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Analysis of beryllium to biomolecule binding using a metal specific fluorescent probe and competitive assay

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ABSTRACT

Studying metal–biomolecule interactions is critical to the elucidation of the molecular basis of the biological functions and toxicity of metals. In the present study, a competitive fluorimetric approach has been developed to measure the apparent affinity of biomolecules for Be^{2+} by using a Be^{2+}-specific fluorigenic probe (10-hydroxybenzo[*h*]quinoline-7-sulfonate, HBQS). Under physiological conditions, HBQS coordinates with Be^{2+} in a molar ratio of 1:1 and results in a fluorescence shift from 580 nm for HBQS to 480 nm for the Be–HBQS complex associated with significant fluorescence enhancement. When a beryllium ligand is present in the mixture of Be^{2+} and HBQS, the competition of ligand against HBQS for beryllium ion binding results in dissociation and thus a fluorescence decrease of the Be–HBQS complex. By titrating ligand and monitoring the dose-dependent decrease of Be–HBQS complex fluorescence at 480 nm, the apparent affinity between ligand and Be^{2+} can be derived. Applying this simple approach, the apparent affinities of various nucleotides and the iron-storage protein ferritin for beryllium ion have been determined. In particular, the apparent dissociation constant of Be^{2+} and adenosine 5′-triphosphate (ATP) was also validated by an electrospray ionization mass spectrometric (ESI-MS) method. The general applicability of the proposed competition assay was further demonstrated using FluoZin-1, a zinc fluorescent indicator, in a binding study for Zn^{2+} and bovine serum albumin. This newly developed competitive fluorimetric assay provides a sensitive, simple, and generic approach for affinity estimation of metal and biomolecule binding.

1. Introduction

Metals play important roles in metabolism, biosynthesis, signal transduction, muscle contraction, nerve transmission, respiration, and many other biochemical activities [1]. While some metals are essential nutrients, others are highly toxic, even in trace amounts [2]. Metal intoxication has been documented and studied especially in neurotoxicity, genotoxicity, cytotoxicity, and carcinogenicity [3]. As metals exert their biological functions or toxicity via binding to ligands including proteins, enzymes, nucleotides, and nucleic acids, studying metal and biomolecule interactions is critical not only to the elucidation of the underlying molecular basis of biological functions and metal-associated diseases but also to the design of efficient therapeutic strategies.

Beryllium plays important roles in the nuclear, aerospace, electronics, and mechanical industries because of its excellent chemical and physical properties. However, beryllium and its compounds are highly toxic [4]. Three lung diseases can result from inhalation of beryllium aerosols: acute pneumonitis, chronic beryllium disease (CBD), and possibly lung cancer. Occupational and environmental exposure to beryllium continues to be a public health concern [5,6]. It has been reported that THP-1 differentiated macrophages can take up a substantial amount of beryllium and the major cellular uptake pathway is through phagocytosis of the particulate form of the beryllium compound [7]. Being a divalent metal ion with high charge density, beryllium exhibits good potential for binding to biomolecules rich in oxygen donor groups. Many of the studies on Be^{2+} and ligand complexation have relied strongly on solubility studies, potentiometric titration, and nuclear magnetic resonance (NMR) data to model solution speciation and estimate formation constants for predicted compounds [8,9]. However, the high concentration requirement (sub-millimolar to millimolar range) for the ligand greatly limits the application of these approaches for studying beryllium coordination with biomolecules. Consequently,

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few binding data have been reported for beryllium coordination with ligands.

