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# Chromofluorescent Indicator for Intracellular $Zn^{2+}/Hg^{2+}$ Dynamic Exchange

**Jeong Won Lee,[†] Hyo Sung Jung,[†] Pil Seung Kwon,[‡] Jong Wan Kim,*[,‡] Richard A. Bartsch,[§] Youngmee Kim,[||] Sung-Jin Kim,[||] and Jong Seung Kim*[,†]**

*Department of Chemistry, Korea University, Seoul 136-701, Korea, Department of Laboratory Medicine, Dankook University Hospital, 330-715, Cheonan, Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409, and Department of Chemistry and Division of Nano Sciences, Ewha Womans University, Seoul 120-750, Korea*

*wan1818@paran.com; jongskim@korea.ac.kr*

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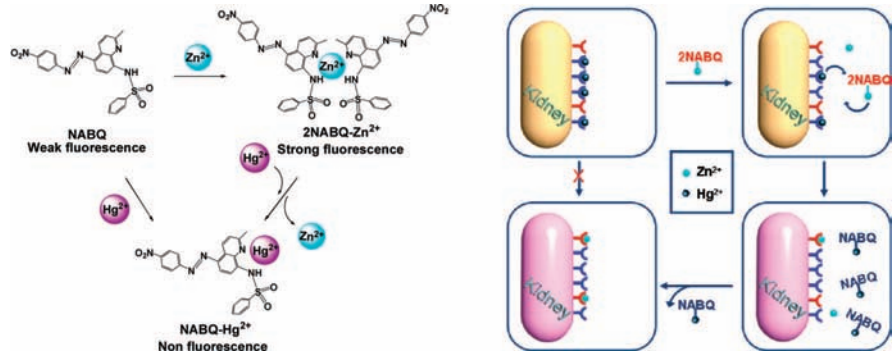
## ABSTRACT




**The fluorescence of NABQ increases remarkably in the presence of $Zn^{2+}$ and is quenched by $Hg^{2+}$. As shown by confocal imaging, NABQ−$Zn^{2+}$ can penetrate cells, where the bound $Zn^{2+}$ is exchanged for $Hg^{2+}$. This results in the concomitant export of $Hg^{2+}$ from the cells, showing that NABQ can act as a $Zn^{2+}$ carrier and as a $Hg^{2+}$ extracting agent in living cells.**

Mercury is extremely toxic, and the widespread contamination of the environment with this mineral has a variety of natural and anthropogenic sources.[1] The accumulation of mercury to high concentrations in the liver, kidney, and spleen leads to DNA damage, mitosis impairment, and nervous system defects.[2] Intense attention has been focused on the development of sophisticated techniques for the determination and removal of $Hg^{2+}$ from living systems.[3]

Zinc is an important mineral for protein synthesis and for the regulation of cell production in the immune system.[4] Zinc deficiency causes unbalanced metabolism, which in turn can induce retarded growth in children, male and female reproduc-

[†] Korea University.
[‡] Dankook University Hospital.
[§] Texas Tech University.
[||] Ewha Womans University.

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tion problems, low blood sugar, poor bone growth, brain disorders, and high blood cholesterol, etc.[5] Another important additive effect of zinc in our bodies is illustrated by zinc-binding proteins, such as metallothionein, which function in lead detoxification by sequestering lead within the enterocyte.[6]

Fluorescence monitoring is receiving great interest because of its simplicity, selectivity, and sensitivity toward specific metal cations in living organisms.[7] As one of several fluorescence sensing mechanisms, ICT (intramolecular charge transfer) has been widely developed for ion sensing, molecular switching, and fluorescent labeling because ICT-based molecules have a long-wavelength emission which is very sensitive to structural changes.[8] We have developed a variety of ICT-related fluorescence materials that are capable of selectively sensing specific cations or anions in aqueous media.[9] Especially, we have sought to develop molecular systems that are capable of supplying beneficial minerals to animals while eliminating toxic metal ions. This led us to the development of a new and promising ICT-based molecule, 5-(4-nitrobenzene diazo)-8-benzenesulfonamidoquinaldine (NABQ), which can be used to simultaneously supply $Zn^{2+}$ and remove $Hg^{2+}$ from living cells with high selectivity (see the graphical abstract). The dual sensitivity scaffolds (nitroazobenzene−*chromophore* and benzenesulfonamido quinaldine−*fluorophore*) of NABQ provide explicit information by color and fluorescence changes upon metal cation complexation, which can be applied to biological systems, such as living cells.

