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Purification of His-tagged proteins using Ni^{2+}–poly(2-acetamidoacrylic acid) hydrogel

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ABSTRACT

In this study, a new matrix for immobilized metal affinity chromatography (IMAC) using poly(2-acetamidoacrylic acid) (PAAA) hydrogels complexed with Ni^{2+} was developed for the purification of the recombinant histidine-tagged green fluorescence protein (His6–GFP). The Ni^{2+}-complexed PAAA hydrogel was prepared by polymerizing 2-acetamidoacrylic acid (AAA) and 2,2′-[(1,4-dioxo-1,4-butanediyl)diamino] bis(2-propenoic acid) (DBDBPA) with potassium persulfate in DMSO, followed by Ni^{2+} complexation. Confocal laser scanning microscopy was used to determine the binding of His6–GFP to the Ni^{2+}–PAAA hydrogel in three-dimensional space. Photoluminescence spectroscopy revealed an 81% binding efficiency of His6–GFP to the Ni^{2+}–PAAA hydrogel yielded with a recovery of 59%. The specificity of His6–GFP binding to Ni^{2+}–PAAA hydrogel was compared with that of the PAAA hydrogel without Ni^{2+}. His6–GFP was purified directly from the cell lysate with Ni^{2+}–PAAA hydrogel matrix but the PAAA hydrogel without Ni^{2+} had no effect. The major advantage of the Ni^{2+}–PAAA hydrogel system over current methods, such as Ni–nitrilotriacetic acid (NTA) agarose beads, was the simple and low-cost procedure for preparing the matrix.

1. Introduction

The immobilized metal affinity chromatography (IMAC) technique employs the strong affinity between the target proteins and metals for protein purification. The metal-binding amino acids (e.g. histidine, cysteine, tyrosine, etc.) were genetically engineered on the targeted proteins to be used as a strong and efficient binding moiety in IMAC with immobilized metal ions (e.g. Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, etc.). Various ligands (e.g. iminodiacetic acid (IDA), nitrilotriacetic acid (NTA), etc.) were used to chelate the metal ions on solid supports. IMAC was first introduced by Porath et al. [1–3] in 1975. Since then, it has become the most widely used technique for purifying recombinant proteins. The benefits of IMAC in handling various recombinant proteins are the high selectivity, high recovery yield of the target proteins, high protein loading, and complete regeneration of the solid supports [4–6].

Extensive studies have been carried out to further improve the efficiency of the IMAC system with increased selectivity, a simple experimental procedure, and lower cost [7]. IMAC system based on the cellulose-membrane was proposed for the purification of recombinant proteins [8,9], where the pressure drop was decreased with increasing accessible flow rate. Another IMAC system using magnetic nanoparticles as an immobilization material were also developed [10–13]. Due to their small size and high surface area, magnetic nanoparticles demonstrate superior characteristics, such as good dispersibility as well as rapid and effective binding of biomolecules, compared to conventional micrometer-sized resins or beads. The binding and elusion of bioparticles from metal affinity ligands in polyacrylamide-based macroporous hydrogel was first reported by Galaev et al. [14]. Thus far, agarose, cellulose beads, polymer resins, and magnetic nanoparticles are generally used as solid supports in IMAC purification systems [3,12,13].

Hydrogels are crosslinked polymers that remain in an expanded matrix formation without dissolving in water. The water-absorbing capacity of hydrogels has led to their use in various technological areas, such as materials for contact lenses, matrices for cell-encapsulation, and devices for the controlled release of drugs or proteins [14–18]. Hydrogels are also widely used as support-

