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A quick and colorful method to measure low-level contaminations of paramagnetic Ni²⁺ in protein samples purified by immobilized metal ion affinity chromatography

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Abstract

Isotopic labeling of recombinantly expressed proteins is generally required for investigation by modern NMR methods. Purification strategies of the labeled proteins often include the use of a poly-histidine affinity tag (his-tag) and immobilized metal ion affinity chromatography (IMAC). Described herein are rapid and inexpensive qualitative and quantitative assays to determine the concentration of paramagnetic Ni²⁺ in protein samples purified by IMAC. Both qualitative and quantitative colorimetric methods detect the amount of Ni²⁺ via the color change produced when a [Ni(PAR)_n]²⁺ (PAR = 4-(2-pyridylazo)resorcinol, n = 1, 2) complex is formed. The qualitative assay provides a rapid visual test for the presence of Ni²⁺ in the low micromolar range in a sample of interest. The usefulness of the spectroscopic quantitative assay is illustrated by: (i) detecting a 12 μM Ni²⁺ contamination in a NMR sample containing 950 μM of the 7.5 kDa α₃W protein purified by a standard his-tag Ni²⁺/IMAC approach and (ii) showing that the ¹⁵N-HSQC spectrum of the α₃W NMR sample, containing one paramagnetic Ni²⁺ ion per 80 protein molecules, displays clear line broadening of both water and protein spectral lines. We also (iii) measured Ni²⁺ release during the equilibration, wash and elution steps of three commonly used Ni²⁺/IMAC resins when following manufacturer protocols. The concentration of Ni²⁺ detected in elutes of the three resins ranged from 2 μM to nearly 1 mM.

Keywords

Immobilized metal ion affinity chromatography; IMAC; Ni²⁺ leakage; 4-(2-pyridylazo)resorcinol; PAR; protein metal ion contaminants; purification of his-tagged proteins

1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy has an inherently low sensitivity and NMR-based structural biology studies typically require tens of milligrams of purified material. Protein production generally involves recombinantly expressed proteins and purification strategies based on affinity chromatography. Proteins engineered to contain a

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poly-histidine affinity tag (His-tag) and purified using immobilized metal ion affinity chromatography (IMAC) represents a major protein production strategy in protein NMR and generally across the biophysical and biochemical research fields. IMAC resins are often charged with paramagnetic metal ions. For example, paramagnetic Co^{2+} , Ni^{2+} or Cu^{2+} are the most commonly used metal ions for purification of His-tagged proteins. All charged IMAC resins have a propensity for metal ion leakage, although it varies depending on the specific chemical properties of the IMAC system and the solution conditions used. Thus, co-purification of sub-stoichiometric amount of a metal ion non-specifically bound to the target protein presents a significant possibility. This situation is of particular concern for the protein NMR field since even a very minor contamination of a paramagnetic species may give rise to undesirable spectral perturbations such as line broadening of water and protein resonances. Here we present quickly and easily conducted qualitative and quantitative colorimetric assays to check Ni^{2+} /IMAC purified protein material for micromolar contaminations of Ni^{2+} .

IMAC separates proteins present in the solvent phase according to their affinity for metal ions such as Fe^{3+} , Co^{2+} , Ni^{2+} , Cu^{2+} and Zn^{2+} that have been immobilized by a metal-chelating agent covalently bound to the stationary matrix. The IMAC approach was based on the known affinities of aqueous histidine and cysteine to coordinate transition metal ions (Hearon, 1948) and the novel idea of using immobilized metal ions to selectively bind peptides and proteins with exposed histidine and cysteine residues. In the proof of concept study, Porath et al. used iminodiacetate (IDA) as the chelating agent to fix Ni^{2+} and Cu^{2+} to agarose and successfully demonstrated fractionation of human serum proteins using the developed M^{2+} -IDA/agarose resins (Porath, Carlsson, Olsson & Belfrage, 1975). The IMAC protein purification approach was validated and further developed over the following years and gained general recognition (Sulkowski, 1985, Porath, 1992). Hochuli et al. introduced nitrilotriacetate (NTA) as a metal-chelating agent and showed that the new Ni^{2+} -NTA/sepharose resin had a high specificity for peptides and proteins containing adjacent histidines (Hochuli, Döbeli, & Schacher, 1987). In a subsequent publication, mouse dihydrofolate reductase (DHFR) was engineered to contain poly-histidine tags and efficient isolation of the expressed DHFR/His-tag fusions on the Ni^{2+} -NTA/sepharose resin was shown (Hochuli, Bannwarth, Döbeli, Gentz & Stüber, 1988). Thus, the application of recombinant techniques and the concept of engineered IMAC affinity tags (Smith, Furman & Pidgeon, 1987, Smith, Furman, Ingolia & Pidgeon, 1988, Hochuli et al., 1988) refined the IMAC methodology towards the well-established and widely used protein purification tool it is today.

