

Application of Metal Coordination Chemistry To Explore and Manipulate Cell Biology

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1. Introduction

Our desire to understand how the individual molecules that make up cells organize, interact, and communicate to form living systems has led to the burgeoning field of chemical biology, an interfacial area of science that combines aspects of chemistry (the study of matter and its transformations) and biology (the study of living things and their interactions with the environment). The defining feature of chemical biology is the use of chemical approaches and small molecules to interrogate or manipulate biology.^{1,2} These small molecules are synthetic or naturally occurring ones that, for example, bind to DNA to affect protein expression levels, bind to proteins to inhibit their function, interact with lipids to alter membrane integrity, or become fluorescent in response to a metabolic event. Because small molecules can affect biochemical function, there is a clear link between chemical biology and pharmacology and medicine.³ While small molecules are usually implied as being organic compounds,⁴ inorganic small molecules also have a long



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history in both biology and medicine. Ancient civilizations used gold and copper for healing purposes, and the modern era of drug discovery was ushered in when arsenic-containing salvarsan was discovered as an antisyphilis agent to become the world's first blockbuster drug.⁵ Inorganic compounds should therefore not be overlooked in the realm of chemical biology, since their distinctive electronic, chemical, and photophysical properties render them particularly useful for a variety of applications.^{6–8}

What are the properties of metal ions that impart utility to biology? Because inorganic elements comprise the bulk of the periodic table, the diversity of these properties is

likewise broad and has been thoroughly covered by several books in the field of bioinorganic chemistry.^{9–11} A brief summary of the general chemical properties of metals is given below.

1. **Charge.** Metal ions are positively charged in aqueous solution, but that charge can be manipulated depending on the coordination environment so that a metal complexed by ligands can be cationic, anionic, or neutral.
2. **Interactions with ligands.** Metal ions bind to ligands via interactions that are often strong and selective. The ligands impart their own functionality and can tune properties of the overall complex that are unique from those of the individual ligand or metal. The thermodynamic and kinetic properties of metal–ligand interactions influence ligand exchange reactions.
3. **Structure and bonding.** Metal–ligand complexes span a range of coordination geometries that give them unique shapes compared with organic molecules. The bond lengths, bond angles, and number of coordination sites can vary depending on the metal and its oxidation state.
4. **Lewis acid character.** Metal ions with high electron affinity can significantly polarize groups that are coordinated to them, facilitating hydrolysis reactions.
5. **Partially filled d-shell.** For the transition metals, the variable number of electrons in the d-shell orbitals (or f-shell for lanthanides) imparts interesting electronic and magnetic properties to transition metal complexes.
6. **Redox activity.** Coupled with the variability of electrons in the d-shell is the ability for many transition metals to undergo one-electron oxidation and reduction reactions.

Biology has taken advantage of these chemical properties of metals to perform several functional roles, which are summarized in Table 1. This is by no means an exhaustive list but rather a primer to highlight important themes. Some metal ions, particularly the alkali and alkaline earth metals, are stable in aqueous solution as cations, making Na^+ , K^+ , and Ca^{2+} ideal for maintaining charge balance and electrical conductivity.¹⁰ On the other hand, the distinct architectures accessible via metal–ligand bonding interactions impart important structural roles to metal ions that encompass both macroscopic structural stabilization, as in biomineralized tissues,¹² and molecular structural stabilization, as in proteins and nucleic acids that are stabilized in a preferred fold by metal ions.^{13–16} Metal–ligand bonding is also significant in its reversibility. For example, Nature takes advantage of reversible binding of metal ions like Ca^{2+} and Zn^{2+} to proteins or other storage repositories in order to propagate various biochemical signals.^{13,17} Metal ions themselves can be their own signal to adjust DNA transcription, as in the case of metalloregulatory proteins.^{18,19} Reversible metal–ligand coordination is also exploited to bind and release molecules to and from a metal center, a prime example being O_2 binding and release from hemoglobin.

The reactivity of metallic centers in biology rests mostly in their Lewis acid or redox-active characters. Metal centers that are strong Lewis acids can activate coordinated ligands for reactivity, so for example, a water molecule coordinated to a Zn(II) center becomes a potent nucleophile for amide bond hydrolysis of a protein substrate.²⁰ In terms of redox activity, a wide variety of transition metals that can access variable oxidation states are found incorporated as enzyme

Table 1. Functional Roles of Inorganic Elements Found in Biology with Selected Representative Examples

function	inorganic element	representative examples	ref
charge balance	Na, K, Ca	K ⁺ channels responsible for electrical conduction in nervous systems	10
structure (macroscopic)	Ca, Si	biominerals in bone, teeth, and shell	12
structure (protein structure)	Zn, Ca	Zn finger proteins, extracellular Ca proteins	13, 16
structure (nucleic acid structure)	Mg, Mn	Mg ²⁺ stabilization of tRNA and the hammerhead ribozyme	14, 15
signaling	Ca, NO, Zn	release of Ca ²⁺ , Zn ²⁺ , and NO instigates diverse biochemical signaling pathways	13, 17, 21
signaling to DNA	Hg, Cu, Zn, Pb, As, Sb, Cd, Ni, Fe, Mn, Co, Ag	metal-responsive transcriptional regulators (metalloregulatory proteins)	18, 19
acid–base catalysis	Zn, Fe, Ni, Mn, Mg	hydrolysis reactions carried out by carboxypeptidase, purple acid phosphatase, urease, arginase, etc.	20
atom or group transfer	V, Fe, Co, Ni, Cu, Mo, W	dioxygen transport (hemoglobin), alkyl group transfer (cobalamin)	11
electron transfer	Fe, Cu, Mo	iron–sulfur proteins, cytochromes, blue copper proteins	22
redox catalysis	V, Mn, Fe, Co, Ni, Cu, W	enzymes involved in oxygen metabolism, nitrogen fixation, radical formation	23

Table 2. Functional Roles of Inorganic Elements Applied to Biology with selected Representative Examples and Reference to the Relevant Section of This Review

function	inorganic element	representative examples	sections
structure	Ru, Rh, Pt	kinetically inert complexes serve as structural scaffolds that interact in novel ways with biomolecules	7.3, 8
signaling	Ca, NO	compounds that release Ca, NO, or other signaling molecules	5, 6
acid–base catalysis	Co, Zr, Pt, Ln	complexes that induce hydrolytic cleavage of biomolecules	7.4
visualization:			
luminescence	Tb, Eu, other Ln	subcellular imaging agents	10
magnetic resonance	Gd, Mn	metal-responsive MRI imaging	9
radioactive	^{99m} Tc, ⁶⁴ Cu, others	SPECT, PET imaging	(see refs 30, 31)
X-ray	Os, Ln	heavy atom derivatives for biomolecular X-ray structure determination	11.5
alteration of metal bioavailability	Fe, Cu, Zn, Ca, other	chelating agents that alter normal metal homeostasis	3, 4, 5
bonding	Zn, Ni, others	metal–ligand bond formation for analyte sensing, metalloprotein inhibition, or protein labeling	6.5, 6.6, 7.1, 11
ligand exchange	Gd, Co, others	change in coordination sphere to increase MRI signal or release bioactive molecule	6.7, 9
electron transfer	Rh, Ru	photoinduced electron transfer to produce potent photooxidants	8
redox	Fe, Cu, others	oxidative degradation of proteins, DNA; chelation to manipulate redox activity	3.1, 3.2, 4, 7.4, 8.4, 11.6

cofactors to carry out oxidation/reduction chemistry. Electron transfer units like cytochromes, iron–sulfur clusters, and blue copper proteins shuttle electrons to other proteins that require redox chemistry for their function, while other redox proteins catalyze multielectron oxidation/reduction reactions directly on a substrate. Examples here involve oxygen metabolism, including the reduction of dioxygen to water by cytochrome *c* oxidase and hydrocarbon oxidation catalyzed by cytochrome P-450 enzymes, to name just a few.

When it comes to applying inorganic compounds to biology, chemists are not restricted to the naturally bioavailable set of metals and can take advantage of the properties of biologically exotic elements, including second and third row transition elements and the lanthanide (Ln) elements. This expansion leads to the list of functional roles of inorganic elements applied to biology shown in Table 2. Many of the functions listed in Table 2 mirror those of Table 1, but applied in novel ways. For example, the structures of kinetically inert metal complexes are found to interact with proteins and nucleic acids in unique ways, and the acid–base and redox activity of native and non-native metals can be harnessed for artificial reactivity. Metal complexes can also impart additional functionality not found naturally. The most

striking addition to the list is in visualization, where the photophysical, magnetic, and radioactive properties of metals make possible studies based on luminescence, magnetic resonance, PET, and SPECT imaging modalities.

This review will explore how the properties of inorganic coordination complexes are applied in the context of inorganic chemical biology, with a particular focus on applications related to cellular trafficking and regulation. We will delve into the structure, bonding, spectroscopy, and reactivity of transition metal coordination compounds and explore how their unique properties can be used as probes and tools to understand or control biological processes. Our discussion will expand on the functions and examples listed in Table 2, which is really only a partial list, because the examples of inorganic and organometallic complexes applied to biology continue to grow. Our focus is on compounds used in cells to understand the trafficking or regulation of something, be it the metal itself or some other molecule or process that is enabled or visualized by a metal complex. Many of the compounds that are discussed have potential applications in medicine, but the reader is referred to other excellent sources for implicit coverage of medicinal and diagnostic uses of metal complexes.^{24–33} There have been

Table 3. Classification of Select Metal Ions and Donor Atoms According to Pearson's HSAB Principle

hard Lewis acids	borderline acids	soft acids
H ⁺ , Li ⁺ , Na ⁺ , K ⁺ , Be ²⁺ , Mg ²⁺ , Ca ²⁺ , Sr ²⁺ , Sc ³⁺ , Ti ⁴⁺ , Zr ⁴⁺ , Cr ³⁺ , Al ³⁺ , Ga ³⁺ , La ³⁺ , Gd ³⁺ , Co ³⁺ , Fe ³⁺	Fe ²⁺ , Co ²⁺ , Ni ²⁺ , Cu ²⁺ , Zn ²⁺ , Pb ²⁺ , Bi ³⁺ , Rh ³⁺ , Ir ³⁺	Cu ⁺ , Au ⁺ , Ag ⁺ , Tl ⁺ , Hg ⁺ , Pd ²⁺ , Cd ²⁺ , Pt ²⁺ , Hg ²⁺
hard Lewis bases	borderline bases	soft bases
F ⁻ , OH ⁻ , H ₂ O, ROH, Cl ⁻ , RO ⁻ , R ₂ O, CH ₃ CO ₂ ⁻ , NH ₃ , RNH ₂ , NH ₂ NH ₂ , CO ₃ ²⁻ , NO ₃ ⁻ , O ₂ ⁻ , SO ₄ ²⁻ , PO ₄ ³⁻ , ClO ₄ ⁻	NO ₂ ⁻ , Br ⁻ , N ₃ ⁻ , N ₂ , C ₆ H ₅ NH ₂ , pyridine, imidazole	RSH, RS ⁻ , R ₂ S, S ₂ ⁻ , CN ⁻ , RNC, CO, I ⁻ , R ₃ As, R ₃ P, C ₆ H ₅ , C ₂ H ₄ , H ₂ S, HS ⁻ , H ⁻ , R ⁻

significant advances in the development of fluorescent probes for monitoring cellular metals. Such molecules are clearly important tools in inorganic chemical biology but will not be covered here, because there are several excellent reviews available.^{34–39} We define a metal complex that is a “probe for biological systems” as one that can be used in ways to teach us about the chemical biology of living cells. It may be used *in vivo* or *in vitro* with the aim of understanding how cells operate. With this definition in mind, we will discuss metal chelators and metal complexes that are being used or have potential to be used to this end, with an emphasis on those that are applied in cellular studies.

2. Principles of Metal–Ligand Coordination Chemistry

The principles governing metal–ligand complex stability and specificity depend on the properties of both the metal ion and the chelating agent, as summarized briefly in the following sections. More comprehensive reviews on ligand design for selective complexation of metal ions in aqueous solution are available.^{40–45} This discussion sets the stage for understanding the properties of the compounds presented throughout this review.

2.1. Donor Atom Preference

The principle of hard and soft acids and bases (HSAB) was developed in 1965 by R.G. Pearson following criteria introduced by Irving, Williams, Arhland, Chatt, and Davies.⁴⁶ The classification is based on an atom's polarizability, where nonpolarizable acids or bases are small with high charge density and are classified as “hard”. Polarizable acids and bases are usually large with low charge density and are classified as “soft”. Acids and bases that have intermediate hard/soft character are classified as “borderline”. The HSAB principle predicts that hard acids prefer hard bases, soft acids prefer soft bases, and borderline acids prefer borderline bases. Pearson's classifications of metal ions (Lewis acids) and their ligands (Lewis bases) are shown in Table 3, which serves as a useful starting point for predicting the preference of metal ions for ligands with various donor groups. For example, soft donor groups such as thioethers (R₂S) and thiolates (RS⁻) prefer soft metal ions, like Cu⁺, whereas hard oxygen donors like carboxylates and phenolates are appropriate for hard metal ions, like Fe³⁺.⁴⁶

In fitting with the Lewis acid–Lewis base description of metal–ligand coordination, it would seem apparent that increasing the Lewis basicity of the donor would enhance metal–ligand bonding. While this principle can be used to tune metal–ligand affinity, other factors must also be considered. For example, increasing the basicity of a phenolate also increases its p*K*_a. Since metal ions compete with protons for ligand binding in aqueous solution, such

an adjustment might actually decrease the effective metal binding at a desired pH. Because of proton competition, overall stability constants (β) do not reflect the actual affinity of a ligand for a metal under biologically relevant solution conditions. A pH-dependent conditional binding constant can be calculated from known β and p*K*_a values.^{47–49} Alternatively, an apparent binding constant (*K*_{app}, also called *K*_{eff} for effective binding constant) can be measured directly as the equilibrium constant under the specified solution conditions of pH and buffer components. For convenience, binding constants are often inverted and discussed as dissociation constants (*K*_d).

2.2. Chelate Rings, Steric Strain, and Preorganization

Polydentate ligands that present multiple donor atoms for metal binding provide greater complex stability compared with monodentate analogs due to the chelate effect. This effect can be maximized if the number and size of the chelate rings are optimized for the size of the cation in a way that minimizes steric strain upon metal binding.^{41,42} The chelate rings formed when two donor groups from the same ligand bind a metal center are most favorable for five- and six-membered rings. Adjacent six-membered rings formed from polydentate ligands, however, can induce unfavorable steric strain that is relieved in ligands containing adjacent five- and six-membered rings.^{41,42} In general, ligands that minimize steric strain in the complex on coordination of the ligand to the metal ion or that preorganize their donor atoms spatially as required for complexation or both are preferred for high-affinity binding.⁴³ Macrocycles that incur minimal strain upon metal complexation can therefore retain their metal ion by tight complexation.

2.3. Complex Geometry

On the basis of its number of valence d electrons, a metal ion may prefer certain binding geometries over others. This preference is based on ligand field stabilization energy (LFSE), a full description of which can be found in standard inorganic chemistry text books.^{50,51} Comparing the geometric preferences of iron, copper, and zinc illustrates the point. The common biologically relevant oxidation states of iron are Fe²⁺ and Fe³⁺, which prefer octahedral and distorted octahedral geometries. Copper, on the other hand, exists primarily as Cu⁺ and Cu²⁺, with Cu²⁺ favoring square planar, square pyramidal, or axially distorted octahedral geometries due to Jahn–Teller distortions of its d⁹ electron configuration. Its reduced Cu⁺ form has a filled d¹⁰ configuration with no preference for geometry based on LFSE and can therefore be found in a range of coordination geometries including two-, three-, and four-coordinate sites. The geometry of the ligand field can influence the redox state of copper. For

example, ligands that impose a tetrahedral arrangement that is unfavorable for Cu^{2+} but reasonable for Cu^+ will destabilize the Cu^{2+} form, shifting the reduction potential more positive in favor of Cu^+ . The ligand-induced change in reduction potential can be used to purposefully select a desired oxidation state. Geometric preferences imposed by the ligand are also important for differentiating Cu^{2+} and Zn^{2+} . Because Zn^{2+} is d^{10} and has no geometric preference based on LFSE, tetrahedral Zn complexes are common.

LFSE is also a factor in the trend observed in the Irving–Williams series of relative complex stabilities of first-row divalent metal ions: $\text{Mn}^{2+} < \text{Fe}^{2+} < \text{Co}^{2+} < \text{Ni}^{2+} < \text{Cu}^{2+} > \text{Zn}^{2+}$. In general, complex stability increases as the ionic radius decreases across the series, but Cu^{2+} shows a sharp spike in stability that can be attributed to LFSE obtained through Jahn–Teller distortion, and Zn^{2+} shows a diminished stability due to a lack of LFSE for its d^{10} configuration. The greater stability of copper(II) complexes compared with zinc(II) complexes revealed by this series indicates an inherent challenge in designing chelating agents that are selective for Zn^{2+} over Cu^{2+} , although sites that impose tetrahedral geometry will prefer Zn^{2+} to Cu^{2+} .

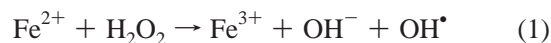
3. Commonly used Chelators To Alter Bioavailability of Metal Ions in Cell Biology

Altering the bioavailability of metal ions by using metal chelating agents is an important strategy for studying cellular processes related to metal ion transport, storage, use, and trafficking. When using live cells in probing for biomolecules related to metal homeostasis, a typical approach is to change the availability of one metal and observe changes in cell processes, especially transcription. Up-regulation or down-regulation of specific genes can indicate the proteins either directly or indirectly related to metal ion handling. The ability to limit the bioavailability of only one target metal is crucial to the integrity of the experiment and interpretation of results. In order to affect the availability of only one metal ion at a time, “selective” metal chelators are often employed, where the selectivity is a measure of its affinity for a particular metal ion over others. Rarely, however, does selectivity imply exclusivity. Many metals are close enough in their HSAB character, their geometric preference, and other properties to make it difficult to select for one specific metal in the presence of others. So while an agent may have a thermodynamic preference for a particular metal ion, it does not mean that it binds that metal to the exclusion of all others. Indeed, the complex equilibria that exist in the compartmentalized environment of a cell (even more so in a whole organism) make metal selection a tricky proposition. The following sections highlight some of the most widely used chelating agents in cell biology and point out potential pitfalls. The structures are shown in Chart 1.

3.1. Iron Chelators

The well-known redox activity of iron makes it a useful metal cofactor for biology but also poses a potential risk because it can participate in redox cycles with oxygen to produce damaging reactive oxygen species (ROS) through the Fenton reaction (eq 1).^{52–54} The catalytic capacity of iron in Fenton reactions requires it to cycle between Fe^{2+} and Fe^{3+} , which means that the reduction potential of the iron center is a key determinant in its ROS production.⁵⁵ Chelating agents that shift the reduction potential to favor either Fe^{3+}

or Fe^{2+} without redox cycling can in principle keep iron from producing ROS. Chelating agents for both oxidation states are discussed below.

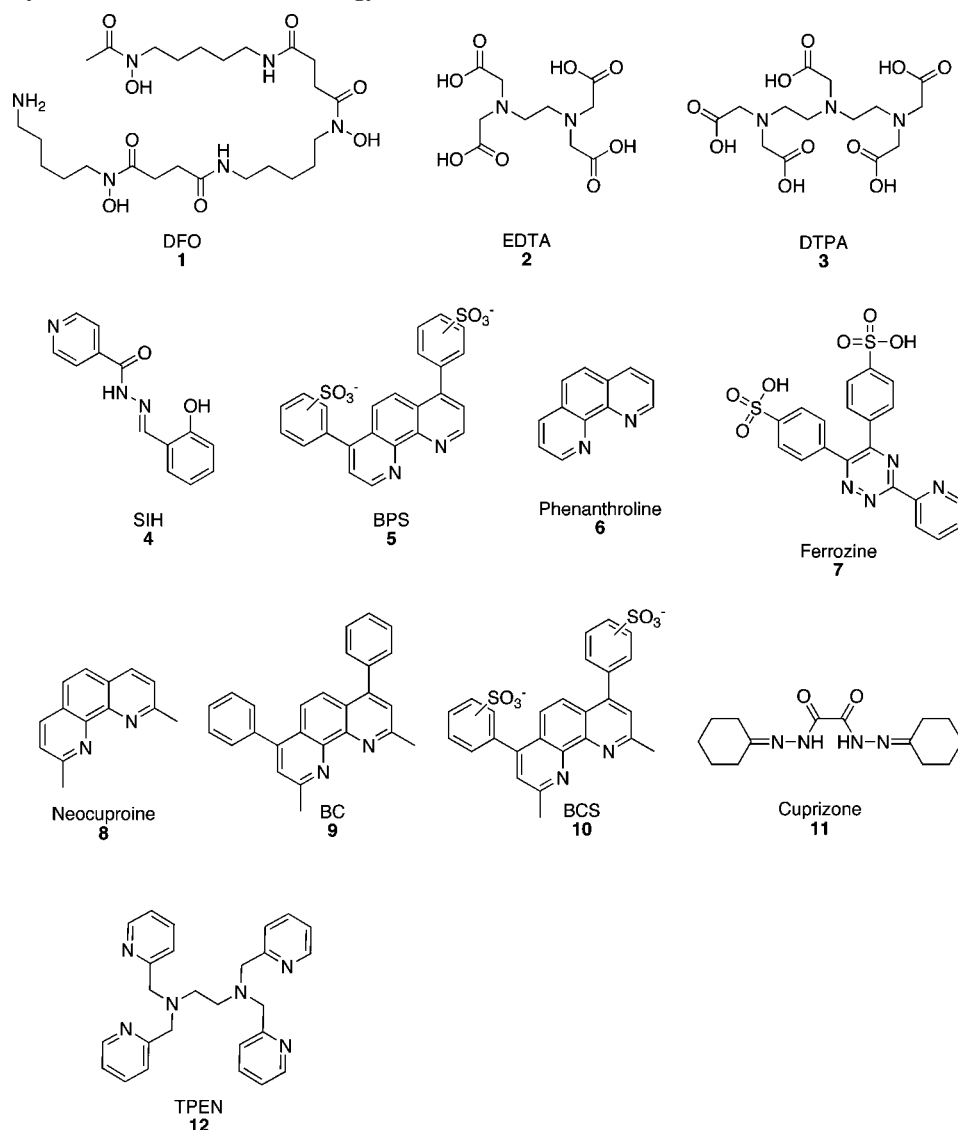


Iron(III) is unique to biological transition metal ions in that it has a high charge-to-radius ratio and as a hard acid forms high-affinity complexes with hard oxygen ligands. Ligands can therefore be designed to have a thermodynamic preference for Fe^{3+} over dicationic metal ions like Cu^{2+} and Zn^{2+} . For a comprehensive discussion on designing ligands for iron chelation, see the review by Liu and Hider.⁵⁶ The iron siderophore literature is also helpful for understanding selective iron sequestration.⁵⁷ It is important to repeat, however, that a high affinity for a particular metal ion does not imply that other metal ions do not also bind with considerable affinity. Most Fe^{3+} chelators also have reasonable affinity for Cu^{2+} and Zn^{2+} , which can lead to depletion of these metals, especially with prolonged exposure to the chelating agent.

A common iron(III) chelator used both in the laboratory and in the clinic is desferrioxamine (DFO, **1**), a naturally occurring siderophore that binds Fe^{3+} with three hydroxamate groups to give a six-coordinate complex. DFO is one of the first chelators used to treat iron overload disorders, and it is still widely used today. DFO is also used in the laboratory for cell studies to control iron bioavailability. It is charged and hydrophilic and does not diffuse easily across the cell membrane.⁵⁸ It is believed to enter the cell only by endocytosis to diminish labile iron pools in the cytoplasm and most drastically inside of endosomes.⁵⁸ It is slow to chelate intracellular iron at room temperature but increases efficiency at 37 °C due to increased endocytosis.⁵⁸ DFO is a potent chelator for Fe^{3+} that inhibits iron-dependent ROS formation and is itself an antioxidant.^{59,60}

The difficulty of DFO in crossing biological membranes and its subsequent slow rate of intracellular iron chelation has spurred the search for other Fe^{3+} chelating agents. General chelators like EDTA (ethylenediamine tetraacetic acid, **2**) and DTPA (diethylenetriamine pentaacetic acid, **3**) have high affinity for Fe^{3+} but also bind several metal ions and are not considered selective. There is a very large body of literature on the design of iron chelators for medical applications (see, for example, the review by Orvig in this issue³² as well as others^{56,61}), so the focus here is rather to highlight select examples with utility for manipulating iron concentrations in cell culture. In this context, the tridentate chelator salicylaldehyde isonicotinoyl hydrazone (SIH, **4**) has emerged as a useful lipophilic chelator that readily crosses biological membranes.^{62–65} It forms a 2:1 ligand/ $\text{Fe}(\text{III})$ complex that inhibits iron-dependent ROS formation.^{66,67} In addition to protecting cells against iron-induced oxidative stress, another application of SIH has been in the determination of labile iron pools in cells by fluorescent chelators. The general protocol for these measurements has been to use a fluorescently labeled, cell-permeable chelator that is quenched upon iron binding, then use SIH to complex iron and compete it away from the probe to restore fluorescence. The extent of restored fluorescence gives an estimate of the chelator-accessible, or labile, iron pool.^{68,69} SIH is not without its drawbacks, however. While its affinity for Fe^{3+} is high, it also complexes Cu^{2+} and Zn^{2+} .⁷⁰ In addition, it has a short half-life in cell culture media and plasma due to hydrolysis of the hydrazone linkage.⁷¹

Chart 1. Commonly Used Chelators for Cell Biology



Chelators for Fe^{2+} are also used to control iron bioavailability. The oxidation states Fe^{2+} and Fe^{3+} differ in terms of their ligand preferences and the ease with which they can be selectively chelated over other metals based on donor atom identity. Because of its lower charge density and larger size, Fe^{2+} is more similar in HSAB character to other biologically relevant transition metal ions, especially Cu^{2+} and Zn^{2+} . Although it may be difficult to select Fe^{2+} over other borderline metals, Fe^{2+} has a preferred ligand field geometry that is different from Cu^{2+} and Zn^{2+} . Fe^{2+} is a d^6 metal that has a favorable LFSE in a low-spin octahedral complex. Chelators with borderline donor groups that can also adopt an octahedral geometry can potentially select Fe^{2+} over other biometals. One chelator that fits these criteria is batho-phenanthroline disulfonic acid (BPS, **5**), a sulfonated water-soluble derivative of phenanthroline (**6**) that is frequently used in cell culture as an iron-limiting reagent.^{72,73} BPS forms a tris-chelate iron(II) complex with octahedral geometry and is membrane impermeable due to its charged sulfate groups.⁷⁴ Because of its large positive reduction potential, BPS is known to cause nonspecific reduction of Fe(III) chelates.⁷⁵ Although it has good affinity for Fe^{2+} , it does complex other metals, especially Cu^{2+} , which is not surprising considering the variable chelate stoichiometry possible with BPS and

related phenanthroline derivatives.⁴⁸ BPS can easily form a bis complex with square planar geometry preferred by Cu^{2+} .

