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Chromium(III) Binding Phage Screening for the Selective Adsorption of Cr(III) and Chromium Speciation

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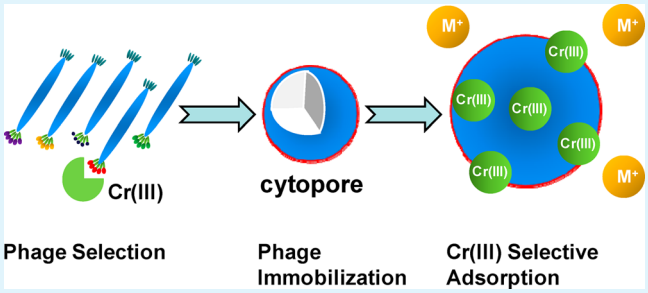
Supporting Information

ABSTRACT: The screening of suitable sorption medium is the key for highly selective solid phase extraction (SPE) of heavy metals. Herein, we demonstrate a universal protocol for producing selective SPE adsorbent through an evolutional approach based on phage display peptide library. By choosing chromium(III) as the model target, immobilized Cr(III) resins are first prepared using Ni-NTA affinity resins for the interaction with NEB heptapeptide phage library. After three rounds of positive biopanning against target Cr(III) and negative biopanning against foreign metal species, Cr(III) binding phages with high selectivity are obtained. The binding affinity and selectivity are further assessed with ELISA. The phages bearing peptide (YKASLIT) is finally chosen and immobilized on cytopore beads for Cr(III) preconcentration. The retained Cr(III) is efficiently recovered by 0.10 mol L^{-1} HNO_3 and quantified with ICP-MS. By loading 4000 μL of sample solution at pH 7.0 for 2 h and stripping with 400 μL of 0.10 mol L^{-1} HNO_3, a linear range of 0.05−0.50 μg L^{-1} is achieved along with an enrichment factor of 7.1. The limit of detection is derived to be 15 ng L^{-1} (3σ, $n = 7$) with a RSD of 3.6% (0.25 μg L^{-1}, $n = 7$). The procedure is validated by analyzing chromium content in a certified reference material GBW08608 (simulate water). In addition, chromium speciation in real water samples is demonstrated. Cr(VI) is first converted into Cr(III), and the latter subjected to the sorption onto the Cr(III) binding phage, followed by elution and quantification of the total chromium amount, and finally speciation is achieved by difference.



KEYWORDS: *phage display peptide library, metal binding peptide, Cr(III), preconcentration, solid phase extraction*

INTRODUCTION

Solid phase extraction (SPE) is an effective analytical sample preparation approach to isolate analytes from the matrix components with a certain extent of preconcentration.[1] The sorbent is no doubt the core that determines the extraction efficiency and specificity. When employing most of the currently available SPE sorbents for heavy metals, especially commercial sorbents, e.g., active carbon, silica, and ion exchange resins, however, sorption is always achieved through nonspecific binding involving electrostatic interaction or ion exchange, which inevitably leads to a low specificity toward target metals.[2] Chelating resins such as Amberlite IRC748 can selectively bind heavy metals in the presence of alkali and alkaline earth metals, but they are unable to discriminate one of them.[3] In the pursuit for producing target-recognition sorbents, certain extents of success have been achieved with molecular imprinting technique.[4,5] On the other hand, the selectivity or sorption capacity could be enhanced by sorbent functionalization with selective moieties.[2,6,7] However, synthetic sorbents always suffer from time-consuming preparation steps and the reproducibility issue. Some studies have been directed to explore a different path inspired by the nature. Microbial cell has a uniform size ranging from nanometer (virus) to micrometer (bacteria and yeast) and diverse metal binding sites including −COOH, −OH, $-HPO_4^{2-}$, SO_4^{2-}, $-RCOO^-$, $R_2OSO_3^-$, $-NH_2$, and −SH.[1,8] The capability of self-reproduction makes it simple to prepare the microbe-based sorbents. In addition, by overexpressing metal binding proteins or metalloregulatory proteins inside the cell or on the cell surface, the binding specificity and capacity of microbial cells for the target metal species can be significantly enhanced.[9−11] A sixfold improvement on Hg^{2+} accumulation was observed after surface display of mercury regulatory protein MerR on *Escherichia coli* surface through ice nucleation protein (INP) anchor with respect to wild-type *E. coli* JM109 cells.[12] In addition, the selective Hg^{2+} binding process was barely influenced by the presence of 100-fold excess of Cd^{2+} and Zn^{2+}. Similarly, by fusing ArsR, which regulates the intracellular arsenic level, to an elastin-like polypeptide in *E. coli*, 5- and 60-fold improvements on the sorption of As(V) and As(III) were achieved, respectively.[13] The overexpression of ArsR in *E. coli* also enhanced the selective bioaccumulation of methylated arsenic species.[14] By employing yeast cell with cadmium binding peptide displayed on its surface as SPE sorbent, a

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highly selective preconcentration approach for trace cadmium was developed.[15,16] The tolerance level toward coexisting ions had an improvement of 25−1000-fold compared with native yeast.