The metal-chelating agent binds the metal ion to the supporting matrix and modulates the metal ion/protein interactions (Porath, 1992). The original IDA and NTA chelators are used in many commercial IMAC resins and protein purification kits including, for example, IDA-containing HiTrap™ products developed by Amersham Pharmacia Biotech and NTA resins from Qiagen, Thermo Fisher Scientific, Bio-Rad Laboratories etc. Commonly used chelators are tridentate (e.g. IDA), tetradentate (e.g. NTA) or pentadentate (e.g. carboxymethylated aspartate used in Co^{2+} -TALON resins, Clontech Laboratories). Upon charging the resin, the chelator binds the added metal ion while leaving some coordination sites available for protein ligation. In general, the tetradentate and pentadentate chelators have higher affinity

for the metal ion (i.e. less metal ion leakage), but they exhibit lower protein binding capacity due to having fewer protein coordination sites. When choosing an IMAC system for a specific protein, there are multiple factors to consider including the protein binding capacity and selectivity of the IMAC resin, suitable protein elution conditions, the potential need for downstream purification steps, and cost. The degree of metal ion leakage is thus only one of several parameters to consider for optimized protein production.

An assay to screen for contaminating Ni^{2+} ions that may co-purify with a target protein should ideally be rapid and easy to perform and require little protein material. Here we used commercially available 4-(2-pyridylazo)resorcinol (PAR) as the active reagent in qualitative and quantitative colorimetric assays to screen for possible Ni^{2+} contaminations in protein samples whose purification protocols include Ni^{2+} /IMAC. The sensitivity of the developed method is illustrated by checking protein NMR samples for low-level Ni^{2+} contaminations. We show that Ni^{2+} released during a typical IMAC purification step co-purifies with the target protein through a subsequent reversed phase HPLC step and affects NMR spectral properties at concentrations as low as one Ni^{2+} ion per 80 protein molecules. The usefulness of the developed method is further illustrated by measuring Ni^{2+} leakage from three commercially available and commonly used IMAC resins. Either protocol described below provides a straightforward method to check a protein purification midstream or an NMR sample and to exclude the possibility of spectral degradation due to a minor contamination of paramagnetic Ni^{2+} .

2. Materials & Methods

CELLSTAR 50 mL light-impermeable centrifuges tubes were purchased from Greiner Bio-One via VWR (catalog number 82051–630). Sodium acetate- d_3 (99 atom % D) was purchased from Cambridge Isotope Laboratories (DLM-3126). His•Bind resin (Novagen) was purchased from EMD Millipore Corporation (69670). Ethylenediaminetetraacetate dipotassium salt extra pure (EDTA, AC209745000), His60 Ni Superflow resin (Clontech Laboratories) (NC0319531), imidazole analytical grade (AC396745000), NaCl extra pure (AC194090010), and Tris-HCl extra pure (AC228030010) were purchased from ACROS Organics via Fisher Scientific. $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ (N73500) and NaH_2PO_4 (S369), both ACS Reagent grade, and GE Healthcare Sephadex G-25 PD10 columns (45–000–148) were from Fisher Scientific. Acetonitrile HPLC plus (34998), cOmplete His-Tag Purification resin (Roche; 5893682001), D $_2$ O (99.9 atom % D; 613444), 2,2-dimethyl-2-silapentane-5-sulfonate (DSS; 178837), NaCl BioXtra (S7653), 4-(2-pyridylazo)resorcinol monosodium salt monohydrate (PAR; 1075330005), and trifluoroacetic acid for HPLC (302031) were purchased from Sigma-Aldrich. 5 mm Precision NMR tubes were purchased from Wilmad via Sigma-Aldrich (Z412015) and Spectrosil quartz cuvettes with 1 (21-Q-1), 2 (21-Q-2), 5 (9-Q-5) and 10 (3-Q-10) mm pathlengths were purchased from Starna Cells Inc. $\alpha_3\text{X}$ proteins are expressed in fusion with thioredoxin (Dai, Tommos, Fuentes, Blomberg, Dutton & Wand, 2002) in *E. coli* BL21-CodonBlue (DE3)-RIL grown on LB or minimal media and purified as described previously (Martínez-Rivera, Berry, Valentine, Westerlund, Hay & Tommos, 2011).

3. Method to determine micromolar Ni²⁺ contaminations in protein samples

3.1 Description of the qualitative visually-based colorimetric assay to screen for Ni²⁺

The qualitative assay described in this section utilizes the observed differences in color between two solutions: a control containing 50 μM of free PAR and a sample containing 50 μM PAR dissolved in the protein solution of interest. The colorimetric scale for this assay is shown in Figure 1 where the Ni²⁺ concentration spans a range of 0 to 37.5 μM in a solution containing 50 μM PAR. As the Ni²⁺ concentration and the nickel to PAR (Ni:PAR) ratio increase, the color of the solution shifts from bright yellow to yellow-orange to coral. Each of the twelve samples shown in Figure 1 was made by titrating together 1 mL of a 100 μM PAR stock solution and 1 mL of a buffered solution containing 0 – 75 μM Ni²⁺. These 1:1 volumetric titrations result in final concentrations of 50 μM PAR and 0 – 37.5 μM Ni²⁺. By following steps 3.2.1 to 3.2.5 described below, protein samples can be screened for micromolar Ni²⁺ contaminations by a simple visual comparison to the colorimetric scale shown in Figure 1.

