamine (Zn-DPA) complexes such as **59** which undergoes a two-fold increase in emission intensity at 460 nm after excitation at 380 nm on addition of ATP. The binding stoichiometry between receptor and analyte was found to be 1:1, with a binding constant of $2.2 \times 10^6\,\mathrm{L\,mol^{-1}}$ for ATP. The binding constants for ADP and AMP are smaller, whereas cAMP is not bound at all. However, phosphate and methyl phosphate also cause a twofold increase in fluorescence intensity which lowers the discrimination ability of **59**. Nevertheless, **59** exhibits different responses to three different nucleotide triphosphates. CTP induces a weaker fluorescence enhancement than ATP whereas GTP quenches the emission. This probe can be employed in aqueous solutions of pH 7.2.

60 and **61** are two more representatives of the zinc-DPA family [96]. They enable probing of ATP in a ratiometric manner. Addition of ATP to **60** and **61** leads to a decrease in emission intensities and a short-wave shift of the emission maxima (from 468 to 441 nm and 473 to 420 nm, respectively). The excitation wavelength lies at 368 nm. Phosphate (Pi), AMP and cAMP do not cause any change in the emission intensity of **61**, whereas pyrophosphate (PPi) has the same effect as ATP. This limits the use of **61** for applications in enzymatic assays where both ATP and PPi are of interest, for example in adenylyl cyclase-based assays where ATP is converted into PPi and cAMP.

Zinc-DPA based receptors can also be embedded in supramolecular hydrogels [97]. Receptor **62** was incorporated into a hydrogel based on a glycosylated amino acetate derivative **63**. The fluorescence of probe **62** (resulting from the dansyl fluorophore) is quenched on addition of ATP. Phosphate and phosphotyrosine also cause quenching of the emission at 512 nm after excitation at 322 nm but to a lesser extent. Noteworthy, these observations are not found in aqueous solution or in an agarose gel which lacks hydrophobic domains. These seem to be essential for



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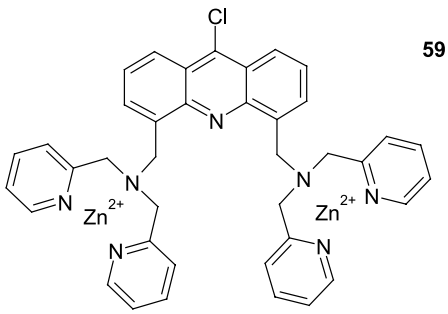
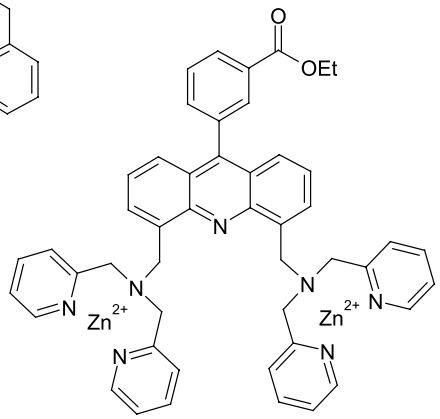


60



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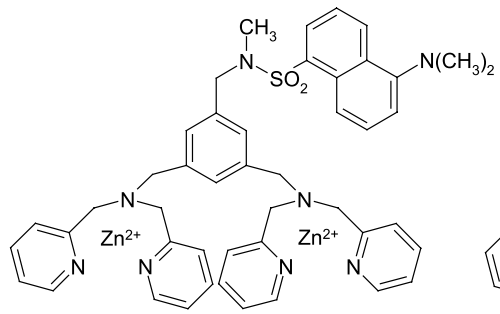
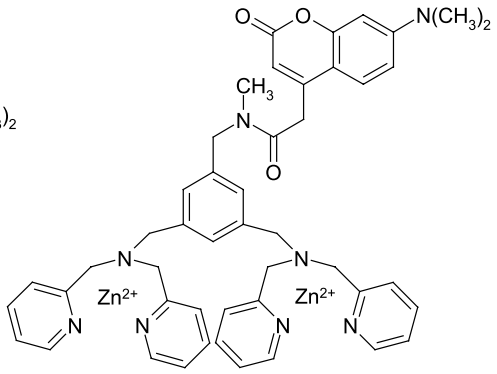


62



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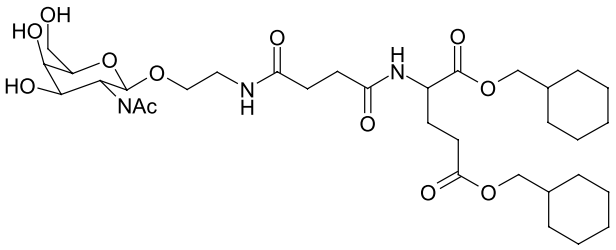
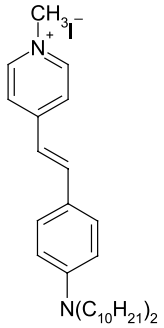


63

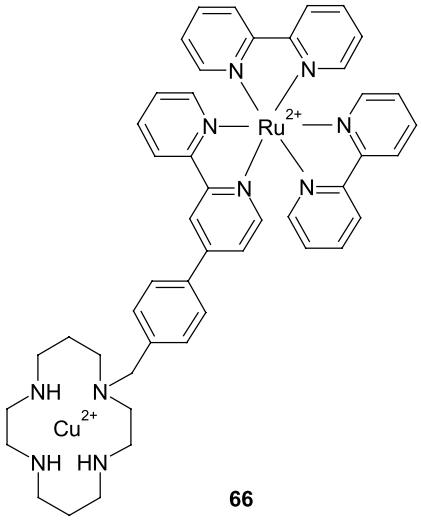


64

the fluorescence to change after analyte binding by **62**. The hydrophilic ATP induces a red shift and a decrease in fluorescence and the hydrophobic phenyl phosphate caused a blue shift with enhanced emission intensity. Obviously, the microenvironment of the receptor-analyte unit seems to have a large impact on the probing capability.

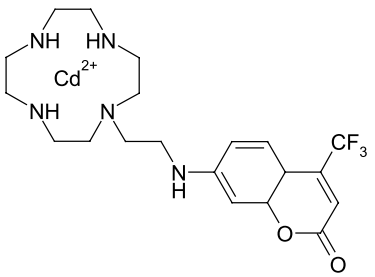
These findings were used [97] to develop a FRET based sensing system. The hydrophobic styryl dye **62** and the receptor **64** (acting as FRET acceptor and donor, respectively), were embedded in a gel matrix of **63** the hydrogel of which has many nano-sized fibers. Two emission peaks were observed at 485 and 569 nm after excitation at 393 nm before analyte addition. The emission intensity at 485 nm was reduced in the presence of phenyl phosphate, whereas the emission peak at 569 nm increased which is typical for FRET systems. This indicated the mobility of **65** in the hydrogel and the recruitment of the hydrophobic analyte-receptor complex to the hydrophobic domains of the hydrogel. In contrast, the emission at 485 nm intensified with increasing ATP concentration while it was reduced at 569 nm. The hydrophilic analyte-receptor complex led to a larger distance between the donor in the hydrophilic cavity and the acceptor being located in the hydrophobic domains of the hydrogel. Hence, the ability for energy transfer in the FRET system was reduced.

Padilla-Tosta et al. [98] reported on a dinuclear system containing a ruthenium(II)-terpyridine core connected to a copper(II)-polyamine unit. The fluorescence of **66** at 650 nm ($\lambda_{exc} = 484$ nm) is quenched in presence of ATP, whilst ADP and GMP did not interfere at all. However, probe **66** has a pH dependent fluorescence, and the detection of ATP is possible between pH 9 and 11 only. At physiological pH values the fluorescence of **66** is quenched. Phosphate and GMP strongly interfere. Furthermore, this probe has only been applied to acetonitrile/water (70:30 v/v) solutions. The fluorescence intensity of **66** is dependent on the distance between the copper(II) unit and the ruthenium(II) core. ATP is thought to bind electrostatically to the copper(II) unit and consequently to alter the geometry in the probe. The altered distance between the two subunits of **66** is responsible for the changes in fluorescence. The effect of ATP or other nucleotides on the fluorescence lifetime of **66** would be worth being investigated.