Ferrozine (**7**) is another commonly used chelator for Fe^{2+} in cell culture to create a “defined Fe medium” or “Fe-depleted medium”.^{76–78} It selects for Fe^{2+} by forming a tris complex of borderline donor atoms in an octahedral binding geometry with a very large binding constant. Like BPS, ferrozine is known to cause nonspecific reduction of Fe(III) chelates.⁷⁵ There is evidence that ferrozine forms ternary complexes with Fe and amino acids, especially histidine, which act as potential antioxidants.⁷⁹

In addition to using chelating agents to limit iron bioavailability, iron salts are also used to supplement cell culture media. An understanding of iron coordination chemistry is also helpful to avoid potential problems. Unchelated Fe^{2+} ion is more soluble and more bioavailable than unchelated Fe^{3+} , both because Fe^{2+} is a substrate for divalent metal ion transporters in mammalian cells and because Fe^{3+} rapidly hydrolyzes in water to give insoluble iron hydroxides. Typical cell culture conditions exist in an oxygen-rich environment, so it must be kept in mind that Fe^{2+} salts added to the culture medium or aqueous buffers are likely to oxidize readily to Fe^{3+} , which may not be soluble. In cell culture media, amino acids or other components in the media likely

keep iron in solution, but addition of simple iron salts to standard laboratory buffers, especially phosphate buffers, will result in insoluble and bio-unavailable iron.

3.2. Copper Chelators

Like iron, the redox chemistry of $\text{Cu}^{2+/+}$ makes it essential to biological processes but also potentially dangerous if it is not handled properly by the cell and becomes available for Fenton-like chemistry to produce ROS. Typically under the oxidizing extracellular environment, copper exists as Cu^{2+} , but in the reducing conditions inside the cell, it likely exists in the reduced Cu^+ oxidation state. Soft character makes Cu^+ unique among the biological metal ions, so it has potential to be selected based on ligand donor groups. In addition, Cu^+ is a d^{10} ion, giving it flexibility in geometric arrangements. This means Cu^+ can adopt tetrahedral, trigonal, or even linear geometries that are disfavored by other metals. Zn^{2+} is also a d^{10} ion but is harder in character than Cu^+ , so can be minimized as an interfering species based on ligand donor choice. Zn^{2+} is also a smaller metal ion, so chelate ring size can be a determining factor.

2,9-Dimethyl-substituted phenanthroline ligands are well-known to select for Cu^+ . Neocuproine (**8**), bathocuproine (BC, **9**), and bathocuproine disulfonate (BCS, **10**) are the three commonly used 2,9-dimethyl-substituted phenanthroline chelates used in cell culture.^{80–86} Unlike phenanthroline, these ligands disfavor octahedral tris-chelate or square-planar bis-chelate coordination modes because of steric interference of the methyl substituents. Instead, when binding to a metal in a bis complex, the metal is forced into a tetrahedral binding geometry with the two chelate ligands nearly perpendicular to each other. This tetrahedral geometry combined with the large bite size of the five-membered chelate ring effectively binds Cu^+ over other metals. However, these ligands do have significant interaction with Cu^{2+} and are known to bind to Cu^{2+} , forcing it into a tetrahedral geometry and inducing its reduction to Cu^+ . BC-bound $\text{Cu}(\text{II})$ is a stronger oxidizing agent by 0.5 V compared with uncomplexed Cu^{2+} , which highlights the fact that these “Cu(I)-selective” chelators are not innocent chelators of Cu^+ .^{87–89} Although they may be able to promote reduction of Cu^{2+} to Cu^+ , once the reduced state is reached, it is stabilized and does not participate in redox cycling.⁹⁰ In fact BCS has been shown to inhibit Cu-dependent redox cycles.⁸⁶

Bathocuproine (**9**) is not very water-soluble, so a sulfonated version (BCS) was developed to give a more soluble derivative.⁷⁴ Because BCS is charged and not membrane permeable, it is commonly used in cell studies as an extracellular Cu-limiting agent. Because Cu^{2+} is the dominant extracellular form of copper, it should be considered that BCS is a potential promoter of Cu^{2+} reduction. Neocuproine (**8**) is more hydrophobic and is used for intracellular and extracellular Cu chelation since it can diffuse over the cell membrane.

Cuprizone (oxalic acid bis(cyclohexylidene)hydrazide, **11**) is another chelator used to selectively bind Cu^{2+} in cell studies;^{82,83} however, the actual nature of its Cu complex has long been under debate. There is strong evidence that cuprizone stabilizes a Cu^{3+} oxidation state in a square planar d^8 complex.^{91–93} The cytotoxic and neurotoxic effects of cuprizone may be related to Cu^{2+} – Cu^{3+} redox cycling induced by this chelator under biological conditions.^{91,94}

3.3. Zinc Chelators

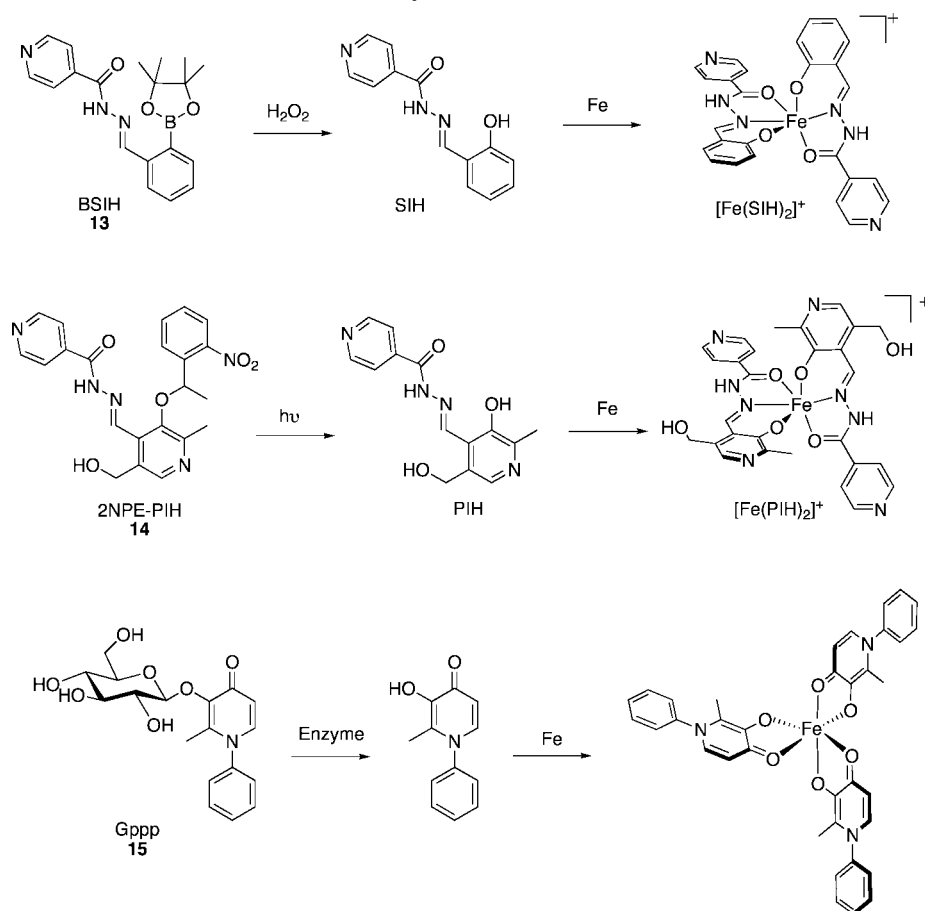
The only relevant oxidation state for zinc is Zn^{2+} . It has borderline HSAB character, and it is d^{10} with no real preference for ligand field geometry. Zn^{2+} is very close to Cu^{2+} in size and charge density and can be difficult to select for over Cu^{2+} . TPEN (*N,N,N',N'*-tetrakis-(2-pyridylmethyl)-ethylenediamine, **12**) is the most common zinc chelator used in the literature;^{85,95–101} however there is an apparent misconception that TPEN is Zn^{2+} -specific. In fact, the affinity of TPEN for Fe^{2+} ($\log K_a$ 14.6) is relatively significant, while its affinity for Cu^{2+} ($\log K_a$ 20.6) is higher than that for Zn^{2+} ($\log K_a$ 18.0).^{102,103} There are several studies that show TPEN affects cellular concentrations of Zn and Cu and that replenishing either metal into TPEN-treated cells will rescue the cells from TPEN-induced apoptosis.¹⁰⁰ Another study shows that levels of Fe are affected in addition to Cu and Zn when TPEN is added to cell culture.¹⁰⁴ Part of the reason that TPEN can have such significant affinity for Zn^{2+} , Cu^{2+} , and Fe^{2+} is its potential variability in chelate binding geometry.¹⁰⁵ TPEN has six possible donor groups, and for Zn and Fe, all six are used to chelate the ion in an octahedral geometry. Cu^{2+} , which is destabilized by octahedral geometry, is chelated by only five of the six possible donor groups and forms a distorted square pyramid with high stability.¹⁰⁵ It is necessary to consider the Cu, Fe, and Zn binding properties of TPEN before interpreting the results of an experiment where TPEN is used as a “selective” chelator.

4. Selectivity by Reactivity or Localization

The challenges associated with selective metal chelation have led to the development of new generation chelating agents that achieve selectivity based on reactivity or localization. Examples are shown in Chart 2. Our own lab has introduced a prochelator strategy that uses the reactivity of oxidative stress to generate metal-binding agents to inhibit further oxidative damage.^{106–108} The concept is based on the hypothesis that oxidative stress is exacerbated by Fenton reactions wherein labile metal ions, particularly iron and copper, react with H_2O_2 to produce damaging hydroxyl radicals (see eq 1). As described in section 3.1, appropriately designed chelating agents can prevent this reactivity, but it is difficult to design ligands that can select for the metal ion causing the damage without altering normal, healthy metal ion distribution. In our prochelator strategy, a metal-binding ligand is masked with a H_2O_2 -sensitive protecting group to prevent metal coordination under normal conditions. Reaction with H_2O_2 converts the prochelator to the chelator, thereby triggering metal sequestration. A boronic ester was selected as the H_2O_2 -sensitive masking group in our first-generation prochelator, BSIH (**13**, isonicotinic acid [2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-benzylidene]-hydrazide), which converts to SIH (**4**) in the presence of H_2O_2 and forms the $[\text{Fe}(\text{SIH})_2]^+$ complex that prevents iron from Fenton reactivity.¹⁰⁶ BSIH itself has only weak interactions with metal ions, whereas SIH strongly interacts with most divalent and trivalent metals. The BSIH to SIH conversion also inhibits copper-catalyzed Fenton reactions,¹⁰⁹ demonstrating that the strategy is not metal specific per se, but rather reactivity specific.¹⁰⁸

BSIH was shown to protect cultured retinal pigment epithelial cells against cell death induced by hydrogen peroxide.¹⁰⁷ Significantly, cells that were not stressed with H_2O_2 remained viable even after repetitive, prolonged

Chart 2. Chelators That Are Selective Based on Reactivity or Localization



exposure to BSIH. Similar treatment of cells with chelating agents SIH and DFO caused significant cell death, presumably due to metal depletion.¹⁰⁷ These initial findings suggest that BSIH discriminates toxic iron from healthy iron.

Another prochelator approach involves masking SIH or PIH (pyridoxal isonicotinoyl hydrazone) with a photoactive nitrobenzyl protecting group to create photocaged iron chelators that release the active ligand only after exposure to UVA light.¹¹⁰ Exposure of 2NPE-PIH (**14**) or 2NPE-SIH to UV light cleaves the protecting group to release PIH and SIH, respectively. Skin fibroblast cells treated with **14** were significantly protected against UVA-induced necrotic cell death at UVA irradiation doses that mimic natural exposure levels of a sunny day.¹¹⁰ The study used an iron regulatory protein bandshift assay and a calcein fluorescence assay to show that intracellular labile iron concentrations increase after exposure to UVA, whereas the labile iron levels are kept in check if the cells are first treated with the caged chelators prior to irradiation.¹¹⁰ The caged chelators represent another promising tactic of getting the protective benefits of chelating agents without the risks of deleterious side effects associated with prolonged exposure of cells to high-affinity metal chelators.

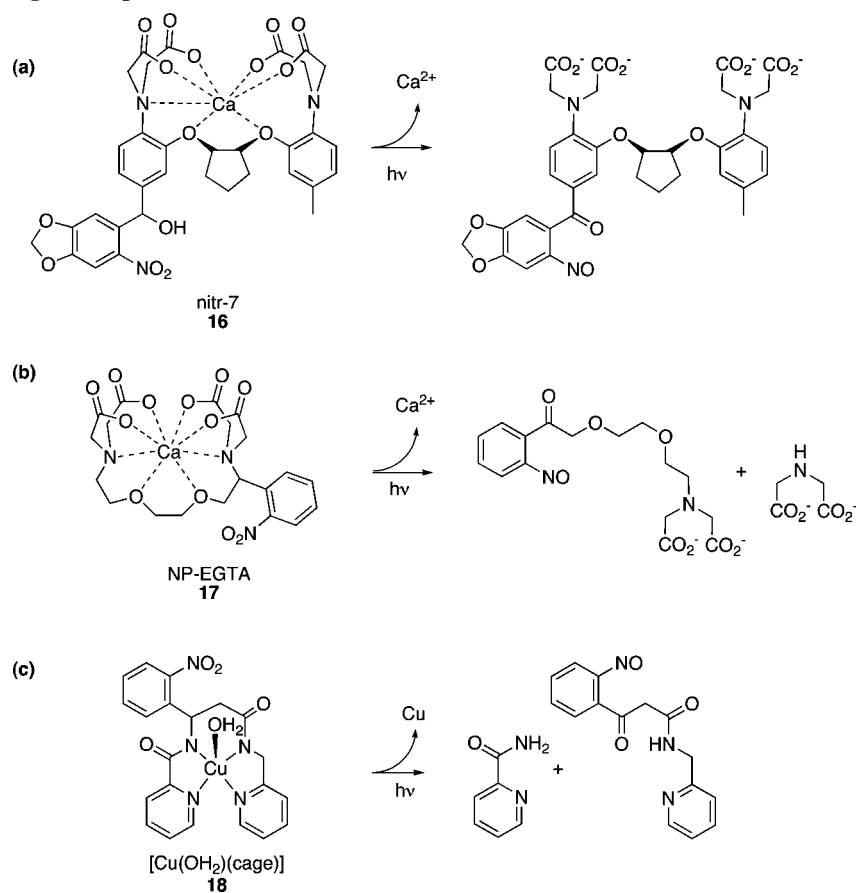
Orvig and co-workers have introduced an alternative prochelator strategy that is based on the premise that glucose transporters can be co-opted to import glucose-tagged molecules into cells. The high concentration of glucose transporters in the brain may provide a pathway to concentrate metal ion chelators across the blood–brain barrier.¹¹¹ In addition to potentially targeting the molecule to the brain, the carbohydrate moiety of the glucoconjugate **15** (3-(*D*-

glucopyranosyloxy)-2-methyl-1-phenyl-4(*1H*)-pyridinone, Gppp) also uses the carbohydrate to mask the metal-binding functionality of the pyridinone, thereby preventing premature metal binding. Once inside the brain, the glucose masking group would be enzymatically cleaved to release the chelator. As a proof-of-principle, it was shown that a broad-spectrum glucosidase from *Agrobacterium faecalis* indeed converts prochelators like **15** into their efficient metal-binding hydroxypyridinone versions.¹¹² A radiolabeled hydroxypyridinone glucoconjugate was also shown to penetrate the blood–brain barrier in a rat brain perfusion experiment, demonstrating adequate cerebral uptake.¹¹² Other strategies of targeting chelating agents for specific diseases are covered in the review by Scott and Orvig in this issue.³²

5. Complexes That Release Metals

While the previous sections focused on using chelating agents to reduce the concentration of bioavailable metals, it is also desirable to manipulate cellular metal ions by increasing their local concentration in a controlled way. The most well-developed compounds in this category belong to a class of molecules known as caged calcium, developed by both Tsien and Ellis-Davies.^{113–115} These reagents operate by using light to trigger a chemical change that reduces a chelator's affinity for Ca^{2+} , thereby providing a sudden release of bioavailable Ca^{2+} . The chelating moieties are based on tetracarboxylate chelators BAPTA and EGTA that bind to Ca^{2+} with high affinity and sufficient selectivity over the most likely competing ions Na^+ , K^+ , and Mg^{2+} . Constructs

Chart 3. Photoactive Caged Complexes That Release Metals



based on EDTA are also available but do not discriminate Ca^{2+} from Mg^{2+} as effectively.

There are two general series of caged calcium, each using a different mechanism to reduce Ca^{2+} affinity, as shown in Chart 3a,b. In the nitr series (nitr-7, **16**), photoactivation converts the 2-nitrophenyl substituent to a more electron-withdrawing group that reduces the donor strength of a metal-binding nitrogen, thereby resulting in a 40-fold decrease in Ca^{2+} affinity.¹¹⁶ In the series of compounds including NP-EGTA (**17**), a photoactive 2-nitrophenyl group is embedded into the backbone of the chelator so that illumination with UV light results in chelator fragmentation and a 40 000–600 000-fold loss in Ca^{2+} affinity, depending on the derivative.^{117,118} There have been hundreds of studies that utilize caged calcium to explore biology. These were recently reviewed by Ellis-Davies and will not be repeated here.¹¹⁵

In an effort to broaden the applicability of caged metals beyond calcium, our group recently introduced a photocleavable pyridal amide ligand that binds to Cu^{2+} with an apparent K_d at pH 7.4 of 16 pM ($[\text{Cu}(\text{OH}_2)(\text{cage})]$, **18**).¹¹⁹ Photolysis of caged copper with 360 nm UV light cleaves the tetradentate ligand to give photoproducts that have only bidentate chelating ability and therefore a reduced affinity for copper. An *in vitro* assay showed that copper-catalyzed hydroxyl radical formation increased 160% following light-induced uncaging, thereby demonstrating that light can be used to trigger the availability and reactivity of copper. Caged copper has yet to be validated in a cellular system, although it was suggested that the affinity may need to be improved to avoid competition with endogenous copper-binding proteins.¹¹⁹

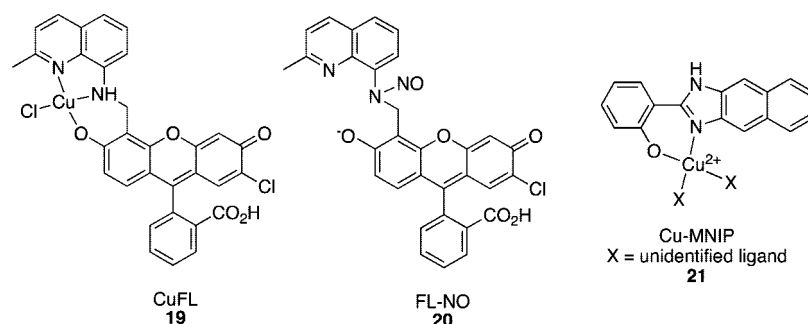
6. Metal Complexes that Bind/Release Small Molecules

6.1. NO and CO

The biological effects of carbon monoxide (CO) and nitric oxide (NO) can aptly be described by the general observation of Paracelsus in the early 16th century that “the dose makes the poison”. Both diatomic molecules are odorless, colorless gases that were long known only as toxic poisons until the discovery in the 1980s of NO as the endothelium-derived relaxing factor. NO is now recognized as an important signaling molecule that impacts a wide range of physiological responses, including blood pressure regulation, neurotransmission, immune response, and cell death.¹²⁰ The knowledge of the biological roles of CO is about 15 years behind that of NO, but it appears to play a role in some of the same pathways as NO in addition to other important processes.¹²¹ In both cases, the concentration dictates the biological effect, with low concentrations being critical for signaling events and high concentrations being toxic.

These gas molecules also have in common rich transition metal coordination chemistry that begets their biological impact. Their biosynthesis is metal-dependent, and in many cases their biological targets are metal centers, typically heme iron. These reactions inform us that transition metal complexes in biological environments can liberate and sequester NO and CO by breaking or forming metal–nitrosyl (M–NO) and metal–carbonyl (M–CO) bonds. It is possible, therefore, to develop artificial small molecule coordination complexes that can detect or manipulate NO and CO in order to study their biological roles, as well as act as therapeutic agents.

