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In-gel detection of His-tagged proteins with 4-methoxy-1,8-naphthalimide based fluorescent probe

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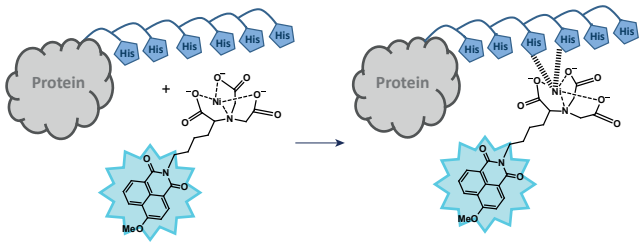
A new derivative of 4-methoxy-1,8-naphthalimide containing a chelating motif of lysine-derived mono-*N*-nitrilotriacetic acid effectively coordinates with Ni^{2+} in a HEPES-buffer solution resulting in the formation of 1 : 1 ligand–metal complex. This demonstrates the possibility to stain His-labeled proteins on a gel after electrophoresis to make the analysis and purification of proteins more convenient.



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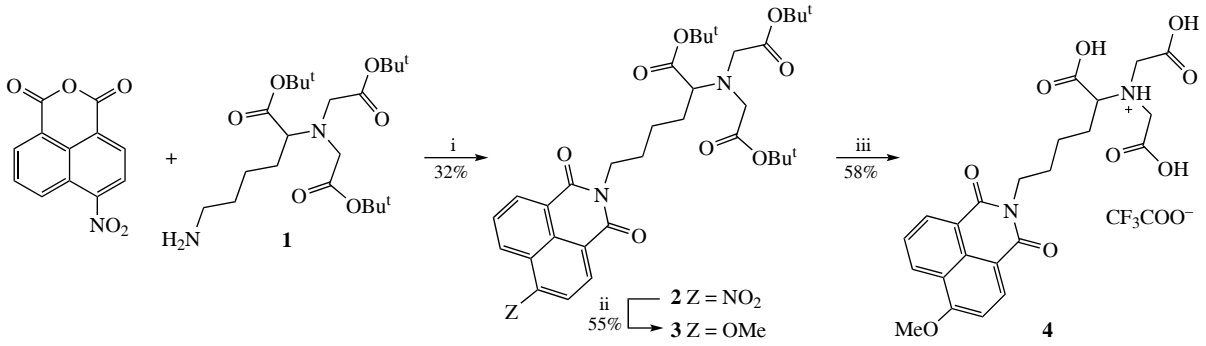
Modern biotechnology methods allow one to modify the DNA of bacteria in order to make them express a particular protein of interest during their life cycle.[1] Such proteins are called recombinant since they are obtained from recombinant (modified) DNA. Recombinant DNA technology is successfully employed to produce vaccines, vector molecules, antibodies, antitumor drugs, and other important proteins used both for research purposes and in the treatment of human diseases (*e.g.* insulin and growth hormone).[1–3] However, in addition to the recombinant protein, bacteria express many other proteins necessary for their life cycle, so the isolation and purification of recombinant proteins is a separate complex task. Detection, isolation and purification of a recombinant protein is simplified if the DNA is modified so that the protein is expressed with a so-called 'affinity tag' at the C- or N-end of the protein.[4,5] Such tags should be small fragments that do not affect the structure and properties of the tagged molecule, have the ability to bind to the specific sorbents, be easily removed by enzymes, and be detectable (using antibodies or other specific reagents). One of the most commonly used affinity tags is polyhistidine (His-tag). The His-tag is a sequence of six histidine residues and ideally satisfies all of the requirements mentioned above.[5]

To detect proteins containing a His-tag at the stage of its production by cells and then during its isolation and purification, gel electrophoresis in a polyacrylamide gel is used.[6] The process is based on the fact that protein molecules in an electric field move along a gel substrate at different speeds, depending on the mass and charge of the molecule (the greater the specific charge of the molecule, the higher its mobility). In the resulting electrophoregram, the proteins are distributed in the form of spots whose location depends on the mobility of the proteins. To distinguish a His-tagged protein from spots of other proteins, after electrophoresis, the gel sample must be kept in a solution containing a reagent that selectively interacts with the His-tag.

The traditional method of detecting proteins with histidine tags is immunoblotting with anti-His antibodies.[7,8] However, this approach is time-consuming and expensive, whereas simplicity and low cost are of key importance for the production and purification of large numbers of proteins. Therefore, the development of new methods for detecting His-tag proteins during electrophoresis is an urgent task. Solving this problem can simplify and reduce the cost of obtaining new protein molecules necessary for a variety of medical applications.