In recent years, a variety of spectroscopic techniques have been developed to study metal and biomolecule interactions, including fluorimetry, UV–vis absorption spectroscopy, circular dichroism (CD), electron paramagnetic resonance (EPR), NMR, electrospray ionization mass spectrometry (ESI-MS), and X-ray crystal structure analysis [10–14]. Among them, fluorescence-based methods have unique advantages of high sensitivity, low sample consumption, and easy operation. The recent advancement in the synthesis of small fluorescent probes that respond to metal ions with appropriate selectivity and sensitivity has enabled researchers to interrogate metal ion chemistry and biology using fluorescence microscopy, flow cytometry, and fluorescence spectroscopy [15–18]. In 2001, a novel fluorimetric reagent for beryllium, 10-hydroxybenzo[*h*]quinoline-7-sulfonate (HBQS) was reported for the highly sensitive and selective determination of ultratrace Be^{2+} in air–dust digestion solution [19]. In the present study, we describe the development of a sensitive, straightforward, and generic competitive approach for affinity estimation of beryllium to biomolecule binding by using HBQS. The assay concept is as follows: HBQS coordinates with Be^{2+} and results in a significant fluorescence enhancement at 480 nm. If a ligand of interest is present in the solution containing Be^{2+} and HBQS, the competition of ligand against HBQS for beryllium binding results in the dissociation and therefore fluorescence decrease of the Be–HBQS complex. By titrating ligand and monitoring the dose-dependent decrease of Be–HBQS complex fluorescence, the apparent dissociation constant of Be^{2+}-ligand binding can be derived. Applying this simple approach, the binding affinities of various nucleotides and the ion-storage protein ferritin with beryllium have been determined. In particular, the apparent dissociation constant of Be^{2+} and adenosine 5′-triphosphate (ATP) binding was also validated by an ESI-MS method. The general applicability of the proposed competition assay was further demonstrated by employing a zinc fluorescent indicator, FluoZin-1, for a binding study of Zn^{2+} and bovine serum albumin (BSA). It is believed that this newly developed competitive fluorimetric assay provides a sensitive, simple, and generic method for affinity estimation of metal binding to biomolecules.

2. Experimental

2.1. Reagents and chemicals

Beryllium sulfate tetrahydrate (>99.99%) and 10-hydroxybenzo[*h*]quinoline (HBQ) were obtained from Alfa Aesar China Co., Ltd. (Tianjing, China). Horse spleen ferritin was purchased from Sigma (St. Louis, MO). FluoZin-1 was obtained from Molecular Probes (Eugene, OR). Zinc sulfate heptahydrate (>99.5%), fuming sulfuric acid (50%), concentrated sulfuric acid (GR), and hydrochloric acid (GR) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Trifluoroacetic acid (TFA, >99.5%) and methyl alcohol (>99.8%) were purchased from Tedia Company Inc. (Fairfield, OH). Tris(hydroxymethyl)aminomethane (Tris-base, >99.9%) and 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES >99.0%) were obtained from Amresco Co., (San Diego, CA). Tris–HCl buffer (adjusted to pH 7.4 with HCl) and HEPES buffer (adjusted to pH 7.4 by NaOH) were diluted to 10 mM by ultra pure water. All other reagents were obtained from Sigma (St. Louis, MO). Buffer solutions were prepared with ultrapure water supplied by a Milli-Q RG unit (Bedford, MA). The standard solution of Be^{2+} ion (100 mM) was prepared by dissolving beryllium sulfate tetrahydrate in 10 mM hydrochloric acid solution. Ten millimolar Tris–HCl (pH 7.4) was used as the dilution buffer.

2.2. HBQS synthesis and purification

HBQS was prepared similarly to a previously reported method [19]. Briefly, 0.5 g of HBQ was gradually dissolved in a mixture consisting of 0.15 mL fuming sulfuric acid (50%) and 1.3 mL concentrated sulfuric acid at below 10 °C. After stirring for 24 h at 8 °C, the mixture was poured over 50 g of crushed ice. A yellow precipitate was filtered off and dissolved in 25–40 mL of ultrapure water at pH 8–10 (typically adjusted with NaOH). Then the solution was heated to boiling and filtered immediately to remove undissolved solid (most of which was unreacted HBQ). The filtrate was diluted to 100 mL, heated to boiling and acidified with hydrochloric acid until slightly cloudy. After settling for 24 h, the suspension was filtered and washed with diluted hydrochloric acid (about 5%) followed by methanol. The final product was purified on a Shimadzu LC-8A preparative liquid chromatography with a C18 Varian Dynamax Column. A gradient mobile phase of water, methanol (from 20% to 40%), and 0.1% trifluoroacetic acid was used (different from the literature method). Lyophilized HBQS was dissolved in ultrapure water to give a stock concentration of 300 μM.