Chart 4. Select Examples of Metal-Based Fluorescent NO Sensors



6.2. Metal-Based NO Sensors

The first metal coordination compounds to provide *turn-on* fluorescence enhancement in response to NO were reported in 2000.^{122,123} These compounds contained cobalt(II) supported by aminotroponimate ligands appended with dansyl fluorophores that are quenched due to their proximity to the paramagnetic metal center. Reaction with NO forms Co(I)-dinitrosyl adducts and induces dissociation of a fluorescent ligand, thereby providing emission enhancement.^{122,123} Over the past decade, a number of metal complexes have appeared that give a fluorescence change in response to NO.¹²⁴ In addition to cobalt(II),^{125,126} these include systems based on iron(II),^{127,128} ruthenium(II),¹²⁹ rhodium(II),¹³⁰ and copper(II)^{131–137} and achieve fluorescence enhancement by a variety of mechanisms in addition to the fluorophore displacement strategy described above. For example, NO-induced reduction of paramagnetic Cu(II) to diamagnetic Cu(I) restores fluorescence of a coordinated fluorophore.¹³¹ Alternatively, reductive nitrosylation of a metal center can produce NO⁺ that reacts with metal-coordinating amines to give noncoordinating *N*-nitrosamines with enhanced emission.¹³⁴

For most of the early attempts at building metal-based NO sensors, the fluorescence responses were restricted to reactions done in organic solvents with high concentrations of NO gas bubbled into a cuvette. In terms of biological applications, other drawbacks of some of these examples include cross-reactivity with O₂ or other species and fluorophores that are not ideal for cellular imaging studies. The recent development of a fluorescein-based copper complex CuFL (Chart 4, **19**) overcomes many of these challenges and has enabled visualization of NO in living cells.¹³⁴ The FL ligand coordinates Cu(II) with an apparent *K*_d of 1.5 μM at pH 7.0 to give a nonfluorescent complex that reacts rapidly and selectively with NO to give an 11-fold increase in fluorescence intensity, while other reactive nitrogen and oxygen species like H₂O₂, HNO, NO₂⁻, NO₃⁻, and ONOO⁻ give no response.¹³⁴ The turn-on response was also obtained by reacting CuFL with *S*-nitrosothiols. The irreversible, NO-induced fluorescence increase was shown through detailed spectroscopic analysis to derive from the *N*-nitrosated product FL-NO (**20**) that results from a reductive nitrosylation mechanism. FL-NO no longer binds to Cu(II) or Cu(I) and has a 7.5-fold higher quantum yield than FL itself, indicating that it is the species responsible for the observed fluorescence signal.¹³⁴ Neither removal of Cu(II) from the CuFL complex nor addition of Cu(I) to FL gives the dramatic enhancement seen for the reaction product of Cu(II)FL and NO, providing further assurance that the response is NO-dependent and not the result of metal release or simple reduction of the probe compound. Given these positive attributes, CuFL was then

shown to be cell permeable and capable of detecting NO produced by both constitutive and inducible NO synthases in living neurons and macrophages.¹³⁴

CuFL has subsequently been used as a key tool to decipher the role of a bacterial NO synthase in the pathogenicity of *Bacillus anthracis*, the causative agent of anthrax.¹³⁸ Fluorescence imaging of macrophages infected with *B. anthracis* spores showed a positive fluorescence response to CuFL within 2 h of infection, while spores lacking their NO synthase gene gave no response to the NO probe at 2 h but a significant response after 18 h. Taken with other control experiments, these data showed that the major source of NO during early stage infection is from the bacterium itself, whereas the later time point is indicative of NO production by the macrophage. The ability to visualize NO production in a time-dependent manner by this metal-based NO sensor helped establish that bacterial-derived NO is a key defense mechanism of bacteria against the host immune response.¹³⁸

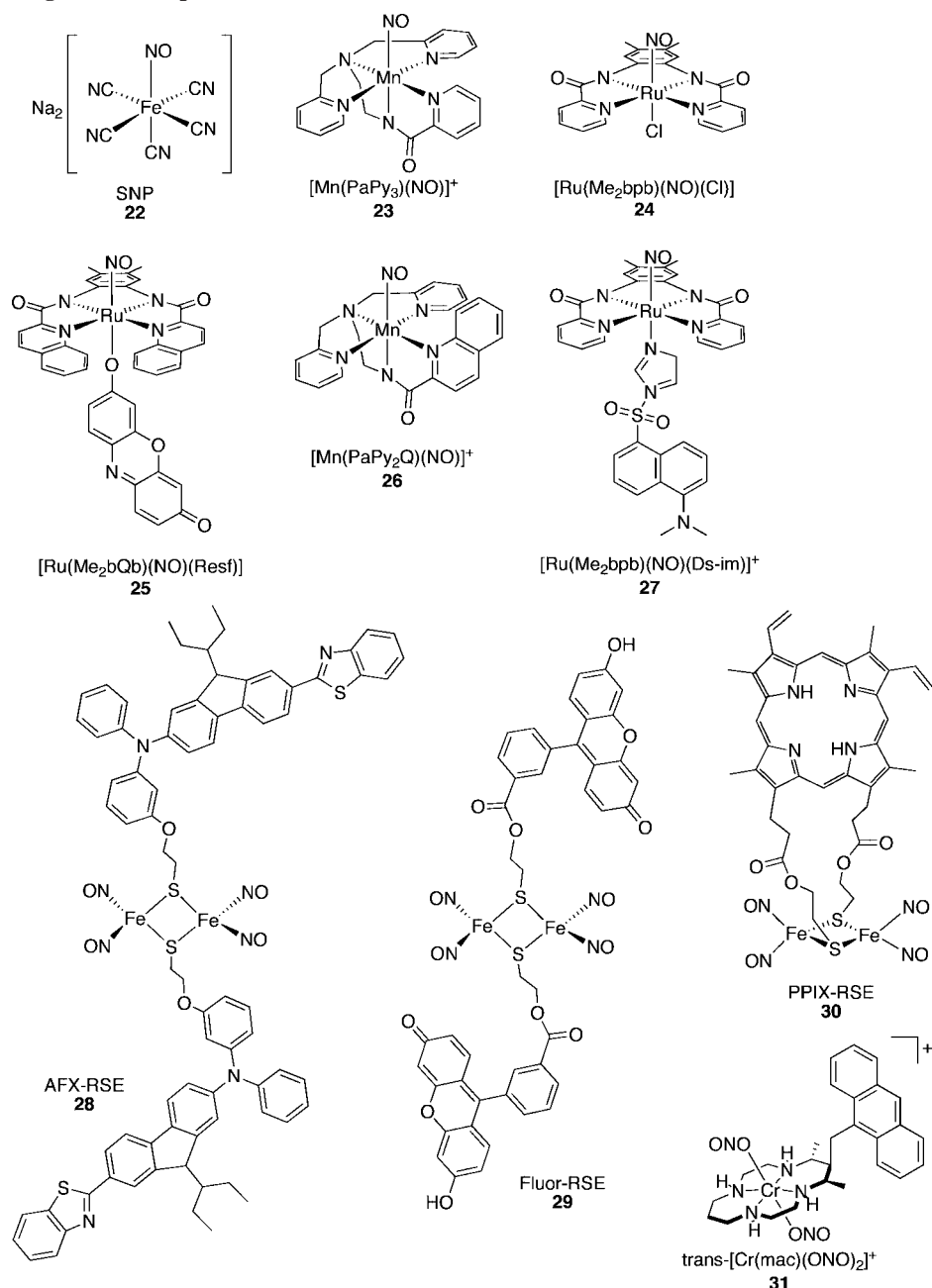
Another Cu(II)-based NO sensor has also appeared recently along with *in vivo* imaging data. The fluorescent compound 4-methoxy-2-(1*H*-naphtho[2,3-*d*]imidazol-2-yl)phenol (MNIP, **21**) binds Cu(II) with 1:1 stoichiometry to give nonfluorescent Cu-MNIP with an apparent *K*_d in pH 7.4 water/DMSO of 0.6 μM.¹³⁵ The fluorescence enhancement observed following reaction with NO was determined to be a similar reductive nitrosylation process as described above, where Cu(I) is released from the complex and one of the nitrogen ligands is nitrosylated. MNIP-Cu was used to show NO production in lipopolysaccharide-activated macrophages and in a model of acute liver injury in mice.¹³⁵

6.3. Metal Complexes as NO-Releasing Compounds

NO-releasing drugs are a broad class of compounds that release NO *in vivo* or *in vitro* to effect a pharmacological outcome.¹³⁹ The first metal-containing NO-releasing drug was sodium nitroprusside (Chart 5, Na₂[Fe(NO)(CN)₅], SNP, **22**), a seemingly simple octahedral coordination complex of Fe(II) with one NO ligand and five cyanide ligands.¹³⁹ Although it is still used clinically for the rapid reduction of hypertension in acute cases, it does have an associated risk of cyanide toxicity and must be administered cautiously.¹⁴⁰

The success of SNP despite its obvious drawbacks associated with its ancillary cyanide ligands has inspired the development of improved coordination complexes for NO delivery. Many of these complexes are based on multidentate chelating ligands that improve the stability of the metal complex and avoid loss of supporting ligands. The focus in recent years has been to design agents that deliver a payload of NO in a controlled and targeted manner.^{141,142} Such

Chart 5. NO-Releasing Metal Complexes



directed release of NO could have applications as anti-infectious agents¹⁴³ or in cancer therapy as sensitizers for γ -radiation or as agents for photodynamic therapy. The known photosensitivity of many metal–nitrosyl compounds makes light an attractive stimulus to release NO from a metal center.

Several photosensitive metal compounds including SNP, Fe–S–NO clusters known as Roussin's salts, and simple Ru complexes like $[\text{Ru}(\text{NO})(\text{Cl})_5]^{2-}$, have been used as controlled sources of NO to elucidate the biological and notably neurophysiological roles of NO in cells and tissues; however, these complexes all suffer from undesirable side reactions.^{144,145} In addition to release of toxic side products like cyanide, the NO is lost prior to photolysis either thermally or by conversion to other NO_x products in the case of the Fe–NO compounds, or the metal itself reacts with biomolecules as in the case of Ru forming DNA adducts.^{144,145} Metalloporphyrins that were studied for their photolytic

properties by Ford in the early 1990s have the advantage of being very stable macrocyclic metal complexes with intense long-wavelength absorptions but suffer thermal instability and oxygen sensitivity in the case of the Fe–NO adducts and complicated back and side reactions in the case of Ru–NO porphyrins.^{141,146} Considerable effort has therefore gone into designing alternative multidentate ligands that support photoactive Mn and Ru nitrosyl adducts,^{142,145} as well as water-soluble Fe complexes,^{147,148} particularly those that can be activated by two-photon excitation.¹⁴¹ These compounds are shown in Chart 5 and described in more detail in the following sections.

6.3.1. NO Donors Sensitive to Visible and Near-IR Light

The Mascharak group introduced the pentadentate ligand PaPy₃H (*N,N*-bis(2-pyridylmethyl)amine-*N*-ethyl-2-pyridine-2-carboxamide), which supports octahedral M–NO com-

plexes by providing four nitrogen atoms around the equatorial plane of the metal with a carboxamide group trans to NO; the Mn complex $[\text{Mn}(\text{PaPy}_3)(\text{NO})]^+$ (**23**) is an example. The strong σ -donor character of the negatively charged carboxamide being trans to NO has proven to be a key feature in the photolability of these complexes, with the nature of the metal center dictating the wavelength of light required to achieve photorelease. The iron complex $[\text{Fe}(\text{PaPy}_3)(\text{NO})]^{2+}$ can be activated by visible light in the 500–600 nm range, but the complex is not stable in aqueous solutions and transfers its NO to thiol-containing compounds even in the absence of light.¹⁴⁹ Replacing Fe with Ru improves the solubility and stability of the complex, because $[\text{Ru}(\text{PaPy}_3)(\text{NO})]^{2+}$ is soluble in water and stable between pH 5 and 9.¹⁵⁰ Photolysis only occurs with UV light, which may limit future in vivo use, but the complex is a useful tool for studying fast reactions of NO with heme proteins.¹⁵¹

The Mn analog $[\text{Mn}(\text{PaPy}_3)(\text{NO})]^+$ (**23**) has proven to be the most interesting among this series, because it is soluble and stable in aqueous buffers and is activated by visible light (500–650 nm) to release NO and form the Mn(II) aqua species $[\text{Mn}(\text{PaPy}_3)(\text{H}_2\text{O})]^+$.¹⁵² Both the Mn and the Ru compounds activate soluble guanylate cyclase activity in vitro in a light- and concentration-dependent manner.¹⁵³ They also elicit a concentration-dependent increase in cGMP in vascular smooth muscle cells, demonstrating that the complexes release NO intracellularly under the control of light. Furthermore, the compounds showed light-dependent vasorelaxant activity in a rat thoracic aortic ring.¹⁵³

The photoactivity of metal nitrosyls requires promotion of an electron from a metal-based molecular orbital to a $\pi^*(\text{NO})$ antibonding orbital; therefore the sensitivity to light depends on the energy of the $\text{M} \rightarrow \pi^*(\text{NO})$ electronic transition.^{154–156} To push the excitation wavelength into the visible and near-IR, the $\text{M}-\text{NO}$ photoband needs to be in this longer wavelength region. As illustrated in the PaPy_3 examples described above, one way to do this is to change the metal. Two other strategies are to change the field strength of the ligand or to attach light-harvesting chromophores to the complex. Both strategies are further explored below.

The ligand H_2bpb (*N,N'*-bis(bipyridine-2-carboxamido)-1,2-diaminobenzene) nicely demonstrates how clever alterations to the coordination chemistry of the complex can tune its photophysical properties. Several variations of this ligand exist; one example is the dimethyl derivative $[\text{Ru}(\text{Me}_2\text{bpb})(\text{NO})(\text{Cl})]$ shown in **24**, which is only sensitive to low-intensity UV light. However, replacing the pyridal arms with quinolines red shifts the $\text{Ru}-\text{NO}$ photoband from 380 to 455 nm, thereby accessing visible light photoactivation.¹⁵⁷ Furthermore, the four-coordinate H_2bpb framework allows an additional donor ligand to be installed trans to NO. By using this open coordination site to attach a light-harvesting dye directly to the metal, additional sensitization is achieved. Resorufin (Resf) is a dye with intense absorption in the visible and a phenolate moiety that enables direct attachment to the Ru center to give $\text{Ru}-\text{NO}$ complexes with strong absorption bands around 500 nm.^{158,159} The combination of the quinoline arms on the ligand and the dye attached to the metal to give $[\text{Ru}(\text{OMe}_2\text{bQb})(\text{NO})(\text{Resf})]$ (**25**) demonstrates that these effects are additive. Adjustment of the ligand frame effectively merges the $\text{Ru}-\text{NO}$ photoband with the intense absorption of the coordinated dye, thereby

increasing the extinction coefficient and sensitizing the compound to visible light photoactivation.¹⁵⁸

Because light penetration through mammalian tissue is mostly restricted to the 700–1100 nm region, it is very desirable to have NO donors that are photoresponsive in this region if they are to be used in applications like photodynamic therapy.¹⁵⁶ Again by clever alterations of the ligand, Mascharak reported that replacing one of the pyridal arms of HpaPy_3 with a more conjugated quinoline arm to give HpaPy_2Q provides a framework that supports $\text{Mn}-\text{NO}$ complex **26** with near-IR sensitization.¹⁵⁶ To demonstrate NO transfer to biological targets, **26** has been immobilized into a biocompatible, polyurethane-coated sol–gel matrix that delivers NO to myoglobin under near-IR light at 780 nm.¹⁵⁶

6.3.2. Trackable NO Donors

In addition to acting as light harvesters to increase the photosensitization of NO donors, the incorporation of fluorescent dyes into NO-releasing compounds has the advantage of providing a tracking signal to monitor cellular distribution. In addition to **26**, the dansyl-containing $[\text{Ru}(\text{Me}_2\text{bpb})(\text{NO})(\text{Ds-im})]^+$ (**27**) also provides a fluorescent handle.¹⁶⁰ Both **26** and **27** are diamagnetic species that retain appreciable fluorescence intensity of the coordinated dansyl or resorufin dyes. Photoinduced loss of NO gives paramagnetic Ru(III) species that quench fluorescence of the coordinated dyes to an extent related to the degree of NO released.¹⁵⁸ Cell culture studies in human breast cancer cells demonstrate the ability to track triggerable NO release from these compounds.^{158,160} The bright red fluorescence in Figure 1 indicates that **25** is taken up by live cells and localizes in the cytosol and nucleus, as evidenced by colocalization with the blue nuclear stain DAPI.¹⁵⁸ Control experiments indicated that the red fluorescence is due to intact **25**, not free resorufin. While there was no sign of apoptosis for cells held in the dark, samples that were subjected to visible light showed signs of nuclear degradation consistent with apoptosis within 4–8 h after illumination (see arrows in Figure 1b). These preliminary cellular studies are consistent with cellular uptake of nontoxic, stable, and fluorescent NO donors that are activated by visible light to release NO, which presumably instigates the onset of apoptosis.

6.3.3. NO Donors Sensitive to Two-Photon Excitation

Roussin's red salt, $\text{Na}_2[\text{Fe}_2(\mu\text{-S})_2(\text{NO})_4]$, has been shown to generate NO photochemically and sensitize hypoxic cells to γ radiation, but it does not have appreciable absorption in the longer visible range desirable for in vivo applications.¹⁶¹ To increase the sensitivity of this class of NO donors to visible and near-IR light, Ford and co-workers have modified Roussin's red salt esters (RSE), $[\text{Fe}_2(\mu\text{-SR})_2(\text{NO})_4]$ with a variety of light-harvesting chromophores, including AFchromophore (**28**, AFX-RSE),¹⁶² fluorescein (**29**, Fluor-RSE),^{163,164} and protoporphyrin IX (**30**, PPIX-RSE).¹⁶⁵ About 85% of the fluorescence intensity is quenched in these compounds, indicating energy transfer from the antenna to the $\text{Fe}-\text{NO}$ cluster, ultimately inducing photochemical release of NO. While the chromophores increase light absorption and improve the rate of NO photorelease at longer wavelengths, the process is not very efficient. However, the advantage of these dyes is their moderate two-photon cross sections that enable the dye-modified clusters to release NO upon two-photon excitation at 810 nm femtosecond pulsed light.¹⁴¹

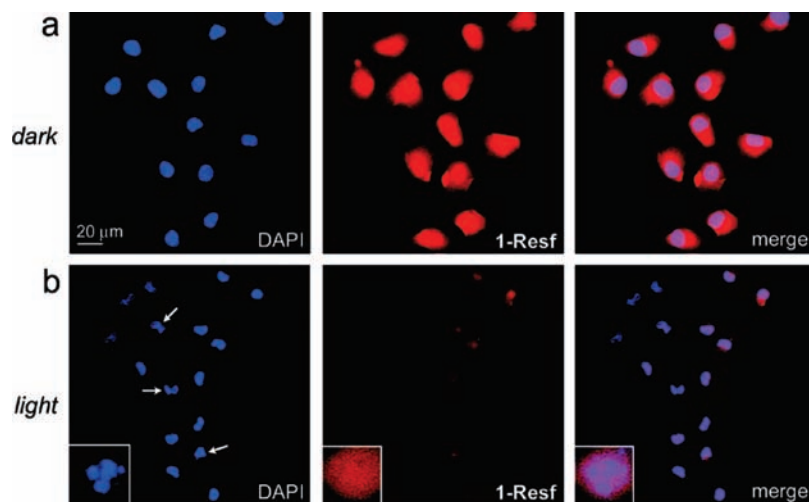


Figure 1. Human breast cancer cells loaded with NO-releasing compound **25** for 1 h and either (a) kept in the dark or (b) exposed for 1 min to visible light. The arrows point to evidence of nuclear degradation. Bottom insets show magnified views of a cell undergoing apoptosis. Reprinted with permission from ref 158. Copyright 2008 American Chemical Society.

While PPIX-RSE is only soluble in organic solvents, Fluor-RSE has modest solubility in water and therefore has potential as a biological NO donor via two-photon excitation. Poly(ethylene glycol) units have also been incorporated onto a RSE cluster modified with a two-photon chromophore to instill water solubility. The complex was shown to have moderate cancer cell killing ability following two-photon excitation.¹⁶⁶

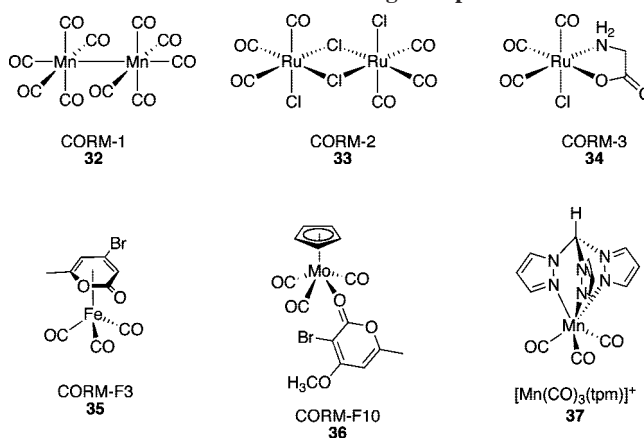
6.3.4. Other NO Donors

In addition to metal nitrosyl complexes, metal nitrito ($M-NO_2$) complexes can also act as precursors for NO release. Examples in this category include Ru supported by bipyridine (bpy) and pyridine (py) spectator ligands, $cis-[Ru(NO_2)(py)(bpy)_2]^+$,¹⁶⁷ and Cr(III) supported by the tetraaza macrocycle cyclam to give the water-soluble $trans-[Cr(cyclam)(ONO)_2]^+$, which release NO upon visible light excitation.¹⁶⁸ To increase the absorption cross-section, pendant chromophores such as anthracene shown in **31** have been attached to the cyclam ring;¹⁶⁹ alternatively, CdSe/ZnS core/shell quantum dots have also served as antennas to sensitize photorelease of NO from $[Cr(cyclam)(ONO)_2]^+$ cations that are electrostatically embedded in the nanoparticle assembly.^{170,171}

6.4. Metal Complexes as CO-Releasing Compounds

CO is produced endogenously in mammalian cells as a byproduct of heme degradation by heme oxygenases (HO), which also release biliverdin and iron as coproducts. Long thought of as a potentially toxic waste product, within the past decade this diatomic gaseous molecule has emerged as an important signaling molecule that influences numerous physiological processes.¹⁷² The basal level of CO in healthy humans is approximately 20 $\mu\text{mol/h}$, but diseases including asthma, cystic fibrosis, and diabetes can significantly elevate this capacity.¹⁷³ In addition to a constitutively expressed HO-2 isozyme, there is also inducible HO-1, which is upregulated in response to both chemical and physical stress.¹⁷² Several protective roles for CO have been identified by increasing the activity of HO-1 or by administering low concentrations of CO gas in cell culture or animal models. These roles include vasorelaxation, inhibition of smooth

Chart 6. Metal-Based CO-Releasing Compounds



muscle cell proliferation, protection against organ transplant rejection, inhibition of platelet aggregation, antiapoptosis, anti-inflammatory, neurotransmission, and protection against ischemic tissue injury.^{172–174}

The expanding possibilities for how heme oxygenase regulates physiological response via CO production along with the possibility that CO could be used therapeutically if appropriately administered has led to a search for small molecules that can deliver CO in a controlled fashion.^{121,173,175–177}

This work has been pioneered by Motterlini. The first transition metal complexes to be investigated for their ability to release CO under physiological conditions were $[Mn_2CO_{10}]$ and $[Ru(CO)_3Cl_2]_2$, which were coined CORM-1 and CORM-2, for CO-releasing molecule 1 and 2 (**32** and **33**, respectively, Chart 6).¹⁷⁸ These compounds were shown to transfer CO in vitro to myoglobin and promote vasodilation in an isolated aortic ring model, and CORM-2 reduced acute hypertension in rats.¹⁷⁸ The drawback that neither compound is water-soluble inspired a search for molecules with improved biological compatibility.

The most promising CORM to date and the one that has received the most intensive biological evaluation is $[Ru(CO)_3Cl(\text{glycinate})]$, or CORM-3 (**34**).¹⁷⁹ Incorporation of glycine onto the $Ru(CO)_3$ core renders this compound water-soluble. The number of biological tests for CORM-3 are extensive and were recently reviewed.¹⁷⁶ The aqueous solution chemistry of CORM-3 is in fact complex, with the

major species present at physiological pH 7.4 conditions expected to be $[\text{Ru}(\text{CO})_2(\text{CO}_2)\text{Cl}(\text{glycinate})]^{2-}$ or $[\text{Ru}(\text{CO})_2(\text{CO}_2\text{H})(\text{OH})(\text{glycinate})]^-$, which result from attack of hydroxide onto a coordinated carbonyl.¹⁸⁰ The half-life of CO loss from CORM-3 varies dramatically depending on the solution conditions: in distilled water, it is 98 h, whereas it shortens to under 4 min in human plasma.¹⁷⁷ The difference in rates of CO liberation is likely a consequence of the mechanism of CORM-3 CO loss, which has been suggested to involve replacement of the labile glycinate and chloride ligands by other components in solution.^{121,177} In plasma or cellular environments, competing ligands like cysteine or glutathione could act as strong trans-labilizing ligands that induce CO loss. The fact that the rate of CO release can be tuned depending on the trans ligand portends that further optimization of this system could result in compounds where exchange of the trans ligand itself could be manipulated under certain conditions to instigate CO loss.

In addition to Mn and Ru, Fe carbonyl complexes are also being investigated for their CO-releasing properties. The homoleptic compound $[\text{Fe}(\text{CO})_5]$ was dismissed early on as a biologically viable candidate because photolysis to release CO caused undesirable precipitation.¹⁷⁸ The water-soluble $[\text{CpFe}(\text{CO})_3]\text{Cl}$ also suffered from precipitation following CO loss, but substitution of the cyclopentadienyl ring results in water-soluble compounds of the type $[(\text{C}_5\text{H}_4\text{CO}_2\text{Me})\text{Fe}(\text{CO})_3]^+$ with promising biological activity.¹⁸¹ A series of CORMs containing 2-pyrone have also been prepared in the hope that the pyrone moiety might facilitate cell membrane transport and intracellular distribution.^{182,183} Examples include CORM-F3 (**35**) and the Mo complex CORM-F10 (**36**). In these compounds, the rate and extent of CO release could be tuned by varying the halogen directly attached to the 2-pyrone ring. With a CO release rate of 3.4 $\mu\text{M}/\text{min}$ in a DMSO/phosphate buffer, CORM-F10 is one of the fastest CO releasers to date.¹⁸²

As with the case of photoactive NO-releasing molecules, photoactive CO-releasing molecules are attractive for directed and triggerable CO release in high concentration at a localized site. Toward this goal, photoreactive $\text{Mn}(\text{CO})_3$ units stabilized by a tris(pyrazolyl)methane ligand to give $[\text{Mn}(\text{CO})_3(\text{tpm})]^+$ (**37**) have been introduced.¹⁸⁴ This compound was shown to release two equivalents of CO to myoglobin in solution following irradiation at 365 nm. It was also shown that the compound was taken up via passive diffusion by human colon cancer cells and induced photo-initiated cytotoxicity.¹⁸⁴ Importantly, the compound was not cytotoxic in the dark.