An alternative to antibodies is immunoblotting with chelating tags based on *N*-nitrilotriacetic acid (NTA) loaded with nickel ions.[9] NTA is a well-known chelator for nickel, and the Ni^{2+}–NTA complex effectively coordinates with proteins containing the His-tag.[10] This phenomenon is widely applied to isolate large amounts of His-tag proteins using Ni^{2+}–NTA-containing chromatography columns used in immobilized metal affinity chromatography (IMAC).[9,11,12] Combining the NTA motif with an organic dye in one molecule makes it possible to obtain a reagent that selectively binds to His-tagged proteins. To date, several labels based on nitrilotriacetic acid have been described for His-tags, but most often they use red and green dyes.[13–15] Application of such reagents requires careful selection of the source of excitation, which complicates the experiment, and is more attractive from the viewpoint of biological research rather of a routine use in electrophoresis.

Herein, we present the synthesis and investigation of a fluorescent label for His-tagged proteins based on nitrilotriacetic acid and 4-methoxy-1,8-naphthalimide fluorophore. Derivatives of 1,8-naphthalimide are actively used in various fields of science and technology due to the simplicity of their chemical



Scheme 1 *Reagents and conditions*: i, EtOH, Δ, 14 h, ii, MeONa, MeOH, Δ, 6.5 h, iii, CF_3CO_2H, MeOH/H_2O, 80 °C, 22 h.

modification and their excellent photostability.[16–19] Naphthalimide dyes bearing 4-positioned alkoxy groups are characterized by high luminescence efficiency even in aqueous medium,[20] and have been applied as components of optical chemosensors[20–23] and fluorescent labels.[24] In addition, such compounds absorb in the UV-region, so their fluorescence can be excited by means of UV transilluminators widely used in laboratory practice, which makes a procedure of analyzing the electrophoregram convenient and simple. In this paper, we focused on the synthesis of the special fluorescent label (Scheme 1), the study of its optical properties and complexation with Ni^{2+} in a buffer solution, and the use of the label for selective gel staining and analyzing with a UV transilluminator.

The phenomenon of quenching fluorescence of compounds by paramagnetic metal ions (including Ni^{2+}) has been described in literature.[25–27] Therefore, when designing the structure of the label, it was decided to implement a spacer consisting of several methylene groups between the fluorophore and Ni^{2+} binding site. Such a spacer would ensure shielding of naphthalimide from the ionophoric fragment so as the fluorescence quenching effect could be reduced. Thus, available[28] N_α,N_α-bis(carboxymethyl)-L-lysine **1** (see Scheme 1) was used for imidation of 4-nitronaphthalic anhydride to obtain compound **2**. Nitro derivative **2** was further treated with sodium methoxide in methanol to give intermediate **3**. At the final step, the *tert*-butyl groups in triester **3** were removed by treatment with trifluoroacetic acid.

After the electrophoresis, proteins are fixed on the polyacrylamide gel by treatment with an acidic solution. The plate is then washed with a buffer solution with pH 7.4–7.6 in order to create a neutral environment in which further binding of the His-tags with the labels becomes possible. Therefore, optical properties of **4** were studied in a neutral HEPES buffer (pH 7.4). Spectral characteristics of **4** are given in Table 1, the absorption spectra and the normalized fluorescence spectrum are depicted in Figure S10 (see Online Supplementary Materials). Compound **4** demonstrates a broadband absorption spectrum in the range from 300 to 500 nm with a maximum at 374 nm, which allows exciting the fluorescence of the dye in a wide wavelength range. The fluorescence maximum is located at 462 nm and corresponds to the blue emission light. An excellent fluorescence efficiency is worth noting (in a HEPES-buffered aqueous solution $\varphi^{fl} = 30\%$). At the same time, a high value of the Stokes shift would provide a good contrast of light spots on the electrophoregram.

Table 1 Optical characteristics of compound **4** and complex (**4**)·Ni^{2+} in HEPES buffer solution (pH 7.4, 0.01 M).

Compound	λ^{abs}_{max}/nm	$\lambda^{fl}_{max}(\lambda_{ex})$/nm	$\varepsilon_{\lambda_{max}}$/ dm^3 mol^{-1} cm^{-1}	ν/cm^{-1}	φ^{fl} (%)	log$K$
4	375	463 (375)	6599	5093	35	–
(**4**)·Ni^{2+}	375	463 (375)	3854	5068	28	5.6±0.4

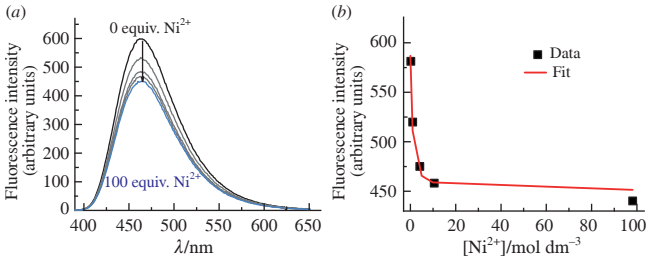


Figure 1 (*a*) Changes in fluorescence spectra of **4** with adding Ni^{2+} to a solution in HEPES buffer, $C_4 = 1 \times 10^{-5}$ mol dm^{-3}, excitation 375 nm. (*b*) Fluorescence intensity at 670 nm *vs*. the Ni^{2+} concentration.