The upper detection limit for Ni²⁺ is set by the final PAR concentration in the sample. With a Ni²⁺ to PAR binding stoichiometry of 1:2, the upper limit for Ni²⁺ detection is predicted to be around 25 μM for a solution containing 50 μM PAR. When the final concentration is above 25 μM Ni²⁺, essentially all of the PAR molecules are coordinated to Ni²⁺ and the samples will be coral in color. Section 3.3.1 provides a more detailed discussion of the binding behavior of PAR to Ni²⁺.

Increasing or decreasing the concentration of the PAR stock solution will raise or lower the Ni²⁺ detection limit, respectively. There are two advantages to carrying out the qualitative assay with a final concentration of 50 μM PAR: (a) the intensity of the colors allows them to be readily distinguished from one another and (b) the samples from the qualitative assay are at ideal concentrations for the spectroscopic quantitative assay described in Section 3.5., should such analysis be needed.

3.1.1 Preparation of a 100 μM PAR stock.—25.55 mg of 4-(2-pyridylazo)resorcinol monosodium salt monohydrate (molecular weight 255.21 g mol⁻¹) was weighed using a micrometer balance and dissolved in 100 mL of milliQ water using a volumetric flask to give a 1.001 mM PAR solution. 5 mL of 1.001 mM PAR was diluted to 50 mL using milliQ water in a volumetric flask to give 100 μM PAR. PAR stock solutions were stored in 50 mL black and opaque polypropylene tubes designed for light exclusion. No spectral changes were observed for 100 μM PAR/milliQ stock solutions recorded on the day of preparation and one month later.

3.1.2 Tip for measuring small masses.—It is good practice to weigh masses that are well within the bounds of the balance you are using. If possible, use a calibrated micrometer balance for measuring out mg quantities of PAR.

3.1.3 Optional preparations of Ni²⁺ and Ni²⁺/PAR solutions.—1.319 g of NiSO₄•6H₂O (molecular weight 262.84 g mol⁻¹) was dissolved into 100 mL of Buffer A (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) using a volumetric flask to give a 50.2 mM Ni²⁺ stock solution. Serial dilutions using volumetric glassware and calibrated automatic pipettors were performed to obtain a concentration series ranging from 1 – 75 μM Ni²⁺ in Buffer A.

To generate a colorimetric series like the one shown in Figure 1, titrate 1 mL of the 100 μM PAR stock and 1 mL of a Ni²⁺ standard into a 5 mL microcentrifuge tube or glass vial. Solutions prepared in our laboratory maintain their color for several months. Importantly, [Ni(PAR)_n]²⁺ (n = 1, 2) solutions should be buffered and have a pH in the 7 – 10 range to prevent precipitation.

3.2 Protocol for the qualitative visually-based colorimetric assay to screen for Ni²⁺

3.2.1 Prepare the PAR stock solution: Prepare 100 μM PAR in milliQ water or in a pH 7 – 10 buffer (see Section 3.1.1).

3.2.2 Prepare the free PAR control: Titrate 1:1 volumetric ratios of the 100 μM PAR stock and the buffer used for the protein sample into a translucent vial. The final pH should be > 7.0. The control should be a vibrant yellow in color.

3.2.3 Prepare the protein-PAR sample: Titrate 1:1 volumetric ratios of the 100 μM PAR stock and the protein sample into a translucent vial. Gently mix and let the protein-PAR sample sit for 5 – 10 minutes or until its color does not change. The final pH should be > 7.0.

3.2.4 Observe differences in color: Visually compare the PAR control and the protein-PAR sample against a white background. The Ni²⁺ concentration in the protein sample can be estimated from Figure 1 or using a colorimetric series prepared using a buffer system of choice (see Section 3.1.3).

3.2.5 Tips for low and high Ni:PAR ratios.—It can be challenging to visually detect color changes associated with small Ni:PAR ratios (0.1:10 – 0.3:10, see Figure 1). In this situation, it is better to use the quantitative assay described below for a more accurate determination of the Ni²⁺ concentration. When the Ni:PAR ratio is 4:10 or greater, the samples will be coral in color as shown in Figure 1. This indicates that all, or nearly all, PAR molecules are coordinated to Ni²⁺. If step 3.2.4 results in coral colored samples, dilute the sample at least 4× and repeat steps 3.2.3 and 3.2.4 until the protein-PAR sample is yellow to yellow-orange.

3.3 Description of the spectroscopic assay to quantitate possible Ni²⁺ contaminations

3.3.1 Spectroscopic and binding behavior of free PAR and nickel-bound PAR.—The properties of PAR make it a generally useful indicator for determining micromolar concentrations of transition metal cations (Mⁿ⁺) in aqueous samples (McCall & Fierke, 2000). PAR is a tridentate ligand known to coordinate metal ions in Mⁿ⁺ to PAR stoichiometries of 1:1 and 1:2, as illustrated in Figure 2. The resulting Mⁿ⁺/PAR complexes

