A novel metal adsorbent composed of hexa-histidine tag and carbohydrate-binding module on cellulose

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Abstract

We developed a novel metal adsorbent composed of bio-based materials, cellulose and a protein. The approach involved the immobilization of hexa-histidine tag (His$_6$), which shows an affinity for intermediate acid (metal ion) in Hard and Soft Acids and Bases (HSAB) theory, on cellulose by fusing with carbohydrate-binding module (CBM). The results show that CBM-His$_6$-bound cellulose has adsorption selectivity reflecting the original properties of His$_6$. Additionally, we prepared three configurations of CBM-His$_6$ proteins, which were subsequently immobilized on a filter paper for Ni$^{2+}$ ion adsorption. Of these configurations, we found the protein containing two His$_6$ tags at each terminus (N- and C-) of CBM exhibited the highest metal adsorption ability. Furthermore, XPS analysis confirmed the binding of Ni$^{2+}$ ions on the cellulose.

Keywords: Carbohydrate-binding module; immobilization; metal affinity tag; transition metals
Introduction

Metal recovery using microorganisms has been continuously studied and is considered as a green and sustainable adsorption system using biomolecule.\(^1\) Biosorption using microorganisms shows a high adsorption capacity, but the selectivity is usually low. We focused on peptides that selectively adsorb metal ions. Some kinds of biomolecules such as peptides can specifically binds to metals and metal ions with high selectivity.\(^2,^3\) This property provides the possibility that metal ions can be separated and recovered by using these biomolecules. However, one of the problems in this approach is the fact that peptides are normally soluble in water and difficult to recover from aqueous solutions even after the complexation with metal ions.

Cellulose is an inert, non-toxic, and renewable material, and is the most abundant biopolymer on earth.\(^4\) The environmental burden of this material would probably be negligible when discarded since cellulose is naturally decomposed. Additionally, cellulose is a physically and chemically robust biomaterial and can be used as a reliable matrix for immobilization of biomolecules. If metal ion-binding peptides can be immobilized on cellulose, it would work as a metal adsorbent, which can be recovered from aqueous solution. **We thus propose a new concept of bio-based adsorbent composed of metal ion-binding peptide and cellulose.** However, the modification of cellulose with metal ion-binding peptide is usually difficult because cellulose is an inert material and the functional groups in cellulose are basically non-reactive. Carbohydrate-binding module (CBM) is a domain, which shows the binding property to cellulose and is widely found in cellulose-degrading enzymes.\(^5,^6\) A wide variety of biomolecules can be immobilized on cellulose by using the affinity of CBM for cellulose.\(^7,^8,^9\)

In the present study, hexa-histidine tag (His\(_6\)) is genetically fused with CBM. His\(_6\)
exhibits an affinity for transition metals such as Ni\(^{2+}\) ions and has been extensively used as a “His\(_6\)-tag” for protein purification in immobilized-metal affinity chromatography.\(^{10}\) The His\(_6\)-fused CBM is adsorbed on cellulose, filter paper, to recover metal ions (Fig. 1). We have prepared different configurations of CBM-His\(_6\) proteins and examined their properties in terms of cellulose-binding ability and Ni\(^{2+}\) ion adsorption/desorption efficiencies.

**Experimental**

*Construction of vectors and protein expression*

The gene for CBM3 (simply named, CBM) in cellulosomal scaffolding protein A (Accession, Q06851) of *Clostridium thermocellum* was cloned from the genome of the bacteria by PCR using a set of primers F1 and R1 (Table S1, Supporting Information). The amplified gene was subcloned into pET-22b(+) expression vector by In-Fusion at *Nde* I and *Xho* I sites to add His\(_6\)-tag at C-terminal position of CBM, named CBM-His\(_6\) (one His\(_6\) tag at the C-terminus of CBM) (Fig. 2). In addition, expansin-like protein EXLX1 (EXP) from *Bacillus subtilis* (deposited as YoaJ protein, GenBank: AAB84448.1) was also cloned and tagged with His\(_6\), named EXP-His\(_6\) (one His\(_6\)-tag at the C terminus of EXP), using a set of primers F2 and R2 to compare cellulose-binding ability with CBM-His\(_6\). Furthermore, other types of His-tagged CBM (Fig. 2) were constructed on the basis of CBM-His\(_6\) such as CBM-His\(_6\)-His\(_6\) (two His\(_6\) tags at the C-terminus of CBM, named tandem His\(_6\)), and His\(_6\)-CBM-His\(_6\) (one His\(_6\)-tag at the N-terminus and another at the C-terminus, named both ends His\(_6\)) using a set of primer F3 and R3, and primer F4 and R4, respectively.