Nevertheless, not all heavy metals have their corresponding metalloregulatory proteins.[17] Besides, the soft-ligand (−SH and $-NH_2$)-rich nature makes metal binding proteins more keen on binding with soft metals including Hg(II), Cd(II), and Cu(I), rather than hard and borderline metals such as Al(III), Cr(III), and Ni(II).[1,18] A more universal approach to obtain highly selective and self-reproducible sorbent is necessary. Phage display peptide library technique is an evolutional approach via high-throughput selection of peptide ligands of diversity,[19] which provides promising potentials for this purpose. The filamentous bacteriophage M13 is the most commonly used vector for constructing random peptide libraries. Variant peptides with diversity of up to 10^9 are expressed on the coating protein of M13, while their corresponding DNAs encoding each peptide remain inside the proteinous cylinder coat. By repeated biopanning against desired target, phages with peptide ligands for target can be selected; the peptide sequences are then identified by DNA sequencing of the corresponding phage. The phage display peptide library technique has been widely applied in protein−protein interaction study, small molecule drug discovery, immune response investigation, and binding peptide selection.[20] Due to the intrinsic binding nature of peptide with heavy metals, several metal binding peptides were also selected through this technique. Peptides binding specifically with nickel,[21] cadmium,[22] and lead[23] were selected and verified. These metal binding peptides have further been expressed on microbial cell surface for enhanced metal uptake.[22,24] Lead binding peptide selected from heptapeptide library has been used to modify porous gold electrode for electrochemical sensing for lead.[25] However, most of the studies focused on the application of the peptide selected from phage display library, while none of them explored the performance of phages bearing metal binding peptide as SPE sorbent. In fact, phage bearing metal binding peptide is an ideal SPE sorbent: the dense proteinous cylinder coat makes M13 phages robust and thermally and chemically stable and offers various binding sites for heavy metal sorption.[26] The small size (6.5 nm × 900 nm) also endows it with higher specific surface area in comparison to other microbial cells.[27] Most importantly, the binding specificity toward target could be readily regulated through biopanning procedure. All these features make M13 phages suitable as SPE sorbent for heavy metal preconcentration and separation.

Chromium plays a paradox role in nature, with Cr(III) being indispensible cofactors in enzymes and Cr(VI) being toxic and carcinogenic even at low dosage.[28,29] Once entered into cells, Cr(VI) is easily reduced to Cr(III) by intracellular reductant including ascorbic acid and glutathione (GSH).[29,30] Therefore, the measurement of Cr(III) in body fluids can offer helpful information when the human body is exposed to Cr(VI). Cr(III) is a typical hard metal which does not bind to natural metal binding protein.[18] In addition, no metalloregulatory protein has been reported specifically for chromium.[17] In this respect, Cr(III) is chosen as the target metal species in the present study. A Cr(III)-binding peptide is screened through biopanning of a phage display heptapeptide library, and its affinity toward Cr(III) is evaluated with an enzyme-linked immunosorbent assay. The self-reproducible "bio-nanowire" bearing Cr(III)-binding peptide at the tail is assembled onto microcarrier cytopore beads, which are then used for the selective preconcentration/separation of Cr(III).

EXPERIMENTAL SECTION

Chemicals and Materials. Unless otherwise specified, all the chemicals used in the present study are analytical reagent grade obtained from the Sinopharm Chemical Reagent Co. (Shanghai, China). A phage display peptide library for biopanning of heptapeptide together with its host cell *E. coli*. ER2738 (Ph.D., Phage Display Peptide Library Kit) is purchased from NEB (New England Bio-Laboratories, U.S.). Horseradish peroxidase-conjugated anti-M13 antibody and 2,2′- azinobis(3-ethylbenthiazoline-6-sulfonic acid) (ABTS) used for ELISA are received from GE healthcare (U.S.) and Sigma-Aldrich, respectively. Ni-NTA-Sefinose resin used for metal-loaded resin preparation is obtained from Bio Basic Inc. (Canada), and the centrifugal filter device with MWCO of 100 kDa employed to isolate phages from eluate is obtained from Millipore (U.S.). M13 phage DNA extraction kit for phage ssDNA extraction is purchased from BioTeke Co. (Beijing, China). Cytopore microcarrier beads (dry bead size 200−280 μm) for phage immobilization are obtained from GE healthcare.

LB medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl in 1 L of deionized (DI) water, pH 7.0−7.2) with 20 mg L^{-1} tetracycline is used for host cell culture and phage amplification. In order to avoid wild-type phage contamination, all solutions used are sterilized either by autoclave procedure (121 °C, 20 min) or passing through 0.22 μm sterilizing filter, and the pipet tips equipped with filter cartridge are used throughout.