typically display good water solubility at neutral to alkaline pH and high molar extinction coefficients ($\epsilon_{\text{max}} \sim 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). The pH dependence of the spectral properties of free PAR and M^{2+} /PAR, including Co^{2+} , Ni^{2+} and Cu^{2+} -coordinated PAR, has been reported previously (Kocyla, Pomorski & Kr̩el, 2015). PAR has three acid-base groups with aqueous $\text{pK}_a(1)$, $\text{pK}_a(2)$, and $\text{pK}_a(3)$ values of 2.9, 5.4 and 12.1, respectively, as displayed in Figure 2. The mole fraction of the mono-protonated species is > 0.95 for the pH 7.0 – 10.5 range (Kocyla et al., 2015) and this state exhibits a characteristic absorption maximum at 413 nm, as shown in Figure 3A (black spectrum). We determined a ϵ_{413} of $32,900 \pm 500 \text{ M}^{-1} \text{ cm}^{-1}$ for PAR in milliQ water. This value was obtained by using a $100 \mu\text{M}$ PAR stock made as described in Section 3.1.1 and preparing a concentration series ranging from 2 – $100 \mu\text{M}$ PAR via large-volume serial dilutions. Cuvettes with pathlengths of 0.2, 0.5 and 1.0 cm were used such that the optical density (OD) did not exceed unity. An ϵ_{413} was determined from the linear dependence in the $\text{OD}_{413}/\text{pathlength}$ values versus the PAR concentration. Upon binding to M^{2+} , the $\text{pK}_a(3)$ group deprotonates and the M^{2+} /PAR system exhibits a significantly red-shifted spectrum with an absorption maximum in the 490 – 510 nm range depending on the specific M^{2+} ion (Gómez, Estela, Cerdà & Blanco, 1992; McCall & Fierke, 2000, Kocyla et al., 2015). Spectral changes induced by the Ni^{2+} to PAR complexation are shown in Figure 3A (colored spectra).

$[\text{Ni}(\text{PAR})_2]^{2+}$ formation is governed by the following equilibria:

Recently, Kr̩el and coworkers presented M^{2+} /PAR titration curves obtained at multiple points across a pH 7.0 – 9.9 range (see Figure 4 in Kocyla et al., 2015). Mn^{2+} , Cu^{2+} , Zn^{2+} , Pd^{2+} , Cd^{2+} and Hg^{2+} to PAR complexation reactions were characterized by titration curves with significant curvature and pH dependence. This behavior is consistent with an increasing presence of the mono-coordinated $[\text{M}(\text{PAR})]^{2+}$ species as the M^{2+} :PAR ratio increases and approaches a 1:2 value. In contrast, Ni^{2+} to PAR and Co^{2+} to PAR complexation gave rise to linear and pH-insensitive titration curves. Thus, free PAR and double-coordinated $[\text{Ni}(\text{PAR})_2]^{2+}$ are the dominant species when PAR is in excess to Ni^{2+} and the $[\text{Ni}(\text{PAR})_2]^{2+}$ complex is highly stable at neutral to alkaline pH (dissociation constant $< 7 \times 10^{-13} \text{ M}^2$, Kocyla et al., 2015).

3.3.2 Determining effective molar extinction coefficients and the upper Ni^{2+} detection limit.—Figure 3A shows a series of spectra measured for samples containing $50 \mu\text{M}$ PAR and 0 – $50 \mu\text{M}$ Ni^{2+} in 25 mM NaH_2PO_4 , 150 mM NaCl buffer at pH 8.0. The OD at the absorption maxima for free PAR (413 nm) and nickel-bound PAR (495 nm) are plotted as a function of the Ni^{2+} concentration in Figure 3C. The difference spectra displayed in Figure 3B were obtained by subtracting the spectrum recorded for the $0 \mu\text{M}$ Ni^{2+} sample from the spectra collected from the 0 – $50 \mu\text{M}$ Ni^{2+} samples. Figure 3D displays the resulting difference OD (ΔOD) values, reflecting the depletion of free PAR at 408 nm and the formation of nickel-bound PAR at 495 nm, as a function of the Ni^{2+} concentration. The

titration plots exhibit a clear linear dependence in the 0 – 20 μM Ni^{2+} range. The slopes of the displayed linear fits correspond to the effective molar extinction coefficients (ϵ^{eff} , panel C) and the effective difference molar extinction coefficients ($\Delta\epsilon^{\text{eff}}$, panel D). The ϵ^{eff} and $\Delta\epsilon^{\text{eff}}$ values capture changes in the PAR absorbance due to formation of $[\text{Ni}(\text{PAR})_n]^{2+}$ ($n = 1, 2$) as the Ni^{2+} content increases. We found $\epsilon_{413}^{\text{eff}} = -55,000 \pm 400 \text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon_{495}^{\text{eff}} = 72,000 \pm 200 \text{ M}^{-1}\text{cm}^{-1}$ for the 25 mM NaH_2PO_4 , 150 mM NaCl, pH 8.0 buffer system used here. The difference spectra analysis produced consistent numbers with $\Delta\epsilon_{408}^{\text{eff}} = -54,000 \pm 400 \text{ M}^{-1}\text{cm}^{-1}$ and $\Delta\epsilon_{495}^{\text{eff}} = 72,000 \pm 200 \text{ M}^{-1}\text{cm}^{-1}$. Effective ϵ and $\Delta\epsilon$ values for other aqueous Ni^{2+} /PAR systems have been reported previously ($\epsilon_{495}^{\text{eff}} = 72,000 \text{ M}^{-1}\text{cm}^{-1}$ for a 50 mM NH_3 solution, Gómez, Estela, Cerdà & Blanco, 1992; $\epsilon_{495}^{\text{eff}} = 61,100 \pm 400 \text{ M}^{-1}\text{cm}^{-1}$ for 50 mM HEPES, 100 mM NaCl, pH 7.4, Kocyla et al., 2015).