2.3. Fluorimetric titration

Fluorescence measurements were performed on an FM4P TCSPC Spectrofluorimeter (Horiba Jobin Yvon). To determine the apparent dissociation constants of metal ions binding to fluorescent probes, 1 μM HBQS (0.5 μM Fluozin-1) was incubated with various concentrations of Be^{2+} (Zn^{2+}). To study the interactions between metal ions and biomolecules, 1 μM HBQS and 2 μM Be^{2+} (0.5 μM Fluozin-1 and 2 μM Zn^{2+}) were incubated with various concentrations of biomolecules. The reagent mixture was incubated at 25 °C for 3 h prior to the fluorescence measurement. Samples containing HBQS and FluoZin-1 were excited at 380 nm and 492 nm, respectively. The fluorescence intensity was normalized for easy comparison. The apparent dissociation constants were measured by fitting the titration curves to the relevant reaction models (see Section 3 for details) using DynaFit version 3.0 (BioKin Ltd., Pullman, WA). Origin 8.0 (Microcal Software, Inc., Northampton, MA) was used for data presentation.

2.4. Mass spectrometric analysis

ESI mass spectra were recorded on a MicrOTOF-Q II mass spectrometer (Bruker) using negative ion mode. The capillary voltage was set at 3200 V and the end plate offset potential was set at −500 V. Dry gas (180 °C) flow rate was 3 L/min and the nebulizer gas pressure was 0.7 bar. The samples were continuously infused into the ESI chamber at a flow rate of 400 L/h using a KD Scientific syringe pump. Data for each sample was acquired for 0.5 min in the mass range between m/z 300 and 1000. All solvents were HPLC grade. A buffer solution containing 50% 10 mM ammonium acetate and 50% methanol (pH 7.4) was used for the preparation of samples to be analyzed by ESI-MS. In order to quantify ATP concentration from its peak height in the mass spectrum of ATP and Be^{2+} mixture, a calibration curve was first constructed. For the samples of ATP, the concentration of ATP was increased from 0 μM to 4 μM while the concentration of AMP as internal standard was kept constant at 2.0 μM. The ATP peak height in the mass spectrum was first normalized to the signal from the internal standard and then the normalized signal of ATP was plotted against ATP concentration to generate the calibration curve. In the titration experiment, 2 μM ATP and 2 μM adenosine 5′-monophosphate (AMP, internal standard) was incubated with various concentrations of beryllium sulfate tetrahydrate (0–10 μM) at 25 °C for 1 h. From the peak height ratio of ATP/AMP in the mass spectrum and the preceding calibration curve, the concentration of unbound ATP in each of the

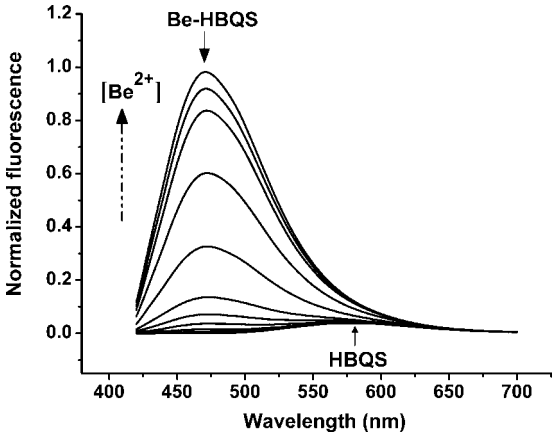


Fig. 1. Fluorescence emission spectra of the Be–HBQS chelation system. Measurements were performed in 10 mM Tris–HCl, pH 7.4. λ_{ex} = 380 nm, [HBQS] = 1.0 μM, [Be^{2+}] = 0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 100, 200 μM.

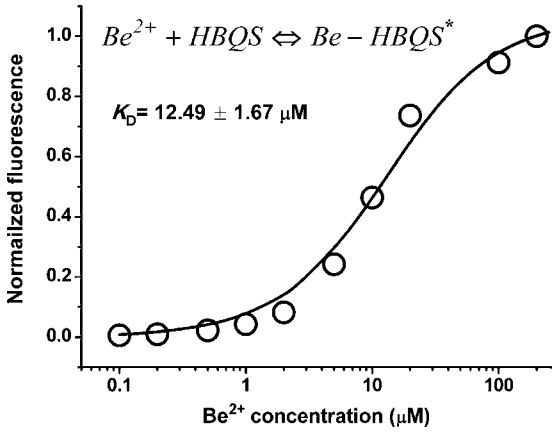


Fig. 3. Affinity determination of Be^{2+} and HBQS binding by fluorescence titration at fixed wavelength mode (λ_{ex} = 380 nm, λ_{em} = 480 nm). ○: Be^{2+} titration data. —: Fitted curve.

titrated samples can be determined, then the equilibrium concentration of Be–ATP complex can be calculated. The concentration of Be–ATP complex was plotted as a function of added beryllium concentration, and the apparent dissociation constant was obtained by fitting the titration curve to the 1:1 reaction model using DynaFit.