6.5. Metal Complexes That Bind Phospho Anions

The charge and Lewis acid character of metal ions make them attractive components of sophisticated anion receptors wherein a metal ion is anchored by a ligand that allows open coordination sites for anion binding. The ligand scaffold also provides added molecular recognition for anion selectivity or reporter groups for colorimetric or fluorescence sensing.^{185–187} As an example, Anslyn introduced a tris[(2-pyridyl)methyl]amine Cu(II) complex that positions guanidinium groups in ideal positions to recognize a phosphate anion above the metal center (Chart 7, **38**).¹⁸⁸ While these compounds have not been used in a cellular setting, they have been applied for measuring phosphate concentrations in biological fluids like serum and saliva by using an indicator-displacement

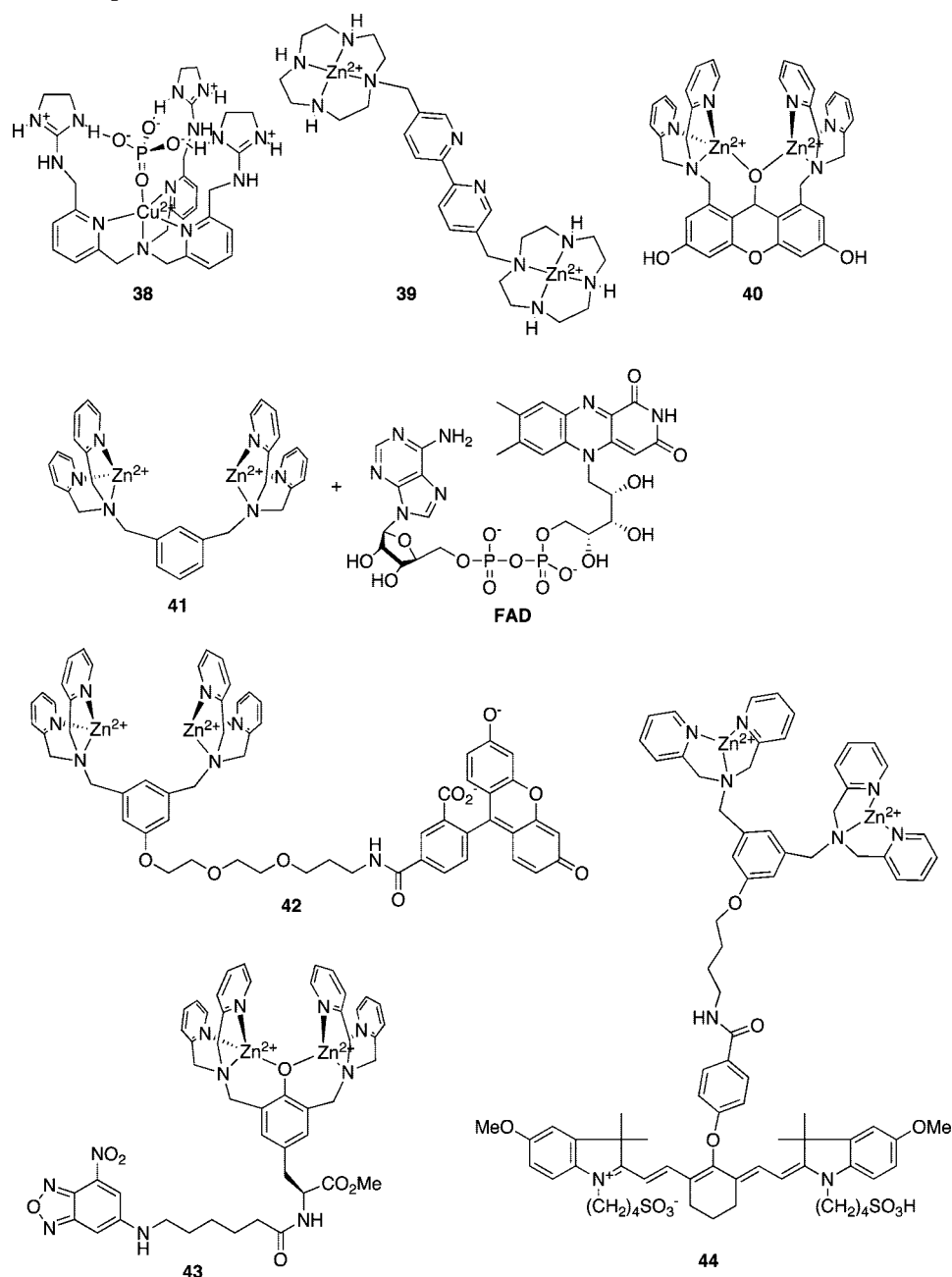
strategy where phosphate displaces fluorescein from the host–guest complex to provide the signal readout.¹⁸⁹

Coordination complexes of Zn(II) are more broadly explored than those of Cu(II) and offer the advantages of being non-redox-active and typically nonquenching toward attached fluorescence dyes. Work by Kimura showed that Zn(II) complexes of the macrocyclic tetraamine cyclen (1,4,7,10-tetraazacyclododecane) form 1:1 complexes with various anions in aqueous solution at neutral pH. Modifications of this motif have led to receptors for phosphate, thymidine nucleotide, and other biologically relevant anions in aqueous solution.^{190,191} A supramolecular sensor that arranges six Zn^{2+} –cyclen units around a $\text{Ru}(\text{bpy})_3$ core is capable of discriminating inositol triphosphate (IP_3) from mono- and diphosphates in aqueous solution.¹⁹² Three of the cyclen-appended bipyridyl ligands, shown as **39** in Chart 7, bind to Ru to form the sensor. The Ru core acts as both a template to arrange the Zn sites optimally for IP_3 binding and as a luminescent reporter for IP_3 sensing. The supramolecular compound suffers from photodegradation but shows a creative approach for the selective detection of an important second messenger for intracellular signal transduction.

In addition to cyclen, dipyrldylamine (DPA) Zn(II) complexes are also promising recognition sites for biological anion sensing.¹⁸⁶ Many of these dinuclear Zn–DPA complexes incorporate a chromophore either into the backbone of the ligand or exogenously for indicator displacement that enables detection of anions like phosphate,¹⁹³ pyrophosphate,^{194–197} and ATP^{198,199} in aqueous solution. More recently, the concept of using metal complexes as host–guest recognition systems for anion detection has moved beyond detection in aqueous solution to the more complicated intracellular applications. Notably, Hamachi introduced a xanthene-bridged bis(Zn–DPA) compound (**40**) that has a remarkable fluorescence enhancement upon binding to polyphosphate anions and is effective for fluorescence imaging of intracellular ATP stores in living cells.²⁰⁰ The fluorescence turn-on mechanism of **40** derives from the recovery of conjugation in the xanthene ring upon polyphosphate binding to the di-Zn site. In the absence of polyphosphate and in neutral buffered solutions, the DPA-bound Zn(II) centers bind and activate a water molecule for nucleophilic attack on the xanthene ring, forming the μ -oxygen atom that destroys the xanthene conjugation. The probe can detect low micromolar concentrations of ATP. Because **40** does not penetrate cell membranes, it was modified by acetylation at both hydroxyl groups to generate a cell-permeable version that converts to **40** intracellularly by esterases. In Jurkat cells **40** showed a particulate pattern of fluorescence that matched images obtained with quinacrine, an existing probe of ATP stores. Importantly, images of cells exposed only to the xanthene–DPA ligand without Zn showed a fluorescence response that was spread over the entire cytosol, suggesting that it is indeed the Zn-bound form of the probe that operates as the ATP recognition domain. While this agent has significant discrimination for polyphosphates over mono- and diphosphates, as well as other anions, it does not have significant discrimination among polyphosphate anions including other nucleoside triphosphates, pyrophosphate, and inositol triphosphate.²⁰⁰

Instead of incorporating a fluorophore into the bis(Zn–DPA) construct, receptor **41** works as a probe of flavin adenine dinucleotide (FAD) by taking advantage of the intrinsic fluorescence of the isoalloxazine ring system of the

Chart 7. Examples of Phosphate Anion Binders



flavin, which is normally quenched by intramolecular ring stacking with the adenine.²⁰¹ Binding of FAD to **41** disrupts this interaction and increases the quantum yield approximately 7-fold. The autofluorescence of riboflavin and flavin mononucleotide (FMN) are not affected by **41** because they do not contain an appropriate binding site for the dinuclear Zn-DPA probe, which therefore provides a platform for the selective targeting of FAD over other flavin-containing species. To demonstrate that **41** could be used as a cellular probe, Hong and co-workers showed that it caused a significant enhancement in the autofluorescence of granules in human white blood cells, which are known to have a high FAD content.²⁰¹

The utility of the dinuclear Zn-DPA systems for binding phosphate anions has further been explored for the selective detection of anionic phospholipids for several biological applications. The Smith group discovered that Zn-DPA coordination complexes have a selective affinity for mem-

branes enriched in anionic phosphatidylserine and that fluorescently modified versions like **42** could be useful for assays of programmed cell death.²⁰² The appearance of phosphatidylserine on the outer surface of the cell membrane is an early sign of apoptosis that provides a means for differentiating healthy vs apoptotic cells. The assay is frequently done with the protein annexin V, although small molecule probes could overcome some limitations of the protein-based assay. Fluorescence imaging of Jurkat cells treated with the apoptosis-inducing anticancer drug camptothecin and subsequently with **42** showed selective staining of cell surfaces on the apoptotic cells, whereas normal cells showed no staining.²⁰² Furthermore, intracellular fluorescence was not observed, indicating that **42** does not permeate membranes.

A related Zn-DPA construct has also been used to selectively label bacterial cells over mammalian cells. The selectivity is again based on the recognition of anionic phosphates by dinuclear Zn-DPA constructs. Whereas mam-

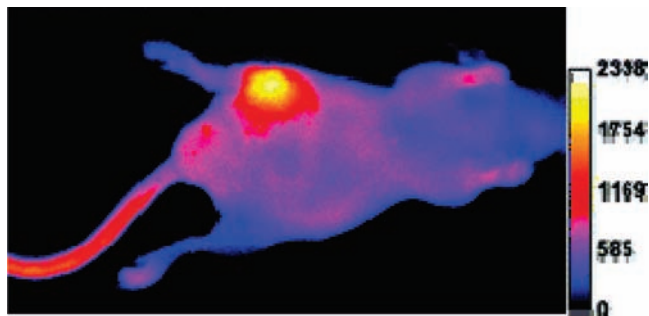


Figure 2. Optical image of a mouse with a *S. aureus* infection in the left rear thigh muscle 18 h after tail-vein injection of carbocyanine-modified bis(ZnDPA) **44**, a probe designed to detect anionic phospholipids found in bacterial cell walls. Scale represents relative fluorescence in arbitrary units. Reprinted with permission from ref 204. Copyright 2006 American Chemical Society.

malian cell surfaces are composed mostly of zwitterionic phospholipids, the surfaces of bacterial cells contain a high percentage of anionic phospholipids. The Smith group therefore explored the differential interactions of a family of complexes based on the 2,6-bis(zinc(II)-dipicolylamine)phenoxide construct.²⁰³ An NBD-tagged version is shown in **43**, but other derivatives have been made in which the alkyl substituent trans to the phenoxide is varied. Whereas lipophilic versions partition into zwitterionic vesicles and are moderately toxic to mammalian cells, a hydrophilic version that contains just a methyl substituent off the phenoxide is not toxic to mammalian cells but is very effective at killing the pathogenic bacterium *Staphylococcus aureus*. The antibiotic action appears to be caused by depolarization of the bacterial cell membrane, which is proposed to occur as a result of large fluctuations in local charge following the binding of cationic Zn-DPA complexes to anionic phospholipids head groups.²⁰³ Fluorescence imaging studies of the fluorophore-labeled version **43** show that staining of both *S. aureus* and *E. coli* is localized to the cell walls, which suggests that these complexes might be useful for detecting and imaging bacteria.²⁰³

Indeed, incorporating a carbocyanine dye that is sensitive to tissue-penetrating near-IR light onto a Zn-DPA construct enabled *in vivo* imaging of bacterial infection in a living animal.²⁰⁴ Nude mice were infected with *S. aureus* by injection of bacteria into the thigh muscle. The NIR fluorescent probe **44** was introduced 6 h postinfection into the bloodstream via a tail-vein injection, and anesthetized animals were irradiated with 720 nm light for imaging. The fluorescent probe accumulated at the site of infection, with the maximum signal contrast reached after 18 h (Figure 2).²⁰⁴

6.6. Metal Complexes for Phosphoprotein Detection

The interest in developing sensors that can selectively detect protein phosphorylation stems from the central role that this post-translational modification plays in signal transduction pathways and a host of other biological processes.^{205,206} Phosphate groups are installed onto a protein's serine, threonine, or tyrosine amino acid side chains by protein kinases and removed by phosphatases. The difference between the negatively charged, moderately sterically demanding phosphomonoester compared with the neutral hydroxyl group of the unmodified amino acid provides a means of using chemistry to differentiate phosphorylated peptide or protein sequences from their unphos-

phorylated analogs. Metal complexes in particular are attractive components of kinase/phosphatase sensors because they can couple a strong metal–phosphate interaction (as demonstrated in the previous section) to a change in fluorescence as a spectroscopic readout of phosphorylation state. Chart 8 shows examples of metal-dependent sensors for protein phosphorylation that are based on Zn(II), Ca(II), Mg(II), and Ln(III) complexes. The goal is to create probes that can selectively recognize a phosphoprotein of interest and thereby visualize the activity of specific protein kinases and phosphatases in real time in living cells, tissues, and even whole organisms.^{206,207} While this ultimate goal has yet to be realized, the probes described below have achieved some success in biological applications.

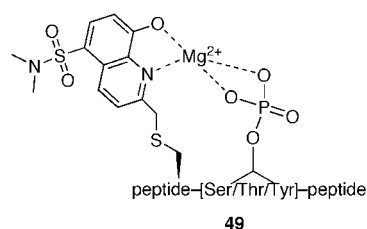
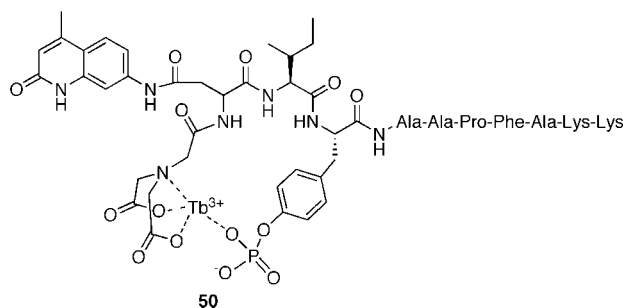
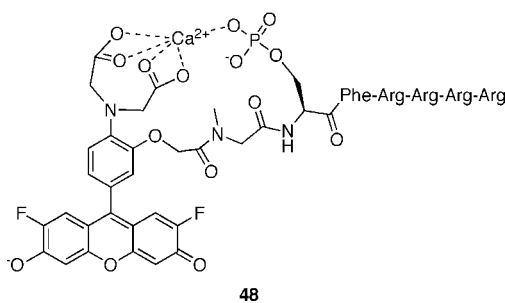
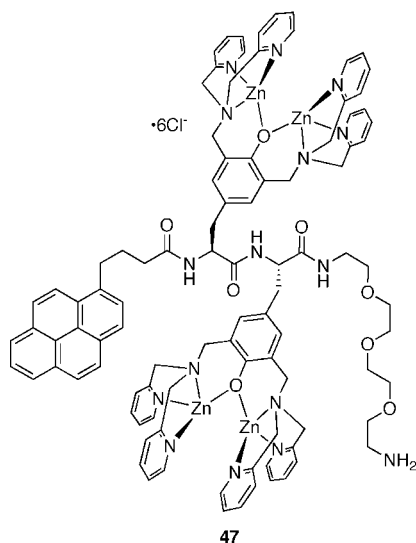
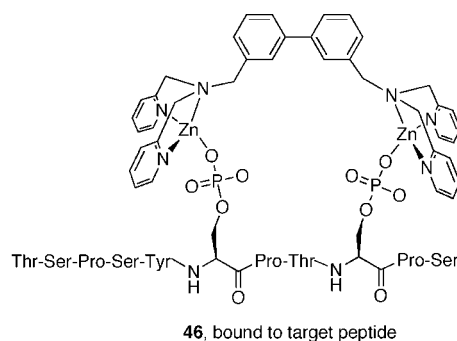
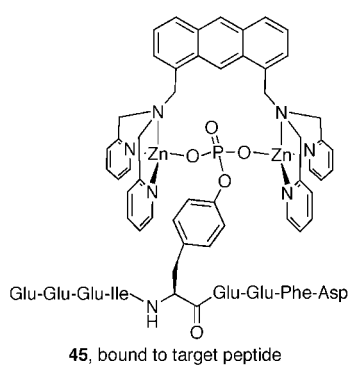
6.6.1. Phosphoprotein Detection by Zinc Complexes

In furthering the anion receptor capability of the Zn-DPA unit, Hamachi has developed a family of probes that recognize and sense phosphopeptides.²⁰⁸ The first probes contained two Zn-DPA units spanned by an anthracene bridge, one example is shown in **45**. Given the similarity of **45** to the ATP sensors described above, it is not surprising that **45** also has a high affinity for ATP and ADP ($K_{app} > 10^7 \text{ M}^{-1}$) in addition to phosphate monoesters but weak affinity for other anions including carbonate, sulfonate, nitrate, acetate, and chloride.^{209,210} The K_{app} of **45** for NaH_2PO_4 and phosphotyrosine is $\sim 10^5 \text{ M}^{-1}$, whereas its affinity for select phosphopeptides increases 1 or 2 orders of magnitude depending on the peptide sequence. A comparison of a series of short peptides with sequences based on various kinase recognition domains revealed that receptor **45** has the highest affinity for peptides with the greatest overall net negative charge. For example, **45** has a K_{app} of $9 \times 10^6 \text{ M}^{-1}$ for the phosphotyrosine (pTyr)-containing sequence Glu-Glu-Glu-Ile-pTyr-Glu-Glu-Phe-Asp, the consensus sequence of the protein kinase, v-Src. Peptides with fewer negative charges or overall positive charge elicit weaker fluorescent changes by the Zn-DPA probes. This correlation between overall net charge of the phosphopeptide and its affinity for the probe presumably arises from a favorable electrostatic attraction between the tetracationic dinuclear Zn-DPA receptor and negatively charged peptides. The unphosphorylated version of the v-Src peptide does not induce a fluorescence change of **45**, proving that the phosphate functionality is required for molecular recognition by the probe.

The dinuclear design of these receptors is critical to their function, since mononuclear versions show very little affinity for phospho species. An X-ray crystal structure of **45** bound to phenyl phosphate shows the phosphate unit bridging the two Zn(II) centers by providing a separate O ligand to each metal²¹⁰ (as indicated by the binding mode of p-Tyr to **45** in Chart 8), thus providing a structural basis for the cooperativity of the dinuclear site in recognizing phosphomonoester functional groups.

The cooperativity of the dinuclear site is also critical for the fluorescence sensing mechanism of **45**. Binding of the d^{10} ion Zn^{2+} to the receptor increases fluorescence by canceling the photoinduced electron transfer of the benzylic amine that otherwise quenches the appended anthracene fluorescence. The receptor is not maximally fluorescent unless both sites are saturated with Zn^{2+} , a situation that is facilitated by the presence of phosphate derivatives.²¹⁰

Chart 8. Metal Complexes for Phosphoprotein Detection



While the anthracene-linked Zn-DPA sensors have yet to be utilized in cell culture, they have been used to monitor phosphatase activity *in vitro*.²¹⁰ The emission intensity of **45** in the presence of a substrate peptide Asp-Ala-Asp-Glu-pTyr-Leu-Ile-Pro-Asn-Asn-Gly, a fragment of the epidermal growth factor receptor, decreased over time upon addition of protein tyrosine phosphatase 1B. In addition, the anthracene-linked Zn-DPA probes have also shown utility as selective phosphoprotein stains for gel electrophoresis analysis.²¹¹ The method was able to brightly stain phospho-ovalbumin and phospho- α -casein without staining four other nonphosphorylated proteins under photoillumination of a polyacrylamide gel that had been soaked with a solution of the Zn-DPA probe. The relatively simple staining method could find general applicability for deciphering the phosphoproteome by using 2D gel electrophoresis.²¹¹

In order to increase the selectivity of the Zn-DPA probes for specific phosphoproteins, Hamachi optimized the length of the spacer connecting the Zn-DPA units so that each Zn(II) could bind to an adjacent phosphomonoester, as demonstrated in **46**.²¹² They found by X-ray crystallography that the

biphenyl unit of **46** disposes the two Zn centers at a distance of 11.5 Å, which matches fairly well with the 9.7 Å distance found between the phosphate groups on Ser6 and Ser9 on the C-terminal domain of RNA polymerase II (CTD peptide).²¹³ Indeed, **46** binds the CTD peptide in a 1:1 fashion with a K_{app} of $8 \times 10^6 \text{ M}^{-1}$ as determined by isothermal titration calorimetry. By using a fluorescence polarization assay with a fluorescently labeled CTD peptide substrate, it was found that **46** effectively inhibited the protein-protein interaction between the CTD peptide and the Pin1 WW domain with a K_i of $1.8 \times 10^6 \text{ M}^{-1}$.²¹³ This work demonstrates that the well-placed metal-ligand interactions between the Zn-DPA coordination complexes and target phosphorylated regions can be utilized to disrupt protein-protein interactions and may be effective for disrupting signaling cascades in living cells that rely on such interactions. A second strategy for increasing selective detection of specific phosphoproteins is a hybrid approach that uses a combination of an artificial Zn-DPA receptor coupled to the Pin1 WW domain.²¹⁴ The doubly phosphorylated CTD peptide binds cooperatively to the hybrid sensor by interacting one of its

pSer residues with the WW domain and the other with the Zn-DPA unit.

In a slightly different application from phosphoprotein detection, Zn-DPA complexes have also shown an ability to deliver phosphopeptides and other phosphate derivatives intracellularly. The highly cationic tetranuclear complex **47** with sufficient lipophilic character was found as the optimal carrier vector to shuttle anionic phosphorylated peptides into cells via endocytosis.²¹⁵ The intracellular fluorescence of fluorescently tagged phosphopeptides was found to increase 4–6-fold in the presence of **47** compared with the absence of the metalloceptor.

6.6.2. Phosphoprotein Detection by Calcium and Magnesium Complexes

In addition to Zn²⁺, Ca²⁺ and Mg²⁺ are also effective at enhancing the fluorescence signal of designed probes via phosphorylation-dependent chelation. The design of **48** is based on Ca²⁺ indicators developed by Tsien that rely on Ca²⁺ coordination to turn on fluorescence.²¹⁶ To convert this idea into a kinase sensor, Lawrence and co-workers replaced one of the iminodiacetate arms with a serine-containing peptide that is a substrate for protein kinase C.²¹⁷ The unphosphorylated probe has low affinity for Ca²⁺ and is therefore weakly fluorescent, but phosphorylation increases the binding affinity of the receptor and turns on the signal. Optimization of the linker connecting the fluorophore and the peptide provided probe **48** with a 23-fold increase in fluorescence after phosphorylation.

Imperiali and co-workers have developed a family of kinase probes that incorporate a sulfonamido-oxine amino acid (Sox) strategically embedded in kinase recognition domains such that phosphorylation improves the chelation of Mg²⁺ to enhance Sox fluorescence 4–12-fold.²⁰⁷ These probes have been validated for the measurement of kinase activities in crude mammalian cell lysates.²¹⁸ The original designs required a conformationally constrained peptide β -turn to position Sox appropriately for cooperative binding of Mg²⁺ with the phosphorylated amino acid.²¹⁹ More recently, they have greatly expanded the scope of Sox-based kinase sensors by developing a Sox derivative called C-Sox that can alkylate a cysteine residue with the chromophore (**49** in Chart 8).²²⁰ This strategy has allowed the probe to be incorporated into a larger number of kinase recognition domains with improved specificity. The probes are amenable for high-throughput screening for kinase inhibitors and, because they are sensitive under conditions of physiological Mg²⁺ levels, may be amenable to future in vivo applications.²²⁰

6.6.3. Phosphoprotein Detection by Lanthanide Complexes

Long-lived emission lifetimes and sharp emission bands are some of the photophysical properties that make lanthanide (Ln) ions attractive for incorporation into biological probes. The trivalent Ln³⁺ is the most common oxidation state across the series. Being hard acids, they have a strong preference for negatively charged, oxygen-rich coordination environments. Not surprisingly, they interact strongly with phosphate-containing groups. Our lab has shown that the phosphorylation state of a peptide can dramatically increase its affinity for metal ions.^{221,222} As a consequence, Tb³⁺ can be an effective reporter of phosphorylation status.^{221,223} In these

cases, the peptides are short sequences resembling calcium-binding loops where the phosphorylated amino acid either is embedded within the peptide, as in DPDNEA(pY)EMP-SEEG,²²¹ or is the anchoring metal-ligating residue near the end of the peptide, as in DKNADGWIDRA(pS)LA,²²³ where pY and pS are phosphotyrosine and phosphoserine, respectively. Since lanthanides are poor absorbers, sensitizing chromophores are required to achieve luminescence. In these peptide examples, the pY itself or a tryptophan (W) residue provides sufficient sensitization to observe luminescence.