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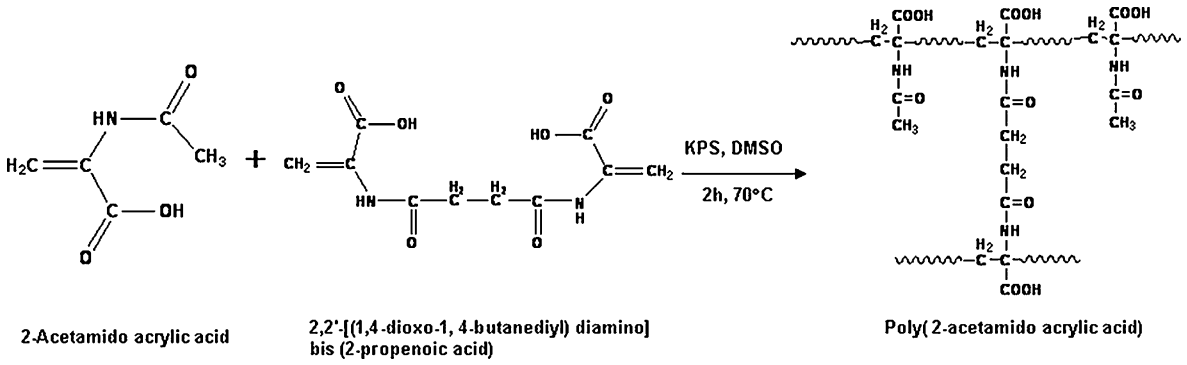

Scheme 1. Synthesis of the PAAA hydrogel.

ing materials for the immobilization or separation of proteins. For example, histidine-tagged Kinesin was immobilized on the surface of a modified 2-hydroxyethyl methacrylate (HEMA) hydrogel as a result of the affinity between the histidine residues and a metal ion–NTA complex [19].

Hydrogels behave like an elastomer. However, their dimensions can be controlled by their degree of swelling, which can also be controlled either by the chemical potential of the aqueous phase or by the crosslinking-density of the hydrogel. A hydrogel with metal affinity ligands was recently introduced as a protein purification media [20,21]. This paper reports the possibility of fully utilizing the three-dimensional structure of a hydrogel for the purification of recombinant proteins. To achieve this, a Ni ion-complexed poly(2-acetamido acrylic acid) hydrogel with controllable functionality was synthesized. The novel hydrogel could be used efficiently for the purification of 6×His-tagged proteins. The major advantage of this new hydrogel-based IMAC system over the conventional two-dimensional purification system was the efficient utilization of the three-dimensional structure of the polymer chains for protein purification.

2. Experimental

2.1. Materials

2-Acetamidoacrylic acid (AAA) (97%) and 2,2′-[(1,4-dioxo-1,4-butanediyl) diamino] bis(2-propenoic acid) (DBDBPA) (97%) were synthesized using previously reported procedures [22]. Imidazole (99.0%) and Nickel(II) chloride hydrate (99.9%) were purchased from ALDRICH and used as received. Potassium persulfate (99.0%) was supplied by ACROS ORGANICS (USA). Dimethyl sulfoxide (DMSO, 99.0%) was obtained from JUNSEI (Japan). Ni–NTA HisBind Resin was obtained from Novagen.

2.2. Synthesis of poly(2-acetamido acrylic acid) hydrogel

AAA (500 mg; 4 mM), 2,2′-[(1,4-dioxo-1,4-butanediyl)diamino] bis(2-propenoic acid) (DBDBPA) (25 mg; 0.10 mM) and potassium persulfate (5 mg; 0.02 mM) were dissolved in 1.50 ml DMSO. The poly(2-acetamidoacrylic acid) (PAAA) hydrogel was formed after heating the reaction mixture to 70 °C for 2 h, and the product was washed thoroughly with distilled water (Scheme 1).

2.3. Ni^{2+}-complexed PAAA hydrogel (Ni^{2+}–PAAA hydrogel)

The PAAA hydrogel (30 g) was immersed in 100 ml of a 0.1 M solution of nickel chloride ($NiCl_2$), and continuously stirred at room temperature for 24 h. The Ni-complexed hydrogel was rinsed with 100 ml of distilled water to remove the unbound or weakly bound Ni^{2+}. The amount of nickel in the Ni^{2+}-complexed PAAA hydrogel was determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES).