Stock solutions of Cr(III)/Cr(VI) (1000 mg L^{-1}) are prepared by dissolving an appropriate amount of $CrCl_3{\cdot}6H_2O/K_2Cr_2O_7$ in 0.1 mol L^{-1} HNO_3. Working standards of various concentrations are obtained by serial dilution of the stock solutions. The pH value of the sample solution is adjusted to pH 7.0 by using 0.1 mol L^{-1} HNO_3 and/or 0.1 mol L^{-1} NaOH. Deionized water (18.2 MΩ cm, 25 °C) is used throughout.

Instrumentation. An Agilent 7500a inductively coupled plasma mass spectrometer (ICP-MS, Agilent Technologies Inc.) is used for the determination of chromium amount by choosing an isotope of ^{53}Cr, and the operation parameters for ICP-MS are given in Table S1. A microplate reader (BioTek SynergyH, U.S.) is used for the absorbance measurement in ELISA assay. The pH values of solutions used in the present study are monitored with a pH meter (Thermo 868, U.S.).

The Preparation of Metal-Chelating Resin. Cr(III) immobilized resin is prepared by replacing the preloaded Ni(II) by Cr(III) on the Ni-NTA-Sefinose resin. Specifically, in order to strip Ni(II) off the Ni-NTA-Sefinose resin, the resin beads are first washed with sufficient EDTA solution (0.5 mol L^{-1}, pH 8.0) until the resin turned from blue to colorless. It is then rinsed three times with citrate buffer solution (pH 4.4) and stored at 4 °C for future use. The metal-free resin is labeled as M^- resin.

To prepare Cr(III) immobilized resin, 1 mL of M^- resin suspension is incubated in 5 mL of Cr(III) solution ($Cr(NO_3)_3{\cdot}9\ H_2O$, 1.0 mmol L^{-1}) overnight with gentle shaking. It is then rinsed six times with citrate buffer solution (pH 4.4) and stored at 4 °C. Resins loading other metals are prepared in a similar procedure, except that the metal salts (M^+ salts) are replaced by $Fe(NO_3)_3{\cdot}9\ H_2O$, $CuSO_4{\cdot}5\ H_2O$, $ZnSO_4{\cdot}7\ H_2O$, $CdCl_2$ (anhydrous), and $HgCl_2$ (anhydrous) and are labeled as M^+ resin.

Screening Cr(III) Binding Phage with High Selectivity. In order to obtain phages with high selectivity toward Cr(III), both positive screening and negative screenings are necessary. Figure 1 illustrates the detailed schematic of the whole screening procedure for Cr(III) binding phage.

For the purpose of eliminating phages that bind with resin beads rather than the immobilized Cr(III) ions, negative screening against M^- resin is conducted at first. One hundred microliters of the phage library (~2.0×10^{12} virions, library complexity (unique clones) = ~2.8×10^9 as claimed by NEB) is dissolved in 900 μL of citrate buffer

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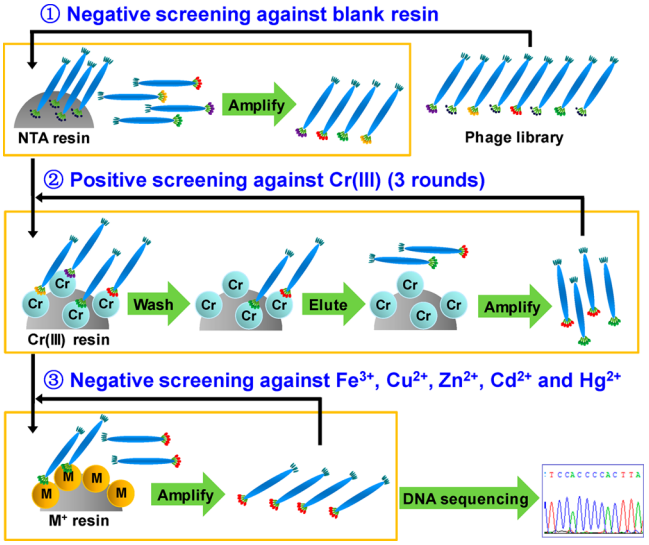

Figure 1. Schematic diagram illustrating the whole biopanning procedure.

solution (pH 4.4). It is then mixed with 100 μL of M^- resin beads. The mixture is allowed to shake for 25 min (100 rpm, 25 °C). Afterward, the supernatant is collected and further amplified. Meanwhile, 10 μL of the supernatant is used for phage titering using X-gal/IPTG agar plates. The amplified phages are purified by precipitation with PEG/NaCl (20% (w/v) poly(ethylene glycol) (PEG)-8000, 2.5 M NaCl) according to the standard protocol provided by NEB.

The amplified phages are further treated with positive screening against immobilized Cr(III) resin. One hundred microliters of the amplified phage (~5.0×10^{12} virions) is dissolved in 900 μL of citrate buffer solution (pH 4.4) and mixed with 100 μL of Cr(III) resin beads, followed by shaking for 25 min (100 rpm, 25 °C). The supernatant is discarded, and the resin is washed twice with citrate buffer solution (pH 4.4) to remove the loosely bonded phages. One milliliter of EDTA solution (0.5 M, pH 8.0) is then added and allowed to shake with resin beads for 10 min (200 rpm, 25 °C). The eluate is further centrifuged with a centrifugal filter device with MWCO of 100 kDa (5000 rpm, 15 min, 4 °C) to separate the phages with coeluted Cr(III) ions. Phages retained on the filter membrane are carefully collected, amplified, and titered for conducting the next round of positive screening. For obtaining phages with high affinity with Cr(III), three rounds of positive screening are conducted.