The addition of Ni^{2+} to the solution does not cause any changes in the absorption spectra of the dye (Figure S11), whereas a slight decrease in the **4** fluorescence is observed (the quantum yield drops from 35 to 28% in the presence of 100 equiv. Ni^{2+}). Changes in the emission spectrum of **4** were investigated by spectrophotometric titration (Figure 1). It was found that the experimental dependence of the fluorescence intensity of the **4** solution at 670 nm on the Ni^{2+} concentration was in the best agreement with the calculated curve if the formation of one type of complex following the equilibrium

$$\mathbf{4} + Ni^{2+} \rightleftharpoons (\mathbf{4})\cdot Ni^{2+}$$

was taken into account [see Figure 1(*b*)]. The calculated logarithm of the stability constant (log$K$) of the complex (**4**)·Ni^{2+} was estimated to be 5.6±0.4 (see Table 1).

The affinity of mono-NTA derivatives to the His-tag is well-studied: binding constants are in the millimolar range of values.[14,28] Therefore, after exploring the binding of compound **4** to nickel(II) cations, we tested the practical application of staining a proteins with or without His-tag in polyacrylamide gel. The bacterial endonuclease in two variants, with and without His-tag, was employed as a model protein. Proteins BSA (Bovine Serum Albumin without His-tag) and TEV (Tobacco Etch Virus protease with His-tag) were used as negative and positive controls, respectively. A solution of **4** containing 5 equiv. of Ni^{2+} was used. The results of staining in the form of an image from a UV-transilluminator are shown in Figure 2.

According to Figure 2, the negative control without a His-tag does not show blue fluorescence after staining (line *1*). For the bacterial endonuclease without a His-tag, a low-intensity fluorescence spot is observed (line *4*), probably as a result of nonspecific binding of the dye to this protein. In contrast, His-tagged bacterial endonuclease demonstrates an intense luminescence when excited in the UV-region (line *3*) comparable to the positive control (line *2*). Figure S12 (see Online Supplementary Materials) shows the results of staining gel with the same set of proteins with a commercially available dye for His-tags produced under the InVision trademark. The structure of InVision^{TM} is a commercial classified information, however, it

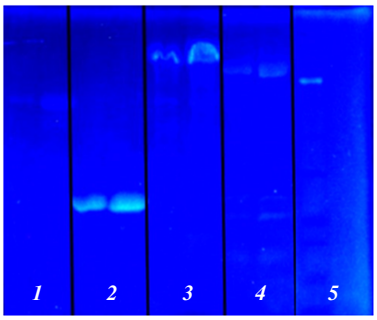


Figure 2 Detection of His-tagged reference proteins by **4**: (*1*) BSA (Bovine Serum Albumin); (*2*) TEV (Tobacco Etch Virus protease); (*3*) His-tagged bacterial endonuclease; (*4*) bacterial endonuclease without His-tag; (*5*) molecular mass marker.

is known that it is also a mono-NTA fluorescent label. The staining was performed in compliance with the procedure recommended by the manufacturer. We have revealed that the InVision^{TM} solution also selectively stains His-tagged proteins (line *3* in Figure S12), however in the case of the bacterial endonuclease without a His-tag, an intense luminescence is observed (line *4*), which indicates a greater contribution of non-specific interactions for InVision^{TM} than for **4**.

Thus, a new derivative of 4-methoxy-1,8-naphthalimide **4** containing a *N*-nitrilotriacetic acid moiety was synthesized. It demonstrates a broadband absorption from 300 to 500 nm and effectively fluoresces in HEPES-buffer solution. Compound **4** exhibits chelating properties for the Ni^{2+} cation with a logarithm of the binding constant of 5.6 ± 0.4 and retains intense fluorescence after coordination with nickel ion. In-gel staining with **4** dye solution makes it possible to detect His-tagged proteins on a gel after electrophoresis using a simple staining procedure, as well as a UV-transilluminator, a device widely used in laboratory practice. Therefore, using **4** simplifies the procedure for analyzing and purifying His-labeled proteins.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.71267/mencom.7775.

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