To increase the sensitization and the metal affinity, Sames incorporated a carbostyryl chromophore and an iminodiacetate chelating motif onto peptides that contain a tyrosine residue within a recognition domain for src and abl protein tyrosine kinases (**50**).²²⁴ The peptide-based probe can detect both kinase (phosphorylation) and phosphatase (dephosphorylation) activity by diluting aliquots of the enzyme assay into buffers containing either Tb³⁺ or Eu³⁺. The probe may be useful for in vitro evaluation of kinase and phosphatase inhibitors.²²⁴

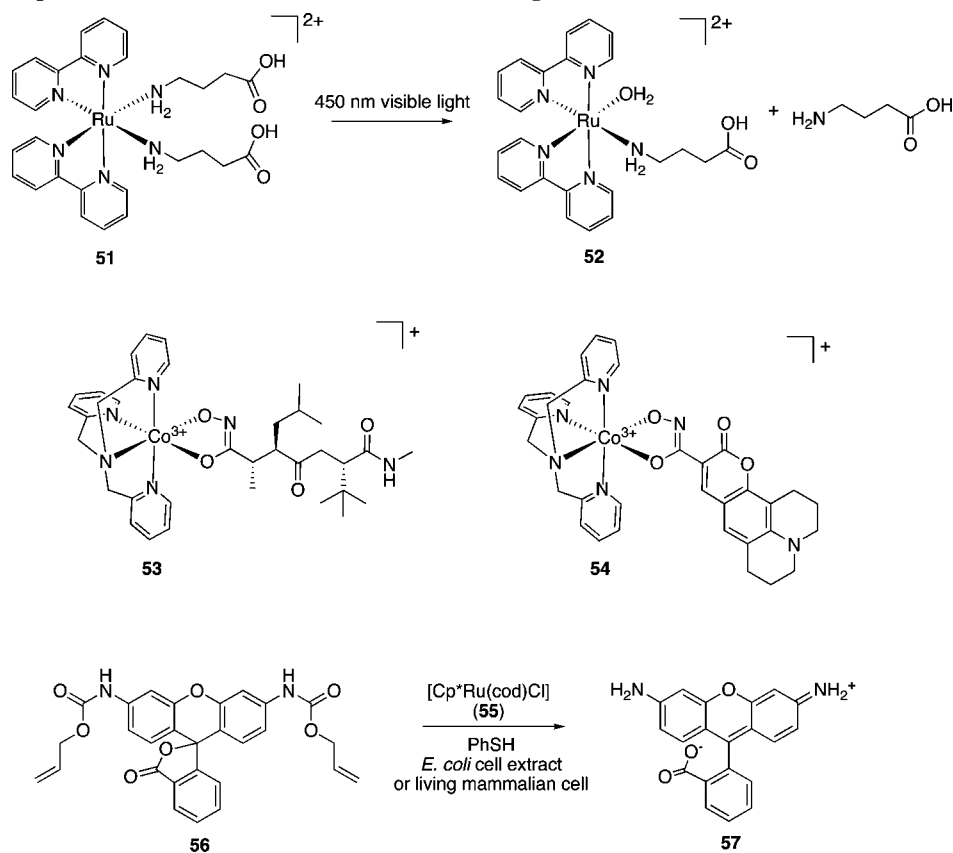
6.7. Metal Complexes That Release Bioactive Small Molecules

6.7.1. Photorelease of Bioactive Molecules

Coordinating a bioactive molecule to a metal center is an attractive way to mask the activity of a molecule of interest. Subsequent rupture of that metal–ligand bond by a specific trigger could in principle release the desired molecule in a controlled way. Etchenique and colleagues have applied this general principle in their development of ruthenium polypyridine complexes as inorganic photolabile caging groups for several amine-containing neurotransmitters. In the example **51** shown in Chart 9, two molecules of γ -aminobutyric acid (GABA) coordinate to a Ru(II) center supported by two bipyridine ligands to form the caged complex [Ru(bpy)₂(L)₂]²⁺, where L is GABA or one of several amine compounds tested, including serotonin, tryptamine, butylamine, and tyramine.²²⁵ Amino acids like glutamate can also be caged by replacing one of the bpy ligands with a tridentate tris(pyrazolylmethane) ligand to give a Ru complex with only one available open coordination site. This strategy forces the amino acid ligand to bind through its amine functional group and avoid a bidentate mode that would be recalcitrant to photorelease.²²⁶ Excitation of these molecules with 450 nm light into a metal–ligand charge transfer band of the Ru(bpy) core induces heterolytic bond cleavage of one of the monodentate ligands, thereby releasing one equivalent of the free amine. As monitored by NMR, the photoreaction is clean, providing only the monoaquo complex [Ru(bpy)₂(L)(H₂O)]²⁺ (**52**) as the other photoproduct. The quantum efficiency of the uncaging photoreaction was determined to be 0.03.

Incorporation of triphenylphosphine (PPh₃) into the coordination sphere to form [Ru(bpy)₂(PPh₃)(GABA)]²⁺ with only one amine ligand coordinated increases the quantum yield to 0.21, which is slightly higher than that of the more conventional nitrobenzyl ester photolabile protecting groups that have been used to cage GABA and other other biomolecules.²²⁷ Conventional, organic photocaging groups require UV excitation that could cause cellular photodamage. An advantage of the Ru-based caging groups is their longer wavelength photoreactivity with visible light. In addition, it

Chart 9. Metal Complexes That Release Bioactive Amine-Containing Small Molecules



has been shown that the Ru-based caged compounds can undergo two-photon excitation at 800 nm.²²⁸

To date the Ru-based cages have been applied in two biological settings. Current flow was measured by voltage-clamp recordings of frog oocytes expressing the GABA_c receptor, a ligand-gated ion channel that opens for Cl⁻ flow only upon activation by GABA. When applied in the dark, the Ru-caged GABA complex [Ru(bpy)₂(PPh₃)(GABA)]²⁺ caused no change in Cl⁻ currents. Pulses of visible light, however, resulted in measurable increases in current flow, consistent with release of GABA, which is then free to activate the GABA_c receptor.²²⁷ In another example, action potentials of a leech neuron were measured by a micropipet electrode inserted into isolated neurons that were exposed to [Ru(bpy)₂(4AP)]²⁺, where 4AP is 4-aminopyridine, a compound known to increase neuronal activity by blocking certain K⁺ channels. Action potentials increased when the neurons were exposed to both the Ru compound and flashes of visible light, consistent with photorelease of 4AP to stimulate neuronal response.²²⁹

6.7.2. Reductive Activation and Ligand Exchange

Reductive activation or ligand exchange is an alternative strategy of releasing a bioactive compound that is deactivated by coordination to a metal center. In principle, kinetically inert metal ions like Co³⁺ could be used as chaperones to deliver bioactive molecules intracellularly by releasing them upon reduction to the more labile Co²⁺ oxidation state.²³⁰ This strategy has been explored with a variety of Co(III) coordination complexes with amine-containing mustard compounds that are known alkylating agents.^{231,232} Some of this family of compounds show hypoxia-selective cytotoxicity, suggesting that the more reducing environment of

hypoxic tumors favors reduction of the metal complex and release of the cytotoxic agent. Details of this mechanism are still not clear, and more recent work suggests that ligand exchange is the more likely mechanism.²³⁰ More recently, Hambley has shown that the matrix metalloproteinase inhibitor marimastat can be incorporated into a Co(III) coordination complex (**53**) and used as a prodrug to inhibit tumor growth.²³³ Unfortunately, both free marimastat and the metal-bound prodrug also potentiated tumor metastasis relative to controls.

Given the promise of using cobalt as a drug chaperone and the clear need to understand its mechanisms of cellular distribution and prodrug activation in order to rationally design effective agents, complexes with fluorescent ligands such as **54** were developed.²³⁴ In the example shown, a hydroxamic acid derivative of coumarin-343 mimics the hydroxamic acid motif of marimastat. In the Co(III) form, the fluorescence of the metal-bound coumarin is quenched but is restored upon ligand release, be it by reduction or ligand exchange. Fluorescence microscopy studies showed that the subcellular distribution of the fluorophore was altered by coordination to the metal complex. Because the intact metal-fluorophore emission is weak, the intensity of the images suggests that the fluorophore is released from the metal, while the different staining patterns of the metal complexes vs the free coumarin suggest that metal complexation influences its cellular distribution. These studies importantly demonstrate that metal complexes can effectively chaperone bioactive molecules and traffic them in different ways compared with the uncomplexed agents, which may have important ramifications for altering the pharmacology of small molecules. Furthermore, these studies provide a strategy for monitoring the cellular trafficking and reactivity

of cobalt coordination complexes. Further studies aimed at understanding how to control the localization and release of active agents from these kinds of complexes remain an important goal.

6.7.3. Catalytic Release of Bioactive Molecules

Meggers and co-workers have developed a different strategy for releasing amine-containing molecules inside cells. Rather than relying on breaking a metal–ligand bond to release the small molecule, they are developing organometallic complexes as biocompatible catalysts to carry out intracellular organic transformations. Although the aqueous, aerobic environment of a cell presents significant challenges for most organometallic complexes, $[\text{Cp}^*\text{Ru}(\text{cod})\text{Cl}]$ ($\text{Cp}^* = \text{pentamethylcyclopentadienyl}$, $\text{cod} = 1,5\text{-cyclooctadiene}$) (**55**) was found to be tolerant of such conditions and able to catalyze the cleavage of allylcarbamates in the presence of excess thiophenol to give amines.²³⁵ To show that such a transformation could be conducted in a mammalian cell, a bis-allyloxycarbonyl-protected rhodamine (**56**) was incubated with cultured HeLa cells. The protected rhodamine derivative is not fluorescent, but treatment with the Ru catalyst and thiols deprotects it to give highly fluorescent rhodamine **57**, as shown in Chart 9. Treatment of the cells with the Ru catalyst caused a 3.5-fold increase in fluorescence within the cytoplasm, as observed by confocal fluorescence microscopy. Addition of thiophenol to the cell medium increased the fluorescence 10-fold. Cell viability was not affected by the treatment with the Ru compound, thiophenol, or the caged rhodamine.²³⁵ These encouraging results suggest that there is significant scope for further development of organometallic complexes used in biology. Indeed, the growing field of bioorganometallic chemistry offers exciting opportunities to uncover compounds with unique biological properties.^{33,236,237}

7. Metal Complexes as Enzyme Inhibitors

Small molecules that are capable of selectively inhibiting a particular enzyme are powerful tools for deciphering an enzyme's biological role. If the enzyme is involved in disease, its inhibitors become important drug candidates. While most inhibitors are organic compounds, inorganic coordination chemistry provides attractive, and perhaps too often overlooked, strategies for enzyme and protein inhibition. Metal-binding compounds and metal complexes themselves both offer unique properties that contribute to enzyme inhibition and are not found in conventional organic molecules. Metalloproteins, which comprise one-third of all proteins, are obvious targets for inhibition based on coordination chemistry, but metal complexes can also target nonmetalloproteins.

Because the metal cofactor of a metalloprotein is critical for function, removing or replacing the metal are very effective means of abrogating activity. Chelators that strip metals from their active sites, however, are usually nonspecific and provide little advantage for developing inhibitors targeted for a specific enzyme. Likewise, substituting a native metal ion with an inactive, non-native one can help in studying an isolated metalloenzyme *in vitro*, but is a difficult strategy to implement in the complex environment of a cell or whole organism. In contrast, compounds that bind directly to an active site metal ion can inhibit enzyme activity by blocking access of the substrate to the catalytic site. Additional interactions like hydrogen bonding and hydro-

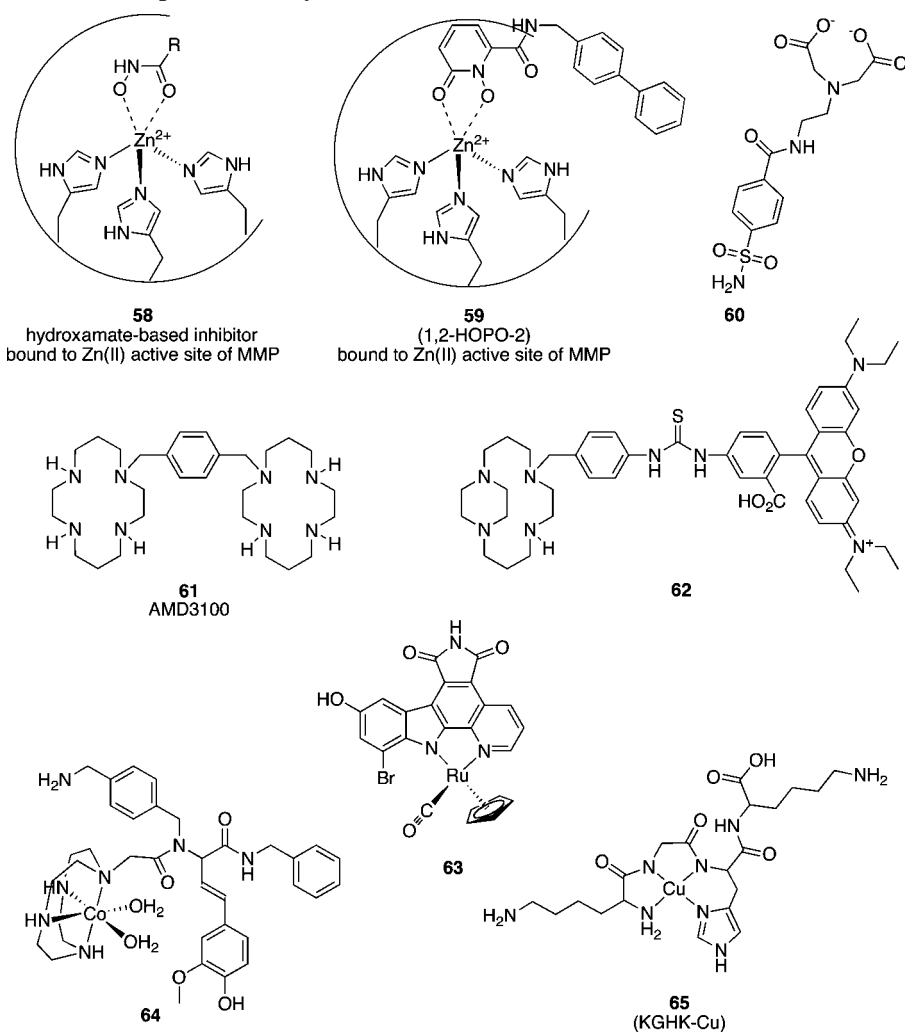
phobic contacts between the inhibitor and the protein can increase specificity of the inhibitor for the protein pocket. For proteins that do not contain a metal site, intact metal complexes can take advantage of their shape and organic framework to bind to proteins with high specificity through outer-sphere interactions. The inhibitor–protein interaction can be further strengthened if an amino-acid side chain from the protein binds directly in the inner sphere of the metal complex to anchor the inhibitor in place. In some cases, the metal ion may not be part of the administered compound but is instead picked up endogenously from the biological milieu. Finally, the reactivity of metal complexes can be harnessed to develop catalytic inhibitors that incorporate aspects of molecular recognition for specificity along with hydrolytic or oxidative reactivity to inactivate proteins through chemical modification.

The use of metal complexes as enzyme inhibitors was reviewed by Louie and Meade in 1999.²³⁸ Since that time, the sophistication in terms of the design and understanding of metal complexes as enzyme inhibitors has increased substantially for a handful of examples, as emphasized in a recent perspective on the topic.²³⁹ These examples, however, still represent only a small minority of compounds compared with conventional, organic inhibitors. Rather than provide exhaustive coverage of all chelator-inhibited enzymes, the following discussion provides select examples to highlight four strategies for using inorganic and organometallic chemistry for targeted enzyme inhibition: (1) metalloprotein inhibitors that use metal binding interactions as a significant component of their molecular recognition, (2) compounds that recruit endogenous metal ions to increase their affinity for protein binding and inhibition, (3) inert metal complexes that provide novel scaffolds for binding protein active sites, and (4) metal complexes that catalytically inactivate proteins. The structures of compounds discussed below are shown in Chart 10.

7.1. Inhibitors with Metal-Binding Headgroups for Targeting Metalloproteins

Carbonic anhydrase, matrix metalloproteinases (MMP), histone deacetylases (HDAC), and anthrax lethal factor are some of the examples of zinc(II)-dependent hydrolytic enzymes that are attractive drug targets for a wide range of diseases.²⁴⁰ Inhibitors of these mononuclear Zn(II) enzymes typically contain two components: an organic backbone that forms noncovalent, hydrogen bonding, hydrophobic, and electrostatic interactions with the protein surface, and a metal-binding headgroup that coordinates the active-site Zn(II). A significant amount of work has gone into optimizing the backbone fragments to impart specificity for a particular enzyme isoform, while less effort has focused on the zinc-binding group, which is typically a hydroxamic acid moiety, as shown in **58**.²⁴¹ In recent years, Cohen and co-workers have been reversing that trend by taking a bioinorganic approach to identify alternative zinc-binding groups. Crystal structures of small chelators coordinated to a Zn(II) model complex that mimics the three histidine ligands in the active site of MMPs were used in conjunction with computational modeling to identify promising zinc-binding groups that could be extended into full-length MMP inhibitors.²⁴² A range of chelating moieties have been investigated, including pyrones, thiopyrones, hydroxypridinones, hydroxypyridinethiones, dipyrildylamine, and aza-macrocycles.²⁴³

Chart 10. Examples of Metal Complexes As Enzyme Inhibitors



An example of a potent, nonhydroxamate inhibitor of MMPs that came out of this process is 1,2-HOPO-2 (**59**), where a hydroxypyridinone is the zinc-binding group, with a bulky biphenyl backbone.²⁴⁴ Interestingly, when a series of inhibitors containing the same biphenyl backbone but different hydroxypyridinone or hydroxypyridone headgroups was evaluated for selective inhibition against eight different MMP isoforms, it was revealed that even subtle differences in the zinc-binding group can influence isoform selectivity.²⁴⁴ The factors influencing the altered selectivity are likely a combination of altered sterics and hydrophobicity of the substituted pyrone/pyridinone rings, the pK_a of the chelating units, and the strength and orientation of the zinc-bound chelator, which will influence the positioning of the backbone into the protein site. The results of this study highlight the importance of understanding both the inorganic and organic aspects of inhibitor design.

MMP inhibitors are known to suffer from nonspecific interactions with off-target metalloenzymes, a general problem with any chelator-based approach. One of the drawbacks of conventional, hydroxamate-based inhibitors is their indiscriminant nature, having equivalent or higher affinity for iron and copper compared with zinc. In order to identify potential cross-reactivity of chelator-based inhibitors, a cell-based screening method was developed in a macrophage model that compares the relative activities of five different zinc and iron metalloenzymes in the presence of inhibitors.²⁴⁵ This protocol identified dipyriddyamine and triazacy-

clononane as promising zinc-specific binding groups. It also found that 1,2-HOPO-2 (**59**) showed some cross-reactivity with heme-dependent enzymes, while a hydroxypyridone analog did not.

In order to develop isozyme specific inhibitors of carbonic anhydrases, Srivastava and Mallik connected iminodiacetic acid (IDA) units to aryl sulfonamides to create two-prong inhibitors such as **60**.^{246,247} Arylsulfonamides have been known since the 1940s to inhibit carbonic anhydrase by binding directly to the Zn(II) and displacing the coordinated hydroxide in the enzyme active site. The second prong of **60** was added to bind Cu^{2+} so that the Cu-IDA unit could bind to surface-exposed histidine residues that are unique to select isozymes. Crystal structures of **60** bound to human carbonic anhydrases I and II validate this strategy and demonstrate that a metal-binding headgroup can couple with a protein-binding metal complex to enhance the overall effect of the inhibitor.²⁴⁸

7.2. Inhibitors That Recruit Endogenous Metals To Increase Potency

AMD3100, the bicyclam compound **61** containing an aromatic bridge connecting two tetraaza-macrocycles, has a history of over 20 years in drug development.²⁴⁹ It was first identified for its potency as an anti-HIV agent. Although clinical trials as an AIDS treatment were discontinued due to severe side effects, **61** (now marketed as Mozobil)

continues to show promise for other diseases, in particular for hematopoietic stem cell transplantation for treatment of non-Hodgkin's lymphoma and multiple myeloma. Compound **61** has been shown to be a highly specific antagonist for the CXCR4 chemokine receptor, a transmembrane protein in the G-protein-coupled receptor family that assists entry of HIV into cells and anchors stem cells in the bone marrow.²⁴⁹ Binding of **61** to CXCR4 therefore blocks viral entry into the host cell and also releases stem cells from the bone marrow into the peripheral blood where they can be harvested for transplantation.

The elements of molecular recognition responsible for the tight binding of **61** to CXCR4 were initially thought to derive principally from hydrogen-bonding interactions between the bicyclam N–H groups and specific carboxylic acid side chains on the protein. The affinity of **61** for CXCR4 was shown to increase 7-, 36-, and 50-fold when Cu²⁺, Zn²⁺, and Ni²⁺, respectively, were inserted into the bicyclam rings,²⁵⁰ suggesting that the metal complexes provide additional binding interactions that improve affinity. While cyclam has a higher thermodynamic binding affinity for Cu²⁺ than Zn²⁺ (log *K* = 27.2 vs 15.5), the higher relative concentration of available Zn²⁺ in blood plasma (reported at 19 μM) coupled with the faster kinetic rate for cyclam–Zn²⁺ complex formation at physiological pH suggests that AMD3100 likely exists as the Zn-bound form in vivo.²⁵¹

While there are no X-ray structures available for the transmembrane CXCR4 receptor, the crystal structure reported by Sadler and co-workers of Cu-cyclam and Cu₂-bicyclam bound to lysozyme provides some insight into protein–metallocyclam interactions.²⁵² The structures demonstrate that carboxylate side chains can indeed directly bind to open sites on the metal complex. Further interactions are formed by hydrogen bonding between the cyclam NH and protein carboxylates, as well as hydrophobic contacts to adjacent tryptophan residues. All of these interaction types are likely present when **61** binds to its target CXCR4. Furthermore, the cyclam rings are found in two configurations, a folded *cis* configuration and a planar *trans* configuration.

Metal-bound cyclam complexes can adopt several configurations that depend on the *cis* or *trans* arrangement of coordination sites not used by the ring nitrogens as well as the stereochemistry around each chiral nitrogen donor atom. Archibald has introduced configurationally fixed macrocycles that in principle present only one configuration to the protein to optimize metal–aspartate coordination that is the key to the increased protein binding interaction of the metal-loaded form of **61**.²⁵³ They have recently shown that the Cu(II) complex of fluorescently tagged, configurationally restricted tetraazamacrocycle **62** competes effectively against anti-CXCR4 monoclonal antibodies and may be useful in drug screening assays.²⁵⁴

7.3. Inert Metal Complexes as Enzyme Inhibitors

Instead of using a coordinate covalent bond between a metal center and a protein side chain to anchor a metal complex into a protein active site, it is also possible to use a substitutionally inert metal center as a structural building block to orient organic ligands in three-dimensional space to create architectures that are difficult to obtain with organic-only scaffolds. The power of this strategy has been successfully demonstrated by Meggers, who has assembled a number

of very potent and selective protein kinase inhibitors from a family of related organometallic Ru core structures.^{239,255,256} The example shown in **63** is highly selective for glycogen synthase kinase 3 (GSK-3).^{257,258} The selectivity in this case was achieved as a result of a structure–activity study that identified substituents on the ligands that improved GSK-3 selectivity over a panel of 57 different kinases. Variations of **63** have been shown to target other kinases, including Pim-1, MSK-1, Pak-1, and P13K, all with low nanomolar binding.²³⁹ Over 500 protein kinases with conserved ATP-binding sites, which are also inhibitor target sites, are encoded in the human genome making selective inhibition very challenging. Subtle optimization of the ligands oriented around Ru has enabled this family of compounds to complement the ATP binding sites in unique ways to differentiate one active site from another. In many cases, cocrystal structures of inhibitor-bound enzymes reveal the purely structural role of the metal and the variation in contacts between the substituents and enzyme active sites.^{257,259,260} Figure 3 shows the crystal structure of GSK-3 with a derivative of **63** bound. The biological effects of several Ru-based inhibitors have been tested in mammalian cell culture, *Xenopus* embryos, and zebrafish embryos.^{258,261,262}

7.4. Metal Complexes as Catalytic Protein Inactivators

In the previous examples, structural aspects of metal–ligand bonding provide functionality to these metal-based inhibitors. While their inorganic nature makes them unique compared with organic inhibitors, they still work by a conventional mechanism of competitive inhibition at a protein active site. It is possible, however, to use the reactivity of metal complexes to cause irreversible destruction of a protein target with substoichiometric amounts of the inhibitor. This strategy of catalytic inactivators couples a protein-targeting moiety for molecular recognition with a metal complex capable of either hydrolytic or oxidative reactivity.^{263,264} While the focus here is on protein inhibitors, it is important to mention that similar strategies have been used to couple DNA- or RNA-targeting agents with metal complexes for inducing damage to nucleic acids.²⁶⁴

The ability of metal ions and complexes, especially strongly Lewis acidic species, to hydrolyze unactivated amide bonds is well-known; a thorough review on this topic recently appeared.²⁶⁵ In order to direct such reactivity to a specific protein of interest, Suh conjugated a Co(III) cyclen complex known to effect amide bond hydrolysis²⁶⁶ to a variety of protein-targeting domains that were uncovered by screening combinatorial libraries.²⁶⁷ For example, **64** was discovered from a library of candidates that was screened for the ability to cleave peptide deformylase, a target for antibacterial drugs.²⁶⁸ While the rate of reactivity was modest, it was shown that a substoichiometric amount of the compound caused cleavage of the target protein, as revealed by mass spectrometry.²⁶⁸ A similar combinatorial approach was used to identify Co(cyclen) conjugates to target Aβ peptides and oligomers.²⁶⁹ Four Co(cyclen) conjugates decorated with benzothiazine derivatives were found that cleave Aβ monomers and oligomers at submicromolar concentrations. It was suggested that such a strategy could be used to reduce the amount of toxic protofibrils of Aβ that are implicated in amyloid formation in Alzheimer's disease.²⁶⁹ The same four compounds were subsequently found to be effective cleavage

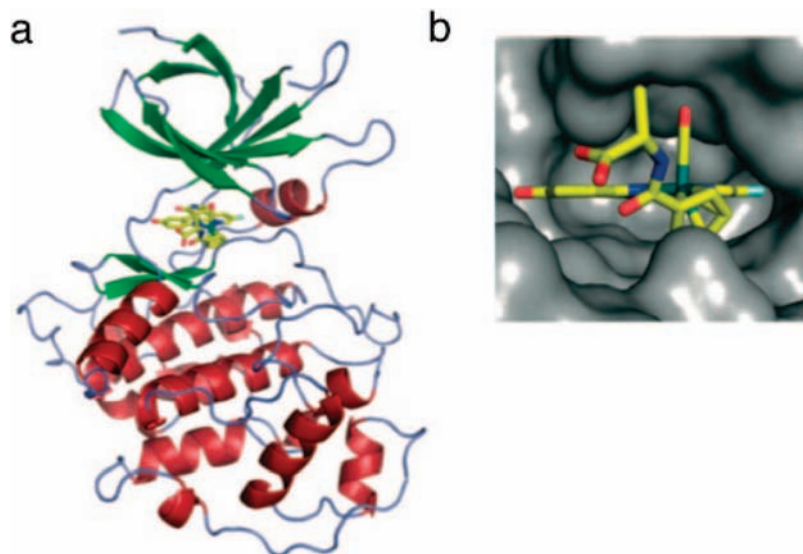


Figure 3. Crystal structure of glycogen synthase kinase 3 with the ruthenium compound (R_{Ru})-NP549 (a derivative of **63**) bound in the ATP-binding site: (a) overview of the complete structure; (b) close up of (R_{Ru})-NP549 in the binding pocket. Reproduced with permission from ref 257. Copyright 2008 Wiley-VCH Verlag GmbH & Co. KGaA.

agents against human islet polypeptide, another amyloidogenic peptide.²⁷⁰

In an alternative strategy, Cowen has shown that Cu(II) complexes of short peptides based on the ATCUN (amino-terminal copper/nickel) motif are effective inhibitors of several zinc metalloenzymes. The peptide Lys-Gly-His-Lys (KGHK) bound to Cu(II) as shown in **65** was found to inhibit angiotensin- and endothelin-converting enzymes (ACE and ECE, respectively), both important in cardiovascular and blood pressure regulation.^{271,272} None of the apo-peptides were effective and neither were metalloptides lacking the C-terminal lysine. It was therefore hypothesized that the Cu(II)-bound form mimics some of the molecular recognition elements of the popular ACE inhibitor lisinopril, which uses a carboxylate to coordinate directly to the active-site zinc(II) and a lysine that fits into a protein subpocket. The observation that thermolysin, another Zn(II)-dependent metalloprotease, is not inhibited by [KGHK-Cu]⁺ but is inhibited by [CGHK-Cu]⁺ also implies that the amino acid sequence provides target selectivity to the metalloptides.²⁷³

Under normal conditions in an *in vitro* inhibition assay, [KGHK-Cu]⁺ demonstrates competitive inhibition of ACE with a K_I of 4 μM .^{271,272} In this respect, the metalloptide behaves as a classical inhibitor. However, when the assay is performed with subsaturating concentrations following a preincubation step and addition of ascorbic acid, the inhibitory activity of the metalloptide was enhanced several fold, suggesting a change in its mechanism of action. The enzyme-inhibition profile supports a catalytic multiturnover inactivation pathway. No protein cleavage was observed, eliminating the possibility of hydrolytic reactivity. Although the mechanism is not fully elucidated, the authors suggest that a metal-based oxidant such as a Cu²⁺-associated hydroxyl radical or a Cu³⁺ oxo may be the agent responsible for catalytic inactivation.^{271,272} Further evidence for protein oxidation as the mechanism of enzyme inactivation was provided in a study of the Cu(II) complex of a Gly-Gly-His peptide tagged with a sulfonamide for targeted inhibition of carbonic anhydrase.²⁷⁴ In this case, mass spectrometric analysis of the protein following treatment with the inhibitor and ascorbic acid revealed that specific protein residues were oxidized, mostly histidine side chains that are 5–20 Å from the active

site, where the inhibitor is presumed to localize.²⁷⁴ This is a promising result that demonstrates the feasibility of metal-based reagents selectively oxidizing and inactivating target proteins. There remains significant scope in further developing such compounds. Toward this goal, it was recently shown that an iron-based catalyst could oxidize amino acids in water.²⁷⁵ Further research along these lines is warranted in order to develop metal-based agents to a point where they could be used in a cellular or animal model system.