The linear region of the titration data shown in Figure 3 also defines an upper detection limit of 20 μM Ni^{2+} when the concentration of PAR is 50 μM . The lower detection limit for our method will be discussed in Section 4.1. In general, as long as the Ni^{2+} :PAR ratio is $\geq 4:10$, ϵ^{eff} can be used to determine the Ni^{2+} concentration without the risk of underestimating the amount of Ni^{2+} in a given sample. The ϵ^{eff} and $\Delta\epsilon^{\text{eff}}$ values listed above were determined for 25 mM NaH_2PO_4 , 150 mM NaCl, pH 8.0. The experimental data set shown in Figure 3A and the analysis illustrated in Figures 3B – D can be reproduced using other buffer conditions, if required for a specific protein of interest. A key advantage of using effective ϵ and $\Delta\epsilon$ values is that one need not delineate the individual concentrations of $[\text{Ni}(\text{PAR})]^{2+}$ and $[\text{Ni}(\text{PAR})_2]^{2+}$ for an accurate determination of the Ni^{2+} concentration.

3.4 Description of the spectroscopic assay to quantitate possible Ni^{2+} contaminations

The quantitative colorimetric assay involves a spectroscopic comparison of two solutions: a control containing 50 μM free PAR and a sample containing 50 μM PAR dissolved in the protein solution of interest. Spectra of the two samples are recorded and a difference spectrum is obtained by subtracting the PAR spectrum from the protein-PAR spectrum. Using Beer-Lambert's law and the effective ϵ_{495} obtained in section 3.3.2, the concentration of nickel-bound PAR can be calculated. Samples prepared for the qualitative protocol can be utilized as-is for the quantitative protocol described below (Section 3.5).

Different solvent conditions (buffer, pH, polarity, ionic strength) can influence the spectral properties of PAR and may cause shifts in peak maxima by a few nm. A 1–2 nm variance is not unusual for different spectrometers. Thus, make sure to use values at the observed peak maximum for the (protein-PAR - PAR) difference spectra to make the Ni^{2+} concentration calculations. Using a 100 μM PAR stock solution sets an upper threshold of 20 μM for the Ni^{2+} content to be reliably measured. The final pH should be ≥ 7.0 for the PAR control and the protein-PAR samples studied.

3.5 Protocol for the spectroscopic assay to quantitate possible Ni²⁺ contaminations

3.5.1 Prepare the PAR stock solution: Prepare 100 μ M PAR in milliQ water or in a pH 7 – 10 buffer (see Section 3.1.1).

3.5.2 Prepare the free PAR control: Titrate 1:1 volumetric ratios of the 100 μ M PAR stock and the buffer used for the protein sample into a translucent vial. The final pH should be 7.0. The control should be a vibrant yellow in color.

3.5.3 Prepare the protein-PAR sample: Titrate 1:1 volumetric ratios of the 100 μ M PAR stock and the protein sample solution into a translucent vial. Gently mix sample and let stand for 5 – 10 minutes or until color does not change. The final pH should be 7.0.

3.5.4 Prepare the spectroscopic blank: Titrate 1:1 volumetric ratios of the solution used to prepare the 100 μ M PAR stock and the buffer used for the protein sample.

3.5.5 Follow Step 3.2.5 if the protein-PAR sample is coral in color.

3.5.6 Record UV-Vis spectra and obtain a difference spectrum: Measure the spectra of the PAR control and the protein-PAR samples from 200 – 700 nm using 0.5 cm cuvettes. If using a double-beam spectrometer (recommended) remember to use the blank sample prepared in step 3.5.2. Subtract the PAR control spectrum from protein-PAR spectrum and note the difference in absorbance at 495 nm (ΔAbs_{495}).

3.5.7 Use Beer-Lambert's law to calculate the concentration of Ni²⁺:

$$[\text{Ni}^{2+}] = \frac{\Delta\text{Abs}_{495}}{\ell \times \epsilon_{495}^{\text{eff}}} \times D = \frac{\Delta\text{Abs}_{495}}{0.5 \times 72000} \times 2$$

where ℓ is the cuvette pathlength (in cm), $\epsilon_{495}^{\text{eff}}$ is the effective difference extinction coefficient at 495 nm ($\text{cm}^{-1} \text{M}^{-1}$), Abs_{495} is the difference in absorption at 495 nm (OD), defined as $\Delta\text{Abs}_{495} = \text{Abs}_{495}^{[\text{Ni}(\text{PAR})_n]^{2+}} - \text{Abs}_{495}^{\text{PAR}}$, and D is the dilution factor. Use D = 2 if the screened protein material/sample was only diluted in the 1:1 volumetric titration of step 3.5.3. If the screened protein material/sample underwent additional dilutions, correct D accordingly.