3. Results and discussion

3.1. Spectral characteristics, stoichiometric ratio, and apparent dissociation constant measurements for Be–HBQS complex

The fluorescence emission spectra of Be–HBQS chelation system were examined by incubating 1.0 μM of HBQS with various concentrations of Be^{2+}. As shown in Fig. 1, on beryllium binding the fluorescence maximum shifts from 580 nm for HBQS to 480 nm for the Be–HBQS complex along with a significant fluorescence enhancement. The fluorescence of Be–HBQS complex continues to rise with the increase in Be^{2+} concentration until approaching saturation. Because the fluorescence emission spectrum of Be–HBQS complex overlaps with that of HBQS, therefore at the HBQS band of 580 nm, the fluorescence signal generated by Be–HBQS complex is much stronger than that of HBQS.

It has been reported that under strongly alkaline conditions (pH 12.0), the composition of the Be–HBQS chelate is 1:1 [19]. The stoichiometric binding of HBQS to Be^{2+} under physiological conditions was determined using the continuous variation method. As shown in Fig. 2a, for the measurement using the continuous variation method, the total concentration of Be^{2+} and HBQS was kept constant at 2 μM. The concentration of Be^{2+} was varied from 0.2 to 1.8 μM while the HBQS concentration was varied from 1.8 to 0.2 μM. It was found that in 10 mM Tris–HCl pH 7.4, HBQS chelates with Be^{2+} in a molar ratio of 1:1. According to a recent report by McCleskey and Scott [20], a tetrahedral geometry is suggested for one HBQS and two H_2O coordinating with one Be^{2+} (Fig. 2b). By monitoring the fluorescence of the Be–HBQS complex at 480 nm and fitting the beryllium equilibrium titration data to the reaction model: Be^{2+} + HBQS ⇔ Be–HBQS * (* represents that the Be–HBQS complex is fluorescent at the monitoring fluorescence) (Fig. 3), the apparent dissociation constant between beryllium and HBQS binding was determined to be 12.49 ± 1.67 μM using the DynaFit program [21]. It should be noted that all the fitted curves presented in the figures were generated by the DynaFit program.

3.2. Development of a competitive fluorimetric assay for Be^{2+} and biomolecule binding study

The conceptual design of our new fluorimetric approach for beryllium and biological ligand interaction study is depicted in Scheme 1. HBQS coordinates with Be^{2+} and results in a fluorescence shift from 580 nm for HBQS to 480 nm for the Be–HBQS complex.

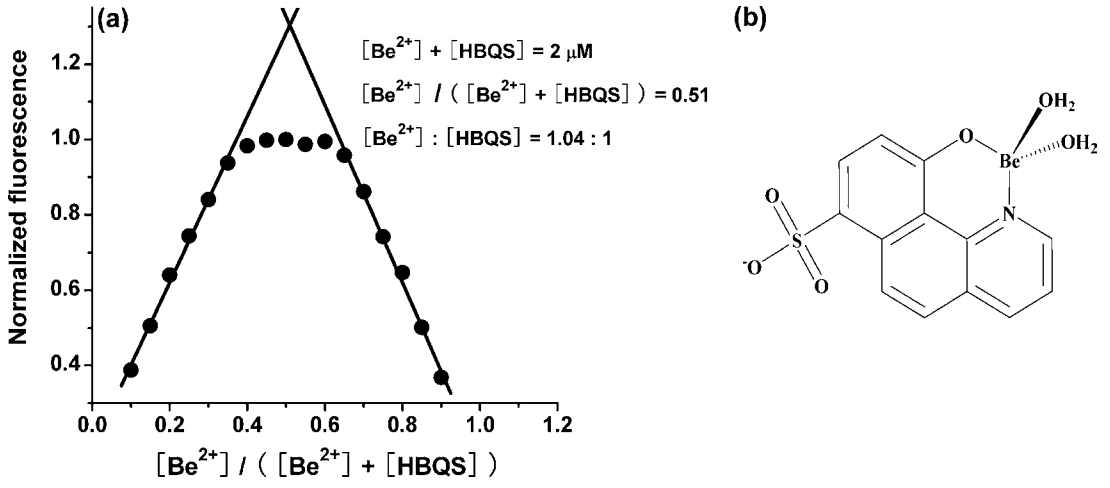
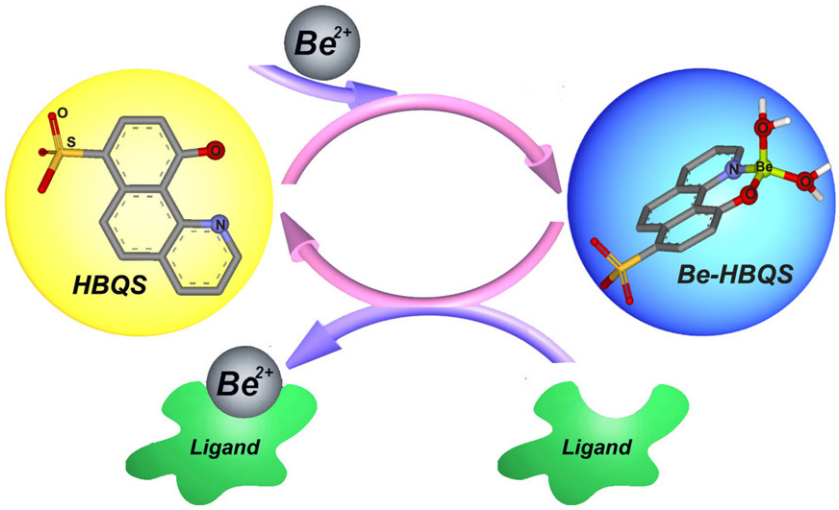