As is known, phages binding with Cr(III) might also have affinity to other metal species. Hence, negative screening against other metal ions is conducted. The procedure is similar to that for negative screening against M^- resin, except that M^- resin is replaced by M^+ resin. In short, 10 μL of the phage solution is dissolved in 500 μL of citrate buffer solution (pH 4.4) and is then mixed with 100 μL of M^+ resin beads, followed by shaking for 25 min (100 rpm, 25 °C). Thereafter, the supernatant is collected and further amplified. Considering the phage titering results (Table S2), negative screenings against Fe^{3+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Hg^{2+} are conducted for 1, 2, 1, 2, 1 rounds.

DNA Extraction and Sequencing. Among the Cr(III) binding phages that went through the whole biopanning procedure, 15 blue plaques are randomly selected from the final titering plate, and the individual phage clones are amplified. The phages' ssDNA are extracted using an M13 phage DNA extraction kit and sequenced by Shanghai Sangon Biotech Co. (Shanghai, China). The corresponding amino sequences of the selected phages are then derived.

Enzyme-Linked Immunosorbent Assay (ELISA) of the Selected Cr(III) Binding Phages. The specificity of the selected phages toward Cr(III) is testified using ELISA assay. Briefly, 100 μL of the amplified individual phage clones (1.2×10^{11} pfu mL^{-1}, diluted with citrate buffer at pH 4.4) is mixed with 200 μL of M^+ resins. Ten micromoles of M^+ salts is employed to prepare the metal loading resins. After incubation at room temperature for 1 h, the resin beads are washed with citrate buffer solution three times to remove the loosely bonded phages. One milliliter of horseradish peroxidase (HRP)-conjugated anti-M13 antibody (1:5000 in citrate buffer, pH 4.4) is then added to allow incubation with phages bonded to the resin beads for 1 h. After washing with citrate buffer (pH 4.4, containing 0.5% Tween 20) three times, 400 μL of a substrate solution (220 mg L^{-1} ABTS, 0.17% H_2O_2 (30%), 50 mM sodium citrate, pH 4.0) is added to allow incubation for 1 h. ABTS is oxidized by H_2O_2 under the catalysis of HRP, resulting in a green product with a λ_{max} at 405 nm. The absorbance of the supernatant at 405 nm is recorded with a microplate reader.

Immobilization of Cr(III) Binding Phages onto Cytopore. Cr(III) binding phages are immobilized on the surface of microcarrier beads cytopore with the following procedure: 10 mL of suspended cytopore microcarrier beads (10 mg mL^{-1}, rinsed with DI water and sterilized) is added to 10 mL of the selected phage dispersion (~10^{13} pfu mL^{-1}, pH 7.5) with gentle stirring overnight (250 rpm, 37 °C). The phage-loaded microcarriers are then rinsed with DI water to remove the loosely bonded phages and resuspended in 10 mL of DI water to give a final concentration of ~10 mg mL^{-1} for future use.

Preconcentration and Separation of Trace Cr(III). It is demonstrated that the Cr(III) binding phage beads also exhibit certain extent retention of Cr(VI). Thus, Cr(VI) should be pre-eliminated before Cr(III) adsorption. This is achieved by incubating the sample solution with the cytopore beads which only retain Cr(VI) at pH 7.0.

Two hundrerd microliters of the immobilized phage are settled by gravity, and the supernatant is discarded. Four milliliters of sample solution is mixed with the immobilized phage and incubated in a vortex mixer for 2 h for complete adsorption of Cr(III). After quick gravity settling, the supernatant is discarded, and 400 μL of nitric acid (0.1 mol L^{-1}) is added as eluent under vigorous shaking for 20 min. The eluate is completely separated from the beads by gravity settling, and the concentration of Cr(III) in the eluate is determined by ICP-MS. For the interference tests, increasing amounts of coexisting species are added to the Cr(III) standard solutions, and then the adsorption/elution procedures are followed as described previously. The tolerance level for a certain kind of coexisting species is identified as the maximum concentration of coexisting species that possess no interferences within an error range of ±5%.

Samples and Sample Pretreatment. Certified reference material GBW08608 (simulate water, National Research Center for Certified Reference Material (NRCCRMS), Beijing) used for procedure validation is diluted and adjusted to pH 7.0 before being subjected to the above adsorption process. Snow water and tap water collected from NEU campus are used for further chromium analysis. Water samples are filtered through a 0.22 μm membrane filter and adjusted to pH 7.0 before being subjected to the above adsorption process. The analysis of Cr(VI) content is facilitated by first measuring Cr(III) as described in the previous section followed by quantifying the total amount of chromium after converting Cr(VI) into Cr(III) via reduction by ascorbic acid (1 g L^{-1}).[31] The concentration of Cr(VI) is obtained by difference.