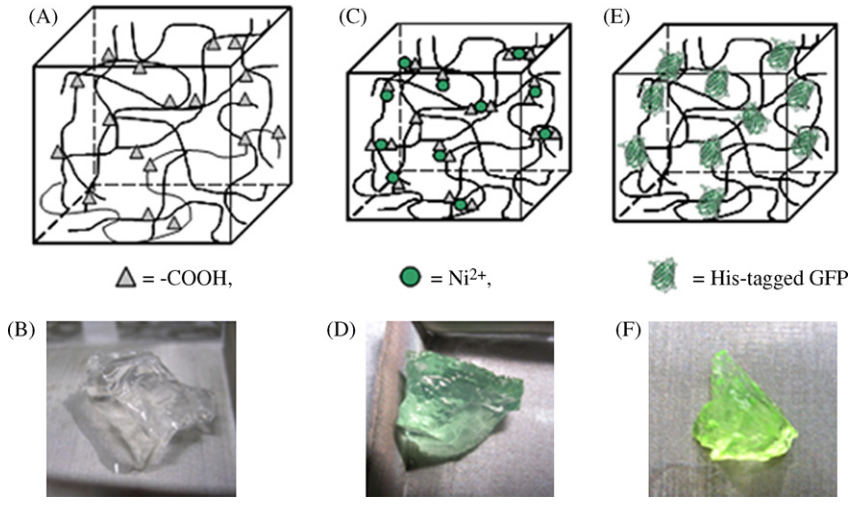

Fig. 1. Schematic diagram and images of the PAAA hydrogel (A and B), Ni^{2+}–PAAA hydrogel (C and D), and His6–GFP/Ni^{2+}–PAAA hydrogel (E and F).

2.4. Determination of swelling ratios

The PAAA hydrogel was almost fully swelled with distilled water over a 7-day period. The water on the surface of the hydrogel was absorbed with tissue paper, and the hydrogel was weighed. The hydrogel was then vacuum-dried and weighed again. Three samples of each hydrogel were measured to calculate the average value. The swelling ratio was calculated as follows:

$$\text{Swelling ratio} = \frac{(\text{wet hydrogel weight} - \text{dry hydrogel weight})}{\text{dry hydrogel weight}}$$

2.5. Expression and purification of His6–GFP

E. coli BL21 (DE3) harboring pET19-EGFP to express the hexahistidine tagged EGFP at the N-terminus was grown to an OD600 of 0.6 at 37 °C in 100 ml of LB medium containing 100 μg/ml of ampicillin. Subsequently, 0.05 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added for induction, and grown for an additional 6 h at 20 °C. The cells were recovered by centrifugation before being lysed. Briefly, the centrifuged cell pellet was resuspended in 5 ml of a lysis buffer and incubated at room temperature for 10 min. The resulting suspension was centrifuged for 20 min at 4 °C at 9000 g. The supernatant was collected and incubated with 5 mg of Ni–NTA HisBind Resin for 3 h at 4 °C. The resin was loaded onto the column, and washed with 3 ml × 4 ml of a washing buffer (50 mM phosphate buffer (PB), at pH 8.0, 300 mM NaCl, 40 mM imidazole). Subsequently, His6–GFP was eluted with 1 ml of an elution buffer (50 mM PB, at pH 8.0, 300 mM NaCl, 250 mM imidazole). The imidazole in the eluted solution was removed by dialysis filtration. The protein fractions were analyzed by SDS-PAGE (12% acrylamide gel), showing that the purity of purified His6–GFP was more than 95%.

