Cell Imaging

DOI: 10.1002/anie.200801965

Three-Coordinate Ligand for Physiological Beryllium Imaging by Fluoresence**

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Beryllium is an extremely light, stiff metal with a wide spectrum of applications in industry. Pure beryllium metal, alloys, salts, as well as metal oxides have been extensively utilized in nuclear, aerospace, and electronic industries.[1] However, Be and its compounds are highly toxic, and exposure to particulate Be can lead to chronic beryllium disease (CBD). A recent NIOSH (National Institute for Occupational Safety and Health) report estimates that 26500 current DOE and DOD employees and up to 106000 workers in the private sector of the United States have potentially been exposed to beryllium.[2] CBD is a cell-mediated immune response characterized by the development of lung granulomas and progressive pulmonary fibrosis, which occurs in up to 6–20% of subjects exposed to beryllium or its salts.[3–6] Even though the nature and effects of CBD have been well-studied, how beryllium triggers such a specific immune response remains a mystery.[5,7] The ability to track Be in cellular studies could provide very useful information on how the immune system is able to selectively recognize the Be^{2+} cation and why the immune response continues into granuloma formation. Selective fluorescent indicators have been reported, and an ASTM approved fluorescent method for Be detection has recently been commercialized.[8–12] These advances have made it possible to detect low levels of Be with small sample sizes, providing the ability to determine Be concentrations in Be–protein binding experiments. However, to our knowledge none of the reported fluorescent sensors have been shown to work in a physiological phosphate medium. A selective fluorescent sensor capable of working in a phosphate media would open the possibility for imaging in a biological system. Such a beryllium-staining agent would open up the possibility for a wide range of biological experiments to gain further insights into the understanding of how CBD develops and advance our understanding of the lung immunology in general.

Previous research has included binding of beryllium by bidentate ligands to make [BeL] and [BeL_2] species with chelating ligands such as chromotropic acid and 2,3-dihydroxybenzoic acid, which was initially designed for polynuclear [Be_2L] complexes.[13–18] Recent work has suggested that Be binding occurs through the displacement of strong hydrogen bonds.[19] Strong hydrogen bonds occur when the distance between the two heteroatoms, typically O–H···O or O–H···N, is shorter than the sum of the van der Waals radii and the energy barrier to hydrogen transfer between two atoms is on the order of the O–H vibrational zero-point energy. The strong hydrogen bond provides two advantages. First, the O···X distance in a strong hydrogen bond is in the range 2.4–2.8 Å, which brings two oxygen atoms into a predefined chelating site for the beryllium atom that corresponds very well to the intraligand oxygen–oxygen atom distances of 2.26 to 2.86 Å observed in known beryllium structures in the Cambridge Structural Database.[20] Second, the strong hydrogen bond provides a low barrier pathway to displace the proton without breaking a strong covalent O–H bond by shifting the proton to the more acidic site as Be interacts with the basic oxygen center.[21] We have used this strategy to select a new tridentate fluorescent agent for binding beryllium.

Polypyridines have been widely used as polydentate chelating ligands. Among the polypyridines, the coordination chemistry of polydentate chelating ligands containing mixed pyridine–phenol donors is a very popular target of study.[22–25] The ligand 2,6-bis(2-hydroxyphenyl)pyridine (BHPP) was chosen because it has two basic phenol ligands that could potentially form strong hydrogen bonds with the central pyridine ring. The pyridine ring provides an acidic site that acts as low kinetic barrier pathway to shuttle the protons off the phenol rings, and the two basic phenolates should bind strongly to the Be center. The resulting tridentate [Be(bhpp)] complex was intended to limit the kinetic accessibility of the Be center in phosphate medium. BHPP has recently been studied for its binding to Cu and Zn in nonaqueous media.[26] Herein we demonstrate that this ligand is able to bind Be selectively at neutral pH in the presence of phosphate and is a promising candidate for cellular tracking of Be.