RESULTS AND DISCUSSION

Biopanning for the Cr(III) Binding Phages. The correct target metal immobilization procedure is a key point in the biopanning process for the selection of target binding phages. Immobilized Cr(III) with free ligands is prepared by replacing the preloaded Ni(II) with Cr(III) on the commercial immobilized metal affinity chromatography (IMAC) resin Ni-NTA agarose beads. Chromium(III) exists in the form of hexaaqua complex in aqueous medium, i.e., $(Cr(H_2O)_6)^{3+}$.[32] Its four coordinating positions are occupied by binding to NTA through N, O, O, O atoms, while leaving another two coordinating positions preoccupied by water molecules to be

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replaced or exchanged by phages. Meanwhile, the acidity of the reaction mixture is maintained at pH 4.4 throughout the whole biopanning process for the purpose of avoiding metal precipitation.[29]

A complete biopanning procedure consists of prenegative screening against blank NTA resin, positive screening against Cr(III), and postnegative screening against the other metal species. The negative screening against blank NTA resin eliminates those phages nonspecifically bound to the functional groups on NTA resins other than Cr(III). After this procedure, ca. 55% of the phages from phage library remains in the supernatant for the next round of biopanning. Thereafter, three rounds of positive screening against Cr(III) is conducted, during which process EDTA is used to strip the phages off the immobilized Cr(III) resin. It is worth mentioning herein that the screening condition is more stringent in the third round with respect to the first two rounds due to the reduced amount of Cr(III) loaded, in order to increase the binding affinity of the selected phages. Following the positive screening procedure, the negative screenings against some of the representative transition and heavy metals, e.g., Fe^{3+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Hg^{2+}, are conducted successively to eliminate phages that have affinity with other metals. The increase of phage recovery (Table S2, output/input) after each round of screening indicates that the phages which do not bind to other metal species are enriched effectively.

Specificity of the Selected Phages to Cr(III). After the whole biopanning procedure, 15 monoclonal Cr(III) binding phages are randomly selected for DNA sequencing, and 11 sets of peptide sequences are obtained as listed in Table 1. No consensus motif in common is found among the 11 identified peptides. However, a predominant feature of all the 11 Cr(III) binding peptides is the relative high content of −OH containing amino acid and nonpolar amino acid. The phenomenon is in accordance with HSAB (hard and soft acids and bases) theory, in which −OH ligands are considered as hard bases and Cr(III) is considered as hard acid that binds preferentially with hard bases.[33] High content of nonpolar amino acid might contribute indirectly to the formation of favorable spatial structure for Cr(III) binding due to the peptide folding caused by hydrophobic interaction. It is worth noting that as a frequently encountered amino acid in the naturally existing metal binding proteins, cysteine is not shown in all of the 11 peptide sequences, probably due to the fact that reductive condition is not specially maintained during the biopanning process.

Table 1. Peptide Sequence of the Selected Cr(III) Binding Phages and Their Amino Acid Property Analysis

no.	peptide sequence	property of each amino acid[a]	frequency of −OH containing amino acid	frequency of nonpolar amino acid
1	ATNKITK	nopbnob	2	2
2	SKVGYPT	obnnono	3	3
3	YKASLIT	obnonno	3	3
4	ALGGVAM	nnnnnnn	0	7
5	QSPRSIS	ponbono	3	2
6	ANLWPDG	npnnnan	0	5
7	GPLSHKG	nnnobbn	1	4
8	NNNWPWT	pppbnno	1	2
9	IQMTDIA	npnoann	1	4
10	SRHLHEW	obbnban	1	2
11	APVTSMK	nnnoonb	2	4

[a]n: nonpolar amino acid (A, V, L, I, F, W, M, P, G); p: polar and uncharged amino acid (C, N, Q); o: −OH containing amino acid (S, T, Y); a: acidic and polar amino acid (D, E); b: basic and polar amino acid (K, R, H).

The binding affinity with Cr(III) of the 11 identified peptides is determined with ELISA assay. Given that the absorbance at 405 nm of the final product in ELISA assay is proportional to the amount of the phages that bind with Cr(III) resin, it is thus used for judging the Cr(III) affinity of phages/peptides. Figure 2 shows the normalized $Abs_{405\ nm}$ of the final product of the 11 selected phages. It can be seen that most of the selected phages bearing these Cr(III) binding peptides (except for P1 and P7) showed higher affinity with Cr(III) with respect to the phage library. This confirms the effectiveness of the biopanning process. Four out of 11 of the selected phages with relative higher affinity, i.e., P3, P5, P6, and P8, are further compared in terms of binding selectivity. Figure 3 compares the affinity of the four phages for both the target, e.g., Cr(III), and the other metals, Zn(II), Hg(II), Fe(III), Cd(II), and Cu(II). It is clearly seen that these four peptides bind selectively with Cr(III) over other metals tested and P3 shows a relatively higher affinity for Cr(III) and an overall lower affinity for other metals among the