2.6. Binding and recovery of His6–GFP using Ni^{2+}–PAAA hydrogel ($His6–GFP/Ni^{2+}$–PAAA hydrogel)

The Ni^{2+}–PAAA hydrogel (0.1 g) was equilibrated with 10 ml of a phosphate buffer solution (pH 8.0) for 2 h, and the excess PB was removed. The hydrogel was then added to a protein solution containing His6–GFP. The hydrogel was incubated for 2 h at 4 °C with constant shaking, and the supernatant was removed from the hydrogel by centrifugation (3000 rpm, for 5 min at 4 °C). The hydrogel was transferred to the column and washed with 3 ml × 4 ml buffer solution (50 mM PB, pH 8.0, 300 mM NaCl, 40 mM imida-

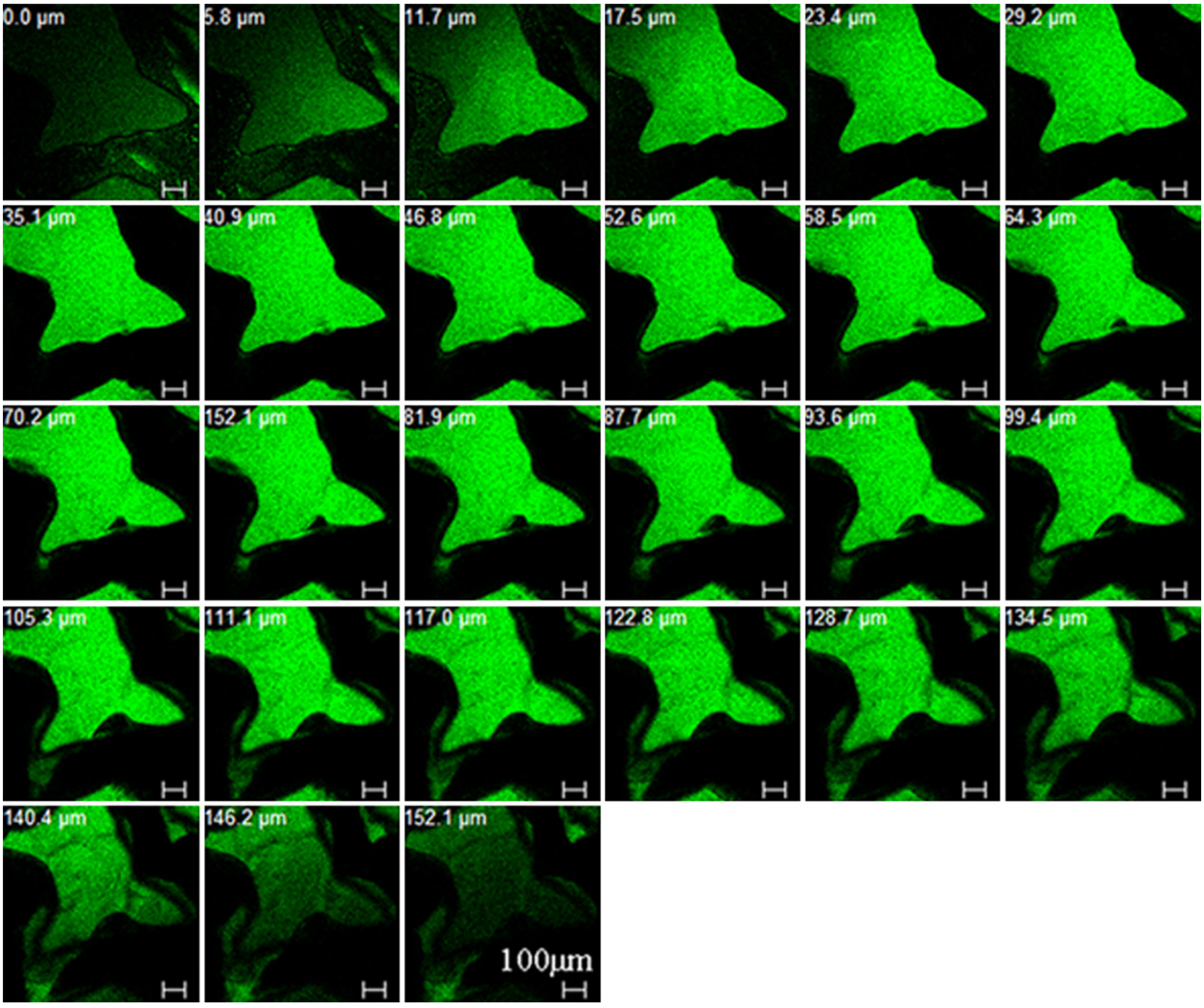

Fig. 2. Images of the His6–GFP bound to Ni^{2+}–PAAA hydrogel from CLSM. The green fluorescence images of the His6–GFP/Ni^{2+}–PAAA hydrogel were obtained from the cross-sections of the depth measurements. Each image shows 5.8 μm sectional views of the His6–GFP/Ni^{2+}–PAAA hydrogel (scale bar = 100 μm).

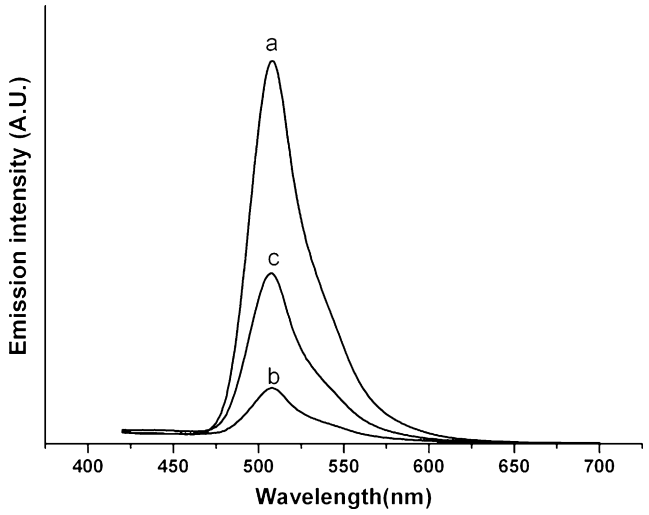

Fig. 3. Fluorescence spectra showing the changes in the emission intensity of His6–GFP in the solutions before (curve a) and after (curve b) bound with the Ni^{2+}–PAAA hydrogel. The emission intensity of the His6–GFP released in the eluent after treating the His6–GFP/Ni^{2+}–PAAA hydrogel with 250 mM imidazole solution is shown in curve (c).

zole) to remove the non-bound proteins. The bound His6–GFP was eluted with 1 ml of a 250 mM imidazole buffer solution.

2.7. Characterization