66

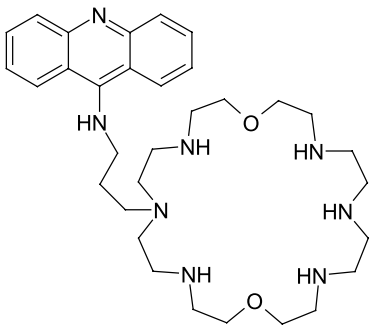
The Cd(II) ion complex **67** is coordinated by a cyclen unit carrying a 7-aminocoumarin group which can chelate Cd(II) as the fifth ligand [99]. Due to the relatively weak coordination of the coumarin it can easily be replaced by anions such as ATP, ADP, and AMP. Addition of AMP to **67** shifts the excitation maximum from 349 to 380 nm (at an emission wavelength of 500 nm). ADP and ATP have even higher affinities for **67**. Nevertheless, this receptor can be applied to the discrimination of AMP and cAMP since the latter cannot be detected by this probe. Probe **67** was utilized in a phosphodiesterase activity assay at near-neutral pH. Phosphodiesterase activity was detected by probing the increase of AMP due to the hydrolysis of cAMP.



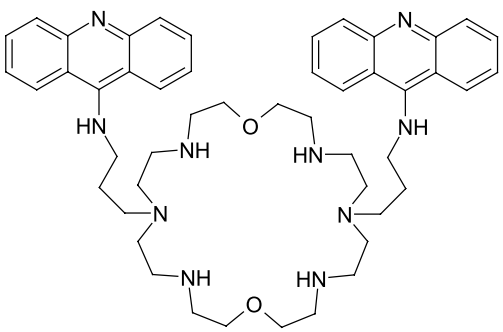
67

All receptors for adenosine phosphates presented so far benefit from the presence of a metal cation that undergoes electrostatic and/or coordinative interaction with phosphate. Several other synthetic probes have been synthesized that do not possess a chelating unit. Lehn et al. reported macrocyclic polyamines covalently linked to acridine groups (**68** [100] and **69** [101]). Both receptors carry four protons on the cyclic amine groups at neutral pH. These are responsible for the recognition of phosphates. The fluorescence of **68** and **69** is intensified by 150% and 250%, respectively, on addition of ATP. Excitation and emission wavelengths were set to 412 nm and 450 nm, respectively. The stoichiometry between ATP and receptor was determined to be 1:1. The interaction is both electrostatic (between the phosphate groups of ATP and the ammonium groups of the receptor) and of the stacking type (between the adenine group of ATP and the acridine unit on the receptor). The latter accounts for the immense fluorescence enhancement. However, the fluorescence is not constant over time in the case of **68** due to the hydrolysis of the triphosphate group of ATP and the release of ADP. **69** does not show such a behavior. ADP also causes an increase in fluorescence of **69** by 78%, whereas the fluorescence enhancement by 9% after addition of AMP is comparably small. A discrimination between ATP and other triphosphate nucleotides is hardly possible. The increase in emission intensity is 209% and 178%, respectively, in the case of CTP and UTP. Hence, **69** can also be applied to the detection of CTP and UTP.

Cudic et al. [102] also introduced (bis)phenanthridinium based receptors such as **70** which shows a higher fluorescence quantum yield than the probes **71**–**74**. The emission intensity is reduced due to the stacking interactions between the phenanthridinium

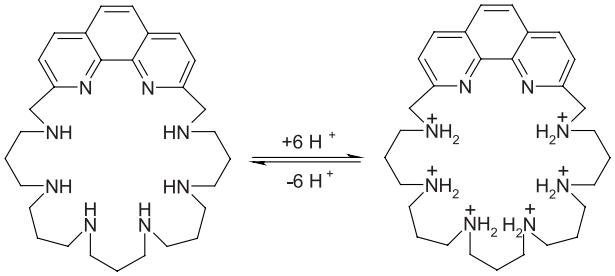


68

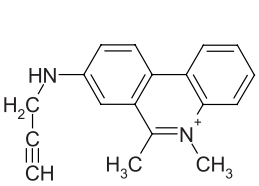


69

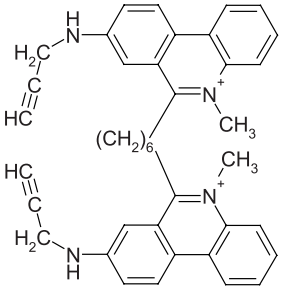
units of the latter probes. Addition of ATP to any of the fluorophores **70**–**74** causes a quenching of their fluorescence. Excitation and emission wavelengths are located at around 280 and 530 nm, respectively. These receptors lack specificity for ATP since each shows almost the same stability constants for ADP, AMP, GMP, CMP, UMP and TMP. Therefore, **70**–**74** can be utilized as overall probes for nucleotide detection.



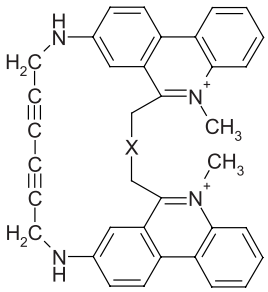
75A **75B**



70



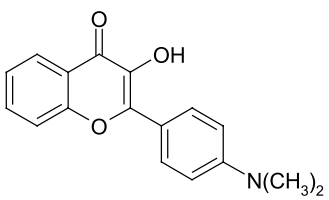
71



72: X = $(CH_2)_4$
73: X = $(CH_2)_6$
74: X = *p*-C_6H_4

Phosphonucleotide anions can be recognized [103] by a cyclic polyammonium unit connected to a fluorescent phenanthroline unit. Receptor **75A** is hexaprotonated at below pH 7.0 (**75B**) and exhibits the typical emission band of phenanthroline at 365 nm. The aliphatic ammonium groups deprotonate at higher pH values and then give rise to quenching by electron transfer processes. Hence, measurements have been performed at pH 6.0 in order to warrant complete protonation of the aliphatic amino groups of **75A**. The binding of ATP comes about by electrostatic and hydrogen-bonding interactions. π-stacking interactions additionally contribute to the strong ATP-receptor interaction. The emission at 365 nm at an excitation wavelength of 270 nm is quenched after ATP binding. This is said to be due to a partially induced proton transfer from the ammonium groups to the phosphate moieties of ATP which enhances the quenching efficiency of the polyammonium unit. ADP and AMP cause a 10% decrease in emission intensity. Quenching by maximally 30% is observed for CTP, while GTP and TTP only show minor quenching properties. Hence, the recognition of ATP by **75B** is relatively selective but the method suffers from the shortwave excitation and emission.

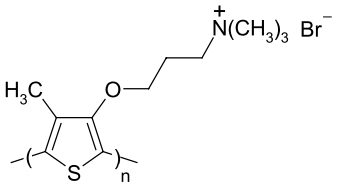
The flavone derived probe **76** was developed by Pivovarenko et al. [104] for the selective detection of ATP which induces a shift of the excitation maximum from 400–410 to 470–480 nm at an emission peak of 554 nm. In presence of various other mono-, di- and triphosphates (ITP, GTP, UTP, ADP AMP, GMP) no such shift is observed. The emission of **76** at 554 nm in presence of ATP increases significantly without any shift of the emission maximum. However, the selectivity of **76** for ATP is limited because increased fluorescence also is induced by certain other mono-, di- and triphosphates. The analyte-receptor interaction arises from intermolecular stacking between the adenine unit and **76**. Furthermore, the phosphate residues of ATP can interact with the positive part of the dipole in **76**. The measurements were carried out at pH 7.4.



76

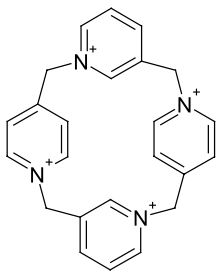
ATP also can be detected [105] with the help of polythiophene derivatives such as **77** which displays

intrinsic fluorescence at 536 nm after excitation at 445 nm. The emission intensity is quenched by up to 84% on addition of ATP, and the emission band is slightly broadened. ADP and AMP quench by 55 and 12%, respectively. The LOD for ATP is in the order of 10^{-8} mol L^{-1} at pH 7.4.