The proteins were overexpressed in *E. coli* BL21(DE3) by inducing with 0.1 mM IPTG at 15°C for 24 hours. The expressed proteins were purified by a
nickel-nitriloacetic acid (Ni-NTA) affinity column (Bio-Scale Mini Nuvia IMAC, Bio-Rad).

**Adsorption of protein to filter paper**

The cellulose-binding ability of the proteins to cellulose was examined. Whatman paper No. 1 filter paper (21 mg) was used as a model for cellulosic material and added to the purified protein solution (0.02 μmol/mL, 1 mL), followed by shaking at 160 rpm and 25°C for 2 hours. After centrifugation (15,000×g, 4 min), the protein concentration in the supernatant was measured by Bradford assay to calculate the protein adsorption on cellulose. Bovine serum albumin (BSA), which would show no cellulose-binding ability, was used as a control.

**Adsorption of metal ions on protein-bound filter paper**

To examine the metal binding property of the protein-bound filter papers, the filter papers were immersed in Mg^{2+}, K^+, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Au^{3+} ions solution (100 nmol/mL for each metal in 0.1 mol/L Tris-HCl, pH 8, 21 mg cellulose/mL, 1 mL), followed by shaking at 160 rpm and 25°C for 2 hours. After removal of the protein-bound cellulose by centrifuge, the concentration of metal ions in the supernatant was measured by ICP-AES (ICPE-9820, Shimadzu Co, Japan) to calculate the amount of metal ions adsorbed.

**SEM and XPS analysis of CBM-His$_6$-bound filter paper**

The filter paper was analyzed by SEM (JSM-IT200, JEOL Ltd., Japan) before and after CBM-His$_6$ adsorption (48 h) to assess the morphology of the filter papers. Furthermore, the CBM-His$_6$-bound filter paper was analyzed by XPS (AlK$_\alpha$ radiation) (JPS-9200,
JEOL, Japan), along with unbound normal filter paper as a control.

Desorption of metal ions from the adsorbent and the reusability

The desorption of Ni\(^{2+}\) ions and the reusability of CBM-His\(_6\)-bound cellulose in metal adsorption were investigated. After adsorption of metal ions on CBM-His\(_6\)-bound cellulose, Ni-bound filter paper was gently shaken (160 rpm, 25 °C) in 5 mmol/L EDTA solution (pH 8, 1 mL) for 2 hours to desorb Ni\(^{2+}\) ions from the cellulose, followed by measurement of Ni\(^{2+}\) ions concentration in the solution by ICP-AES to evaluate the desorption efficiency. After Ni\(^{2+}\) ion desorption, EDTA was removed by washing the filter paper adsorbed with protein with distilled water. One cycle includes adsorption and desorption process, and five cycles of experiments were continuously performed in the same manner.

Results and discussion

Construction of vectors and protein expression

The expression vectors for CBM-His\(_6\), EXP-His\(_6\), CBM-His\(_6\)-His\(_6\), and His\(_6\)-CBM-His\(_6\) were successfully constructed, which are confirmed by DNA sequencing. SDS-PAGE analysis after the expression of these proteins in *E. coli* DH5\(_\alpha\)(DE3) revealed that the target proteins were expressed in a soluble form and were successfully purified with Ni-NTA column.

Adsorption of proteins on filter paper

In order to choose a suitable protein for immobilization on cellulose, we compared the adsorption property of CBM-His\(_6\) and EXP-His\(_6\), along with BSA as a control, on filter paper (Fig. 3). CBM-His\(_6\) and EXP-His\(_6\) were adsorbed onto the filter paper with the
amount of 0.914 and 0.643 nmol/mg, respectively, whereas BSA showed negligible adsorption. This indicates that the adsorption of CBM-His$_6$ onto the filter paper is due to cellulose-binding property of CBM. EXP is a cellulose-expanding protein found in *B. subtilis*, which is called bacterial expansin and has a cellulose-binding ability similar to CBM. The difference in cellulose adsorption ability between CBM-His$_6$ and EXP-His$_6$ would result from the original cellulose-binding properties of these proteins reported in the literature.$^{11}$ The amount of CBM on filter paper was quantitatively evaluated by the fusion of GFP, and the amount of adsorbed CBM-His$_6$ is almost consistent with the report. Based on the result, we selected CBM-His$_6$ as a basic structural model for immobilization and used in subsequent experiments.