The photoluminescence (PL) spectra were recorded using an Ocean Optics HR4000CG Composite-grating spectrophotometer with an excitation wavelength at 395 nm. Confocal Laser Scanning Microscopy (CLSM) was performed using a LSM 5 PASCAL, Zeiss with an argon ion laser (488 nm). ICP-AES was carried out using a MLAN 6100 (PERKIN ELMER, USA).

3. Results and discussion

Fig. 1(A and B) shows the PAAA hydrogel synthesized from 2-acetamido acrylic acid (AAA) and 2,2′-[(1,4-dioxo-1,4-butanediyl)diamino] bis(2-propenoic acid) (DBDBPA). The main (inner) part of the PAAA hydrogel structure consisted of hydrogen bonds between the hydrogel matrix and water. This PAAA hydrogel could swell up and extend to more than 100 times its dry weight. PAAA hydrogels have been utilized in a variety of biotechnological areas on account of their water-absorbing capacity. Ni^{2+} was complexed with the fully swollen hydrogel in distilled water after a $NiCl_2$ treatment. After incubating the PAAA hydrogel with Ni^{2+}, the colour of the hydrogel changed to a blue–green, and its volume decreased by approximately 15%, indicating successful complexation between Ni^{2+} and the carboxylic groups of PAAA (Fig. 1(C and D)). ICP-AES showed that the number of the Ni^{2+} ions per carboxylic acid group in the dry Ni^{2+}–PAAA hydrogel was 0.183. Fig. 1(E and F) shows the His6–GFP bound to Ni^{2+}–PAAA hydrogel in an aqueous environment. After the binding process, the colour of the hydrogel changed to bright-green, confirming the successful binding of His6–GFP to the Ni^{2+}–PAAA hydrogel.