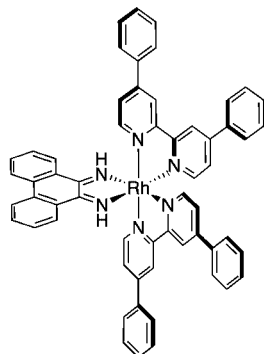
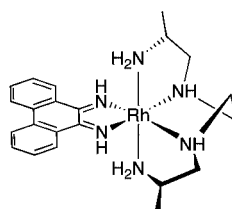
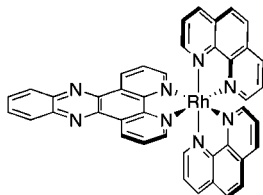
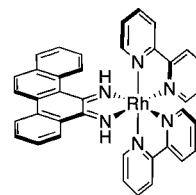
8. Metal Complexes for Probing DNA

An ongoing effort toward unlocking biological information contained within DNA is the pursuit of molecules that can specifically interact with, label, or cleave oligonucleotide sequences. DNA sequences of interest can be specifically targeted by taking advantage of sequence-dependent changes in overall three-dimensional shape and surface electronic distribution. Metal complexes are especially well suited for DNA interaction because the electropositive metal center is innately attracted to the negatively charged phosphate backbone of DNA. Metal centers also serve as unique three-dimensional structural scaffolds, which can be adjusted to fit into the base stacks and grooves of targeted DNA sequences.^{276,277}

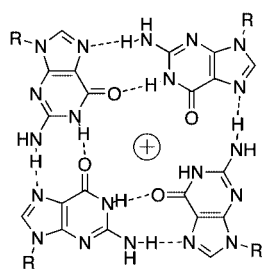
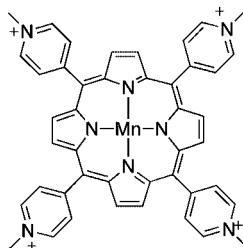
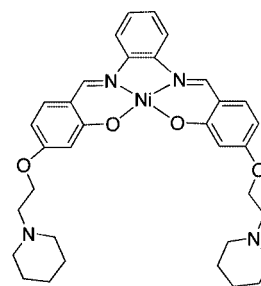
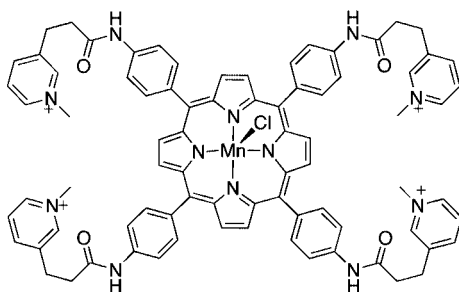
Metal complexes that are useful for structural recognition of DNA must have rigid three-dimensional structure, as fluxional behavior would negate selectivity based on shape recognition. The stereochemistry of the complex, if applicable, can provide an element of enantioselectivity, because DNA itself is chiral. In addition, unique chemical properties of metal ions can be exploited to label or cleave selected DNA sites; however, *in vivo* probes must also be substitutionally inert so that they remain intact within the cell. The complexes that have shown the most utility as DNA probes are the ones with interesting chemical and photophysical properties, serving as DNA footprinting agents or as specific luminescent probes for DNA sequence or structure. Redox-active Cu and Fe, for example, can induce oxidative strand scission, whereas Lewis acidic Zn can promote hydrolytic cleavage, and Ru, Os, and Rh can be exploited for their interesting photophysical properties as

Chart 11. Metal Complexes for Probing DNA

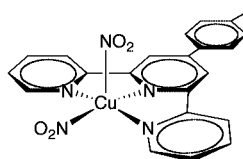
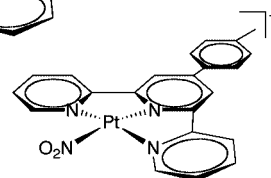
a) Complexes that recognize mismatched DNA

 Δ -[Rh(DPB)₂phi]³⁺ (66) Δ - α -[Rh(R,R)-Me₂trien]phi]³⁺ (67) Δ -[Rh(phen)₂dppz]³⁺ (68) Δ -[Rh(bpy)₂chrisi]³⁺ (69)

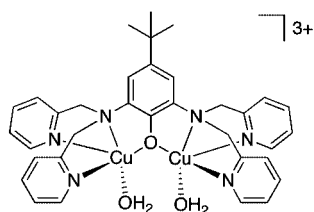
b) Complexes that recognize G-quadruplex DNA

70
planar G-quartet found
in G-quadruplex DNA71
(MnTMPyP4)72
(Ni(II)-salphen)

73

74
(Cu-ttpy)75
(Pt-ttpy)

c) Complexes that recognize single-double strand junctions



76

probes. The following section will focus on novel metal complexes (see Chart 11) that have potential for *in vivo* use to recognize specific DNA sequences or structures.

8.1. Sequence-Specific DNA Probes

Barton and co-workers have taken advantage of the small variations in shape and functionalities of the DNA major

groove in order to design octahedral intercalating complexes that select for specific DNA sequences.^{276,277} Intercalation is a DNA binding mode in which a planar aromatic group inserts and π -stacks between two adjacent bases in the core of a DNA double helix. Coordination complexes that contain at least one large planar aromatic ligand are capable of this noncovalent interaction, as first demonstrated by Lippard in

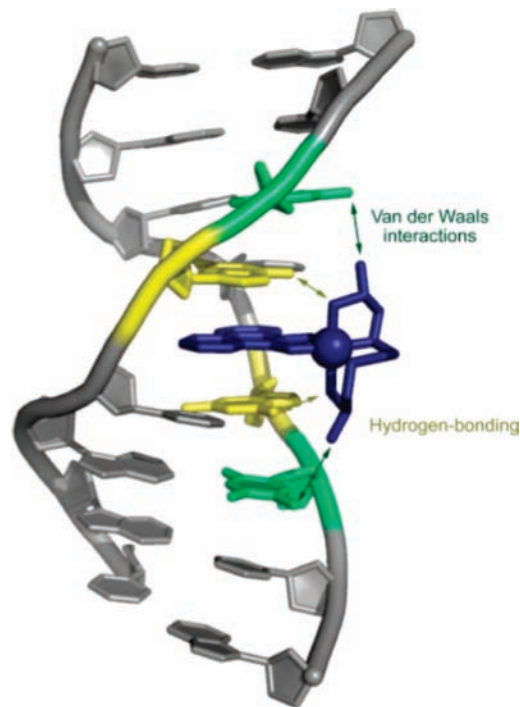


Figure 4. Crystal structure of the metallo-intercalator **67** bound to target sequence 5'-TGCA-3'. Note the intercalation of the aromatic phi group, the hydrogen-bonding interactions between the axial amines and the O6 of guanine, and the contacts between the pendant methyl groups of the ligand with the methyl groups of thymine. Reprinted with permission from ref 281. Copyright 1993 American Chemical Society.

1976 for a square planar platinum complex.²⁷⁸ Octahedral metal complexes can be designed to bind specific sequences by using a large planar aromatic intercalating ligand for DNA binding and ancillary ligands for shape and functional group recognition within the major groove. The enantioselectivity of a chiral complex for right-handed B-DNA over left-handed Z-DNA is mostly steric in nature and is achieved through bulky ancillary ligands. Small ancillary ligands like phenanthroline and bipyridine are nondiscriminatory, whereas complexes with bulkier ligands like DPB (4,4'-diphenylbipyridine) are enantioselective. For example, Δ -[Rh(DPB)₂(phi)] (**66**, Chart 11) readily intercalates B-DNA at the sequence 5'-CTCTAGAG-3', but the Λ -enantiomer is inactive, even in 1000-fold excess.^{279,280} On the other hand, the Λ -enantiomer will bind to left-handed (and less stereoselective) Z-DNA. It is noteworthy that **66** will also inhibit the *Xba*I restriction enzyme, which shares the same recognition site.^{279,280}

Sequence selectivity is also achieved by matching the functional group on the ancillary ligands with those positioned in the intercalated major groove. For example, Δ - α -[Rh((R,R)-Me₂trien)(phi)]³⁺ (**67**) was designed to recognize 5'-TGCA-3' via hydrogen bonding between the axial amines and the O6 of guanine, as well as by van der Waals contacts between the pendant methyl groups and the methyl groups of thymine.²⁸¹ These contacts are highlighted in the crystal structure shown in Figure 4.

Using both shape and functional group recognition, Barton has developed an entire series of site-specific DNA binders.²⁷⁷ In addition to their impressive site-selectivity, these complexes employ the useful photophysical properties of Ru and Rh to visualize and cleave DNA, respectively. Octahedral Ru complexes containing a dppz intercalating ligand show

solvatochromatic luminescence in organic solvents.^{282–284} One of the best studied examples of these molecular light switch complexes is [Ru(phen)₂(dppz)]³⁺ (**68**). In aqueous solvent, hydrogen bonding with the endocyclic N of dppz deactivates the excited state and quenches fluorescence. When DNA is added to solution, however, the complex shows its characteristic luminescence by intercalation into the base stack, shielding its dppz ligand from any interaction with the H-bonding solvent. Ru-dppz complexes have good cellular uptake but so far show little selectivity for DNA over other hydrophobic cellular components.^{285,286}

Rh has been exploited for its photoactivated DNA strand cleavage abilities.²⁸⁷ This reaction is useful because it enables direct footprinting of the site of intercalation. Irradiation with short wavelength light (313–325 nm) in the presence of dioxygen prompts formation of an intercalating ligand-based radical that abstracts a hydrogen atom from an adjacent deoxyribose ring, leading to direct strand scission.²⁸⁷

8.2. Metal Complexes That Recognize Mismatched DNA

A particularly impressive achievement in the area of DNA recognition has been in the selective recognition of mismatched DNA. Mismatched base pairs are a result of errors in DNA replication. While cells have mismatch repair machinery, unchecked mismatches lead to permanent genetic mutations. Almost 80% of hereditary nonpolyposis colon cancers and 15–20% of biopsied solid tumors are linked to mutations in the cell's mismatch repair machinery.^{288,289} Barton has extended the application of octahedral Ru and Rh coordination complexes into the design of metal coordination compounds that are selective for DNA base-pair mismatches. Metal complexes using the expansive 5,6-chryssene quinone (chrisi) ligand, like [Rh(bpy)₂(chrisi)₂]³⁺ (**69**), can bind mismatched base pairs at least 1000 times stronger than matched base pairs.²⁹⁰ In addition, enantioselectivity is observed, with the Δ -enantiomer binding and photocleaving well, while the Λ -enantiomer is almost completely inactive toward B-DNA. The rational design of a mismatch-selective complex is to use a planar aromatic ligand that is too wide to fit into a normal DNA major groove. The larger ligand cannot intercalate into normal matched DNA, but in the case of a mismatch, the duplex is thermodynamically destabilized. This destabilization allows the large ligand to eject the mismatched bases and replace them with its own inserting ligand.

A Rh mismatch complex similar to **69** attached to a cell-penetrating polyarginine peptide and fluorophore was tested in cells and shown to localize in the nucleus.²⁹¹ The question of whether the complex is specifically binding to DNA in vivo is yet to be answered.

8.3. Metal Complexes That Recognize G-Quadruplex DNA

Certain guanine-rich sequences in DNA are known to form higher order structures called G-quadruplexes (G4-DNA). These structures are formed by hydrogen bonding between guanines of the same or different strands to form a planar G-quartet (**70**). The resulting structure can be stabilized by the presence of a monovalent cation bound in the center of the tetrad and can be further stabilized by π stacking with other G-quartets.^{292,293}

All vertebrate chromosomal DNA ends with telomeres containing repeats of the sequence d(TTAAGGG), known to have quadruplex-forming potential. Other sequences with quadruplex-forming potential are overexpressed in promoter regions of diverse organisms. Specifically, 370 000²⁹⁴ such sequences have been recognized in humans and 40% of all known promoter regions have at least one.²⁹⁵ The enrichment of telomeres and promoter regions with quadruplex-forming potential sequences makes them likely candidates in genetic regulation. Despite their rigorous study *in vitro*, motivation to study these structures and their biological implications has been spurred by recent evidence of their *in vivo* significance.^{293,296–298} Development of new methods not only to distinguish G4-DNA from duplex DNA but also to discriminate between the polymorphs of G4-DNA is necessary. Metal complexes are especially promising for G-quadruplex targeting; however, it is important to note that these complexes are only in the beginning stages of development and are not as evolved as metal complexes that bind duplex DNA.

Several metal complexes have been reported to interact with G-quadruplex with some selectivity. Notable examples include Mn-TMPyP4 (**71**), which shows nearly 10-fold preference for G4-DNA over duplex DNA and modest telomerase inhibition,²⁹⁹ followed by Ni(II)-salphen (**72**)³⁰⁰ and Mn(II)-porphyrin (**73**)³⁰¹ complexes, with 50- and 1000-fold selectivity, respectively.

Square-pyramidal geometry of the metal complex has been shown to be a major factor in the selectivity for quadruplex over duplex DNA. For example, the simple square pyramidal complex, Cu-tpy (**74**) is highly selective for G-quadruplex in comparison to square-planar Pt-tpy (**75**).³⁰² The tpy ligand in the Pt complex can stack on the outside of G-quad, as well as insert into double-stranded DNA. On the other hand, apical ligands of square-pyramidal complexes prevent intercalation into duplex DNA without interfering with its G-quadruplex association.³⁰²

Just as the unique properties of metal complexes have been successfully used to probe duplex DNA, the metal complexes discussed above show promise for probing quadruplex DNA to help elucidate the potential structures that exist and the significance of G-quadruplex structures in live cells.

8.4. Metal Complexes That Recognize Single–Double Strand Junctions

Single strand (ss)/double strand (ds) DNA junctions are another type of structure existing in telomere regions in a wide variety of species. Some DNA-binding proteins that recognize ss/ds junctions in telomeres are known to have functions in telomere maintenance and capping. Other instances of ss/ds junctions occur during replication, are a major component of RNA secondary structure, and are known to occur at hairpin loops and Holliday junctions. Single/double strand junctions are yet another DNA structure with implications in disease and represent a potentially rich source of new understanding of *in vivo* DNA function.

A class of binuclear and trinuclear copper complexes has demonstrated exquisite selectivity in oxidizing DNA at ss/ds DNA junctions.^{303–307} Complexes such as **76** are also sequence-selective, preferring to oxidize guanine at the *n* and *n* + 1 position of the single-stranded DNA directly extended from a duplex region. Site specificity derives in part from the coordination of at least one copper to a guanine. Interestingly, efficient cleavage is not observed for ss or ds

DNA alone, nor is cleavage observed for GG–GG mismatches flanked on both sides by matched duplex DNA.³⁰⁴ The authors predict that the flexibility of the DNA strand is a factor in the ss/ds junction selectivity. The myriad of possible conformations of ss DNA may effectively compete against its productive association with the metal complex, and duplex DNA may not be flexible enough to accommodate the required conformation. A ss/ds junction may offer unique proximity of the two single strands yet offer flexibility to assemble with the copper complex.³⁰⁴ Although these ss/ds targeting complexes have not yet been fully developed for *in vivo* application, the potential application of these and similar metal complexes that can recognize unique DNA structures is apparent.

9. Metal-Responsive MRI Agents

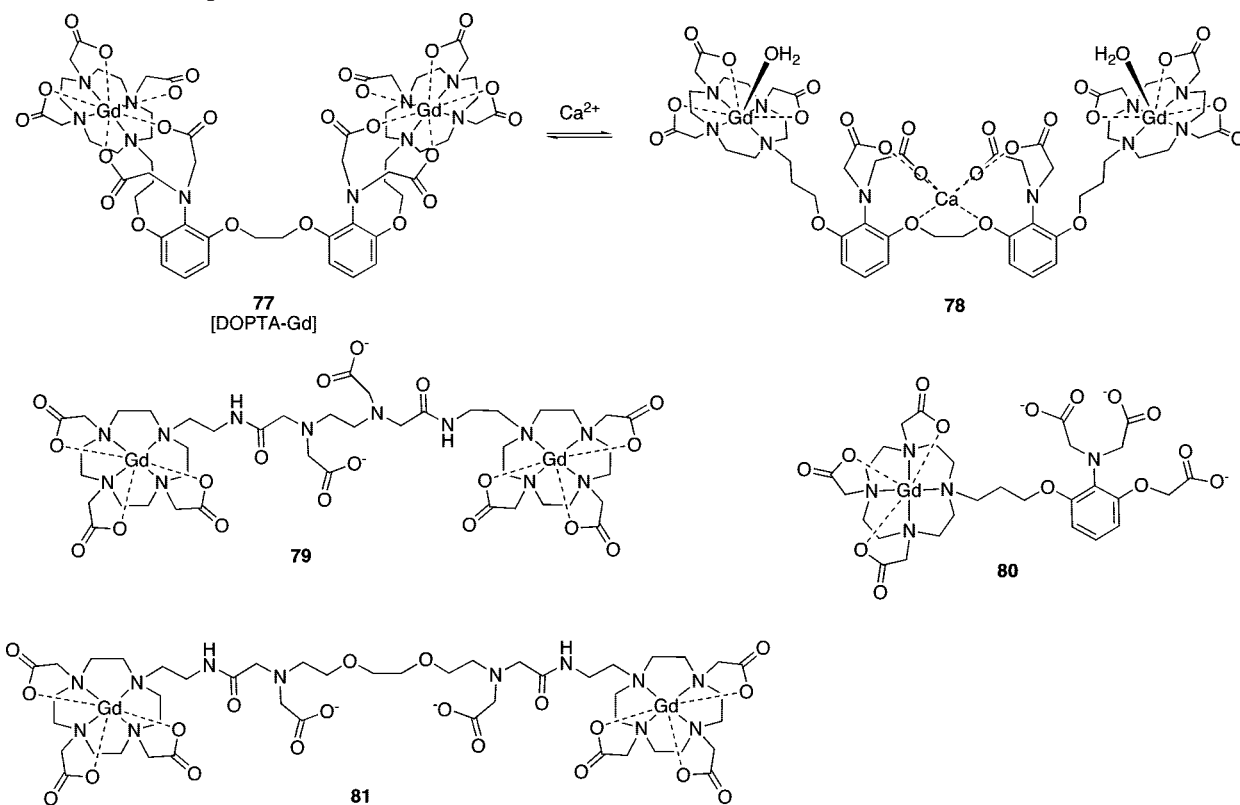
Magnetic resonance imaging (MRI) provides three-dimensional images of opaque biological structures with relatively high spatial resolution ($\sim 10 \mu\text{M}$) in a noninvasive procedure, making it an increasingly popular imaging modality. Because a MR image derives from the nuclear magnetic resonance of water protons, the contrast in the image depends on the local concentration of water and on the longitudinal (T_1) and transverse (T_2) relaxation times of its protons.³⁰⁸ A radio-frequency pulse inverts the magnetization vector of water protons from their preferential alignment with the external magnetic field. The time required for the spins to realign with the field is characterized by T_1 , which can be significantly reduced if the spins are in contact with a local paramagnetic center, thereby brightening the image. Coordination complexes that leave open coordination sites for water molecules to access the inner sphere of paramagnetic metal ions (particularly Gd^{3+} with seven unpaired f electrons, but also high-spin Fe^{3+} and Mn^{2+} with five d electrons) are therefore excellent candidates as agents that enhance MR images via a T_1 mechanism.³⁰⁹

Contrast agents that are currently widely used in clinical medicine are nonspecific, extracellular agents. Since the approval of $[\text{Gd}(\text{DTPA})(\text{H}_2\text{O})]^{2-}$ (known commercially as Magnevist) in 1988, research on designing new Gd coordination complexes as improved MR imaging agents has increased dramatically.³⁰⁸ The desire to use MR imaging to understand physiological events has sparked a trend in the design of “smart” contrast agents that change their relaxivity (and therefore their level of contrast enhancement) in response to a biological stimulus or change in environment.^{310,311} Activateable contrast agents have appeared in the research literature that respond to changes in pH,^{312–315} O_2 partial pressure,³¹⁶ small molecules like glutathione³¹⁷ and sialic acid,^{317,318} enzymatic activity,^{319–322} and specific metal ions. While not used clinically, these kinds of responsive agents may become increasingly useful for studying molecular biology and intracellular chemistry.

9.1. MRI Agents for Calcium

The first metal-responsive probe was introduced by Meade in 1999.³²³ The design of DOPTA-Gd (Chart 12, **77**) consists of a BAPTA Ca^{2+} chelating motif onto which Gd(DOTA) macrocycles are fused. In the absence of Ca^{2+} , the carboxylate arms of the central BAPTA bind to the Gd^{3+} centers and occlude H_2O from direct coordination (**77**). Addition of Ca^{2+} shifts the coordination mode and opens the door for H_2O to interact with the Gd^{3+} centers directly (**78**) and

Chart 12. Calcium-Responsive MRI Probes



increase relaxivity. This general concept of flanking a metal-recognition motif with Gd³⁺ chelates in such a way that binding of the target metal ion induces a shift in coordination, which increases water access to Gd,³⁺ has provided the template for a host of other metal-responsive contrast agents. DOPTA-Gd has an apparent K_d of 0.96 μM and was shown to be sensitive to in vitro Ca²⁺ concentrations in the 0.1–10 μM range.³²³ In order to develop probes that would be responsive to extracellular Ca²⁺ in the millimolar range, Logothetis and Toth introduced three families of probes with diminished affinity for Ca²⁺ so that the probes would not be saturated under the target conditions. The chelating motifs for the Ca²⁺ recognition unit for these probes are derived from EDTA (**79**),³²⁴ APTRA (*o*-aminophenol-*N,N,O*-triacetate) (**80**),³²⁵ and EGTA (**81**).³²⁶ The apparent K_d values for Ca²⁺ in buffered aqueous solution are 400, 11, and 200 μM , respectively. All three probes are reported to be selective for Ca²⁺ over Mg²⁺, while compound **81** is also shown to be sensitive to Ca²⁺ in the presence of Zn²⁺.³²⁵ Competition from other metal ions was not reported.