**Adsorption of metal ions on protein-bound filter paper**

After the adsorption of CBM-His$_6$ on cellulose, the CBM-His$_6$-cellulose was directly used for metal adsorption test. In a preliminary experiment, we confirmed that Ni$^{2+}$ adsorption reached equilibrium within 10 min (Figure S1 in supporting information). We investigated the effect of pH on Ni$^{2+}$ adsorption (Figure 4). We found the increase in Ni$^{2+}$ adsorption with pH increase. This would be due to the decrease in protonation of imidazole group ($pK_a = 6.04$) with pH increase. Although higher pH would be better for metal adsorption, some kinds of transition metal ions such as Fe$^{2+}$ are known to be precipitated at pH 9 or higher. Therefore, the following experiments were conducted at pH 8. Figure 5 shows the amount of metal ions adsorbed on CBM-His$_6$-cellulose. The CBM-His$_6$-cellulose exhibited metal binding property. Of the different metal ions tested, Fe$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$ and Zn$^{2+}$ ions showed higher adsorption on the adsorbent compared to other metal ions such as Mg$^{2+}$, K$^+$ and Au$^{3+}$. The results can be explained in terms of coordination and Hard and Soft Acids and Bases (HSAB) theory.$^{12}$ His$_6$-tag
is known to cooperate with Ni$^{2+}$ ions$^{10}$, which is classified into intermediate acid under the HSAB rules. Here, imidazole groups in histidine residue is also classified into intermediate base. This would be the reason for the adsorption of Ni$^{2+}$ ion by CBM-His$_6$-cellulose. As well as Ni$^{2+}$ ion, Fe$^{2+}$, Co$^{2+}$, Cu$^{2+}$ and Zn$^{2+}$ ions are also classified as intermediate acids, leading to efficient adsorption by CBM-His$_6$-cellulose. The stability constants (log K) between histidine and Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, Zn$^{2+}$ ions are 6.92, 8.69, 10.56, 6.63 (the data for Fe$^{2+}$ is not found).$^{13}$ The order of these stability constants are inconsistent with the order of adsorption of metal ions shown in Fig. 5. Since His$_6$ is an oligo peptide with 6 consecutive histidine residues, it may have different adsorption behavior from that of single histidine. Since Mg$^{2+}$ and K$^+$ ions are classified into hard acid, these metal ions were hardly recovered by CBM-His$_6$-cellulose. Since histidine is known to form a complex with soft acid Au$^{3+}$ ion,$^{14}$ a small amount of Au$^{3+}$ ions were adsorbed on CBM-His$_6$-cellulose.

**SEM and XPS analysis of CBM-His$_6$-bound filter paper**

In the present approach, both proteins and metal ions would be bound on cellulose. Figure 6 shows SEM images of filter paper before and after soaking in CBM-His$_6$ solution. We could not see a significant change in the appearance of the surface of the filter paper before and after the adsorption of CBM-His$_6$. CBM is known to swell cellulose fibers when it is bound on cellulosic materials,$^{15}$ however, filter paper seemed to maintain its original microstructure under the present condition.

Figure 7 shows the results of XPS analysis of the surface of CBM-His$_6$-cellulose before and after Ni$^{2+}$ ion adsorption. While no peak for Ni was observed in CBM-His$_6$-cellulose before Ni$^{2+}$ ion adsorption (Fig.7a), new peaks appeared around 860 eV and 880 eV after adsorption, which can be assigned to Ni 2P$_{3/2}$ observed with
AlKα radiation (Fig. 7b). Although the weak peaks for Ni observed are due to the low amount of Ni^{2+} ions on the surface, the XPS data confirms the presence of Ni^{2+} ions bound on the adsorbent.