CLSM was used to demonstrate the binding of His6–GFP in the three-dimensional structure of the Ni^{2+}–PAAA hydrogel (Fig. 2). The hydrogel was analyzed after incubating with purified His6–GFP and washing extensively with PB. The fluorescence intensity could be directly related to the quantity of His6–GFP bound to the hydrogel. The fluorescence images of the surface and vertical cross-section of the hydrogel clearly showed the specific binding of the His6–GFP to the Ni^{2+}–PAAA hydrogel in three-dimensional space. This indicates the potential applications in utilizing the three-dimensional matrix structure of the Ni^{2+}–PAAA hydrogel to purify the histidine-tagged recombinant proteins.

The efficacy of the Ni^{2+}–PAAA hydrogel as a purification system was investigated. The Ni^{2+}–PAAA hydrogel (0.14 g) was incubated with 300 μl (60 μg) of His6–GFP in an Eppendorf tube for 2 h at 4 °C. The His6–GFP/Ni^{2+}–PAAA hydrogel was then loaded into the column. After washing twice with a washing buffer (50 mM PB, pH 8.0, 300 mM NaCl, 40 mM imidazole), the His6–GFP from the His6–GFP/Ni^{2+}–PAAA hydrogel was released with 300 μl of a 250 mM imidazole solution (Fig. 3(curve c)). The eluted His6–GFP was confirmed by SDS-PAGE analysis (data not shown). In this experiment, the binding and eluting of His6–GFP was analyzed quantitatively by their fluorescence emission intensity (Fig. 3). The binding efficiency of His6–GFP to Ni^{2+}–PAAA hydrogel was determined from the emission intensity at 508 nm of residual solution after removal of protein-bound hydrogel. The amount of His6–GFP binding to the Ni^{2+}–PAAA hydrogel was approximately 81%. 59% of the His6–GFP was recovered from the protein-bound Ni^{2+}–PAAA hydrogel after treatment with 250 mM imidazole solution, which was determined from the emission intensity of eluted solution. This suggests that His6–GFP can be captured efficiently by the

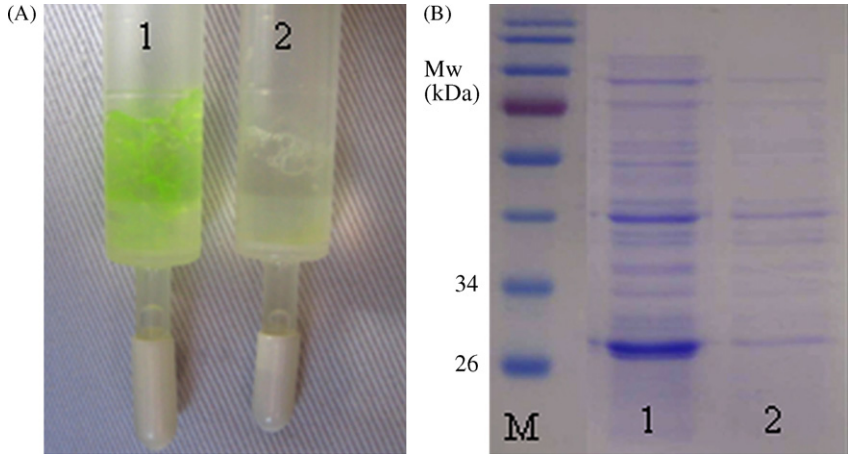

Fig. 4. (A) Binding of His6–GFP to the PAAA hydrogel with (1) and without (2) Ni^{2+}. (B) SDS-PAGE analyses of the purified His6–GFP using PAAA hydrogel with (lane 1) and without (lane 2) Ni^{2+}.