Fig. 2. (a) Stoichiometry determination of HBQS binding to Be^{2+} using the continuous variation method. λ_{ex} = 380 nm, λ_{em} = 480 nm. Buffer: 10 mM Tris–HCl, pH 7.4. (b) Suggested chelation scheme for HBQS coordination with Be^{2+} under physiological conditions.



Scheme 1. Conceptual design of the competitive fluorimetric assay for beryllium and ligand binding study.

Assuming there is a beryllium ligand present in the mixture of beryllium and HBQS, the competition of ligand against HBQS for beryllium binding will result in the dissociation and therefore fluorescence decrease of the 480 nm, the binding affinity of ligand to beryllium can be derived by fitting the titration data to the combined reaction models (1) and (2) using the Dynafit program.

$$Be^{2+} + HBQS \Leftrightarrow Be\text{–}HBQS* \quad (1)$$

$$Be^{2+} + Ligand \Leftrightarrow Be\text{–}Ligand \quad (2)$$

Nucleotides were the first type of biological ligands tested for beryllium binding because of their central role in metabolism. Usually when complexed with metals, nucleotides serve as substrates in many important biological reactions [22]. In previous studies, beryllium fluoride was found able to form an adduct with adenosine 5′-diphosphate (ADP) to inhibit F1-ATPase activity [23,24]. Therefore, it is important to study the interactions between beryllium and nucleotides. Using the proposed competitive assay, binding between ATP and Be^{2+} has been studied. As shown in Fig. 4a, when 2.0 μM of ATP is present in a mixture of 1.0 μM of HBQS with 2.0 μM of beryllium, the fluorescence signal of the Be–HBQS complex decreases by about 50%. Meanwhile, control experiments demonstrate that ATP does not interact with HBQS and ATP itself exhibits negligible background fluorescence at 480 nm. Before curve fitting using DynaFit program, the background fluorescence from free ATP was subtracted. From the ATP titration curve, the derived apparent dissociation constant of ATP for beryllium binding is 0.63 ± 0.11 μM (Fig. 4b).