Desorption of metal ions from the adsorbent and the reusability

We examined the desorption of metal ions from CBM-His_{6}-cellulose (metal recovery) and the reusability of the filter paper. We employed EDTA to remove Ni^{2+} ions from His-tagged proteins bound on a cellulose. In our preliminary test, the desorption of Ni^{2+} ions from the filter paper reached equilibrium within 10 minutes (Figure S2 in supporting information). Figure 8 shows the amount of adsorbed Ni^{2+} ions on the adsorbent (left bars) and that of desorbed Ni^{2+} ions from the adsorbent (right bars) in each cycle. The metal ions could be desorbed by EDTA from CBM-His_{6}-cellulose, and the adsorbent was able to be used in subsequent cycles. However, both metal adsorption and desorption decreased with the recycling process. There would be several possible reasons for this decline. Since EDTA is a strong chelator to remove metal ions from proteins, which could possibly change the protein structure by removing Ca^{2+} ion from CBM,^{16} then CBM-His_{6} protein would be denatured and released from cellulose. We tried to indirectly quantify the amount of CBM-His_{6} on cellulose after recycling tests. The amount of CBM-His_{6} released from cellulose to EDTA solution during Ni^{2+} desorption was measured by Bradford method, and it was found to be 1.34 nmol. Since this value is the amount of CBM-His_{6} released from the filter paper in one cycle, the amount of CBM-His_{6} on the filter paper after 5 cycles can be calculated as 12.5 nmol based on initial protein adsorption (34.9% CBM-His_{6} was released from the filter paper). In another respect, the efficiency would be declined due to the segmentation or breaking down of filter paper by CBM-His_{6}. CBM exhibits, not only cellulose-binding ability, but
also cellulose-swelling ability,\textsuperscript{15} which could possibly lead to gradual collapse of filter paper. Although a significant change was not observed in microstructure of cellulose after contacting with CBM-His\textsubscript{6} as shown in Figure 6, the filter papers actually became shredded during the repeated use with shaking in a metal solution, resulting in a loss of filter paper. In order to improve the reusability, we can take some other strategies such as: the use of packaging system for filter paper or the introduction of amorphous cellulose, which is not affected by CBM.

\textit{The efficiency of different configurations of CBM-His\textsubscript{6}}

To improve the adsorption efficiency, we tried to construct other types of His-tagged CBMs: CBM-His\textsubscript{6}-His\textsubscript{6} (two His\textsubscript{6} tags at the C-terminus of CBM, named tandem His\textsubscript{6}), and His\textsubscript{6}-CBM-His\textsubscript{6} (two His\textsubscript{6} tags, one at the N-terminus and one at the C-terminus, named both ends His\textsubscript{6}) (Fig. 2). Figure 9 shows the adsorption of these proteins on cellulose. CBM-His\textsubscript{6}, tandem His\textsubscript{6} and both ends His\textsubscript{6} were adsorbed onto the filter paper in different quantities (0.914 nmol/mg, 0.711 nmol/mg, and 0.710 nmol/mg, respectively), whereas BSA showed negligible adsorption. CBM-His\textsubscript{6} showed the highest binding ability to cellulose, compared to tandem His\textsubscript{6} and both ends His\textsubscript{6}, which have two His\textsubscript{6} units in their structure. This indicates that the number and the position of His\textsubscript{6} tag in CBM molecule affect the cellulose-binding ability of the proteins, and the introduction of two His\textsubscript{6} tags could slightly inhibit adsorption to cellulose, as observed in tandem His\textsubscript{6} and both ends His\textsubscript{6}. It is assumed that the introduction of His\textsubscript{6} tag to CBM molecules would cause some sort of the conformational change in protein structure, resulting in a slight decrease in adsorbing ability on cellulose.

Figure 10 shows the adsorption of Ni\textsuperscript{2+} ion on the protein-bound celluloses and the desorption of the ions. Adsorption of Ni\textsuperscript{2+} ion from the cellulose indicated that 1.91
nmol/mg, 2.26 nmol/mg and 2.84 nmol/mg of Ni$^{2+}$ ions could be adsorbed to CBM-His$_6$, tandem His$_6$ and both ends His$_6$-bound cellulose, respectively. The results indicated that all the CBM-His$_6$-bound cellulose materials could adsorb Ni$^{2+}$ ions. Both ends His$_6$ exhibited the highest Ni$^{2+}$ ion adsorption, followed by tandem His$_6$ and CBM-His$_6$. Tandem His$_6$ and both ends His$_6$ possess two His$_6$ units in their structure, which leads to higher Ni$^{2+}$ ion adsorption. Both ends His$_6$ exhibited higher adsorption than tandem His$_6$. This is probably due to the location of the two His$_6$ peptides. The two metal-binding sites are closely located in tandem His$_6$ and could possibly be involved in the coordination of one Ni$^{2+}$ ion. On the other hand, when two binding sites are distantly located, each His$_6$ could coordinate a Ni$^{2+}$ ion individually.