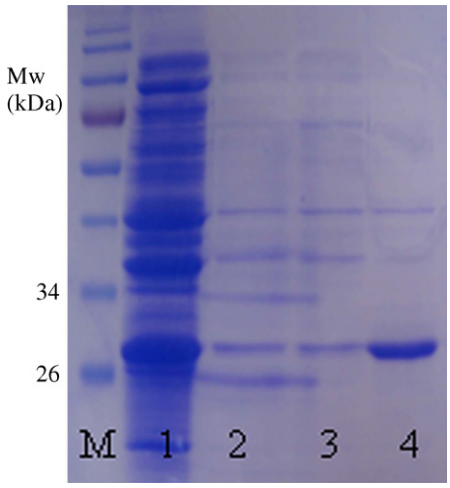

Fig. 5. SDS-PAGE analyses of purified His6–GFP using Ni^{2+}–PAAA hydrogel directly from the cell lysates. Cell lysates (lane 1), Washing fraction of proteins treated with the buffer solution containing 40 mM imidazole (lanes 2 and 3), and eluent from the His6–GFP/Ni^{2+}–PAAA hydrogel with an elution buffer containing 250 mM imidazole (lane 4), Lane M, molecular weight markers.

Ni^{2+}–PAAA hydrogel and is readily released by the addition of concentrated imidazole.

The purification efficacy of the Ni^{2+}–PAAA hydrogel was investigated. In order to determine the amount of non-specific binding of His6–GFP with the Ni^{2+}–PAAA hydrogel, an attempt was made to bind His6–GFP to a PAAA hydrogel with or without Ni^{2+}. The Ni^{2+}–PAAA hydrogel (0.1 g) was incubated with 500 μl of His6–GFP in a test tube for 2 h at 4 °C. The His6–GFP/Ni^{2+}–PAAA hydrogel was then loaded into the column. After washing with a washing buffer (50 mM PB, pH 8.0, 300 mM NaCl, 40 mM imidazole), the His6–GFP from the Ni^{2+}–PAAA hydrogel was eluted with an elution buffer (50 mM PB, pH 8.0, 300 mM NaCl, 250 mM imidazole). Fig. 4(A) shows the His6–GFP bound to the PAAA hydrogel matrix, conjugated with or without Ni^{2+}. As shown in Fig. 4(A, 2), there was no His6–GFP fluorescence in the PAAA hydrogel without Ni^{2+}. SDS-PAGE analysis of the eluents from the PAAA hydrogel with and without Ni^{2+} also showed that the His6–GFP did not bind to the PAAA hydrogel, and only interacted with the hydrogel complexed with Ni^{2+} (Fig. 4(B)). This indicates the formation of specific binding between His6–GFP and the Ni-complexed PAAA hydrogel. In addition, the non-specific interaction between His6–GFP and the functional groups in PAAA hydrogel was quite weak compared with the specific interaction.

The Ni^{2+}–PAAA hydrogel system was used in an attempt to purify the recombinant His6–GFP from *E. coli* BL21(DE3) extracts. The recombinant His6–GFP was induced with IPTG, and the cell lysates were treated as described in Section 2. The recombinant His6–GFP was purified using the soluble fraction of the cell lysates. The purification of GFP without the histidine tag was carried out as a control. Fig. 5 shows the SDS-PAGE data of the cell lysates, washing fraction, and eluted fraction. As shown in Fig. 5, the intensities of the His6–GFP band in SDS-PAGE were analyzed using image analyzing software in Gel-doc 2000 (Bio-Rad). The purity of the His6–GFP band from the eluted fraction was estimated to be approximately 99%. The efficiency of purifying His6–GFP by the Ni^{2+}–PAAA hydrogel system highlights its convenience and simplicity as a purification system for any recombinant His-tagged proteins. These results also confirmed that purification was achieved through specific interactions between the polyhistidine tag and Ni^{2+}.