NABQ was synthesized from 8-nitroquinaldine via a three-step procedure (reduction, sulfonamidation, and diazotization) from 8-nitroquinaldine in an ca. 33% overall yield (Scheme S1). The absorption spectrum of free NABQ gives a characteristic ICT band at 400 nm (Figure S1).

When $Zn^{2+}$ was gradually added, the $\lambda_{max}$ bathochromically shifted by 36 nm to 436 nm, and the solution color changed from yellow to red (Figures S2 and S22). Figure 1a records the fluorescence changes of NABQ (10 $\mu$M) upon addition of chloride salts of various metal cations. Even with highly concentrated $Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$ (~5.0 mM) under physiological conditions,[10] we found no fluorescence changes. However, $Zn^{2+}$ led to an 11-fold increase in the fluorescence at 513 nm. The inset of Figure 1b shows that addition of only 0.2 equiv of $Zn^{2+}$ increases the fluorescence by 90%, and the fluorescence reaches a plateau at 0.5 equiv of $Zn^{2+}$, presumably due to the CHEF effect.[11] This demonstraes that NABQ is a very sensitive and selective $Zn^{2+}$ indicator, as well as a promising tool for the quantitative analysis of $Zn^{2+}$.

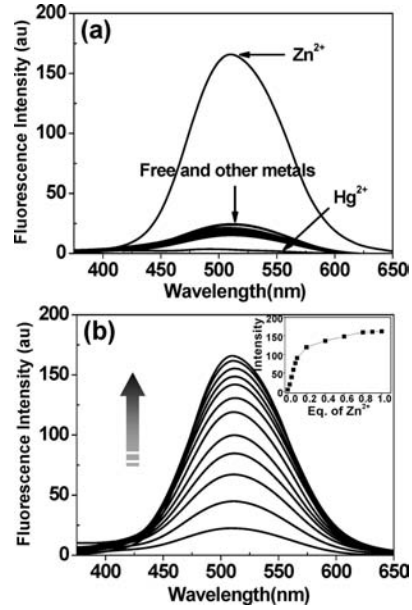


**Figure 1.** Fluorescence responses of NABQ (10 $\mu$M) in $CH_3CN$/$H_2O$ solution (1/1, v/v) at pH 7.4 (0.02 M HEPES) in the presence of (a) chloride salts of various metal cations ($Li^+$, $Rb^+$, $Cs^+$, $Sr^{2+}$, $Ba^{2+}$, $Cu^{2+}$, $Cd^{2+}$, $Hg^{2+}$, and $Zn^{2+}$ (100 $\mu$M, respectively) and $Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$ (5 mM, respectively)) and (b) increasing concentrations of $Zn^{2+}$ (0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, and 10 $\mu$M). Excitation at 365 nm. Inset: fluorescence intensity of NABQ at 513 nm vs equiv of $Zn^{2+}$.

Job's plot analysis gave a maximum at 0.33 mol fraction of $Zn^{2+}$ (Figure S8) indicating the formation of a 2:1 complex between NABQ and $Zn^{2+}$. FAB-MS (Figure S27) with $m/z$ = 958 (2NABQ + $Zn^{2+}$) additionally provides strong evidence for a 2:1 stoichiometry. O'Halloran et al. reported a crystal structure for a quinoline derivative similar to ours in which $Zn^{2+}$ is coordinated to the sulfonamide unit as well as to the quinoline nitrogen in a 2:1 complexation mode.[11]

On the other hand, when $Hg^{2+}$ was added to a solution of NABQ, $\lambda_{max}$ in the UV spectrum was red-shifted by 44 nm to 444 nm. The fluorescence emission of NABQ was quenched markedly by $Hg^{2+}$ (Figure 1a) attributable to $Hg^{2+}$ acting as an inherent fluorescence quenching ion via enhanced spin−orbit coupling.[12]

Figure S23 shows that NABQ could selectively detect $Zn^{2+}$ among the tested metal ions except for $Hg^{2+}$. When 1 equiv