The DOTA-based probes all suffer from a loss of relaxivity in biological media, a response that is likely due to anions in the buffers displacing H₂O from Gd³⁺ in the Ca²⁺ loaded form.³²⁵ Even with this diminished relaxivity, however, the Ca²⁺ response of the EGTA-based probe (**81**) in a complex cell culture medium designed to mimic the brain extracellular medium gave a ~10% change in relaxivity over the 0.8–1.2 mM Ca²⁺ concentration range that might be expected in the brain.³²⁶

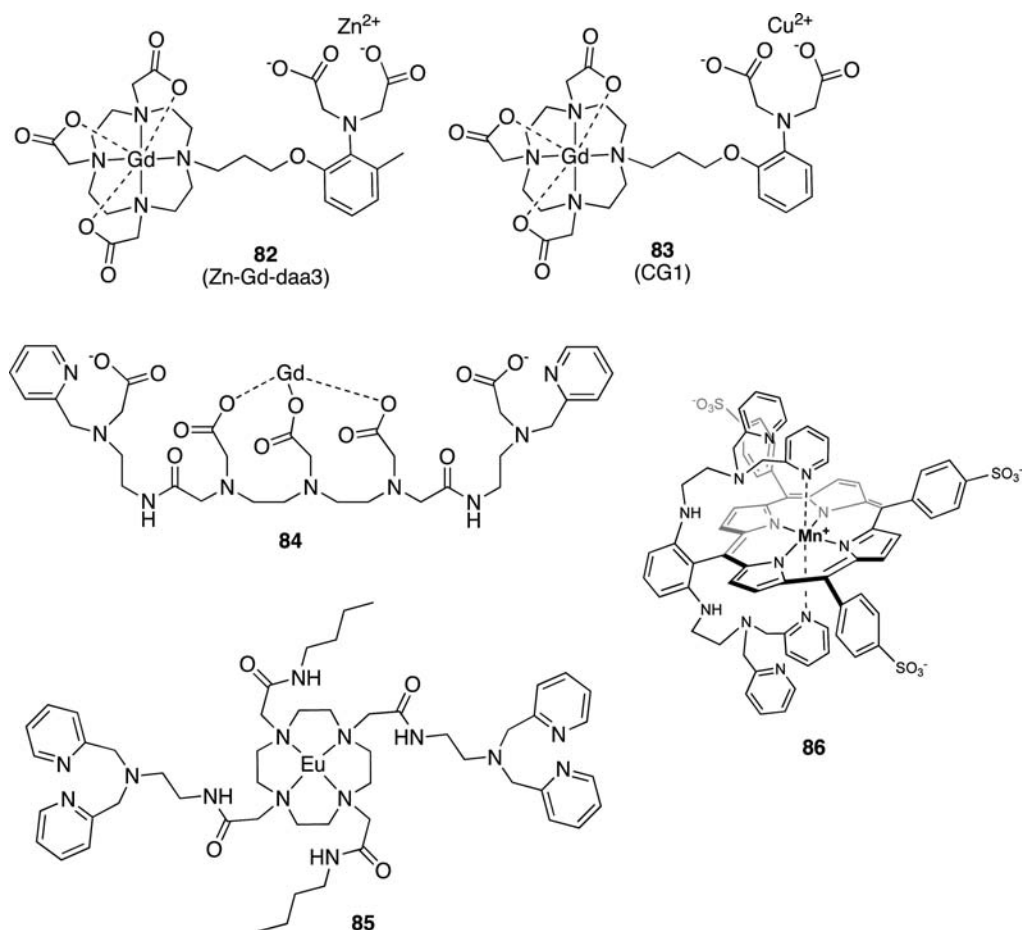
In addition to these Gd-based agents, a different approach that uses superparamagnetic iron oxide nanoparticles for Ca²⁺ imaging has been presented by Jasanoff.³²⁷ The particles are linked to the Ca-binding protein calmodulin and calmodulin-binding peptides. The aggregation of the nanoparticles in the presence of Ca²⁺ results in a change in T_2 signal. The

impermeability of these reagents to cell membranes is a current limitation for their use.

9.2. MRI Agents for Zinc and Copper

One strategy for generating Gd-based probes that are sensitive to metal ions like Zn²⁺ and Cu²⁺ has been to modify the general template of the Ca-responsive probes by decreasing the number of carboxylic acid arms to lower the affinity for Ca²⁺ while retaining affinity for these d-block divalent metals. Two very similar designs have appeared, one by Meade and co-workers, Zn-Gd-daa3 (**82**, Chart 13), that shows a 115% increase in relaxivity in the presence of Zn²⁺,^{328,329} and another by Chang, CG1 (Copper-Gad1, **83**), that shows a 41% increase in relaxivity in the presence of Cu²⁺, both in vitro.³³⁰ As anticipated from the similarity in the structures of these two probes, both compounds suffer from interference of the nontarget metal ion, either Zn²⁺ or Cu²⁺, but are insensitive to Mg²⁺ and Ca²⁺. Intracellular imaging has not yet been reported for these compounds, although **82** was shown to be nontoxic to NIH/3T3 cells and to accumulate intracellularly to a concentration of $\sim 10^{-14}$ mol Gd/cell.³²⁸ Changing the metal receptor from a carboxylate-based ligand as in CG1 to a softer thioether-based ligand set results in later-generation CG sensors with high selectivity for Cu⁺ over competing ions and turn-on responses up to 360%.³³¹

An alternative approach for a Zn-dependent MR sensor was introduced by Nagano wherein the Gd-chelate forms the central core of the molecule, with flanking Zn²⁺-binding units composed of either dipicolylamine or a combination of pyridal and carboxylate ligating arms, as shown in **84**.^{332,333} The disadvantage with this strategy is that Zn-binding

Chart 13. MRI Sensors Sensitive to Zn^{2+} and Cu^{2+} 

displaces H_2O from the Gd^{3+} center, resulting in a decrease in relaxivity that provides a less appealing bright-to-dark signal.

A different take on designing a Zn binding site above the plane of a lanthanide chelate was introduced by Sherry as a PARACEST contrast agent.³³⁴ Unlike the Gd^{3+} -based agents that rely on changes in the water relaxation rates to achieve contrast, PARACEST reagents (paramagnetic chemical exchange saturation transfer) respond to changes in water exchange rates at paramagnetic centers. Incorporating two dipicolylamine units across a Eu^{3+} -chelated macrocycle to give **85** provides a compound that shows a significant change in the CEST spectrum upon addition of Zn^{2+} . The increase in the water exchange rate is attributed to a Zn-bound water or hydroxide positioned above the Eu^{3+} that catalyzes proton exchange between the Eu^{3+} -bound water molecule and bulk solvent.³³⁴

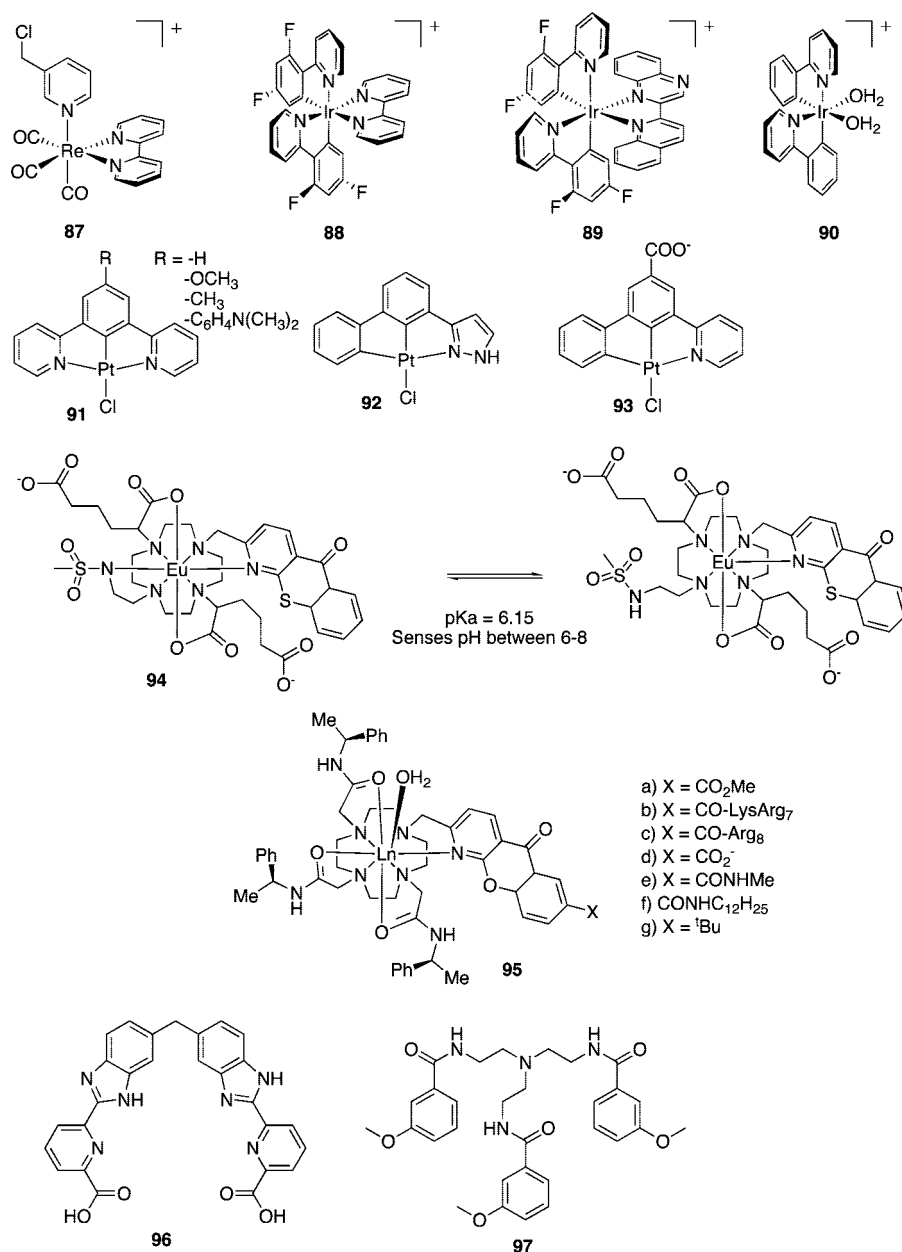
Yet another approach of placing Zn binding sites in proximity to a paramagnetic center was recently introduced by Zhang et al.³³⁵ In this case, dipicolylamine units are situated above and below a water-soluble porphyrin ring. The metal-free form operates as a fluorescence sensor for Zn^{2+} , whereas Mn^{3+} insertion into the porphyrin converts the molecule to a MRI sensor with relaxivity changes sensitive to Zn^{2+} (**86**). A significant advantage of this sensor is its water solubility and cell-permeability. The compound was shown to have little cytotoxicity to HEK-293 cells, wherein it localizes predominantly in the nuclear fractions rather than the cytosol. Importantly, this is the first reported example of a cell-permeable MRI sensor for intracellular Zn^{2+} . In aqueous solution, addition of Zn^{2+} results in a darker MR

image; however, in cells the T_1 -weighted images increase brightness. These somewhat conflicting results indicate that the mechanism of the Zn-induced relaxivity changes is not at present well understood. It seems likely that **86** could be interacting with other biomolecules inside cells in a Zn-dependent fashion that results in the increased relaxivity not seen in aqueous solution.

10. Luminescent Metal Complexes for Cellular Imaging

Fluorescence microscopy is a powerful, high-resolution technique for biological imaging, and luminescent molecular probes make it particularly useful for probing cellular biology.³³⁶ Fluorescent proteins^{337–339} and heterocyclic aromatic dyes like fluorescein, rhodamine, and others have been indispensable tools for observing the cellular localization of biological and other molecules of interest.³⁴⁰ However, a continuing quandary is in distinguishing between autofluorescence of the analyzed sample and that of a luminescent dye meant to image a particular cellular component. This problem can be avoided if the molecular probe absorbs and emits light at distinctly different wavelengths (i.e., large Stokes shift) such that autofluorescence can be easily differentiated from signal luminescence. Alternatively, if the probe has a long excited-state lifetime on the order of hundreds of nanoseconds to milliseconds, time-resolved luminescence microscopy (TRLM) can be used to eliminate autofluorescence.^{341,342} Quantum dots and several luminescent metal complexes have these characteristics. Semiconductor quantum dots have been used in tracking cell mobility and

Chart 14. Luminescent Metal Complexes for Cellular Imaging



have the advantage of tunable luminescence based on particle size; however their large size limits their application in probing small cellular organelles and structures. Metal complexes, on the other hand, can have large Stokes shifts and long excited-state lifetimes, are usually small in size, and can be designed for low toxicity, good cellular uptake, and targeted intracellular localization. In addition, metal complexes have easily recognizable line spectra, can emit over a broad range of the visible spectrum, and are relatively resistant to photobleaching. Over the past 15 years, there has been great progress in the design of luminescent metal complexes for biological imaging. Several examples of luminescent metal complexes targeted to cellular components or biological receptor sites by covalent linkage to biological recognition moieties have been reported and are promising for biological imaging and biochemical assays.^{343–348}

10.1. Luminescent Transition Metal Complexes

Some examples of membrane-permeable luminescent Ru complexes that target DNA by noncovalent interaction were

discussed previously in section 8. These complexes are specifically designed to target DNA; however they have demonstrated nonspecific luminescence of various hydrophobic cellular components and could potentially serve as more general *in vivo* luminescent stains.

Rhenium tricarbonyl complexes have luminescence properties that have long been postulated but only recently demonstrated as useful *in vivo* probes.^{345,349,350} Their luminescence stems from a metal-to-ligand charge transfer triplet excited state (³MLCT), and they have characteristic properties of a large Stokes shift (187 nm), long fluorescence lifetime (131 ns), and good quantum yield.³⁵¹ The application of a ³MLCT luminescent agent for specific targeting of a cellular component has been recently demonstrated with the tricarbonyl rhenium(I) cation **87** (Chart 14). Studies of **87** in yeast cells and human MCF-7 cells showed that this membrane-permeable dye localizes to the mitochondria.³⁵⁰ The selective localization is likely due to reaction of the chloromethyl group with thiols that are concentrated in mitochondria. A

similar mechanism is suggested for the commercial mitochondrial stain MitoTracker.^{352,353}

The first demonstration of phosphorescent iridium(III) complexes used as cell-permeable dyes for imaging live cells was also reported recently.³⁵⁴ The two Ir(III) complexes **88** and **89** emit bright green and bright red light, respectively, are membrane-permeable, and have low cytotoxicity up to 100 μM over 24 h. Both complexes have excited-state lifetimes on the order of 1 μs , have low susceptibility to photobleaching, and have large Stokes shifts (>170 nm) with emission in the visible region following excitation at 360 nm. In addition, their exclusion from the nucleus and specific staining of the cytoplasm was observed.³⁵⁴ Another cyclometalated Ir(III) complex, **90**, contains weakly coordinated solvent molecules that readily exchange with histidine ligands and thereby allow for protein staining of histidine-rich proteins in SDS-PAGE gels.³⁵⁵

Several nontoxic, chemically stable luminescent Pt complexes have recently been reported as suitable for live-cell imaging with two-photon excitation and TRLM. In one example (**91**), variations in the R group allow for tuning of emission over a wide range of the visible spectrum from blue-green to orange.³⁵⁶ The R-group also allows a site for potential conjugation to biomolecules. The complex is photostable and has two-photon excitation luminescence properties. Instead of absorbing one high-energy photon in the near-UV, the complex can be excited by absorbing two low-energy photons in the 780 nm range. Two-photon excitation is advantageous because low-energy light is less damaging to biomolecules. Also, because biological samples have increased optical transparency³⁵⁷ at near-infrared (NIR) wavelengths, deeper penetration, and excellent spatial resolution can be attained. The luminescent properties of these complexes are attributed to an excited state of predominantly triplet intraligand $\pi-\pi^*$ character, but with sufficient contribution from the metal to achieve long luminescent lifetimes greater than 100 ns.^{358,359} Application for live-cell imaging of **91** was tested in three cell lines and showed no cytotoxicity at 10 μM over 24 h. The complexes readily diffused across membranes and localized preferentially in the nucleus. An incubation time of only 5 min was necessary to achieve maximum intracellular emission.³⁵⁶

The Pt complex **92** also shows two-photon luminescent properties with a relatively long excited-state lifetime on the order of 1 μs in organic solvent.³⁶⁰ The two-photon cross section for **92** was measured to be 20.8 GM, which is higher than the value suggested for biological samples (0.1 GM),³⁶¹ and also greater than that reported for **91** (4 GM).³⁵⁶ Experiments using human lung carcinoma, cervical carcinoma, and human nasopharyngeal cell lines showed strong green luminescence after incubation with the complex (5 $\mu\text{g}/\text{mL}$) for 5 min, with low cytotoxicity observed over 6 h. Both luminescent platinum complexes **91** and **92** represent a new way to visualize cells using two-photon excitation, and both complexes have lifetimes sufficient for time-resolved techniques. Another cyclometalated Pt(II) complex **93** has shown application in staining proteins in 1D and 2D SDS-PAGE analysis as well as live cell imaging.³⁶²

10.2. Luminescent Lanthanide Complexes

In their mission to develop optical probes that report on conditions of the intracellular environment and that can be targeted to specific cellular locations, Parker and colleagues have developed luminescent lanthanide complexes capable

of signaling changes in pH,^{363–366} carbonate,^{367–369} citrate,^{370,371} and urate³⁷² concentrations in live cells.³⁷³ In addition, the complexes are promising for use in two-photon microscopy.³⁷⁴ By design, these complexes report on the target analyte via changes in at least two emission bands, allowing for ratiometric analysis that is independent of probe concentration.³⁶⁵ Each probe is based on a lanthanide coordination complex of a cyclen macrocycle equipped with a Ln-sensitizing antenna chromophore and an analyte-sensing moiety held in close proximity or directly coordinated to the Ln metal center. The emission spectrum varies as a function of analyte-induced changes in the Ln coordination environment. A luminescent probe sensitive to changes in pH between 6 and 8 in biological media is shown as an example (**94**).³⁶⁵ At higher pH, the deprotonated species has a higher ratio of europium luminescence intensity at 680 nm over 587 nm, whereas the protonated species emits more strongly at 587 nm.³⁶⁶ The probe shows good cellular uptake and low toxicity, with an estimated error of 0.2 pH units. Although this sensitivity should be improved before it is applicable to measuring intracellular pH for analytical purposes, this probe is a promising step toward an in vivo pH meter.³⁶⁵

The chromophore in these Ln-cyclen complexes not only influences the luminescent properties but also functions as a recognition element for cellular uptake, efflux, and localization.^{375–377} Depending on the chromophore identity, the luminescent cyclen complexes can show relatively slow (>30 min) or fast (<15 min) uptake and efflux and localize in various cellular compartments. A systematic study compared the in vivo profiles of a number of complexes of core structure **95** with variations in substitutions on the azaxanthone sensitizer moiety.^{375,376} Carboxymethyl (**95a**) and cationic peptide (**95b,c**) substituents resulted in fast uptake and showed no cytotoxicity, with localization in cytosolic macropinosomes and perinuclear endosomes/lysosomes, respectively. In contrast, when the substituent was a negatively charged carboxylate (**95d**) or methyl amide (**95e**), slow cellular uptake was observed. Hydrophobic substituents (**95f,g**) caused membrane destabilization resulting in high toxicity. In addition, changing the azaxanthone moiety to azathioxanthone (**94**)^{369,378–380} or another group^{380–384} or varying the linker also altered the cellular uptake, localization, and cytotoxicity.³⁶⁹ Figure 5 shows two examples of different cellular distribution of these related complexes. Very clearly, the in vivo behavior of cyclen lanthanide probes is defined by the nature of the chromophore, and the authors hypothesize that protein binding to the sensitizing moiety regulates these processes.^{376,384}

Toward the development of bifunctional lanthanide probes,³⁸⁵ Bünzli and co-workers have developed a class of highly luminescent bimetallic helicate complexes suitable for in vivo fluorescent imaging.^{386–390} Derivatives of the homoditopic ligand 6,6'-[methylenebis(1-methyl-1*H*-benzimidazole-5,2-diy)]bis(4-[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]pyridine-2-carboxylic acid) (**96**) self-assemble with Ln³⁺ ions to form complexes having the form [Ln₂(L)₃]. The Ln ions are bound in an ideal nine-coordinate, tricapped trigonal prismatic environment and are well-shielded from quenching effects of solvent or other high vibration oscillators. Helicates formed with Eu(III) display bright luminescence with millisecond excited-state lifetimes. They are thermodynamically stable with negligible dissociation in biological media.^{386,391} The complexes can easily be substituted with polyethylene groups in the 2- or 4-positions of the pyridines or on the

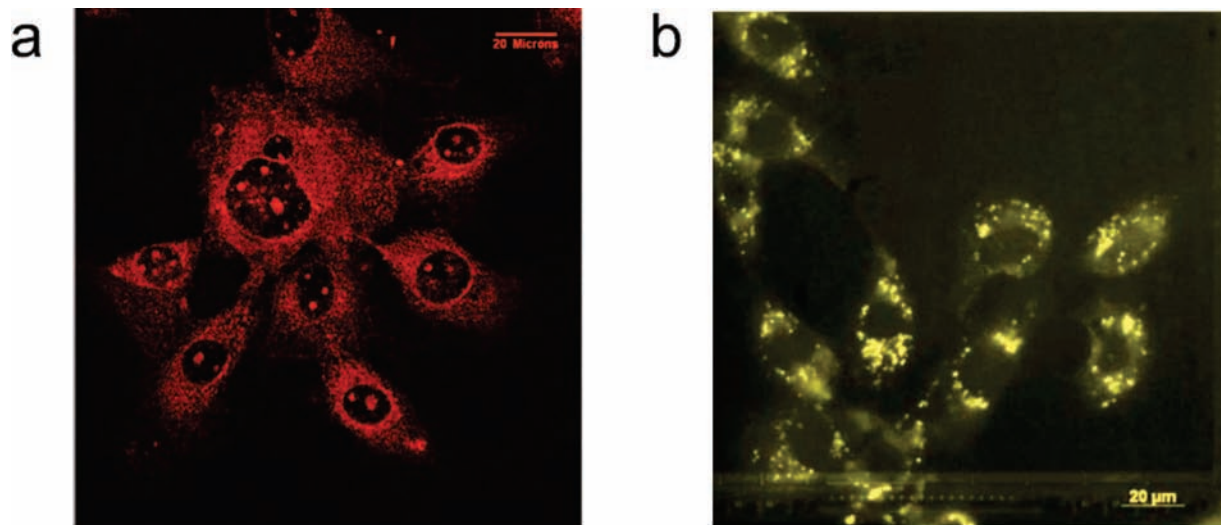


Figure 5. Microscopy images of NIH-3T3 cells loaded with two Ln-cyclen complexes bearing different sensitizing moieties and demonstrating different cellular distribution: (a) cells incubated with the azathioxanthone complex **94** show typical localization to nucleosomes and ribosomes; (b) cells incubated with Tb-**95a**, bearing a carboxymethyl-substituted azaxanthone moiety show localization of the probe to cytosolic macropinosomes. Reprinted with permission from refs 366 and 376. Copyright 2007 and 2008, respectively, Royal Society of Chemistry.

benzimidazole N's to achieve water solubility or for potential covalent coupling to biological molecules. Interestingly, variation among these substituents has little effect on cellular localization or uptake profiles, unlike the cyclen complexes discussed above. However, substitution in the 4-pyridyl position results in higher overall quantum yields than complexes substituted at the benzimidazole position. For this reason, the 4-pyridyl-substituted helicenes are more promising for *in vivo* application.³⁹⁰ The low cytotoxicity of the helicenes is demonstrated by HeLa cells being unaffected when treated with up to 500 μM for 24 h.³⁸⁹ Bright Eu(III) emission can be seen in cells after incubation with concentrations above 10 μM in as little as 15 min. Localization in vesicles indicates uptake via a lysosomally directed or recycling endosomal pathway.³⁸⁹ One drawback of these helicenes is their short excitation wavelength (330–365 nm), although they are photoluminescent with two-photon excitation.³⁷⁴

A new luminescent stain for the cytoplasm of living cells has been generated by using three-photon excitation of a terbium (Tb) complex. The ligand *N*-[2-[(bis(2-[(3-methoxybenzoyl)amino]-ethyl)amino)ethyl]-3-methoxybenzamid (**97**) forms [Tb(L)(NO₃)₃] polymeric complexes.³⁹² The probe is noncytotoxic at 20 $\mu\text{g/L}$, resistant to photodegradation, and must absorb at least three photons at 800 nm to demonstrate multiphoton-induced emission. Three human cell lines were incubated with the complex for up to 24 h. After incubation, the cells demonstrated green fluorescence in the cytoplasm in as little as 1–2 min of exposure to high intensity light, although up to 60 min of exposure was needed for some cells to be visualized.³⁹²

11. Using Coordination Chemistry To Label Proteins

In addition to *in vivo* probes targeted to specific cellular compartments, site-specific labeling of proteins is another powerful technique for visualization of cellular processes. Fluorescent proteins of many colors have been extremely popular and useful for tracking expression and localization of fusion proteins; however their large size can interfere with

protein folding, trafficking, and function. The metal coordination properties of specific amino acid sequences have been exploited to create much smaller fluorescent labels via affinity tags. In a metal-chelation approach to fluorescent labeling, peptides with high affinity for metal ions are paired with fluorescent labels containing a chelated metal ion with open coordination sites available for simultaneous binding to the peptide tag. In this way, metal–ligand bonding joins together the peptide-tagged protein and the fluorescent label. Alternatively, luminescent metal ions themselves are the reporting functionality, as in the case of lanthanide-binding tags (LBT). Small peptide affinity tags can be easily fused to a protein of interest without interfering significantly with the protein character.³⁹³

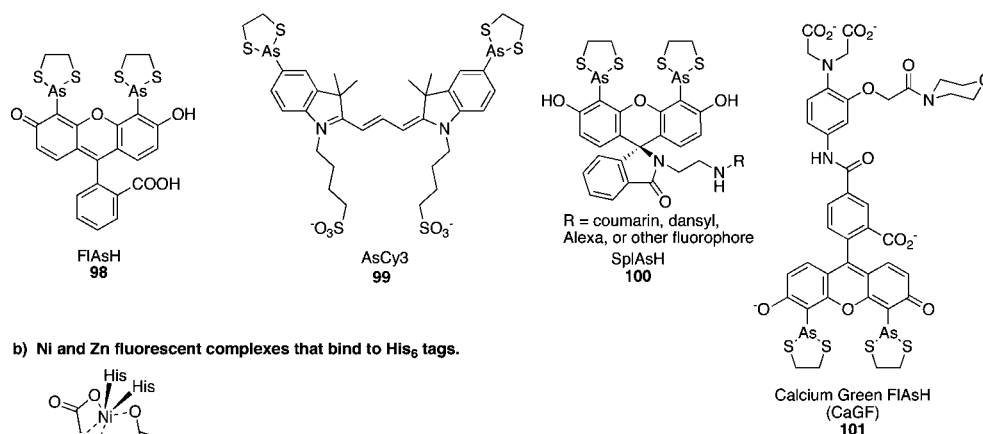
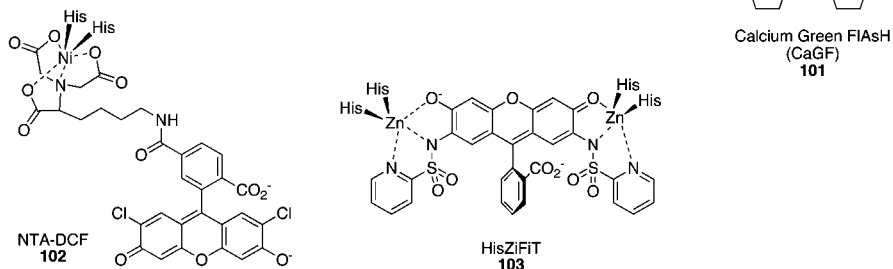
In general, there are four types of metal affinity tags that have been developed to label proteins with luminescent reporters; each will be discussed further below, and examples are shown in Chart 15. Each peptide tag consists of a dense region of metal-binding amino acids, particularly cysteine, histidine, or aspartic acid or combinations (LBT). It is important to note that this approach is not limited to the use of a fluorescent marker, because any group can be attached to the metal-binding synthetic probe.