4. Conclusions

This paper reports the synthesis of Ni-complexed PAAA hydrogel and the successful application of its three-dimensional structure for the purification of His-tagged GFP. The main advantage of the Ni-complexed PAAA hydrogel over current methods, such as Ni–NTA agarose beads, was the simple and low-cost hydrogel preparation procedure. It is expected that the production cost of the Ni-complexed PAAA hydrogel would be significantly less than that of the commercial Ni–NTA agarose beads, which require several synthetic steps. Furthermore, the high hydrophilicity, low non-specific protein adsorption, and sufficient capturing groups of the metal–PAAA hydrogel highlight its suitability as a novel material in IMAC.

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References

[1] J. Porath, J. Carlsson, I. Olsson, G. Belfrage, Nature 258 (1975) 598.
[2] J. Porath, B. Olin, Biochemistry 22 (1983) 1621.
[3] J. Porath, B. Olin, B. Granstrand, Arch. Biochem. Biophys 225 (1983) 543.
[4] G.S. Chaga, J. Biochem. Biophys. Methods 49 (2001) 313.
[5] V. Gaberc-Porekar, V. Menart, J. Biochem. Biophys. Methods 49 (2001) 335.
[6] E.K.M. Ueda, P.W. Gout, L. Morganti, J. Chromatogr. A 988 (2003) 1.
[7] V. Gaberc-Porekar, V. Menart, Chem. Eng. Technol. 28 (2005) 1306.
[8] H.-L. Hu, M.-Y. Wang, C.-H. Chung, S.-Y. Suen, J. Chromatogr. B 840 (2006) 76.
[9] C.-S. Chen, S.-Y. Suen, S.-Y. Lai, G.R.-L. Chang, T.-C. Lu, M.-S. Lee, M.-Y. Wang, J. Virol. Methods 130 (2005) 51.
[10] D.-B. Shieh, C.-H. Su, F.-Y. Chang, Y.-N. Wu, W.-C. Su, J.R. Hwu, J.-H. Chen, C.-S. Yeh, Nanotechnology 17 (2006) 4174.
[11] A. Ditsch, J. Yin, P.E. Laibinis, D.I.C. Wang, T.A. Hatton, Biotechnol. Prog. 22 (2006) 1153.
[12] I.S. Lee, N. Lee, J. Park, B.H. Kim, Y.-W. Yi, T. Kim, T.K. Kim, I.H. Lee, S.R. Paik, T. Hyeon, J. Am. Chem. Soc. 128 (2006) 10658.
[13] H. Gu, K. Xu, C. Xu, B. Xu, Chem. Commun. (2006) 941.
[14] A.S. Hoffman, Adv. Drug Deliv. Rev. 54 (2002) 3.
[15] M. Casolaro, S. Bottari, Y. Ito, Biomacromolecules 7 (2006) 1439.
[16] K.H. Bae, J.J. Yoon, T.G. Park, Biotechnol. Prog. 22 (2006) 297.
[17] L.A. Haines, K. Rajagopal, B. Ozbas, D.A. Salick, D.J. Pochan, J.P. Schneider, J. Am. Chem. Soc. 127 (2005) 17025.
[18] S.V. Vinogradov, T.K. Bronich, A.V. Kabanov, Adv. Drug Deliv. Rev. 54 (2002) 135.
[19] T. Yu, Q. Wang, D.S. Johnson, M.D. Wang, C.K. Ober, Adv. Funct. Mater. 15 (2005) 1303.
[20] F. Plieva, B. Bober, M. Dainiak, I.Y. Galaev, B. Mattiasson, J. Mol. Recogn. 19 (2006) 305.
[21] E. Efremenko, Y. Votchitseva, F. Plieva, I. Galaev, B. Mattiasson, Appl. Microbiol. Biotechnol. 70 (2006) 558.
[22] H. Tanaka, T. Suzuka, K. Hada, Y. Tezuka, Polym. J. 32 (2000) 391.