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of $Zn^{2+}$ was added to a solution of the NABQ−$Hg^{2+}$ complex, no restoration of fluorescence emission was observed (Figure S4). In reverse order experiments, introduction of other cations into a solution of NABQ−$Zn^{2+}$ did not result in fluorescence quenching (Figure S24). Thus, only the addition of $Hg^{2+}$ to the solution of NABQ−$Zn^{2+}$ gave fluorescence quenching. Also, Figure S25 illustrates that when $Hg^{2+}$ was added to a solution of NABQ−$Zn^{2+}$ containing various metal ions fluorescence quenching was observed. This is consistent with a greater binding affinity of NABQ for $Hg^{2+}$ than $Zn^{2+}$.

In titration experiments with $Hg^{2+}$, we found that a solution of NABQ−$Zn^{2+}$ exhibits more pronounced quenching than that of a solution of free NABQ (Figure 2b). Thus, the

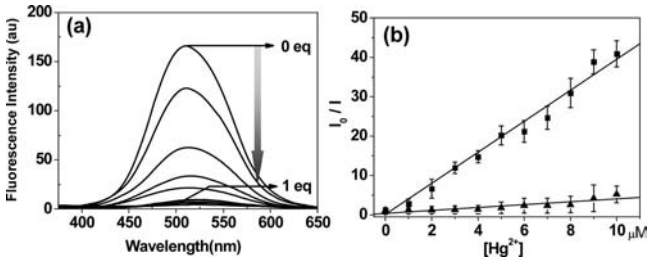



**Figure 2.** Fluorescence changes of 2NABQ−$Zn^{2+}$ (5.0 $\mu$M) in $CH_3CN/H_2O$ solution (1/1, v/v) at pH 7.4 (0.02 M HEPES) upon addition of (a) various concentrations of $HgCl_2$ (0−10 $\mu$M) with excitation at 365 nm. (b) Fluorescence intensity decrease ($I_0/I$) at 510 nm of 2NABQ−$Zn^{2+}$ in the presence of increasing concentrations of $Hg^{2+}$ (−■−) and NABQ alone in the presence of increasing concentrations of $Hg^{2+}$ (−▲−).

NABQ−$Zn^{2+}$ complex displays greater selectivity and sensitivity for $Hg^{2+}$ than does free NABQ, suggesting that it could be employed as a $Hg^{2+}$ indicator. NABQ−$Zn^{2+}$ is found to have a detection limit of $5.62 \times 10^{-8}$ M (Figure S20) which is sufficient for $Hg^{2+}$ analysis in the case of mercury poisoning symptoms in which mercury levels exceed 1.0 $\mu$M in blood and 3.0 $\mu$M in urine.[13]

To gain insight into the binding mode of NABQ−$Hg^{2+}$, we obtained a single crystal by mixing equivalent amounts of NABQ−$Zn^{2+}$ and $Hg(NO_3)_2$ in MeCN followed by slow *n*-hexane vapor diffusion. The crystal structure shows a coordinative interaction between NABQ and $Hg^{2+}$ with a 1:1 stoichiometry (Figure 3). The $Hg^{2+}$ binds to the quinaldine and sulfonamide nitrogens and to the nitrate. Job's plot analysis shows that NABQ−$Hg^{2+}$ is formed with a 1:1 stoichiometry (Figure S9). The FAB-MS (Figure S28) with $m/z = 748$ (NABQ + $Hg^{2+}$ + $ClO_4^-$) provides additional evidence of a 1:1 stoichiometry.

Scheme 1 summarizes the binding mechanism of NABQ with $Zn^{2+}$ and $Hg^{2+}$ based on fluorescence on−off switching. NABQ exhibits a weak fluorescence but emits strong fluorescence upon $Zn^{2+}$ complexation.[14] Regarding the complexation mode of NABQ−$Zn^{2+}$, the metal ion is believed to coordinate with the nitrogens of the sulfonamide unit and the heterocyclic ring in a 2:1 complexation mode