3.3. Analysis of beryllium and biomolecule binding

Applying this same approach, the chemical reactivity of various nucleotides and the iron-storage protein ferritin with beryllium has been determined and the measured apparent dissociation constants (K_D = ([Be^{2+}][Ligand])/([Be–Ligand])) are listed in Table 1. It should be noted that care was taken to examine possible interactions between HBQS and ligands of interest and no interaction could be discerned from spectral analysis. In cases where the Be–ligand complex exhibits weak fluorescence at 480 nm, the fitting model was modified to accommodate this situation. As shown in Table 1, with the decrease of phosphate number, the binding affinity for Be^{2+} decreased remarkably from ATP to AMP. ADP (K_D = 3.15 ± 0.73 μM) exhibits ~5-fold less affinity for Be^{2+} as compared to ATP (K_D = 0.63 ± 0.11 μM), and AMP (K_D = 1084 ± 360 μM) only displays minute affinity for beryllium. On the other hand, different nucleosides 5′-triphosphates exhibit similar binding affinities for Be^{2+}, suggesting that triphosphate is the functional ligating group. Our results confirm that Be is chelated by adjacent oxygen atoms on adjacent phosphate groups [25]. The high binding affinity between beryllium and ATP implies a role for this interaction in the *in vivo* biochemical toxicity of beryllium. The binding of beryllium to the nucleotides have been studied by Alderighi and co-workers [25] using potentiometric titration. The logarithm of equilibrium constants was 6.52 and 5.97 for ATP and ADP, respectively. In the present study, the binding affinity calculated from the K_D values reported above are $\log_{10} K_{Be\text{–}ATP}$ = 6.2 and $\log_{10} K_{Be\text{–}ADP}$ = 5.5, which are comparable to their results. For AMP and beryllium binding, our measured apparent dissociation constant equals to a $\log_{10} K_{Be\text{–}AMP}$ of 2.96. This value is smaller than that reported by Alderigh et al. ($\log_{10} K_{Be\text{–}AMP}$ = 4.92). This discrepancy could be ascribed to the binding between ligand and Tris cation, which could influence ligand and beryllium binding significantly for a weaker beryllium binder (AMP) rather than for a strong binder (ATP or ADP).

Ferritin, the iron-storage protein in the body, has been demonstrated to bind a large variety of divalent metal ions including Be^{2+}, and to reduce the toxicity of beryllium both *in vitro* and *in vivo* [26]. Ferritin can reactivate the enzymes alkaline phosphatase, Na–K ATPase, and phosphoglucomutase inhibited by Be^{2+} [27]. As we can see from Fig. 5, ferritin competes avidly against HBQS for beryllium binding. Ferritin at 100 nM sequesters all of the 2 μM beryllium from HBQS and completely suppresses Be–HBQS fluorescence. The apparent dissociation constant of ferritin for beryllium binding was calculated to be 10.07 ± 1.16 nM. Our measured high binding affinity between ferritin and Be^{2+} provides strong support for a literature report that a Be–ferritin adduct stimulated proliferation of bronchoalveolar lavage lymphocytes from subjects with CBD at

Table 1
Measured apparent dissociation constants (K_D) for the complexes of Be^{2+} with various nucleotides and ferritin in 10 mM Tris–HCl (pH 7.4) at 25 °C.

Potential ligand	Binding to Be^{2+} K_D (μM)
ATP	0.63 ± 0.11
ADP	3.15 ± 0.73
AMP	1084 ± 360
GTP	0.62 ± 0.03
UTP	0.59 ± 0.03
CTP	0.64 ± 0.08
Horse spleen ferritin	0.010 ± 0.0012