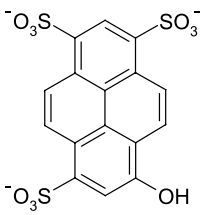


77

Another approach for ATP determination [106] is based on the property of the calixpyridinium tetracation **78** to bind to pyranine (**79**), a strongly fluorescent compound, via electrostatic interactions. Upon binding, the green fluorescence of **79** is quenched in a first step. ATP is capable of forming a strong complex with **78** and thereby displaces **79** whose fluorescence is subsequently recovered. ATP can be determined at pH 7.5 at excitation and emission wavelengths of 440 and 520 nm, respectively. The effect of ADP and AMP is negligibly small due to incomplete displacement of **79** which allows the selective determination of ATP.



78



79

Hagihara et al. [107] reported the use of fluorescent ribonucleopeptide (RNP) complexes for the detection of ATP. The RNP consists of an ATP binding RNA subunit and a peptide modified with a pyrenyl group (Fig. 7). The ATP binding RNP receptors were obtained by in vitro selection of a library of stable RNP complexes. The addition of ATP to an RNP complex yielded increased fluorescence of the pyrenyl group. UTP, CTP or GTP did not interfere due to the high selectivity of the ATP binding domain in the RNA subunit of the RNP.

The same principle was applied to the development of GTP selective RNP complexes by using binding domains for GTP rather than ATP. All measurements were carried out at pH 7.6. Depending on the fluorophore attached to the peptide, fluorescent probes were obtained with excitation and emission maxima that range from 340 to 650 nm, and from 390 to 670 nm, respectively.

The macrocyclic pyrenophane **80** was syntzhesized [108] with the aim to detect nucleotide triphosphates. **80** consists of two pyrene fluorophores connected by two diazoniacrown units. In absence of any analyte the excimer emission of the pyrene fluorophores can be observed at 520 nm after excitation at 370 nm. The excimer emission was reduced on addition of ATP due to π-stacking interactions between the nucleobase and the pyrene fluorophores. Additionally, ATP is recognized as a result of electrostatic interactions between its phosphate moieties and the positively charged diazoniacrowns. The binding constants for GTP, CTP and UTP were close to that of ATP whereas those of mono- and diphosphates were much smaller. Hence, **80** can selectively probe nucleoside triphosphates. All measurements can be carried out in water.

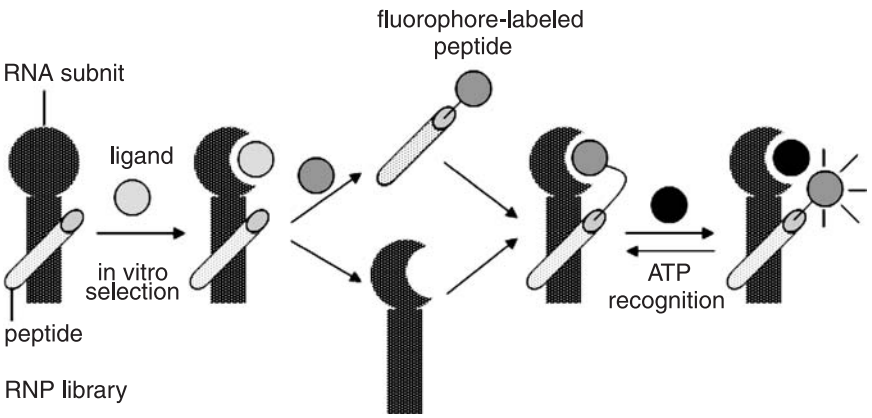
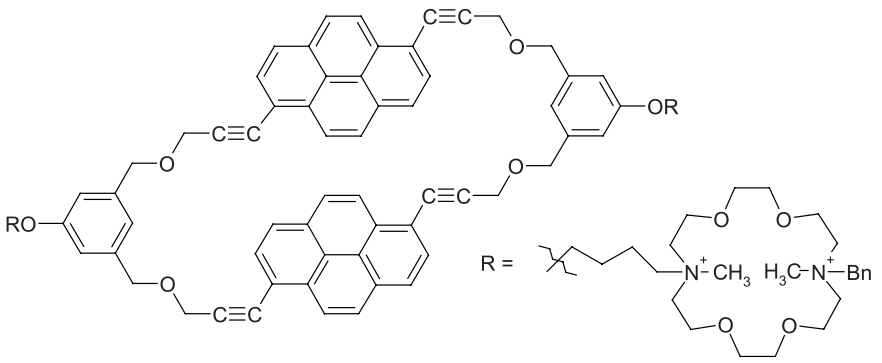


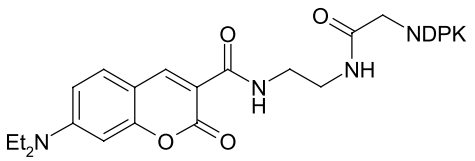
Fig. 7. Identification of ribonucleopeptide (RNP) receptors by in vitro selection of a library of stable RNP complexes against ATP-agarose resins (*ligand*). Subsequent labeling of the peptide unit with a fluorophore allows the detection of ATP binding by the ATP binding domain of the RNA subunit which generates a fluorescent signal



80

Srinivasan et al. [109] published an ADP-specific probe based on the generation of fluorescence after recognition of ADP by an ADP aptamer. Several selection steps during a so-called SELEX process were applied in order to achieve a high discrimination between ADP and ATP. Therein, RNAs that bind to ADP and ATP are separated, and the one with the highest affinity for ADP is amplified. This aptamer was subsequently appended to a hammerhead ribozyme core via a spacer consisting of eight randomized nucleotides. Molecules active in the presence of ADP were identified in several rounds of selection. The introduction of fluorescein at the 3′ end of the allosteric ribozyme and the hybridization of an oligonucleotide with the label dabcyl at its 5′ end leads to the desired fluorescence signaling system. The close proximity of dabcyl to the fluorescein unit prevents fluorescence emission of the fluorescein fluorophore. The self-cleavage of the molecule on ADP recognition leads to the release of the fluorescein labeled oligonucleotide which generates a fluorescent signal at 535 nm. Measurements were carried out under aqueous conditions at pH 8.0. The probe works in the presence of excess ATP and was applied to a kinase assay where ATP is consumed and ADP is formed. The detection limit for ADP is 1.0 µmol L^{-1}.

Nucleoside diphosphate kinases (NDPK) can be utilized for the fluorescent determination of nucleoside diphosphates [110]. NDPKs catalyze the transfer of the γ-phosphate group of a purine nucleoside triphosphate to another purine nucleoside diphosphate. Single cysteine residues were introduced in the NDPK which are necessary for fluorophore attachment. The thiol-reactive and environmentally sensitive fluorophore **81** was covalently attached at the edge of the nucleotide binding cleft of the NDPK in order to gain a fluorescent probing system with excitation and emission wavelengths located at 430 nm and 475 nm, respectively.

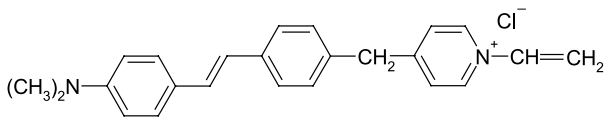


81

It was found that the NDPK-**81** conjugate has a four-fold higher fluorescence intensity on addition of ATP than on addition of ADP. This indicates that the transfer of the γ-phosphate group gives the phosphorylated NDPK-**81** (p-NDPK-**81**) which is a highly fluorescent species. The fluorescence of p-NDPK-**81** is quenched to 25% of its intensity in presence of ADP as a result of phosphate transfer from p-NDPK-**81** to ADP. The presence of ATP has no effect on the emission of p-NDPK-**81**. Since NDPK cannot discriminate between ADP and GDP, this system can probe both dinucleotides. All measurements can be carried out at neutral aqueous conditions. This system benefits from its ability to detect diphosphate nucleotides in presence of an excess of triphosphate nucleotides. This is important for example in experiments with single muscle fibers where initial ATP concentrations are high.