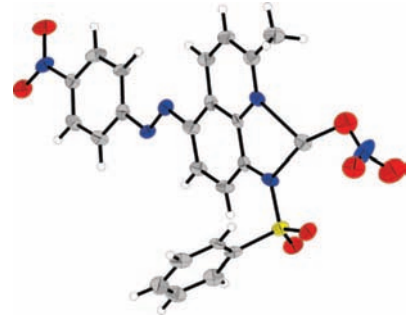
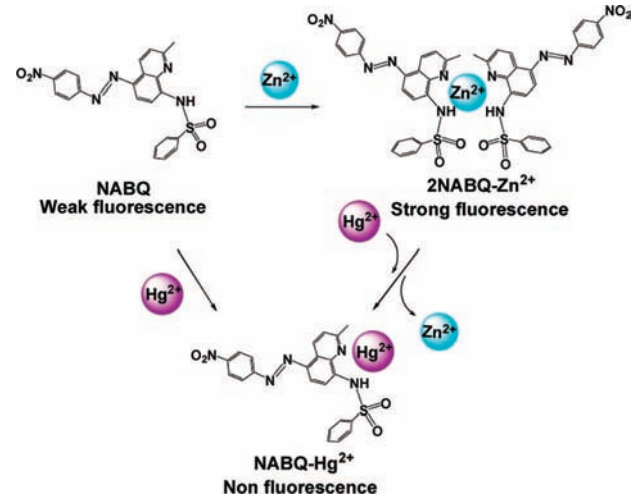


**Figure 3.** Solid-state structure of NABQ−$Hg^{2+}$ ($NO_3^-$) with displacement atomic ellipsoids, which are drawn at a 50% probability level.

**Scheme 1.** Complexation Modes of NABQ with $Zn^{2+}$ and $Hg^{2+}$




analogous to that reported by O'Halloran et al. for a related system. Thus we proposed that $Hg^{2+}$ binding induces $Zn^{2+}$ release from the NABQ−$Zn^{2+}$ complex and the formation of a new complex between $Hg^{2+}$ and NABQ that lacks fluorescence.

Selective monitoring of metal ions in living cells has considerable importance for biological applications. To determine if NABQ could be used in this manner in living cells, kidney cells of monkey (LLC-MK2) were incubated with NABQ (50 $\mu$M) in D-MEM (Dulbecco's Modified Eagle's Medium),[15] in which chloride mineral salt such as $CaCl_2$, $MgCl_2$, KCl, and NaCl, etc. are abundant. Then, its fluorescence changes (Figure S33) were observed by confocal laser microscopy. The cells were incubated with NABQ in the growth medium for 5 min at 37 °C, and the free NABQ

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displayed a weak fluorescent image (Figure S33b). Upon loading with $ZnCl_2$ (50 $\mu$M, only 1 equiv) under the same conditions, a significant increase in the fluorescence of the intracellular area (Figure S33c) was observed, clearly demonstrating that NABQ can be used for $Zn^{2+}$ sensing in living cells.

Interestingly, the exchange of $Hg^{2+}$ for $Zn^{2+}$ in NABQ−$Zn^{2+}$ complexes could also be detected in LLC-MK2 cells (Figure 4). The fluorescence of NABQ−$Zn^{2+}$ (Figure 4b),

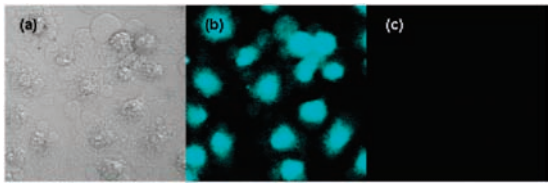


**Figure 4.** Confocal fluorescence images of $Zn^{2+}$ in LLC-MK2 cells (excited wavelength at 405 nm, Zeiss LSM 510 META confocal microscope, ×20 objective lens) in D-MEM. (a) Bright-field transmission image of LLC-MK2 cells with NABQ (50 $\mu$M). (b) Fluorescence image of NABQ−$Zn^{2+}$ (25 $\mu$M) in LLC-MK2 cells and (c) after subsequent treatment with $Hg^{2+}$ (50 $\mu$M).

which was initially intense, eventually disappeared upon treatment of the cells with 1 equiv of $Hg^{2+}$ (Figure 4c). Regarding this fluorescence quenching, intracellular $Hg^{2+}$ could be detected at micromolar concentration levels (Figure S40).