BHPP was synthesized by a modified literature procedure utilizing a Suzuki coupling to increase purity of the final product.[27] The proton NMR spectrum of the free BHPP ligand in DMSO shows a sharp singlet at $\delta = 12.31$ ppm with an integrated area of 2 for both of the phenol protons. This large downfield shift is indicative of protons that are involved

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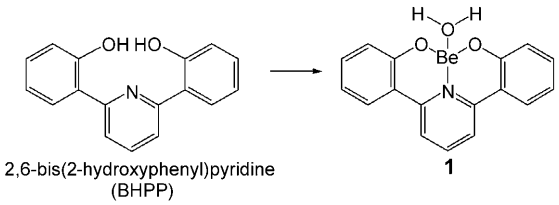
[**] This work was supported by the Laboratory Directed Research and Development program (LDRD) at Los Alamos National Laboratory.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200801965.

in a strong hydrogen bonding interaction. Further, the sharp peak that integrates for two protons suggests that both hydrogen atoms are participating equally in the intramolecular strong hydrogen bond, which implies a structure in which both phenol rings are equally rotated to allow three-centered hydrogen bonding O–H···N···H–O. Reaction of BHPP in ethanol with aqueous $BeSO_4$ gave **1** in good yield (Scheme 1).



Scheme 1. Coordination of Be by the tridentate ligand BHPP.

Bright orange-yellow X-ray quality crystals were obtained by the slow diffusion of diethyl ether into a solution of **1** in dimethylformamide or pyridine. The ^{9}Be NMR spectrum of **1** shows a sharp singlet at $\delta = 4.08$ ppm indicating the presence of a tetracoordinated Be center (Figure 1).

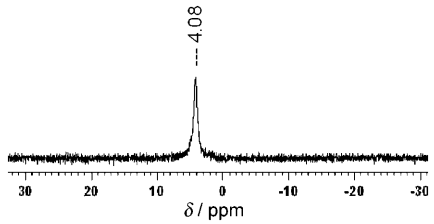


Figure 1. ^{9}Be NMR spectrum of [Be(bhpp)] in D_2O.

The single-crystal X-ray structure (Figure 2) reveals that the Be center is coordinated to both the phenolate oxygen atoms. The remaining coordination sites on the beryllium are taken up by the pyridine nitrogen donor and the oxygen atom from a water molecule. The BHPP ligand is slightly distorted from planarity. The simple 1:1 BHPP:Be binding with a water molecule in the last coordination site is in contrast to the previous coordination complexes. The structures of both the Cu and Zn compounds involve multiple metal centers with bridging phenolates.[26]

The strong hydrogen bond in the free BHPP ligand leads to rapid proton transfer in the excited state which deactivates

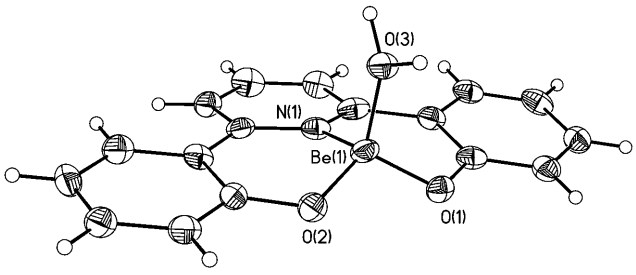


Figure 2. X-ray crystal structure of [Be(bhpp)] complex **1**.

the fluorescence. In the presence of Be these protons are lost as Be binds and the highly efficient fluorescence from the π^* to π singlet transition appears at 470 nm with 360 nm excitation (Figure 3). Fluorescence is readily observed at

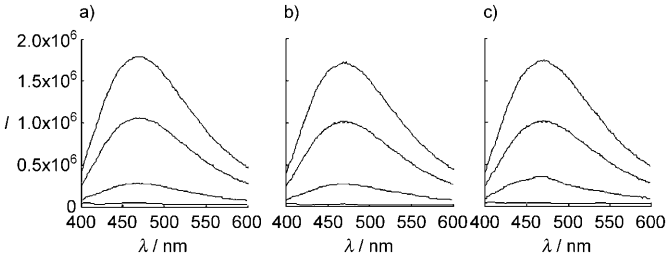


Figure 3. Fluorescence spectra of 100 μM BHPP at pH 7 with Be concentrations of 0, 1, 5, and 10 μM (bottom to top). a) 5 mM HEPES; b) 5 mM phosphate; c) 5 mM phosphate with 200 μM Ca and 120 μM Mg.