Imprinted polymers were employed for the detection of cAMP by Wandelt et al. [111]. The fluorescent indicator **82** readily undergoes polymerization in the presence of cAMP. **82** was earlier used for the detection of purine nucleotides in solution but cannot discriminate between cAMP and cGMP [112]. cAMP is washed out of the polymer matrix after polymerization has been completed, and this results in a template void volume for cAMP. The fluorescent styryl dye **82** shows high sensitivity to nucleotides which makes it an excellent indicator probe for the detection of nucleotides such as

cAMP. The imprinted polymer was deposited as a thin-layer on quartz plates. The addition of cAMP in methanol to the imprinted polymer resulted in a diminished fluorescence at 565 nm after excitation at 469 nm. The extent of quenching on the thin-layer film was much more effective compared to the molecular imprinted polymer obtained in bulk. Moreover, the cross-selectivity of the cAMP imprinted thin-layer polymer was tested with cGMP. There was no change in emission intensity after addition of cGMP which confirmed the high specificity of the imprinted polymer technique and makes it a useful tool for the detection of cAMP.

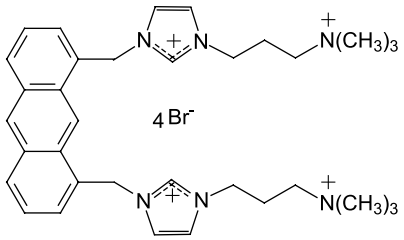


82

Probes for guanosine nucleotides

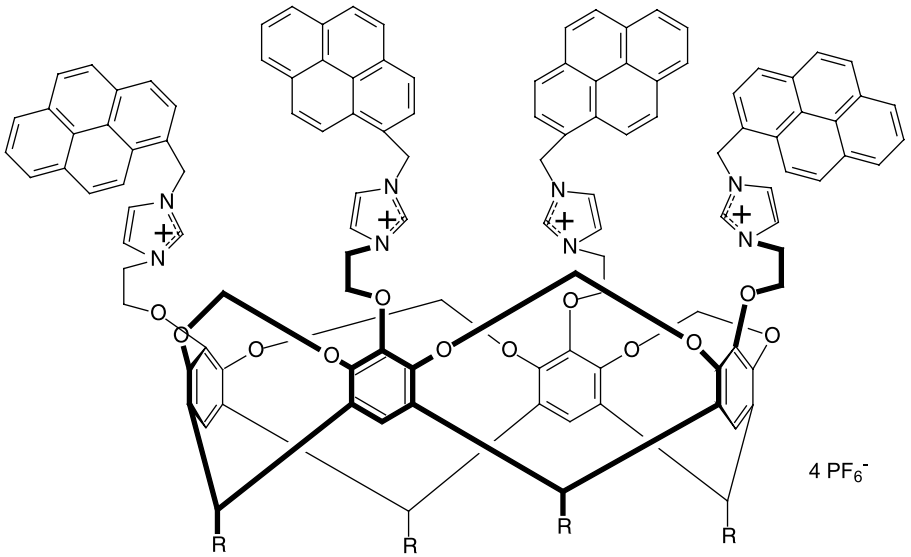
Tb(III) ion forms a fluorescent complex with GTP and was applied to the determination of GTP [113]. In continuation of this scheme [114] the Tb(III)-Gd(III)-*o*-phenanthroline (phen) system was found to enable determination of GTP because the emission intensity of the Tb(III)-phen-GTP system is strongly increased on addition of Gd(III). Fluorescence is read at the typical Tb(III) emission peak at 535 nm after excitation at 300 nm. The respective calibration plot is linear for GTP between 1.0×10^{-9} and 3.0×10^{-5} mol L^{-1}, with a limit of detection of 3.1×10^{-10} mol L^{-1} at pH 9.5. Al(III) and certain other nucleotides (not specified any closer) strongly interfere. Its high sensitivity makes this system suitable for the determination of trace amounts of GTP.

Compound **83** is a fluorescent probe for GTP that works in aqueous solution and can differentiate between GTP and ATP [115]. While the presence of ATP and to a lesser extent ADP and AMP evokes a chelation enhanced fluorescence (CHEF) effect the addition of GTP shows a strong chelation enhanced quenching (CHEQ) effect. Excitation and emission wavelengths are located at 367 and 415 nm, respectively. **83** can be used at pH 7.4. Pyrophosphate and primary phosphate do not interfere. The stoichiometry between **83** and GTP was found to be 1:1. Probe **83** represents the first artificial fluorescent receptor for GTP capable of clearly differentiating between GTP and ATP. However, its use is compromised by the relatively shortwave excitation wavelength.



83

The same group [116] reported on the use of a cavitand for selective probing of GTP. The rather com-

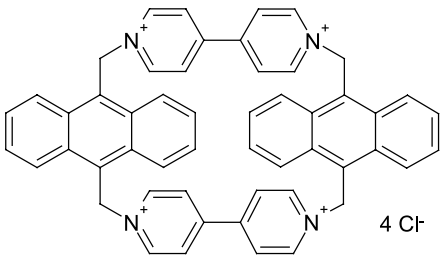


R = CH_2CH_2Ph

84

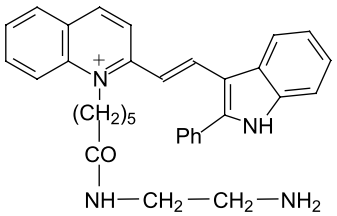
plex fluorophore **84** bears four imidazolium groups and four pyrene groups giving the fluorescent signal. ATP, ADP and CTP exhibited a small CHEF effect, and other phosphate containing anions did not interfere at all. GTP, however, showed a CHEQ effect. The emission intensity of **84** at 480 nm (excitation at 367 nm) is quenched after addition of GTP. The stoichiometry of GTP to **84** is 1:1. The selectivity for GTP over ATP is more than 5. The recognition of nucleotides is achieved by interactions between the positively charged imidazolium groups and the phospho groups in the nucleotides. However, **84** has been used in a 6:4 mixture of DMSO and a 20 mmol L^{-1} aqueous HEPES buffer of pH 7.4.

The indicator displacement scheme was used by Neelakandan et al. [117] for the selective detection of GTP. Receptor **85** binds to the highly fluorescent compound **79** due to its four positive charges. As a result, the emission of **79** is quenched. **79**, however, can be released from **85** by addition of GTP. The fluorescence intensity at 512 nm after excitation at 364 nm is enhanced up to 150-fold by GTP whilst AMP, ADP, CTP and UTP do not induce any change. Although this displacement mechanism is applicable under aqueous conditions at pH 7.4, it has to be taken into account that a fluorescence enhancement was observed in presence of ATP. This is the only nucleotide that interferes but is the most prevalent one and therefore will be present in many samples. For samples free of ATP, however, this scheme can be utilized to selectively determine GTP.



85

The poor capability of most fluorescent probes to discriminate between GTP and ATP still represents a major challenge. Wang and Chang [118] reported on the first turn-on fluorescent receptor for selective detection of GTP. Out of a combinatorial benzimidazolium dye library, a suitable receptor was found in compound **86**. Its optical properties were investigated in presence of the mono-, di- and trinucleotides of adenine, uracil, cytosine and guanosine, respectively. GTP led to an 80-fold increase in fluorescence whereas all other analytes did not, except for ATP which showed a two-fold increase which is negligible in this case. The excitation and emission wavelengths are located at 480 and 540 nm, respectively. The assays can be carried out at pH 7.4. Probe **86** represents the first artificial fluorescent GTP-selective receptor that can be applied under purely aqueous conditions and excited at wavelengths higher than 400 nm at the same time.



86

Probes for cytosine- and uracil-derived nucleotides

Specific probes have been reported rarely. Two fluorescent probes introduced for ATP probing earlier in this review have been shown to respond to CTP and UTP as well: the fluorescence intensity of the Tb(III)-complex with tiron (**57**) is quenched [91], while that of probe **80** [108] is increased in the presence of ATP. The changes are of comparable magnitude on addition of CTP and UTP.

Probes for DNA and RNA

The probing of nucleic acids can be approached in different manners: they can be either recognized by the ribose or phosphate units of the nucleic acid backbone or by intercalator dyes. This review only covers probes that bind to the phospho groups of nucleic acids.