In addition to LLC-MK2 cells, we also tested AMC-HN3, Hep-2, and MDCK cell lines and obtained similar results (Figures S34−S39). Hence, NABQ has potential as an agent for the intracellular imaging of $Zn^{2+}$ and/or $Hg^{2+}$ in living cells based on intracellular metal ion exchange.

To gain insight into the effect of NABQ and NABQ−$Zn^{2+}$ on $Hg^{2+}$ cytotoxicity,[16] we treated LLC-MK2 cells with $Hg^{2+}$, $Hg^{2+}$ and NABQ, and $Hg^{2+}$ and NABQ−$Zn^{2+}$ (untreated cells were used as the control). The percentage of cell viability remaining after cell treatment was taken as a measure of toxicity and is shown in Figure S41. As expected, treatment with $Hg^{2+}$ resulted in serious toxicity as shown by the 90% loss of cell viability after 1 day of cell incubation. Remarkably, cell viability increased to 50% for incubation with $Hg^{2+}$ in the presence of NABQ and to 77% with $Hg^{2+}$ in the presence of NABQ−$Zn^{2+}$. It is notable that NABQ−$Zn^{2+}$ was more effective for the retention of cell viability in the presence of $Hg^{2+}$ than NABQ alone. Thus zinc is capable of controlling mercury distribution followed by protection from mercury toxicity, like metallothionein.[17]

We next determined if $Hg^{2+}$ entrapped by NABQ during the metal ion exchange process in the cell is removed from the cell by performing emission spectroscopy on the supernatants of the cells treated with $Hg^{2+}$, NABQ−$Zn^{2+}$, and $Hg^{2+}$ and NABQ−$Zn^{2+}$, respectively. As shown in Figure S42, in the absence of $Hg^{2+}$ ions, the fluorescence intensity of the supernatants of cells treated with NABQ−$Zn^{2+}$ alone decreased moderately because delivery of NABQ−$Zn^{2+}$ into the cells resulted in a decline in its concentration in the supernatant. Importantly, in the presence of $Hg^{2+}$ in the cells, the fluorescence of NABQ−$Zn^{2+}$ in the supernatant was quenched. This indicates that $Hg^{2+}$ initially adsorbed by the cell was transported outside of the cell after metal ion exchange by formation of NABQ−$Hg^{2+}$.

To probe the intracellular metal ion exchange from NABQ−$Zn^{2+}$ to NABQ−$Hg^{+}$ followed by $Hg^{2+}$ delivery outside of the cell, we carried out cell lyses of LLC-MK2 cells after treatment with $Hg^{2+}$, $Hg^{2+}$, and NABQ, and $Hg^{2+}$ and NABQ−$Zn^{2+}$ (untreated cells were used as the control). The results are shown in Figure S43. NABQ−$Zn^{2+}$ was the most potent species for reducing the $Hg^{2+}$ concentration in the cell (44% of control). Thus, treatment of $Hg^{2+}$-embedded cells with NABQ−$Zn^{2+}$ expels $Hg^{2+}$ from the cell, which is consistent with results reported by Rooney.[17] The graphical abstract summarizes the dynamic metal ion exchange process of NABQ in the cell. In this model, NABQ acts as a dual sensitive metal ion exchanger for the supply of $Zn^{2+}$ to the cell, as well as for extraction of $Hg^{2+}$.

In summary, we have presented in this study new fluorescent agents NABQ and NABQ−$Zn^{2+}$. With a fluorescence technique, NABQ can be used for $Zn^{2+}$ detection. Fluorescence of NABQ−$Zn^{2+}$ is strongly quenched by $Hg^{2+}$ with which the binding constant of NABQ is greater than with $Zn^{2+}$. Confocal imaging shows that NABQ−$Zn^{2+}$ can penetrate cells and engage in reciprocal metal ion exchange with $Hg^{2+}$, resulting in the export of $Hg^{2+}$ from the cells, which is confirmed by cell lyses experiments. This shows that NABQ can act as a $Zn^{2+}$ carrier and as a $Hg^{2+}$ extracting agent in living cells, resulting in increased cell viability in the presence of toxic $Hg^{2+}$. The present observations provide a foundation for new fluorescence probes and target metal deliverer-based biological investigations.

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**Supporting Information Available:** Synthetic details, additional NMR, UV and emission spectral data, and biological test. This material is available free of charge via the Internet at http://pubs.acs.org.

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