less than 1 μM concentration of the [Be(bhpp)] complex at pH 7 in noncoordinating HEPES (*N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid) buffer. Previous Be fluorescent agents have also been able to detect Be at low levels, but often suffer from phosphate interference at pH 7, which limits their use in imaging Be in cells. Previous studies with Be in phosphate media have been limited to acidic conditions to avoid precipitation of BeO.[28] The BHPP complex is able to detect Be at μM levels in 5 mM phosphate (pH 7) with no interference even in the presence of 200 μM Ca and 120 μM Mg (Figure 3).

These results suggest that BHPP has the ability to act as a Be imaging agent under physiological conditions. No fluorescence background is observed even in the presence of a metal cocktail containing 1 mM each of Al, Fe, Cr, Cu, Zn, Cd, and Pb, demonstrating the selectivity of the BHPP ligand for Be in a complex medium. The intensity increases linearly with Be concentration, and a correlation coefficient of greater than 0.995 is obtained in all cases.

To demonstrate the potential of BHPP as a fluorescent Be indicator for cellular studies, we incubated human lung epithelial cells (A549) with control buffer and a mixture of 50 μM $BeSO_4$ and 100 μM BHPP for 2 h. The cells were washed in HEPES three times prior to fixing, and fluorescence studies were performed by using a Zeiss Axiophot microscope. The resulting images (Figure 4) clearly demonstrate that the fluorescence of [Be(bhpp)] can be observed in a cellular medium. The insets showing 100-fold magnification suggest that Be may be preferentially distributed within the cell—a feature worthy of further study.

In summary, we have demonstrated that the strong hydrogen bond in BHPP can be displaced by Be. BHPP binds Be very effectively and selectively at pH 7 in 5 mM phosphate buffer. The background fluorescence remains minimal and no interference is observed in the presence of other metals. The enhanced fluorescence observed with human lung epithelial cells incubated with [Be(bhpp)] demonstrates that BHPP is ideal agent for biological imaging of Be in the study of CBD.

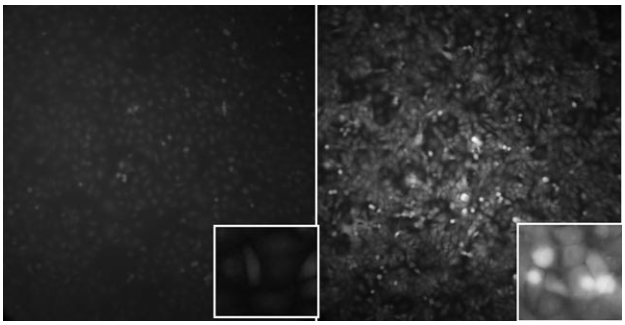


Figure 4. Direct fluorescence analyses in A549 cells from control cultures (left) and cultures exposed to [Be(bhpp)] (right) at 20× magnification. Insets: representative cells at a 100× magnification.

Experimental Section

Direct fluorescence studies: Human lung epithelial cells (A549) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Ham's F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% v/v heat-inactivated fetal bovine serum (Invitrogen), 100 $\mu g\,mL^{-1}$ streptomycin, 100 $U\,mL^{-1}$ penicillin, and 2 mM L-glutamine in a humidified atmosphere (5% CO2) at 37°C. Cells seeded on sterile glass coverslips in six-well cell culture plates were used to study fluorescence induced by the [Be(bhpp)] complex. Cells were incubated with 1) control buffer and 2) 50 μM $BeSO_4$ and 100 μM BHPP for 2 h, subsequent to which growth medium was added to the culture. The cells were washed in HEPES three times prior to fixing and fluorescence studies were performed (excitation: 355 nm emission: 465 nm; blue channel) using a Zeiss Axiophot microscope.

Received: April 25, 2008
Published online: August 7, 2008

Keywords: beryllium · fluorescent probes · immunology · ligand design · luminescence

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