Numerous fluorescent probes for DNA and RNA have been developed based on lanthanide complexes. An overview is given in Table 4. The Eu(III)-tetracycline (EuTC) complex exhibits the typical emission bands for Eu(III) luminescence at 590 and 615 nm with an excitation spectrum peaking at 398 nm. The TC ligand (**3**) forms a 1:1 complex with Eu(III). Double- and single-stranded DNA is capable of enhancing the fluorescence of EuTC while RNA only has a negligible effect on the emission properties [119]. The optimal pH range is between pH 8.0 and 9.7, pH 8.5 being used in most experiments. Since both natural and denatured DNA increase the fluores-

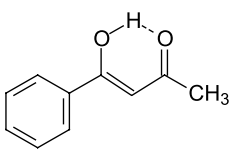
Table 4. Luminescent lanthanide ion complexes for probing nucleic acids

Luminescent probe	Nucleic acid	LOD* [ng mL^{-1}]	pH range	Ref.
Eu(III)-tetracycline	DNA	10	8.0–9.7	[119]
Eu(III)-benzoylacetone-CTMAB	DNA/RNA	0.33/0.99	8.2–8.5	[120]
Tb(III)-norfloxacin	DNA	0.9	6.3–6.7	[121]
Tb(III)-difloxacin	DNA	0.5	7.0–8.0	[122]
Tb(III)-tiron	DNA/RNA	1.0/0.6	6.9	[123]
Tb(III)-1,10-phenanthroline	DNA/RNA	200/100	6.8–7.2	[124]
Tb(III)-**89**	DNA	10	9.0	[125]
Tb(III)-**90**	DNA	3	9.0	[126]
Tb(III)/Gd(III) ions	DNA/RNA	4.3/6.4	8.4–9.6	[127]
Tb(III)/Gd(III)-CPB	DNA/RNA	4.1/3.2	8.5–10.0	[128]
Y(III)-BPMPHD-CTMAB	DNA/RNA	14.0/21.0	5.1–5.8	[129]

* Limit of detection.

cence intensity of EuTC it is suggested that no intercalation type of interaction occurs as is the case for the DNA ethidium bromide interaction. Experiments in presence of both DNA and RNA revealed that RNA does not interfere at all with the determination of both natural and denatured DNA. This renders EuTC highly suitable for selective determination of DNA. Trivalent lanthanide ions have fluorescence lifetimes ranging from 1 to >2 msec. Hence, time-resolved measurements can be performed which resulted in low limits of detection.

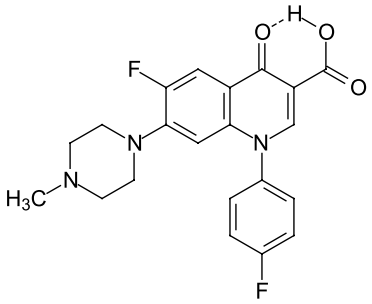
The Eu(III) complex with benzoylacetone **87** exhibits relatively weak fluorescence with line-like emission maxima at 592 and 615 nm after excitation at 350 nm [120]. In presence of cetyl trimethyl ammonium bromide (CTMAB), its fluorescence strongly increases if DNA or RNA are added. No change in emission intensity is observed in absence of CTMAB. Emission intensity has a maximum at pH 8.25. An incubation time of 20 min is required after which the signal reaches its maximum and remains constant for over three hours. RNA induces a comparably strong fluorescence enhancement. This enables the determination of the total amount of nucleic acid present in the sample of interest.



87

Tong et al. [121] described a Tb(III) complex with the quinoline antibiotic norfloxacin **58**. A 1:1 complex is formed which exhibits emission maxima located at 490 nm and 545 nm at an excitation wavelength of 290 nm. The fluorescence at both emission maxima is tremendously intensified on addition of DNA. Under the optimal conditions at pH 6.5 an incubation time of 20 min is required until the signal reaches its maximum and remains constant for one hour. The interactions between the fluorescent probe and DNA are based on both electrostatic binding of phospho groups to Tb(III) and on intercalation by ligand **58**.

The fluorescent determination of DNA has been carried out using a Tb(III) complex with difloxacin **88** [122], a structurally closely related compound to **58**. The probe is excited at 340 nm and the emission is read at 545 nm. The luminescence intensity increases tremendously by about 85-fold on addition of DNA (after an incubation time of 10 min). The long lifetime of the complex enables time-resolved measurements. Hence, the sensitivity is enhanced by off-gating short-lived background luminescence. The fluorescence of Tb(III)-**88** is pH-independent between pH 7.0 and 8.0. This probe has the advantage of a longer excitation wavelength compared to the Tb(III)-**58** complex and has a limit of detection of 0.5 ng mL^{-1} which is one of the lowest reported so far.



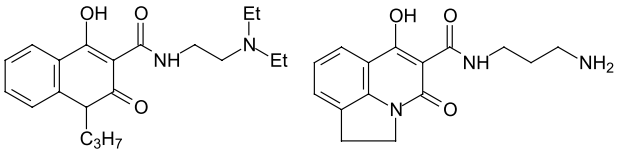
88

Another probing system consists in the fluorescent complex between Tb(III) and tiron (**57**). The typical

emission maxima for Tb(III) at 490 nm and 545 nm after excitation at 317 nm can be observed [123]. Their intensity is quenched by addition of DNA and RNA which is due to competitive binding of these analytes and displacement of the ligand. As a result, the energy transfer from Tiron to Tb(III) is suppressed. Maximal fluorescence appears at pH 6.9 which can be used in such experiments. The determination of RNA is more sensitive than that of DNA which accounts for the lower LOD of RNA compared to DNA.

The Tb(III) complex with 1,10-phenanthroline (**56**) has been applied [124] as a probe for the determination of nucleic acids. Its stoichiometry is 1:2, its excitation and emission maxima are at 289 nm and 490/545 nm, respectively. The emission intensity is amplified rather than quenched in presence of nucleic acids. It is maximal in the pH range between 6.8 and 7.2. RNA intensifies the fluorescence of the Tb(III)-**56** complex much more effectively than DNA. Hence, RNA can be detected in presence of DNA. Up to 40% of DNA does not interfere in the determination of RNA. Additionally, the limit of detection for RNA is smaller than that for DNA. However, the Tb(III)-**56** system has much higher limits of detection compared to other lanthanide-based probes.

Tb(III) complexes with 2-oxo-4-hydroxy-quinoline-3-carboxylic acid derivatives **89** [125] and **90** [126] have been the first quinolone based Tb(III) complexes for the fluorescent determination of DNA. Tb(III) forms 1:2 (metal:ligand) complexes with **89** and **90**. The emission of the probes at the characteristic Tb(III) emission at 545 nm ($\lambda_{ex} = 340$ nm) is highly pH dependent and reaches the maximum fluorescence intensity at pH 9.0. In presence of DNA the emission intensity is enhanced up to 10-fold. Metal ions that mainly occur in biological samples such as Na^+, K^+, Mg^{2+}, and Ca^{2+} only slightly affected the fluorescence of the probes. However, Cu^{2+}, Cd^{2+}, and PO_4^{3-} ions as well as surfactants reduced the emission intensity even at low concentrations. The interaction between DNA and the Tb(III) complexes is said to be based on both electrostatic interactions between the cationic probes and the phospho groups of DNA and on intercalation. Moreover, it is assumed that the protonated side arms of the ligands can form hydrogen bonds with lone electron pairs of nitrogen atoms in the groove of DNA. The detection limits for the Tb(III) complexes with **89** and **90** are 10 and 3 ng mL^{-1}, respectively. It has to be noted that these probes display comparable sensitivities to ethidium bromide but are not cancerogenic.



89 **90**

Tb(III) ion itself can be used for the determination of nucleic acids. Lin et al. [127] reported on a so-called co-luminescence effect. Nucleic acids are capable of forming a complex with Tb(III). The fluorescence intensity of this system is quite low but can be enhanced by addition of Gd(III) ion. Both DNA and RNA can be determined in this fashion at an optimal pH of 8.0. After excitation at 290 nm, the emission is measured at the strongest Tb(III) emission band which is centered at 545 nm. The fluorescence enhancement is slightly greater for DNA than for RNA which also accounts for the slightly lower limit for the quantitation of DNA. The lower limit of detection can be improved by adding cetylpyridine bromide (CPB) as proposed by Li et al. [128]. The system exhibited the same excitation and emission wavelengths but the measurements should be carried out at an optimal pH of 9.0. Both methods require an incubation time (10 and 15 min, respectively) to reach the maximal fluorescence signal which then remains stable for 2 and 4 h, respectively.