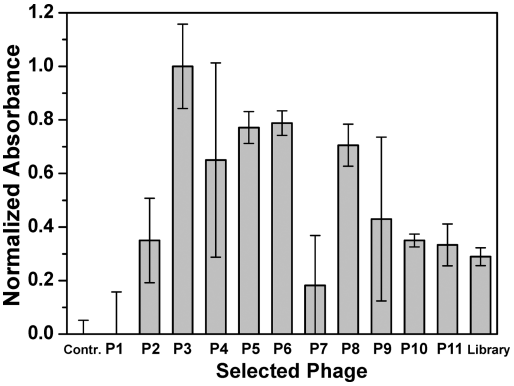

Figure 2. Binding affinity of the selected Cr(III) binding phages with Cr(III). Binding affinity is represented by the normalized absorbance at 405 nm; Cr(III) column without adding any phages is used as control. Details of experimental conditions are given in the section on ELISA assay of the selected Cr(III) binding phages.

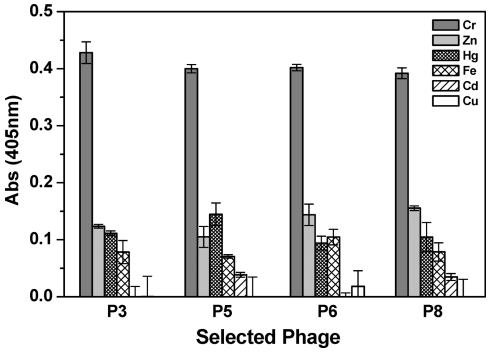

Figure 3. Binding selectivity of the selected Cr(III) binding phages (P3, P5, P6 and P8). Binding affinity toward different metals is represented by the absorbance at 405 nm. Details of experimental conditions are given in the section on ELISA assay of the selected Cr(III) binding phages.

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four peptides. Considering both the binding affinity and specificity/selectivity, the phage bearing P3 is finally chosen for the selective preconcentration and separation of Cr(III).

Immobilization of Cr(III) Binding Phage on Cytopore Beads. The small dimension of M13 phages makes them well dispersed in aqueous solutions but hard to separate from supernatant even under centrifugation at >10 000 rpm. Considering that M13 phages possess negative charge under neutral pH (zeta potential of ca. 31.5 mV at pH 7.5 as determined), they can be easily assembled on the surface of a biocompatible, positively charged, and porous microcarrier cytopore through electrostatic interaction.[15] The loading of phage particles is confirmed by the appearance of brown color in the phage-loaded cytopore after staining with $AgNO_3$ (Figure 4). By employing the immobilized Cr(III) binding phage as SPE sorbent for Cr(III) preconcentration, solid–liquid separation is achieved by gravity settling of immobilized phages within minutes; thus, tedious centrifuge steps are avoided.

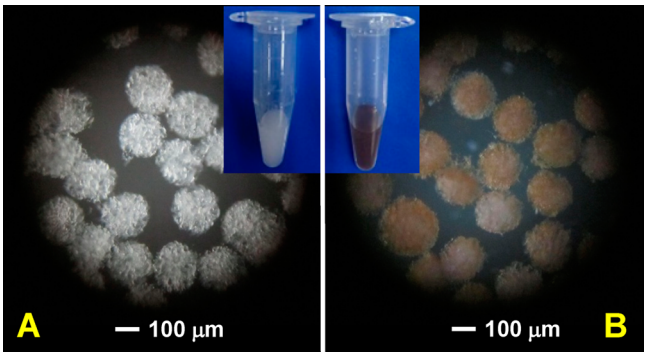

Figure 4. Optical microscopic images of cytopore (A) and Cr(III) binding phage-loaded cytopore (B) after staining with $AgNO_3$ (×100). Inset: photograph of A and B.

Selective Adsorption of Cr(III) by the Immobilized Phages. The pH value of the reaction medium generally plays a crucial role in the biosorption process of heavy metal species due to its pronounced influence on both the binding sites dissociation on phage particle surface and the metal species distribution. The sorption efficiencies for Cr(III) by the immobilized Cr(III) binding phage P3 and the cytopore itself at various pHs are shown in Figure 5. It can be seen that native cytopore barely adsorbs Cr(III) within pH 3–9, mainly due to the electrostatic repulsion. Meanwhile, the sorption efficiency for Cr(III) by cytopore loaded with Cr(III) binding phage improves significantly with the increase of pH value, and a maximum sorption efficiency is reached at pH 7.0. This indicates that the adsorption of Cr(III) originates from Cr(III) binding phage other than cytopore, assuring the binding selectivity for Cr(III). The unfavorable retention of Cr(III) at lower pH values might be attributed to the competitive binding with active sites on the phage particle surface by protons (H^+). For further studies, the selective adsorption of Cr(III) is conducted at pH 7.0.