11.1. Polycysteine Tags

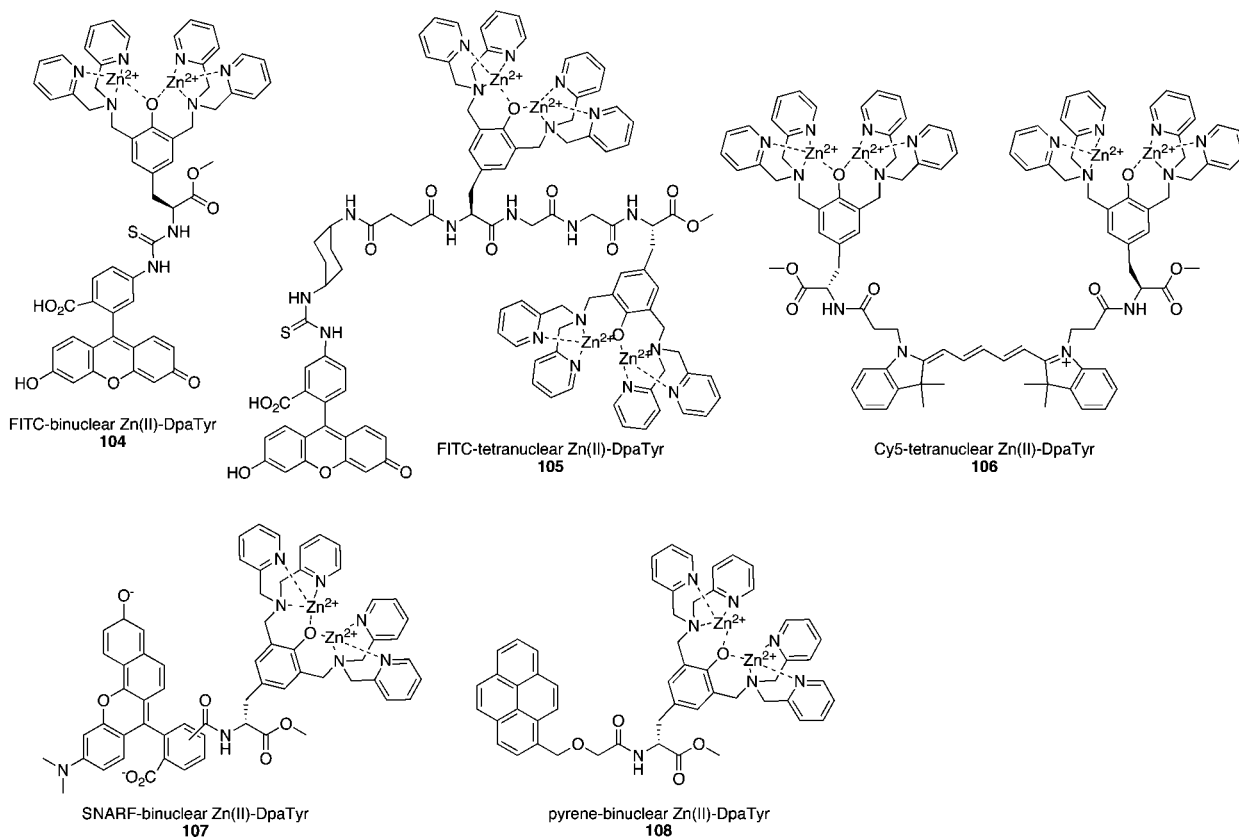
Tsien developed the first metal-chelating affinity tag system in 1998 by pairing a cysteine-rich peptide tag with a biarsenical fluorescein derivative.³⁹⁴ Biarsenical dyes such as 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FAsH, **98**) bind specifically with high affinity to the short peptide sequence (CCPCC).^{394,395} The spacing of the two arsenic (As) ions matches the spacing on the two bicysteine (CC) groups in an α -helical structure so that the thiols of CC may replace the thiols of ethanedithiol (EDT) that mask the fluorescein-bound As. FAsH displays fluorescence ($\lambda_{\text{ex}} = 508$, $\lambda_{\text{em}} = 528$) upon peptide binding. Due to nonspecific interaction with membranes and hydrophobic pockets or thiols, FAsH also displays some undesired background fluorescence despite use of excess EDT and washing steps.³⁹⁶

Chart 15

a) Biarsenical probes that bind to polycysteine sequences.

b) Ni and Zn fluorescent complexes that bind to His₆ tags.

c) Zn-DPA constructs that bind to polyaspartic acids sequences (D4 tags).



Since the development of FIAsh, there have been several variations of the polycysteine tag/biarsenical pair with various colors of emission,^{395,397,398} improved photochemical properties,³⁹⁸ FRET partners,³⁹⁸ and improved affinity tag sequences.^{399,400} The conserved interatomic distance of the As ions ($\sim 6 \text{ \AA}$) in FIAsh derivatives limits the complementary use of two dyes to uniquely label two separate protein sites. To address this problem, a Cy3-based biarsenical dye (AsCy3, **99**) was developed with a larger interatomic distance ($\sim 14.5 \text{ \AA}$)

between As ions capable of binding a peptide sequence with larger spacing between the target CC groups.⁴⁰¹ AsCy3 enables high-affinity binding to the sequence CCKAEACC, showing superior photostability and minimal environmental sensitivity and providing a FRET partner to FIAsh.⁴⁰¹

The development of various colors of biarsenical dyes is expected to promote widespread use of this labeling technique. Although many biarsenical dyes have been reported, structural requirements of both the fluorophore and As ions

has made the scope of compatible dyes narrow and difficult to predict. To further improve the applicability of the polycysteine/biarsenical system, Miller developed a strategy for recruiting any fluorophore to a polycysteine tag.⁴⁰² Based on a modular approach, a fluorescent moiety is separated from the biarsenical targeting moiety in order to remove any restrictions on its structure. The fluorescein-based biarsenical targeting moiety, called SplAsH (**100**), can be conveniently attached to a variety of fluorophores to create different colored emission. In this method, the probe is fluorescent whether or not it is bound to the peptide tag, but it has the advantage that any fluorophore can be used.

A recent advance in biarsenical dyes has come in the form of the Ca^{2+} sensor Calcium Green FAsH (CaGF, **101**), which combines a FAsH moiety with a Ca^{2+} chelator. The binding of Ca^{2+} to CaGF results in a 10-fold increase in fluorescence.⁴⁰³ CaGF-labeled calcium channels have demonstrated that CaGF can be used for precise and fast detection of intracellular Ca^{2+} .⁴⁰³

11.2. Polyhistidine Tags

Peptide tags with six or more sequential histidine residues (His-tag) are known to interact with transition metal complexes and are commonly used for purification of expressed protein by Ni^{2+} -NTA affinity chromatography, where NTA is nitrilotriacetic acid. The affinity of His-tags for metal complexes can also be applied to fluorescently label proteins or other molecules *in vitro*^{404,405} and *in vivo*^{406–408} by introducing a Ni-NTA complex where NTA is derivatized with a fluorophore.

With the Ni-NTA complex present in solution as opposed to being in large excess on a solid support as is the case for protein purification, the relatively low affinity between a His-tagged protein and Ni-NTA ($K_d \approx 1\text{--}20 \mu\text{M}$) becomes a limitation to this approach. Increasing the number of Ni-NTA appendages on the fluorophore increases affinity by 4 orders of magnitude in going from a mono-Ni-NTA probe to one with four Ni-NTA groups.⁴⁰⁴ Increasing the length of the His-tag from His₆ to His₁₀ also improves affinity.⁴⁰⁷

In most cases, the use of paramagnetic Ni^{2+} results in partial quenching and a decrease in fluorescence. Goldsmith et al., however, have reported that the fluorescence of NTA-DCF (**102**) is not significantly influenced by the presence of Ni^{2+} .⁴⁰⁸ This tag does not enter cells either by itself or as its Ni(II) complex; therefore it is useful for imaging extracellular proteins that are expressed with His-tags.⁴⁰⁸

A strategy for avoiding paramagnetic quenching is to replace Ni^{2+} with diamagnetic Zn^{2+} as the metal. Zn^{2+} , which is ubiquitous in biological systems, will also help avoid heavy metal toxicity that can be caused by Ni^{2+} . Tsien developed a novel chelating molecule 2',7'-bis(pyridyl-2-sulfonamido)-4',5'-dimethylfluorescein (histidine-zinc fluorescent *in vivo* tag, called HisZiFiT, **103**), which holds two Zn^{2+} ions in a coordinately unsaturated environment and has affinity for His₆-tags comparable to bis-NTA- Ni^{2+} ligands ($K_d \approx 40 \text{ nM}$). HisZiFiT has been used successfully in live cells to label surface-exposed His-tagged proteins without compromising the integrity of protein structure.⁴⁰⁹

11.3. Polyaspartate Tags

Runs of negatively charged amino acids provide another target as metal affinity tags. Hamachi and co-workers developed multinuclear Zn(II) luminescent complexes con-

sisting of a fluorescein or Cy5 fluorescent moiety attached to one (**104**) or two (**105**, **106**) pendant binuclear Zn-DpaTyr groups, which have affinity for poly(aspartic acid) sequences.⁴¹⁰ Notable here is the similarity in design of these constructs with Zn-DPA constructs mentioned in sections 6.5 and 6.6.1 that target phosphate-containing molecules. The affinity of the binuclear probes for aspartic acid stretches increases by an order of magnitude with each additional aspartic acid residue up to four (D4 tag). Compared with the binuclear Zn(II) complex, the tetranuclear Zn(II) complexes **105** and **106** have affinity 3 orders of magnitude higher when paired with a longer tag (D4-G-D4) capable of interacting with both Zn-DpaTyr groups.⁴¹⁰

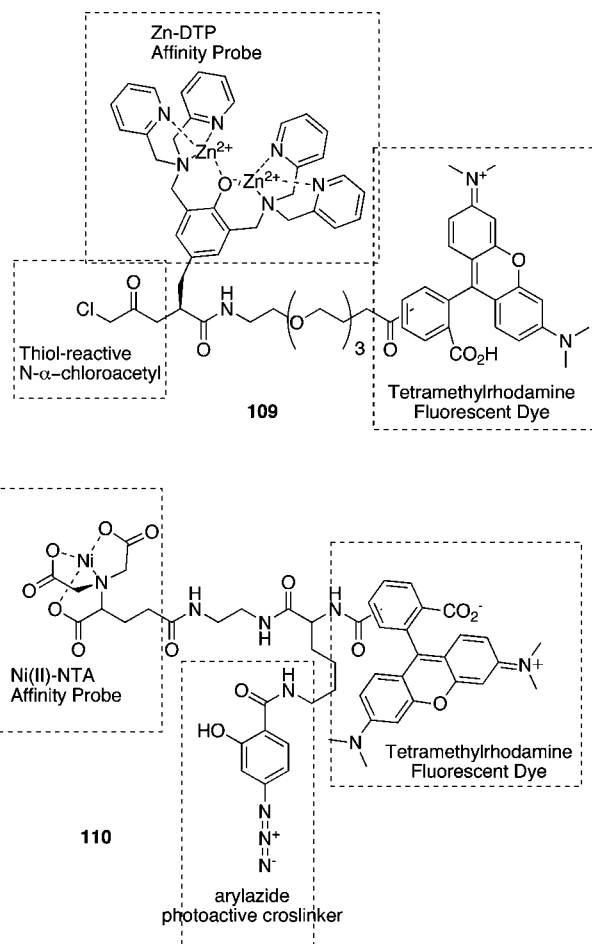
The utility of the D4/Zn-complex system was expanded by creating probes capable of ratiometric analysis.^{411,412} Ratiometric analysis is highly desirable because it helps reduce background signal due to the unbound fluorophore thereby facilitating precise detection and imaging of the protein of interest. In the first example, a binuclear Zn-DpaTyr attached to a pH-sensitive fluorophore (seminaphthorhodafluor (SNARF), **107**) takes advantage of the local acidic pH of polyaspartate tags to create an affinity probe that changes emission wavelength upon binding. In bulk solution, the phenolate form of SNARF emits at 586 nm, and upon binding to a D4 tag, protonation of the phenol shifts emission to 628 nm.⁴¹¹ In a second example, a peptide containing two D4 tags attracts two separate binuclear Zn(II) complexes containing pyrene moieties (**108**) into close proximity. The **108** monomer shows emission at 379 and 407 nm, but when two probe complexes bind to the D4 tag simultaneously, excimer formation results in emission at 472 nm.⁴¹²

11.4. Covalent Protein Labeling Facilitated by Metal Chelation

A drawback to imaging proteins tagged with metal affinity peptides is the high background generated by unbound fluorophores and the reversibility of the metal-tag interaction. To overcome this problem, covalent labeling strategies have been developed that take advantage of the peptide tag to direct metal complexes containing a reactive functional group to a protein of interest, as shown in Chart 16. The reactive group is used to covalently attach the probe thereby achieving site-specific protein labeling without the requirement of an enzyme. In this way, a binuclear Zn-DpaTyr construct **109** bearing a fluorophore and a thiol-reactive chloroacetyl group both targets and reacts with a D4 tag containing a cysteine thiol, resulting in a covalently labeled protein.⁴¹³ The *in vivo* utility of this system was demonstrated in *Escherichia coli* cells with CAAAAAADDDDD-tagged maltose binding protein paired with **109**. Labeling in live cells was not as efficient as labeling *in vitro*, but improvements in the affinity between the tag and probe are expected to improve labeling efficiency.⁴¹³

Hintersteiner et al. also developed a nonenzymatic covalent labeling technique, in this case by using a His-tag as the affinity target.⁴¹⁴ Probe **110** consists of a rhodamine reporter group, a Ni-NTA complex, and a photoactive arylazide cross-linker. After the probe binds to its His-tag target via Ni coordination, UV-light is used to photoactivate the arylazide and form a covalent bond to the protein of interest. This system was applied to label His-tagged proteins on the surface of living cells, where covalently bound probe

Chart 16. Constructs Used for Nonenzymatic Covalent Protein Labeling



remained on the cell surface while reversibly bound probe was easily washed away.⁴¹⁴

11.5. Lanthanide Binding Tags

Rather than using a synthetic chelator attached to a fluorophore to accomplish protein labeling, Imperiali and co-workers developed lanthanide binding tags (LBTs) as genetically encodable peptide sequences optimized for binding Ln ions, where the coordinated Ln itself is the luminescent reporter.⁴¹⁵ In the proper coordination environment, Ln ions have very attractive luminescent properties for biological applications, including sharp emission spectra, large Stokes shifts, and millisecond luminescent lifetimes. For luminescent LBTs, Tb(III) is particularly important because its emission can be sensitized by native tryptophan or tyrosine chromophores. Peptides with high affinity and bright emission for Tb(III) were discovered from combinatorial screens of sequences based on known calcium-binding motifs under 20 amino acids in length.^{415,416} The best sequence to date is FIDTNN DGWIEGDELLLEEG, which binds Tb with an apparent K_d at pH 7.4 of 19 nM and achieves bright luminescence by occluding inner-sphere water molecules that quench emission.⁴¹⁷

The utility of LBT-tagged proteins has so far been demonstrated in vitro. For example, time-resolved luminescence measurements were used to interrogate the protein–protein interactions between Src homology 2 (SH2) domains expressed with an LBT and phosphopeptides labeled with chromophores suitable for luminescence energy transfer with the LBT-bound Tb(III).⁴¹⁸ Beyond luminescence applications,

the properties of protein-bound Ln ions enabled by LBTs also facilitate macromolecular structure determination by both NMR^{419–421} and X-ray crystallography.⁴²² Although the challenge of incorporating Ln ions into tagged proteins in a living cell remains, the ability to genetically encode a protein with a nonintrusive and multifunctional tag is attractive for future cellular applications.⁴¹⁸

11.6. Unnatural Metal-Binding Amino Acids

Labeling systems that employ metal coordination chemistry to unite a synthetic probe with a genetically encoded peptide tag are applicable to a wide variety of proteins and provide general tools for in vivo, site-specific labeling of proteins. These new metal sites are usually limited to the C- or N-terminus of a protein of interest so as not to disrupt the protein's native structure and function. Schultz demonstrated a new way to introduce metal sites into proteins without the use of oligopeptide tags. By incorporating non-natural amino acids directly into prokaryotic and eukaryotic organisms, chemically and photochemically active groups, including metal-binding centers, can be introduced site specifically into proteins.^{423–425} The metal-binding non-natural amino acid bipyridylalanine (BpyAla, **111**) was incorporated into defined sites in a DNA binding protein to directly map its binding site on duplex DNA.⁴²⁶ Importantly, introduction of the metal binding site involved replacement of only one amino acid in the entire protein sequence. In the case of *E. coli* catabolite activator protein (CAP), the replacement of one lysine in the DNA recognition site did not significantly perturb its interaction with its DNA binding

sequence. CAP-K26Bpy-Ala showed site-specific oxidative cleavage at two different sites on the DNA sequence.⁴²⁶ Because introduction of non-natural amino acids requires only simple mutagenesis and because there are numerous potential metal binding non-natural amino acids, redox-active metals could be placed almost anywhere in a protein to map protein–RNA or protein–protein interfaces.

12. Conclusions

As discussed in the previous sections, the application of metal chelating agents, coordination compounds, and organometallic complexes in cell biology offers diverse opportunities for manipulating biological processes. The examples that have been highlighted here are only representative of the significant opportunity that metal complexes offer for exploring the chemical biology of living cells. In describing the principles and trends involved in the design, functionality, and reactivity of inorganic complexes in this context, we hope to inspire further research in the area. In many cases, a creative approach can extend a seemingly simple motif across several applications. The dipicolylamine chelating unit is one example. This basic motif appears in TPEN, which is used as a chelator to alter cellular metal content, as well as fluorescence and MRI-based Zn(II) sensors, and in constructs that use their coordinated metal ion to tag proteins, recognize phosphorylated proteins and other phosphate-containing biomolecules, or bind to specific DNA sequences.

The breadth of these different biological applications for inorganic complexes is exciting, but also presents ongoing challenges. As these kinds of reagents become more frequently used in biological studies, questions of cross reactivity and specificity are likely to increase. The cellular reactivity of any of these complexes will depend on their distribution and relative concentration with respect to competing metal ions that may induce transmetalation, as well as endogenous chelating agents that can compete for the metal ion or form unanticipated ternary complexes that might alter their reactivity. All of these complex equilibria, in addition to factors of stability and metabolism, will affect the specificity of the reagent for the intended biological interaction. Future development will therefore need to focus on understanding these complex processes. In particular, this area will benefit greatly from advances in understanding cellular metal trafficking and regulation.

There is an obvious synergy between inorganic chemical biology and the biology of metal trafficking. Small molecule chemical tools that manipulate cellular metal content and distribution are beneficial for understanding metal trafficking pathways. At the same time, as we better understand these pathways we can develop better strategies for intercepting them, whether it be for overcoming a metal deficiency, reducing a metal overload, or hijacking a native metal into an artificial complex with non-native function, like an enzyme inhibitor or catalytic protein inactivator. Likewise, how do cells handle unnatural metals and metal complexes? For intact metal complexes that are applied to cells or organisms, at what point does the cellular machinery differentiate the cargo from the carrier, and how does this influence where metal complexes are distributed in a cell? Finding answers to these questions will be an exciting journey into the frontier of inorganic chemistry and cell biology.

13. Abbreviations

4AP	4-aminopyridine
ACE	angiotensin-converting enzyme
ADP	adenosine diphosphate
APTRA	aminophenol- <i>N,N,O</i> -triacetate
ATCUN	amino-terminal copper/nickel
ATP	adenosine triphosphate
BAPTA	(1,2-bis(<i>o</i> -aminophenoxy)ethane- <i>N,N,N',N'</i> -tetraacetate acid)
BC	bathocuproine
BCS	bathocuproine disulfonate
BPS	bathophenanthroline disulfonic acid
bpy	bipyridine
CAP	catabolite activator protein
CDK9	cyclin-dependent protein kinase 9
CEST	chemical exchange saturation transfer
cod	1,5-cyclooctadiene
CORM	carbon monoxide releasing molecule
Cp*	pentamethylcyclopentadienyl
CTD	C-terminal domain
Cy3	cyanine dye
cyclam	1,4,8,11-tetraazacyclotetradecane
DFO	desferrioxamine
DMSO	dimethylsulfoxide
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate acid
DPA	dipyridylamine
DTPA	diethylenetriamine pentaacetic acid
ECE	endothelin-converting enzyme
EDT	ethanedithiol
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
FRET	fluorescent resonance energy transfer
G4-DNA	guanidine quadruplex DNA
GABA	γ -amino butyric acid
GSK-3	glycogen synthase kinase 3
HDAC	histone deacetylases
HSAB	hard and soft acid and base
IP ₃	inositol triphosphate
IDA	iminodiacetic acid
LBT	lanthanide binding tag
LFSE	ligand field stabilization energy
MLCT	metal-to-ligand charge transfer
MMP	metalloproteinase
MNIP	4-methoxy-2-(1 <i>H</i> -naphtho[2,3- <i>d</i>]imidazole-2-yl)phenol
MR	magnetic resonance
MRI	magnetic resonance imaging
NIR	near-infrared
NMR	nuclear magnetic resonance
NTA	nitrilotriacetic acid
PARACEST	paramagnetic chemical exchange saturation transfer
PET	positron emission tomography
PIH	pyridoxal isonicotinoyl hydrazone
PPh ₃	triphenylphosphine
pS	phosphoserine
pSer	phosphoserine
py	pyridine
pY	phosphotyrosine
Resf	resorufin
RNA	ribonucleic acid
ROS	reactive oxygen species
RSE	Roussin's red salt esters
SH2	Src homology 2
SIH	salicylaldehyde isonicotinoyl hydrazone
SNARF	seminalphthorhodafluor
SNP	sodium nitroprusside

Sox	sulfonamide-oxine amino acid
SPECT	single photon emission computed tomography
SplAsH	spirolactam arsenical hairpin binder
TPEN	<i>N,N,N',N'</i> -tetrakis-(2-pyridylmethyl)-ethylenediamine
TRML	time-resolved luminescence microscopy.

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