3.5.8 Tip for obtaining good quality difference spectra: The use of a double-beam spectrophotometer is highly recommended. It is important that the buffer composition in the blank, the PAR control and the protein-PAR sample are (near) identical. The difference spectra should have a baseline centered around zero (e.g. see isosbestic point at 445 nm and the 600 – 700 nm region in Figure 3B or Figure 4). Difference spectra not centered on the x-axis indicate that good quality baseline and/or PAR and protein-PAR spectra were not obtained.

3.5.9 Interpreting difference spectra: A negative intensity at ca. 410 nm indicates the depletion of free PAR while the positive intensity at 495 nm indicates the formation of

$[\text{Ni}(\text{PAR})_n]^{2+}$ ($n = 1, 2$). A flat line for difference spectra would indicate equal concentrations of free PAR in the control and in the protein-PAR sample and, consequently, no Ni^{2+} in the latter. Figure 4A and B illustrate these effects, as described in Section 4.1 below.

4. Two examples of useful applications for the developed colorimetric method

4.1 Checking NMR protein material for Ni^{2+} .

Protein NMR samples are often precious and the general guidelines presented above can be modified to preserve material. The following protocol to check and prepare NMR samples works well for the $\alpha_3\text{X}$ family of Ni^{2+} /IMAC purified model proteins developed by the Tommos group (Martínez-Rivera et al., 2011, Berry, Martínez-Rivera & Tommos, 2012, Tommos, Valentine, Martínez-Rivera, Liang & Moorman, 2013, Glover, Jorge, Liang, Valentine, Hammarström & Tommos, 2014, Ravichandran, Zong, Taguchi, Nocera, Stubbe & Tommos, 2017): Place 2.5 mL 50 mM sodium acetate- d_3 , 50 mM NaCl, pH 5.7 buffer in a disposable UV cuvette and titrate freeze-dried $\alpha_3\text{X}$ powder to a protein concentration of about 200 μM . Remove 50 μL for Ni^{2+} analysis. Add EDTA to a final concentration of 220 μM , let the sample sit for 15 minutes, and buffer exchange using a Sephadex G-25 PD10 column equilibrated with 3 mM sodium acetate- d_3 , 3 mM NaCl, pH 5.7 buffer. Remove 50 μL for Ni^{2+} analysis. Freeze-dry the buffer-exchanged sample and dissolve in 500 μL H_2O and/or D_2O containing 75 μM DSS. Check and adjust pH.

Figure 4 shows a Ni^{2+} screen of ^{15}N -labeled $\alpha_3\text{W}$ prepared using this protocol. For the spectroscopic assay, it is important that the blank contains the same final buffer composition as the PAR and protein-PAR samples to ensure good baseline recording and spectral subtraction results. If done carefully and using a double-beam spectrometer, a Abs_{495} of a few mOD can be detected. This is illustrated in Figure 4. A Ni^{2+} content of 2.5 μM was determined for the 50 μL , 200 μM $\alpha_3\text{W}$ aliquot removed prior to the EDTA step (Figure 4A). No Ni^{2+} could be detected for the 50 μL , 130 μM $\alpha_3\text{W}$ aliquot removed after the EDTA step (Figure 4B). More experimental details are provided in the Figure 4 legend. Notably, when screening multiple batches of purified $\alpha_3\text{X}$ proteins we consistently observed the Ni^{2+} content to vary by one order of magnitude even when using the same type of Ni^{2+} /IMAC resin and set of stock solutions. We estimate that the lower detection limit is about 0.3 – 0.5 μM Ni^{2+} for the spectroscopic assay. Practically, the lower limit will vary somewhat depending on the quality of the spectrophotometer, the Ni^{2+} to target protein stoichiometry, protein availability and/or cuvette pathlength used.

4.2 NMR spectral line broadening due to low-level Ni^{2+} contaminations.

Figures 5A and B present ^{15}N -HSQC spectra of $\alpha_3\text{W}$ samples prepared from the same material that was used for the Ni^{2+} screen. The NMR samples were made excluding (Figures 5A and C) and including (Figures 5B and D) an EDTA treatment step. A Ni^{2+} contamination of $\sim 12 \mu\text{M}$, or one metal ion per 80 protein molecules, results in a clearly observable line broadening of both water and protein resonances. The positive and negative contour levels are identical for the blue and the red spectra and emphasize the more prominent background

water streaks (colored black) in the blue spectrum representing the Ni^{2+} -containing sample. Additionally, the average peak height is lower and the peak intensities more uneven in the spectrum from the Ni^{2+} -containing sample (Figure 5C) as compared to EDTA-treated sample (Figure 5D).