Figure 6 shows the effect of reaction time on the sorption efficiency for Cr(III) by the immobilized Cr(III) binding phage. The sorption of Cr(III) on the immobilized Cr(III) binding phages is demonstrated to be a fast process, of which the sorption equilibrium can be reached within 30 min (Figure 6). However, a longer equilibration time is needed when the same amount of Cr(III) is provided in a larger sample volume. This is probably due to the fact that it takes a longer time for the target to reach the pores on cytopore wherein the phages are immobilized. For the ensuing studies, a reaction time of 2 h is chosen in order to ensure sufficient contact time between the phages and Cr(III).

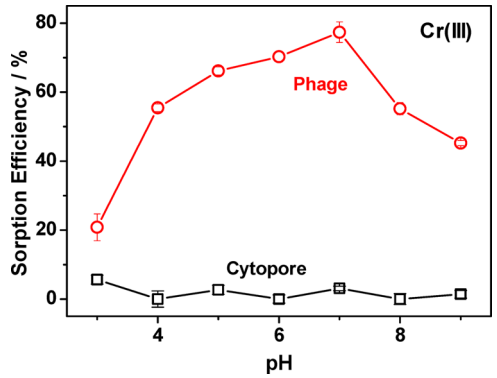

Figure 5. pH dependent sorption efficiency for Cr(III) by the immobilized Cr(III) binding phage (red circle) and cytopore (black square). Sample solution: 2.0 μg L^{-1} Cr(III)/500 μL; the amount of sorbent: 10 g L^{-1}/200 μL; contact time: 30 min.

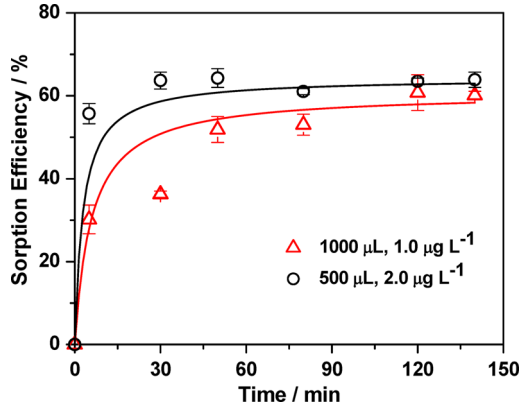

Figure 6. Effect of reaction time on the sorption efficiency for Cr(III) by the immobilized Cr(III) binding phage at 1.0 and 2.0 μg L^{-1}. Cr(III) solution: 2.0 μg L^{-1}/500 μL or 1.0 μg L^{-1}/1000 μL, pH 7.0. The amount of sorbent: 10 g L^{-1}/200 μL.

Evaluation on Cr(III) Preconcentration. Due to the existence of reductive amino acids including cysteine and tyrosine on the phage surface, the reduction of Cr(VI) to Cr(III) tends to take place followed by adsorption on the phage. Figure S1 shows the adsorption efficiency for Cr(VI) by the immobilized Cr(III) binding phage and cytopore at various pHs. A similar trend of sorption for Cr(VI) is observed as that for the adsorption of Cr(III) by the immobilized Cr(III) binding phages as a function of pH. In practice, the presence of Cr(VI) might cause potential interference on the adsorption and preconcentration of Cr(III); thus, for the purpose of evaluating Cr(III) adsorption behaviors, the coexisting Cr(VI) should be removed by native cytopore at pH 7.0.

Considering that lower pH value is not preferential for the retention of Cr(III) on the immobilized phages, nitric acid is preferred as an eluent for stripping of the retained Cr(III). Thus, a selective Cr(III) preconcentration procedure can be developed based on the above observations. The elution efficiency as a function of nitric acid concentration is therefore investigated within a range of 0.01–0.20 mol L^{-1} HNO_3. As

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can be seen in Figure 7, 100% of the retained Cr(III) could be readily stripped off the immobilized phages at a HNO_3

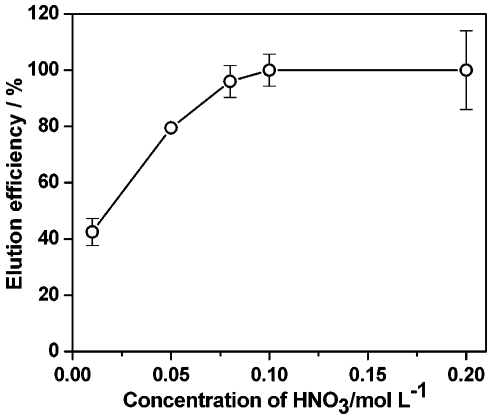

Figure 7. Elution efficiency for Cr(III) retained by the immobilized Cr(III) binding phage as a function of nitric acid concentration. Sample: 2.0 μg L^{-1} Cr(III)/500 μL, pH 7.0; eluent: 500 μL; elution for 20 min under vigorous shaking; sorbent: 10 g L^{-1}/200 μL.

concentration of >0.10 mol L^{-1}. A further increase on the eluent concentration to 0.20 mol L^{-1} contributes nothing to the recovery. Thus, 0.10 mol L^{-1} of nitric acid is chosen for the elution of Cr(III). For facilitating effective and quantitative stripping of the retained Cr(III), a vigorous shaking for 20 min is adopted.