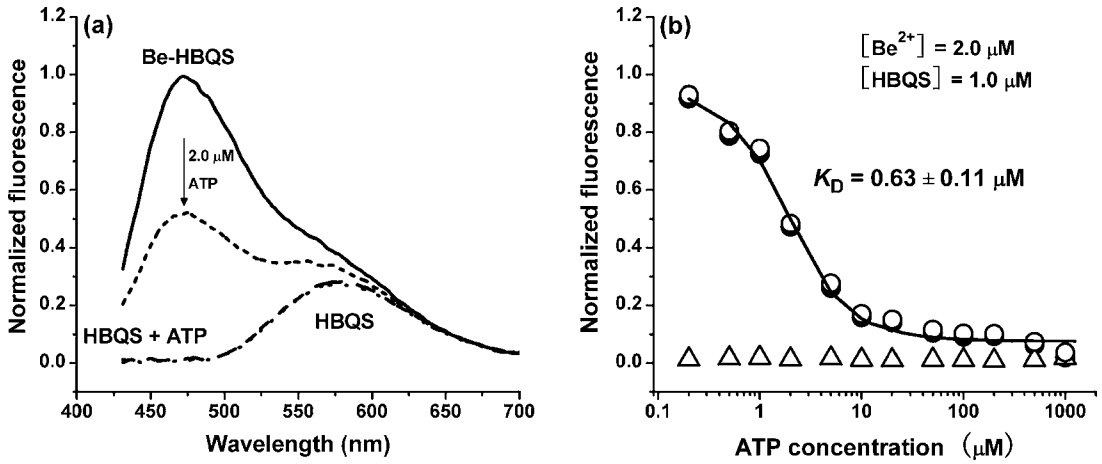


Fig. 4. (a) Fluorescence emission spectra of ATP competing against HBQS for Be^{2+} binding in 10 mM Tris–HCl, pH 7.4. λ_{ex} = 380 nm, [HBQS] = 1.0 μM, [Be^{2+}] = 2.0 μM. (b) Determination of the affinity of ATP for Be^{2+} by measuring the Be–HBQS complex fluorescence versus ATP concentration. λ_{ex} = 380 nm, λ_{em} = 480 nm. ○: Be–HBQS complex fluorescence signal. △: ATP background fluorescence. ●: Net signal. —: Fitted curve.

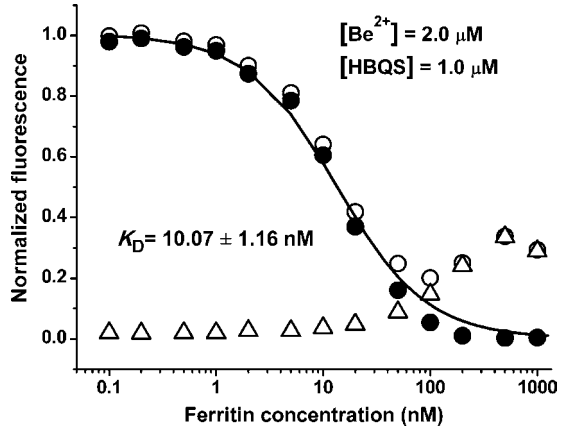


Fig. 5. Determination of the apparent affinity of ferritin for Be^{2+} by measuring the Be–HBQS complex fluorescence versus the titration of horse spleen ferritin concentration. Buffer: 10 mM Tris–HCl, pH 7.4. λ_{ex} = 380 nm, λ_{em} = 480 nm. ○: Be–HBQS complex fluorescence signal. △: Ferritin background fluorescence. ●: Net signal. —: Fitted curve.

concentrations 5–6 orders of magnitude lower than the amounts of beryllium sulfate needed to induce proliferation [28]. The authors suggested that Be–ferritin might serve as a "Trojan Horse" that promotes beryllium antigen presentation and induces Be-specific T cell proliferation.

3.4. Verification of the apparent dissociation constant between ATP and beryllium binding by ESI-MS

In the present study, an ESI-MS method [29] has been used to verify the apparent dissociation constant of ATP and beryllium binding determined by the new competitive fluorimetric assay. An ESI mass spectrum of ATP in 50% 10 mM ammonium acetate and 50% methanol (pH 7.4) is shown in Fig. 6a, in which a major peak at *m*/*z* 506.0 (representing $[ATP–H]^-$) is seen. When ATP is mixed with beryllium ions, a new peak corresponding to a 1:1 ratio of ATP–beryllium complex (*m*/*z* 513.0) appears and the peak height increases with increase of beryllium concentration. The concentration of ATP–beryllium complex can be calculated by the method described in the experimental section with AMP added to each sample as an internal standard. Due to the ~1700-fold affinity