4.3 Measuring Ni^{2+} release from three common IMAC resins.

The $\alpha_3\text{X}$ proteins are purified using two Ni^{2+} /IMAC steps. The first step isolates the expressed thioredoxin-His₆-thrombinsite- $\alpha_3\text{X}$ fusion and the second step separates the thioredoxin-His₆ part from $\alpha_3\text{X}$ following thrombin digestion. The $\alpha_3\text{X}$ -containing flow-through fraction from the second Ni^{2+} column is further purified by reversed-phase HPLC using a semi-preparative C18 column and a linear 30–60% acetonitrile/water/0.1% trifluoroacetic acid gradient. Despite the final HPLC step, Ni^{2+} co-purifies with the $\alpha_3\text{X}$ proteins as described above. To refine the $\alpha_3\text{X}$ purification protocol and minimize Ni^{2+} co-purification, we investigated Ni^{2+} leakage from three IMAC products: His•Bind resin (Novagen; chelator chemistry IDA, stationary matrix agarose, binding capacity 8 mg protein/mL resin), Ni^{2+} preloaded His60 Ni Superflow resin (Clontech; chelator chemistry IDA, stationary matrix Superflow 6 agarose beads, binding capacity 60 mg protein/mL) and Ni^{2+} preloaded cComplete His-Tag Purification resin (Roche; chelator chemistry proprietary; stationary matrix Sepharose-CL 6B, binding capacity 40 mg protein/mL resin). 10 mL of new resin from each brand were prepared, equilibrated, washed and eluted with an imidazole gradient following the manufacturer's instructions. Fractions were continuously collected during the equilibration, wash and elution steps and their Ni^{2+} content measured using the quantitative colorimetric methods described in Section 3.5. The results are presented in Figure 6 and more experimental details are provided in the legend.

There are major differences in Ni^{2+} leakage between the His•Bind and His60 resins and the cComplete resin. Ni^{2+} concentrations on the tens to hundreds of μM range were observed across the equilibration, wash and elute fractions from the His•Bind resin. The Ni^{2+} leakage was lower for the His60 resin with concentrations on the $\sim 6 \mu\text{M}$ to hundreds of μM range. The cComplete resin releases very little with no detectable Ni^{2+} during the equilibration step and $\sim 2 \mu\text{M}$ at the end of the 0 – 250 mM imidazole elution step. Based on this information, the $\alpha_3\text{X}$ protein purification protocol was modified to include His•Bind and cComplete resins for the first and second IMAC steps, respectively. The former is easily charged and stripped and this procedure can be repeated many times without a notable change in the effective binding capacity. This provides the advantage that multiple members of the $\alpha_3\text{X}$ protein family, which typically differ by only one or two residues in their primary sequence, can be purified using a single column without the risk of cross contamination. Introducing the Ni^{2+} pre-charged cComplete resin for the second IMAC step dramatically lowered the Ni^{2+} content of the final HPLC purified material to the point where no obvious NMR spectral perturbations could be observed.

5. Conclusion

Protein production involving expression of his-tagged proteins and/or IMAC-based purification strategies presents the likelihood of contamination by the M^{n+} species used to

charge the resin. Ni^{2+} , and other commonly used transition metals including Co^{2+} and Cu^{2+} , are paramagnetic and their presence in protein NMR samples may introduce unwanted spectral perturbations. The colorimetric assays presented here provide a simple and quick means to determine the presence of Ni^{2+} in low μM concentrations.

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Figure 1.

Colorimetric series made from samples containing 25 mM NaH₂PO₄, 150 mM NaCl, pH 8.0, 50 μM PAR and 0 – 37.5 μM Ni²⁺. The Ni²⁺/PAR titration series displays colors ranging from bright yellow (0 μM Ni²⁺, far left) to coral (37.5 μM Ni²⁺, far right). The series was made by mixing equal volumes of a 100 μM PAR (in milliQ water) stock and 0 – 75 μM Ni²⁺ (in Buffer A) solutions. The Ni²⁺ concentration and the Ni:PAR ratio are printed at the center and the top of each tube, respectively.

Figure 2.

Chemical structures of (1) fully protonated PAR in its free form, (2) mono-coordinated $[\text{Ni}(\text{H}_2\text{O})_3(\text{PAR})]^{2+}$, and (3) double-coordinated $[\text{Ni}(\text{PAR})_2]^{2+}$. Free PAR has three acid-base groups and their $\text{p}K_{\text{a}}$ values are also shown. An ϵ_{413} of $32,900 \pm 500 \text{ M}^{-1} \text{ cm}^{-1}$ was determined for PAR in milliQ water (Section 3.3.1).

Figure 3.

(A) Absorption spectra following a Ni^{2+} /PAR titration series with samples containing 25 mM NaH_2PO_4 , 150 mM NaCl, pH 8.0, 50 μM PAR, and 0 – 50 μM Ni^{2+} . The spectra of free PAR and nickel-bound PAR display a peak maximum at 413 and 495 nm, respectively. The titration series was made by mixing equal volumes (750 μL) of a 100 μM PAR (in milliQ water) stock and 0 – 100 μM Ni^{2+} (in Buffer A) solutions. (B) The Ni^{2+} /PAR titration series displayed as a set of difference spectra obtained by subtracting the free PAR (0 μM Ni^{2+}) spectrum. The difference spectra of free and nickel-bound PAR display a peak maximum at 408 and 495 nm, respectively. (C) Changes in the 413 nm (blue) and 495 nm (red) absorbance as a function of the Ni^{2+} concentration. The linear regions display a slope of $-53,678 \pm 408$ and $71,739 \pm 90$ for the 413 and 495 nm data, respectively. (D) Changes in the 408 nm (purple) and 495 nm (red) difference absorbance as a function of the Ni^{2+} concentration. The linear regions display a slope of $-54,829 \pm 415$ and $71,798 \pm 123$ for the 408 and 495 nm data, respectively. The spectra were recorded on an Evolution 300 UV-Vis double-beam spectrophotometer equipped with a Smart Peltier thermostatted cell holder using the following settings: blank 25 mM NaH_2PO_4 , 150 mM NaCl, pH 8.0, pathlength 0.5 cm, sample volume 600 μL , spectral range 200 – 700 nm, bandwidth 2 nm, step size 1 nm, scan rate 120 nm min^{-1} and temperature 25 $^\circ\text{C}$. Data were processed using Igor Pro 6.37.