Desorption of Ni$^{2+}$ ion from the cellulose indicated that 1.48 nmol/mg, 1.69 nmol/mg and 2.04 nmol/mg of Ni$^{2+}$ ions could be desorbed from CBM-His$_6$, tandem His$_6$ and both ends His$_6$-bound cellulose, respectively. CBM-His$_6$ showed a relatively high desorption efficiency of Ni$^{2+}$ while the removal efficiency was not high in both ends His$_6$. Table 1 summarizes the molar ratios of adsorbed Ni$^{2+}$ ion per adsorbed protein molecule on cellulose and the ratios of desorbed/adsorbed Ni$^{2+}$ ions in three proteins. As for the adsorption ability, tandem His$_6$ and both ends His$_6$, which has two His-tags, showed higher Ni$^{2+}$ ion adsorption. tandem His$_6$ did not exhibit the same adsorption as both ends His$_6$. It would be presumably caused by the position of His$_6$ tag, that is, two His$_6$ tags in tandem His$_6$ are very close and they are responsible for one metal ion adsorption while two His-tags in both ends His$_6$ are positioned distantly, resulting in metal ion capture by each His$_6$ tag. Although we cannot precisely discuss the reaction stoichiometry precisely based only on the obtained result, it implies that one protein could capture more than one Ni$^{2+}$ ion. A reverse trend was found between the adsorption and desorption abilities in the proteins, indicating that excellent
adsorption ability would cause some difficulty in the removal of Ni$^{2+}$ ions.

Conclusions

In the present study, we developed novel metal adsorbents composed of proteins and cellulose and found that the number and configuration of metal ion-binding peptides fused with proteins affected both the adsorption and desorption of Ni$^{2+}$ ions. The bio-based adsorbent can be used to recover various metals by changing the type of peptides according to the target metal. The adsorption capacity and reusability could be improved by designing peptide and/or protein configuration. The present concept of bio-based metal adsorbent would be applied in many fields such as food industry and environmental cleanup to removal of harmful metals.

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Table 1 The molar ratios of adsorbed Ni\(^{2+}\) to proteins on filter paper and the ratios of desorbed Ni\(^{2+}\) to adsorbed Ni\(^{2+}\).

| protein            | adsorbed Ni\(^{2+}\) / protein on filter paper | desorbed Ni\(^{2+}\) / adsorbed Ni\(^{2+}\) |
|--------------------|-----------------------------------------------|------------------------------------------|
| CBM-His\(_6\)      | 2.09                                          | 0.775                                    |
| tandem His\(_6\)   | 3.18                                          | 0.748                                    |
| both ends His\(_6\)| 4.00                                          | 0.720                                    |
Figure Captions

Fig. 1  Conceptual diagram of cellulose-based adsorbent modified with CBM-His₆ fusion protein. PDB ID for CBM: 1NBC.

Fig. 2  Configuration of proteins constructed in this study.

Fig. 3  Adsorption of proteins on a filter paper.

Fig. 4  Effect of pH on Ni²⁺ adsorption (100 nmol/mL Ni²⁺, 21 mg cellulose/mL, 1mL, 120 min, 160 rpm, 25 °C). pH 4-5: 100 mM Acetate-NaOAc, pH 6-7: 100 mM NaH₂PO₄-Na₂HPO₄, pH 8-9: 100 mM Tris-HCl, pH 10: 12.5 mM Borate-NaOH.

Fig. 5  Metal ion adsorption rate on filter paper modified with CBM-His₆ at pH 8.

Fig. 6  SEM analysis of filter paper surface (a: untreated, b: after soaking in CBM-His₆ solution for 48 h).

Fig. 7  XPS analysis of the surface of filter paper after immersion in Ni²⁺ solution (a: without protein, b: with CBM-His₆).

Fig. 8  Recyclability of protein-bound filter paper (left bars: adsorption, right bars: desorption).

Fig. 9  Comparison of adsorption property of His-tagged proteins on filter paper.

Fig. 10  Adsorption (left bars) and desorption (right bars) of Ni²⁺ ions onto/from three types of bio-based metal adsorbents. Adsorption: 100 nmol/mL Ni²⁺ in 0.1 mol/L Tris-HCl, pH 8, 21 mg cellulose/mL, 1 mL, Desorption: 5 mmol/L EDTA, pH 8, 1 mL, 120 min, 160 rpm, 25 °C.
Fig. 1
Fig. 2
Fig. 3

Protein (nmol) per cellulose (mg)

BSA  EXP-His$_6$  CBM-His$_6$
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Protein (nmol) per cellulose (mg)

Fig. 9
Fig. 10