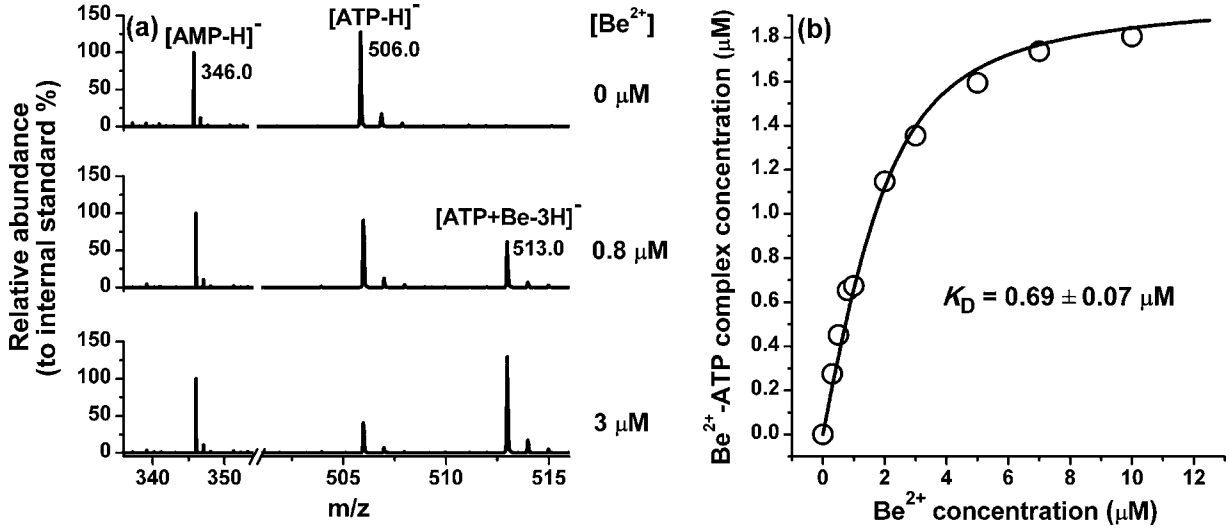


Fig. 6. (a) Negative ion mode ESI mass spectra of the titration of 2.0 μM ATP with various concentrations of beryllium ions. (b) Determination of the affinity of ATP for Be^{2+}. The calculated ATP–beryllium complex concentration is plotted as a function of the added beryllium ion concentration. AMP was added in every sample as an internal standard. Buffer: 50% 10 mM ammonium acetate and 50% methanol (pH 7.4). ○: ATP–beryllium complex concentration. —: Fitted curve.

difference between ATP and AMP for beryllium binding, the disruption of AMP to the binding equilibrium between ATP and Be^{2+} can be ignored. By plotting the concentration of the ATP–beryllium complex against added beryllium concentration and fitting the titration curve to the 1:1 reaction model using the DynaFit program, an apparent dissociation constant of 0.69 ± 0.07 μM was derived (Fig. 6b), which is in very good agreement with the result obtained by the competitive fluorimetric method.

3.5. General applicability of the competitive fluorimetric assay for metal–biomolecule interaction study

The general applicability of the developed competitive fluorimetric approach using a metal specific fluorescent indicator for metal:biomolecule interaction studies is further demonstrated by a Zn^{2+} to BSA binding analysis. In the plasma, approximately two-thirds of the zinc present is bound to albumin. By competitive spectrophotometry, the conditional dissociation constant of Zn^{2+} for BSA binding has been reported as $K_D = 0.1$ μM ($\log_{10} K_{Zn-BSA} = 7.0 \pm 0.02$) at pH 7.74 with the ionic strength at 0.1 [30]. FluoZin-1 is a fluorescent indicator for Zn^{2+}, forming a 1:1 complex with Zn^{2+}. The free dye is only very weakly fluorescent and the fluorescence is enhanced >100-fold upon binding to Zn^{2+} (Molecular Probes Handbook). In the present study, by fitting the titration curve of Zn^{2+} with FluoZin-1 to the 1:1 reaction model using DynaFit, the apparent dissociation constant of Fluozin-1 for Zn^{2+} binding is found to be 20.9 ± 1.9 μM in 10 mM HEPES, 100 mM NaCl, pH 7.4 (data not shown). Then, BSA is titrated into the solution containing Zn^{2+} and Fluozin-1 and the dose-dependent decrease of the Zn–Fluozin-1 complex fluorescence signal was monitored. The apparent dissociation constant of BSA for Zn^{2+} binding is calculated as 0.10 ± 0.03 μM, which is in excellent agreement with the literature report [30]. Compared to literature reported competition assays for metal and protein binding study, this newly developed approach is more sensitive and direct. First, the current approach is based on the analysis of fluorescence changes of the metal–probe complex instead of intrinsic protein fluorescence quenching [31,32] or changes in the UV–vis absorbance of the metal indicator [32–35]. Second, the biological ligand is titrated instead of metal ion [31,36], which makes interpretation of the data more straightforward.

4. Conclusion

In summary, by employing a Be^{2+}-specific fluorescent probe, a simple fluorimetric approach is developed for affinity estimation of Be^{2+} and biomolecule binding. More importantly, this competition assay can be directly applied to the study of any other metal ions interacting with biomolecules as long as a metal specific fluorigenic probe can be provided. Compared to other approaches for metal and biomolecule interaction studies, the newly developed fluorescent competition assay is sensitive, easy to perform, and does not require any derivatization or labelling of biomolecules, which may affect the accuracy of the binding measurements. It is believed that this approach provides a good tool for the study of the biological effects of metal ions.

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