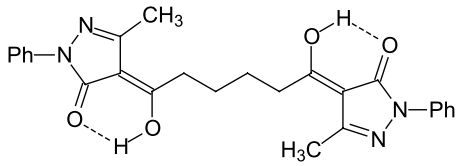
Yttrium(III) ion forms a 1:4 complex with 1,6-bis(1′-phenyl-3′-methyl-5′-pyrazolone-4′-)hexanedione (BPMPHD) **91** [129]. The emission intensity at 446 nm is quenched in presence of DNA and RNA but only after addition of the surfactant CTMAB. The excitation maximum is located at 300 nm. Recognition of nucleic acids by this probe is based on electrostatic interactions, but not on intercalative binding or groove binding. The maximal quenching effect was observed in the pH range of 5.1–5.8, and a working pH of 5.5 was used in all measurements. An incubation time of 20 min is required before a stable signal is obtained. The limit of detection for both DNA and RNA is higher than reported for Eu(III)- and most Tb(III)-based probes. The lanthanide complexes are all able to determine nucleic acids with limits of detection in the ng mL^{-1} region. However, they all suffer from the short-

Table 5. Luminescent probes for the determination of nucleic acids

Luminescent probe	Nucleic acid	LOD[a]	pH	Ref.
Ethidium bromide	DNA	10[b]	3.0–11	[130]
SYBR Green I	DNA	0.08[c]	8.3	[131]
SYBR Gold	DNA/RNA	0.5[b]/480[c]	–	[132, 133]
EvaGreen	DNA	–	7.6	[134, 135]
Hoechst 33258	DNA	5.6[b]	7.3	[136, 137]
PicoGreen	DNA	0.025[b]	7.5	[138]
TOTO, YOYO	DNA	0.5[b]	8.2	[139]

[a] Limit of detection; [b] ng mL^{-1}; [c] [pg].

wave excitation in the UV which often inhibits their application.



91

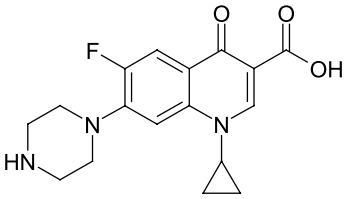
Many other probes have been developed [130–139] for the determination of nucleic acids which are not based on phosphate detection but recognize the characteristic double-stranded (ds) structure of DNA. They can only probe dsDNA with more than 11 base pairs. Since this review is focused on phosphate detection, only a short overview on such probes is given in Table 5. It can be noted that all these probes – for good reasons – have excitation and emission peaks in the visible.

Probes for other biophosphates

This section covers the so-called organic phosphates such as NADP, pyridoxal phosphate, sugar phosphates, and phospholipids.

NADP/NADPH

Jiang et al. [140–142] reported on lanthanide complexes acting as probes for the detection of NADP. Ciprofloxacin **92** and norfloxacin **58** bear an α-carbonyl carboxylic acid group which is ideal for the complexation of lanthanide ions and were used as ligands for Tb(III) ion. Doxycycline **52** contains β-diketonate configuration and is an ideal ligand for Eu(III).



92

The coordination sphere of Tb(III) and Eu(III) in their complexes is not saturated. The free coordination sites are occupied by NADP which bears two bridging and one terminal phospho group and can therefore bind electrostatically to the complexes. As a consequence the energy loss due to O–H vibration of bound water molecules is reduced since these are replaced by NADP. A more effective energy transfer between the ligands and the central lanthanide ions can occur which largely intensifies the lanthanide ion emission. Table 6 summarizes the spectral characteristics, experimental conditions, and LODs for the NADP sensitive lanthanide complexes. Not withstanding the relatively long incubation times (which limit the use of the complexes for kinetic measurements in enzymatic reactions), these probes have been applied to the determination of NADP in synthetic water samples and showed that many other substances did not interfere.

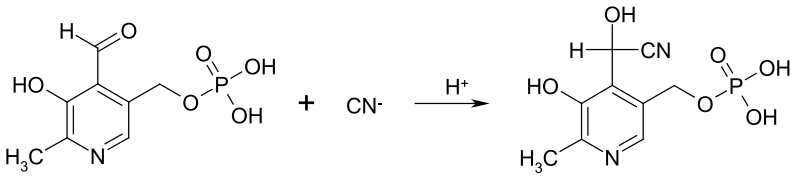
Pyridoxalphosphate

Pyridoxalphosphate (PLP) **93** is one representative of differently phosphorylated vitamin B_6 derivatives all of which act as coenzymes. The method introduced by Bonavita [143] in 1960 has been widely used for the determination of PLP. The addition of KCN to PLP leads to the formation of a cyanohydrine according to Scheme 1. Bonavita reported an increase in fluores-

Table 6. Spectral characteristics, experimental conditions, and limits of detection of various lanthanide complexes for the determination of NADP

Probe	$\lambda_{ex}/\lambda_{em}$ [nm]	pH optimum	LOD* [µmol L^{-1}]	incubation time [min]	Ref.
Tb(III)-ciprofloxacin	335/545	9.0	0.100	25	[140]
Tb(III)-norfloxacin	335/545	8.4	0.068	15	[141]
Eu(III)-doxycycline	385/612	7.6	0.043	20	[142]

* Limit of detection.

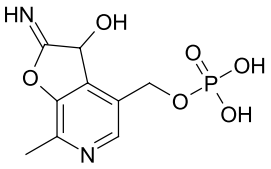


93 94

Scheme 1. Reaction of PLP with cyanide to give the corresponding cyanohydrin

cence at 420 nm after excitation at 358 nm at a pH of 7.55. According to the author, the reaction is completed within 30 min.

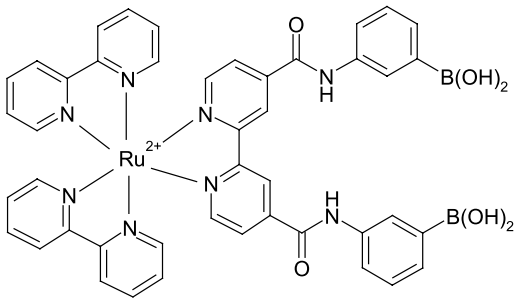
Ohishi and Fukui [144] demonstrated that the fluorescent compound formed was not the cyanohydrin derivative **94**. Rather the product of the reaction is the fluorescent lactone **95**. Bonavita reported a linear range for the fluorometric determination of PLP from 10 to 120 ng mL^{-1}.



95

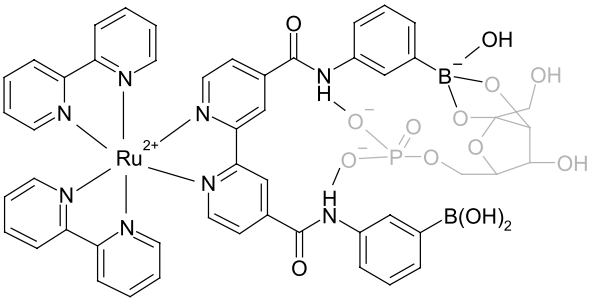
Fructose-6-phosphate, glycerol-phosphates

Boronic acids are known to form covalent complexes with diol groups as they are present, for example, in saccharides [83]. Deetz and Smith [145] made use of the boronic acid-diol-chemistry to develop a fluorescent receptor for fructose-6-phosphate. Probe **96** is based on a heteroditopic ruthenium(II) bipyridyl complex bearing binding sites for both phosphate and saccharides.



96

Addition of fructose-6-phosphate to **96** causes the boronic acid group to form covalent bonds with the saccharide. The interaction is enhanced by the formation of hydrogen bonds between the amide protons and the phospho group **97**. The emission intensity of **96** is enhanced by binding of fructose-6-phosphate. Fluorescence is detected at 637 nm with an excitation wavelength at 470 nm. Receptor **96** can be applied in phosphate buffer of pH 7.3, however, using the latter leads to smaller signal changes than when working in plain water. The interaction between the boronic acid and the diol group on the saccharide is the driving force for analyte-binding. Nevertheless, Deetz et al. showed that the phosphate group also is needed for strong saccharide binding because non-phosphorylated saccharides display smaller association constants. The selectivity is relatively low since there will always be an interaction between **96** and diol groups on other saccharides even though recognition also is accompolished by phospho groups. Moreover, the fluorescence enhancement does not exceed 30%.