Interferences. The interfering effects of some coexisting cationic and anionic species are investigated. At a Cr(III) concentration level of 0.25 μg L^{-1} and within a ±5% error range, the following tolerance ratios are achieved, i.e., 3 180 000-fold NO_3^-, 2 000 000-fold K^+, 400 000-fold Cl^-, HCO_3^-, and Na^+, 200 000-fold Ca^{2+} and $H_2PO_4^-$, 40 000-fold Mg^{2+}, 4000-fold Pb^{2+}, 2000-fold Ni^{2+}, 400-fold Cu^{2+} and Fe^{3+}, 320-fold Al^{3+}, 200-fold Zn^{2+} and Cd^{2+}. For common biological and environmental samples, the contents of the above coexisting species in the sample digests, especially after appropriate dilution, will be well controlled within their tolerate concentration levels.[7] Therefore, the present procedure can be directly adopted for the preconcentration of Cr(III) from real world sample matrixes.

Analytical Performance and Real Sample Analysis. For each round of analysis, 4000 μL of sample solution is adjusted to pH 7.0 and treated with native cytopore for 5 min to adsorb and remove Cr(VI). It is then incubated with 2 mg of immobilized Cr(III) binding phage for 2 h, and the retained Cr(III) is then eluted by 400 μL of nitric acid (0.10 mol L^{-1}) for 20 min. The amount of Cr(III) is determined by ICP-MS. The characteristic analytical performance data for the selective Cr(III) preconcentration procedure are summarized in Table 2. With a sample volume of 4000 μL and an eluent volume of 400 μL, an enrichment factor of 7.1 and a detection limit of 15 ng L^{-1} are obtained within a linear range of 50−500 ng L^{-1}, achieving a precision of 3.6% RSD at 250 ng L^{-1} (n = 7). Table 3 gives a comparison of the analytical performance in terms of detection limit and precision with some other Cr(III) preconcentration procedures.

The present method is validated by analyzing the chromium content in a certified reference material, GBW 08608 (simulate water). It is shown that chromium in GBW 08608 exists only in the form of Cr(III), and the obtained chromium content (31.9 ± 5.0 μg L^{-1}) is in fair agreement with the certified value (31.4 ± 2.0 μg L^{-1}). This observation demonstrates the practical usefulness of the present sample pretreatment protocol. Further validation is performed by analyzing Cr(III)/Cr(VI) contents in a series of water samples along with spiking recoveries. As illustrated in Table 4, favorable recoveries are achieved for 0.06−0.13 μg L^{-1} chromium.

Table 2. Characteristic Analytical Performance Data for the Selective Preconcentration of Cr(III) by the Immobilized Cr(III) Binding Phages with Detection by ICP-MS

parameter	value
sample volume	4000 μL
eluent volume	400 μL
detection limit (3σ, n = 7)	15 ng L^{-1}
RSD (250 ng L^{-1}, n = 7)	3.6%
linear range	50−500 ng L^{-1} (R^2 = 0.9977)
enrichment factor	7.1

Table 3. Comparison of Analytical Performance by Various SPE Sorbents for the Selective Preconcentration of Cr(III) with Detection by ICP-MS

SPE sorbents for Cr(III)	LOD (ng L^{-1})	RSD (%)	ref
restricted accessed magnetic nanoparticles	11.9	7.6	34
Ti-containing mesoporous silica	19	7.3	35
2-nitroso-1-naphthol impregnated MCI GEL CHP20P resin	40	1.6−3.2	36
DPTH-gel Amberlite resin	30	3.2	37
immobilized Cr(III) binding phage	15	3.6	this work

Table 4. Chromium Speciation in Environment Water Samples (n = 3, Confidence Level 95%, Unit: μg L^{-1})

	Cr(III)			Cr(VI)		
samples	found	spiked	recov. (%)	found	spiked	recov. (%)
snow water	0.12 ± 0.05	0.10	100	0.08 ± 0.03	0.10	105
tap water	0.06 ± 0.04	0.10	98	0.13 ± 0.03	0.10	103

CONCLUSIONS

In this study, a highly selective Cr(III) binding peptide (YKASLIT) is selected through biopanning from phage display peptide library. A novel and selective Cr(III) preconcentration and separation protocol based on the sorption of Cr(III) on the immobilized phages bearing Cr(III) binding peptide is thus developed. The practical usefulness of this approach is validated by chromium analysis in certified reference material and chromium speciation in environmental water samples. The observations in the present study provide a universal protocol for the development of solid phase extraction sorbents with tunable selectivity toward targeting metals, by replacing the targets with other metals of interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b05606.

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Tables presenting instrument settings for Cr(III) determination by ICP-MS and the phage titer results of biopanning procedure; figure presenting the pH dependent sorption efficiency of Cr(VI) by the immobilized Cr(III) binding phage and cytopore (PDF)

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Notes

The authors declare no competing financial interest.

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