Figure 4.

Screening for Ni^{2+} in precious material and small volumes. The following protocol can be used if material is scarce: Mix 50 μL protein solution, 50 μL 200 μM PAR in Buffer A and 150 μL Buffer A (or some other pH 7.0 buffer). Prepare the blank, PAR control and protein-PAR samples so that the final buffer composition is the same in the three solutions. A 250 μL sample can be measured accurately in a 0.1 cm pathlength cuvette (nominal volume 400 μL) but confirm that the sample when placed in the cell holder covers the light beam of the spectrophotometer with good margin. The displayed spectra were recorded with sample volumes as low as 210 μL . (A) A ^{15}N -labeled $\alpha_3\text{W}$ preparation (blue spectrum) was screened for Ni^{2+} by subtracting the PAR control spectrum (red; 40 μM PAR) from the corresponding $\alpha_3\text{W}$ -PAR spectrum (orange; 50 μM $\alpha_3\text{W}$ + 40 μM PAR). The difference spectrum (black) displays a Abs_{495} of 4.4 mOD, which corresponds to 0.6 μM Ni^{2+} . This sets the Ni^{2+} content to 2.5 μM in the 50 μL , 200 μM $\alpha_3\text{W}$ aliquot removed for testing. (B) The ^{15}N -labeled $\alpha_3\text{W}$ material was treated with EDTA as described in Section 4.1 (blue spectrum) and then screened for Ni^{2+} . The EDTA-treated $\alpha_3\text{W}$ sample gave rise to identical control (red; 35 μM PAR) and $\alpha_3\text{W}$ -PAR (orange; 35 μM $\alpha_3\text{W}$ + 35 μM PAR) spectra and no Ni^{2+} was detected. Spectral settings as in Figure 3.

Figure 5.

^{15}N -HSQC spectra of (A) a 950 μM $\alpha_3\text{W}$ sample containing $\sim 12 \mu\text{M}$ Ni^{2+} and (B) a 800 μM $\alpha_3\text{W}$ sample with no detectable Ni^{2+} . Panels (C) and (D) display the peak heights for the protein resonances in panels A (blue) and B (red), respectively. The spectra were collected, processed and displayed using identical parameters. Experimental conditions: Bruker Avance 500 MHz spectrometer equipped with a cryoprobe, ^1H dimension spectral width of 10 ppm and 512 complex points, ^{15}N dimension spectral width of 8.8 ppm and 160 complex points, buffer conditions about 23 mM sodium acetate- d_3 and 23 mM NaCl, 75 μM DSS, pH 5.5 ± 0.2 , and temperature 30 $^\circ\text{C}$. The spectra were processed using Felix95 (Accelrys Inc., San Diego, CA), analyzed with NMRFAM-SPARKY (Lee, Tonelli & Markley, 2015) and displayed using Adobe Illustrator (Adobe Creative Cloud).

Figure 6.

Ni^{2+} leakage from three commonly used IMAC resins. (A) A column containing 10 mL of pristine His•Bind resin was rinsed with column volumes 3 (CV) of milliQ water, charged with 5 CV of 50 mM NiSO_4 , and rinsed with 5 CV of binding buffer (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9). The column was then (a) equilibrated with an additional 10 CV of binding buffer, (b) washed with 6 CV of wash buffer (20 mM Tris-HCl, 500 mM NaCl, 60 mM imidazole, pH 7.9), and (c) eluted using a 60 – 1000 mM imidazole in 20 mM Tris-HCl, 500 mM NaCl, pH 7.9, gradient. Panel A displays the elution profile of steps a to c. (B) A column containing 10 mL of pristine Ni^{2+} pre-charged His60 Ni Superflow resin was rinsed with 10 CV of equilibration buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 20 mM imidazole, pH 7.4). The column was then (a) washed with an additional 10 CV of equilibration buffer, (b) washed with 10 CV of wash buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 40 mM imidazole, pH 7.4), and (c) eluted using a 40 – 300 mM imidazole in 50 mM NaH_2PO_4 , 300 mM NaCl, pH 7.4, gradient. Panel B displays the elution profile of steps a to c. (C) A column containing 10 mL of pristine Ni^{2+} pre-charged cComplete His-Tag Purification resin was rinsed with 10 CV of equilibration buffer (50 mM NaH_2PO_4 , 300 mM NaCl, pH 8.0). The column was then (a) washed with an additional 10 CV of equilibration

buffer and (*b*) eluted using a 0 – 250 mM imidazole in 50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0, gradient. Panel C displays the elution profile of steps *a* and *b*.

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