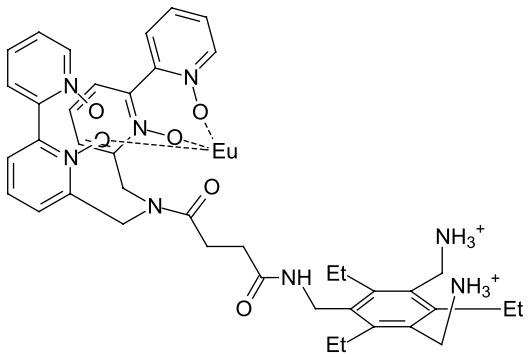


97

Koban and Berhard [146] used uranium(VI) ion for the fluorescent determination of glycerol-1-phosphate. Based on findings that phosphate groups can strongly bind uranium ions at pH < 4, Koban et al. investigated the interaction between glycerol-1-phosphate and uranium(VI). A 1:1 complex is formed at an U(VI) concentration of 10 μmol L^{-1}. The emission maxima are located at 498, 520 and 545 nm after excitation at 266 nm. All measurements were performed in water at pH 4.0. The emission intensity of all peaks is increased after addition of glycerol-1-phosphate due to complex formation. The limit of detection for glycer-

ol-1-phosphate was not reported. The method is unlikely to be specific.

2,3-Bisphosphoglycerate (BPG) can be detected with the Eu(III)-complex **98** reported by Best and Anslyn [147]. The complex displays the characteristic emission peak of Eu(III) at 610 nm after excitation at 260 nm. The addition of BPG to **98** evokes a decrease in emission intensity. However, the probe cannot be used in water/methanol mixtures containing more than 5% water due to complete quenching of the Eu(III) fluorescence. The detection of BPG with **98** was performed in 50% methanol/acetonitrile where 1:1 binding occurs. The ammonium groups in **98** are important for BPG binding which was proven by titration experiments with a control host **99** lacking these ammonium groups. With **98** a 2:1 host to guest binding was observed. Hence, a binding mode of BPG to **98** as shown in **100** was suggested.



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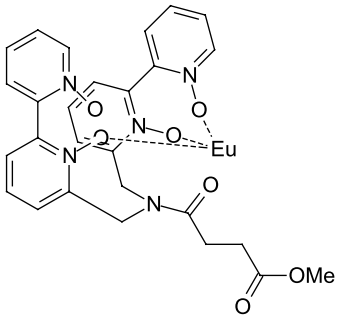

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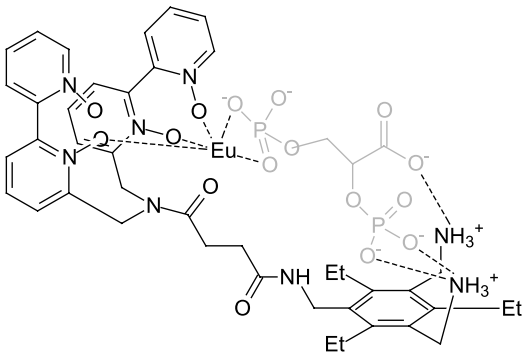
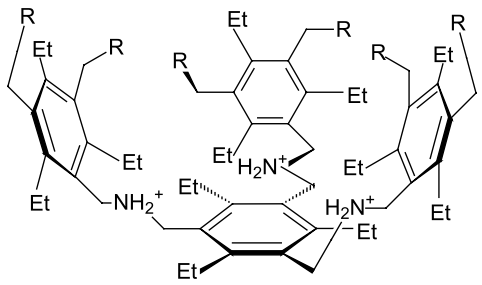


100

Inositol phosphate

Niikura et al. [148] reported a receptor that can be used for the detection of inositol-1,4,5-trisphosphate (IP_3) when combined with a fluorescent signaling unit. On addition of **101** to 5-carboxyfluorescein (**102**), an aggregate between those compounds is formed. In presence of IP_3, **102** is displaced from the aggregate resulting in a shift of the emission maximum from 500 to 493 nm at pH 7.4. The limit of detection under these conditions was found to be $1\,\mu mol\,L^{-1}$. However, **102** prefers a cyclic form in 100% methanol where it is non-fluorescent. Interaction with **101** causes ring opening of **102**, and fluorescence is restored. **102** is released in presence of IP_3 and undergoes cyclization in methanol, thus resulting in reduced fluorescence. The limit of detection is $2\,nmol\,L^{-1}$. In order to overcome the use of methanol, the cyclization under aqueous conditions was induced [149] at pH 4.0 by adding the neutral surfactant Triton X-100. Fluorescence is restored after complex formation with **101** and is decreased again after replacement by **102**. This modulation in fluorescence intensity is very similar to the one found for 100% methanol.



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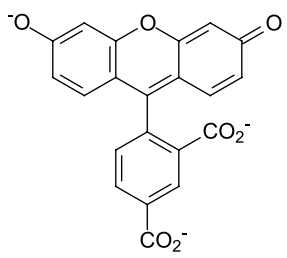

102

Sato et al. [150] presented modified IP_3 receptors (IP_3Rs) for the determination of IP_3. Type 1 IP_3R has a boomerang-like IP_3 binding region which was modified by the attachment of CFP on one side, and of YFP on the other. The two fluorescent proteins act as donor and acceptor, respectively, in a FRET system. The conformation of the CFP and YFP bearing protein is changed on binding of IP_3 which is accompanied by a less effective energy transfer between the donor and acceptor (Fig. 8). Hence, the emission ratio of CFP to

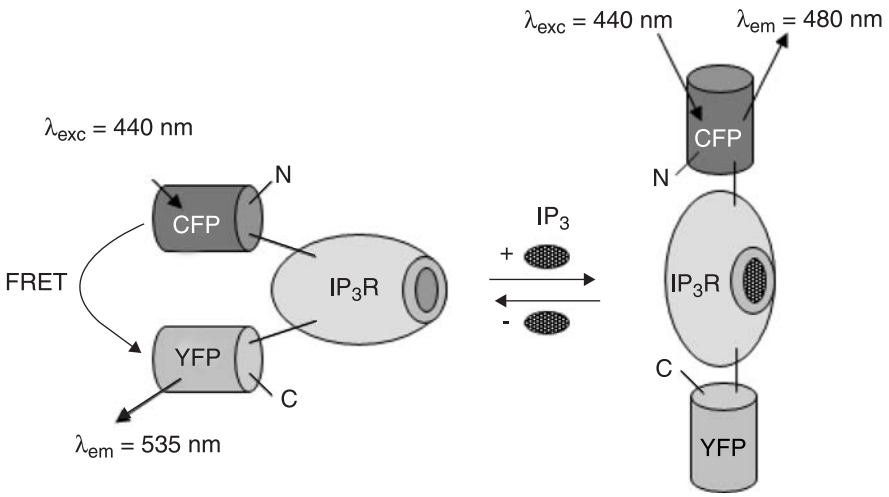

Fig. 8. Principle of the inositol-1,4,5-trisphosphate receptor assay (*IP_3R*) for visualizing inositol-1,4,5-trisphosphate (*IP_3*). The efficiency of FRET from cyan fluorescent protein (*CFP*) to yellow fluorescent protein (*YFP*) is reduced after IP_3 binding to IP_3R due to conformational changes of the receptor

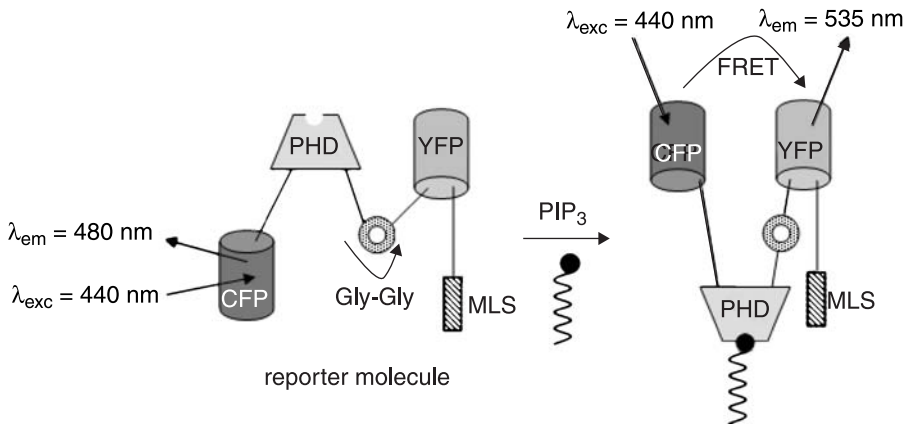

Fig. 9. Scheme for visualizing phosphatidylinositol-3,4,5-triphosphate (*PIP_3*) levels using a complex reporter molecule. Binding of PIP_3 to the pleckstrin homology domain (*PHD*) induces a conformational change of the reporter molecule via a flexible di-glycine motif leading to FRET from cyan fluorescent protein (*CFP*) to yellow fluorescent protein (*YFP*). The reporter molecule can be tethered to a membrane via a membrane localization sequence (*MLS*)

YFP is enhanced as a function of IP_3. The determination of IP_3 was applied to monitor phospholipase C_γ activity. The enzyme is activated by the vascular endothelial growth factor and subsequently produces IP_3 whose formation can be followed by an increase in the ratio of the emission intensities of CFP and YFP, respectively. However, the increase in the emission ratio is maximally 30%.

Further research by Sato [151] in the field of FRET based systems for the determination of secondary messengers brought about a detection ensemble for phosphatidylinositol-3,4,5-triphosphate (PIP_3). In this approach, a PIP_3 binding receptor named pleckstrin homology domain (PHD) is sandwiched by CFP and YFP via rigid α-helical linkers. One of these linkers is modified by a di-glycine group (see Fig. 9). This reporter molecule is then tethered to a membrane by fusion with a membrane localization sequence (MLS) via a rigid α-helical linker. The PHD domain binds the PIP_3 produced which induces a conformational change in the protein due to the flexible di-glycine motif. As a consequence, FRET from CFP to YFP is augmented. The emission ratio of CFP to YFP is measured as a function of PIP_3 concentration.

Lecithin

Lecithin is the most common phospholipid in nature and the main source of choline. Various fluorescent metal complexes were studied by the group of Jiang with respect to their suitability to probe lecithin. Tb(III) complexes with ciprofloxacin [152] (**92**), norfloxacin [153] (**58**), and of Eu(III) with tetracycline [154] (**3**) are capable of probing lecithin by fluorescence. Both Tb(III) complexes exhibited reduced emission intensity in presence of lecithin whereas Eu(III)-tetracycline displayed enhanced fluorescence. The spectral characteristics, experimental conditions, and LODs for these lecithin sensitive lanthanide complexes are summarized in Table 7. It is unlikely that these probes are specific for lecithin.

Table 7. Spectral characteristics, experimental conditions, and limits of detection of various lanthanide complexes for the determination of lecithin

Probe	$\lambda_{ex}/\lambda_{em}$ [nm]	pH optimum	LOD* [$\mu mol\,L^{-1}$]	Ref.
Tb(III)-ciprofloxacin	335/545	5.6	0.344	[152]
Tb(III)-norfloxacin	335/545	6.8	0.254	[153]
Eu(III)-tetracycline	385/612	5.7	0.039	[154]

* Limit of detection.

Current challenges and limitations

This review shows that impressive progress has been made in the area of fluorescent probes for detecting (bio)phosphates. It also reveals that some papers are difficult to assess in terms of their practical applicability because many studies are incomplete and interferences have not been studied in adequate scrutinity.

Affinity vs. Signal Changes. There is still no reasonable interpretation of the fact that the fluorescence of certain probes is quenched while that of others is enhanced, and why the signal changes sometimes are high and sometimes are small. The extent of signal change rarely is related to the affinity (binding) constant of the probe.

Toxicity. Probes are expected not to harm metabolic pathways since most probes are applied to living cells and tissue. This is of particular significance in case of probes containing heavy metal ions or Cd based quantum dots.

Spectroscopic Schemes and Materials Used. The variety of the sensing schemes that has been presented is impressive. Most approaches are based on measurement of fluorescent intensity at a single wavelength or at two wavelengths. The dual emission by FRET pairs is more powerful since it allows ratiometric measurement at two wavelengths. The measurement of fluorescent decay times is less established, as is fluorescence polarization. The exploitation of spectroscopic schemes other than measurement of fluorescent intensities will further contribute to the success of research in this complex area.

Unfortunately, some of the spectroscopic approaches have been presented in an overoptimistic way in that the probes used to demonstrate a spectroscopic scheme to work are not suitable for certain purposes (see the section on UV excitable probes), or cross interferences have not been studied in appropriate detail. Moreover, probes need to exhibit constant emission intensity over a certain period of time in order to enable the real time monitoring of dynamic processes. Other features include the brightness of a probe (defined as $\varepsilon \times QY$) and a response time of less than <1 min. Many probes reported in the literature have response times of 10 min if not more.

Selectivity and Sensitivity. The largest issue still is selectivity. Numerous articles on probes for (bio)phosphates have appeared that do not report on the effect

of conceivable interferents. The selectivity of existing probes is not adequate at present for many purposes, so that they can only be applied to specific situations. In fact, only a minority of all known probes match the conditions needed for phosphate determination in aqueous solution at near neutral pH and at below 50 °C.

Selectivity is, however, not required in situations where reagents are needed to detect the presence of (bio)phosphates in combination with separation methods such as (PAGE) electrophoresis. Rather, a general reagent is preferred here that is capable of staining all species with very low limits of quantification. In fact, specificity would be a disadvantage here.

Determination versus Detection. Many authors confuse the two terms. *Detection* of a species is a qualitative process while *determination* is a quantitative process. It is adequate in many situations to detect presence of a phosphorylated species without knowing its concentration precisely. More often, however, it is desirable to know the exact concentration of a species so that simple detection is not sufficient.

How useful are UV-excitable probes?

Many probes require UV excitation. This may be useful in a research laboratory but is beyond realism when it comes to sensing or probing of chemical species in a biological matrix, not to talk about whole blood. Excitation wavelengths below 400 nm are simply beyond reality. Not only is the filter effect of biological matter very strong at below 400 nm, but also the intrinsic fluorescence so strong that it will blur the signal of a probe. Similar considerations hold for systems that are based on the fluorescence of the excited state dimer (''excimer'') of pyrene. The only real alternative to such approaches consists in the use of the fluorescence resonance energy transfer (FRET) effect. FRET systems work well in the visible and near infrared. On the other hand, more longwave absorbing and emitting probes imply the risk of low solubility as a result of their larger molecular size.

Sensors and Probes. Many authors use the term *sensor* for what in fact is a *probe*. There is, however, a substantial difference. Molecular probes can be used to detect or to determine a chemical species by adding it to the system to be investigated, for example to solutions in microplate wells, in cuvettes, or to cellular systems, but cannot be used to continuously monitor species such as phosphate in blood, groundwater, or other flowing systems, in rapidly growing media, and the like. Sensors are usually obtained by immobilizing a molecular probe in an adequate polymer to give a sensor membrane that protects the probe from undesired effects of the sample, but at the same time is permeable to the analyte. Unfortunately, many molecular probes do not work when incorporated into sensor membranes, and no adequate polymeric sensor membranes have been found for many molecular probes.

Conclusion

In conclusion, the use of probes for (bio)phosphates has given us insight into many processes related to phosphate metabolism and phosphate transport on a molecular or cellular level. On the other side, more specific probes are highly desirable in many situations, and in our opinion only a comprehensive analysis of all the aspects that make a probe an excellent probe will warrant continuous progress. In other words: the design of better and more specific probes is as challenging as it was ever. Once more specific probes and suitable matrix polymers will be available, continuous sensing of (bio)phosphates in blood and other flowing systems may become reality.

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