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Notes

Visual Assay of Total Iron in Human Serum with Bathophenanthrolin Disulfonate-accommodated MCM-41

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A simple visual method for determining the total iron in human serum is proposed based on color development in the nanospace of mesoporous silica MCM-41 and a chromogenic ligand bathophenathroline disulfonate (BPS). Observing the color intensity of a complex between iron(II) and BPS devloped on the MCM-41 material by the naked eye enabled us to quntify iron(II) with a detection limit of 0.5 μM. The BPS-loaded MCM-41 was successfully applied for quantifying the total iron in human serum.

Keywords Mesoporous silica, visual determination, bathophenanthroline disulfonate, iron(II) in serum

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Introduction

The development of molecular sensing systems using mesoporous silica MCM-41 and its analogs has attracted attention, because of nanopore dimensions that enable to accomodate a variety of ions and molecules. Not only enzymes,[1-6] but also small molecules,[7-9] have been accomodated into nanopores or chemically modified nanopores for a variety of purposes, including the design of highly sensitive molecular senisng systems.[10-18] We reported lipid-loaded MCM-41 as a platform for highly sensitive electrochemical and fluorometric detection of analytes.[19,20]

An advantage of using molecule-loaded meosporous silica for chemical sensing is that small moleclules of interest are spontaneously transported and concentrated in the pores. Light can penetrate mesopores to excite internal dyes, as already shown for fluorescence dyes.[20] The pores may be regarded as being microspaces for accomodating ions and molecules. If optical properties can be changed by a chemical interaction of an analyte with chromogenic molecules in the nanopores, a spectroscopic sensing system can be designed.

In the present work, we investigated the accomodation of chromogenic reagents for iron(II) into the nanopores of MCM-41. A chromogenic ligand, bathophenanthroline disulfonic acid (BPS), is loaded on the MCM-41 material and used for naked eye-based visual determinations of total iron in human serum.

Experimental

Materials

1,10-Phenathroline (phen), bathophenanthrolinedisulfonic acid, disodium salt (BPS) and bathophenanthroline (BP) were obtained from Dojindo Laboratories (Kumamoto, Japan). Hydroxyammonium chloride, ammonium iron(II) sulfate hexahydrate, iron(III) chloride, L-ascorbic acid, bilirubin, acetonitrile (AN), tetradecyltrimethylammonium bromide (purity >99%, abbreviated as C_{14}TMABr) and tetraethoxysilane (TEOS) (purity >95%) were obtained from Wako Chemicals. Co. (Osaka, Japan). A solution of 1,10-phenanthroline iron(II) salt (1/40 M ferroin solution) was obtained from Merck (Darmstadt, Germany). 3-Aminopropyltrimethoxysilane (APS) was obtained from Shin-Etsu Chemical Co. (Tokyo, Japan). Tris(2,2′-bipyridine)iron(II) hexafluorophosphate ($[Fe(bpy)_3]^{2+}PF_6$), human serum (type AB, male, H4522-20ML), albumin from human serum (lyophilized powder, 97 – 99%) and γ-globulins from human blood (>99%) were obtained from Sigma Chemical (St. Louis, MO, USA). A hepatitis C virus (HCV) specimen (11486477) of human serum (male, age 41) was obtained from Promeddx (Norton, MA, USA). 96-Well plates (round bottoms) were obtained from Nunc A/S (Roskilde, Denmark). Milli-Q water (Millipore reagent water system, Bedford, MA) was used throughout the experiments.

Preparation of MCM-41 and APS-MCM-41

Mesoporous silica MCM-41 was synthesized according to the reported procedure.[21] Briefly, 2.55 g of C_{14}TMABr as a template was dissolved in 120 g of Milli-Q water and then 9.5 g of aqueous ammonia (25 w/w%, 0.14 mol) was added. Under stirring (400 rpm), 10 g of TEOS was added slowly (1 TEOS: 0.152 C_{14}TMABr:2.8 NH_3:141.2 H_2O in a molar ratio). The resulting precipitate was filtered, washed with 100 ml of Milli-Q water and dried at 90°C. The sample was heated to 550°C (rate 1 K min^{-1}) in air and kept at the temperature for 5 h. The pore diameter was calculated to be 2.8 nm, using a d_{100} value of 3.26 nm.

3-Aminopropyltrimethoxysilane (APS)-modified MCM-41 was also prepared according to a reported procedure.[21] Briefly, a weighed amount (15 mg) of MCM-41 was sonicated in

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2.4 mL of toluene (anhydrous), to which 0.62 g APS was added. After incubation under stirring at 20°C for 60 min, the MCM-41 suspension was centrifuged at 2880*g* at 20°C for 5 min. The precipitates were washed three times with 1.5 mL (each time) of toluene by centrifugation. Finally, the precipitates were air-dried overnights at 90°C. The MCM-41 is hereafter called APS-MCM-41.

Accommodation of BPS on APS-MCM-41

APS-MCM-41 (17.5 mg) was suspended in 0.7 mL of an acetate buffer. After adding 0.7 mL of 93 μM BPS in an acetate buffer (pH 4.7), the mixture was incubated for 1 h. The suspension was centrifuged at 18000*g* and 20°C for 5 min. The precipitates were washed with 1.4 mL of an acetate buffer. The accommodated amount of BPS was determined based on absorbance measurements at 285 nm of the supernatant. The leakage of BPS from APS-MCM-41 by washing with an acetate buffer was negligibly small (Fig. S1, Supporting Information).

Calibration of color intensity

A series of the color intensity was prepared with an iron(II) solution. A 0.10-mL portion (25 mg/mL) of BPS-loaded APS-MCM-41 in an acetate buffer was mixed with 2.0 μL of 14 M hydroxylamine hydrochloride and 98 μL of iron(II) at a known concentration in each well of a 96-well plate. The mixture was incubated for 2 h, during which the MCM-41 material precipitated on the round bottom of the well. The final concentration (0.14 M) of hydroxylamine was sufficient for the color development of an iron(II) complex (Fig. S2, Supporting Information).

Assay of iron in human serum

A 0.20-mL of human serum was mixed with 0.10 mL of 1 M hydrochloric acid, and heated at 95°C for 5 min. Then, 0.10 mL of 100(w/v)% trichloroacetic acid was added. After centrifugation at 18000*g* and 20°C for 5 min, the supernatant (0.20 mL) was mixed with 0.20 mL of 2 M sodium acetate solution. For an assay, 98 μL of the deproteinized human serum was taken in each well and mixed with a 0.10-mL portion of BPS-loaded APS-MCM-41 in an acetate buffer (25 mg/mL) and 2.0 μL of 14 mM hydroxylamine hydrochloride. The mixture was incubated for 2 h. The color intensity was compared by naked eye with those of the standard color prepared with iron(II) at a known concentration.

Results and Discussion

Accommodation of chromogenic reagents and related compounds

The potential of accommodating some chromogenic reagents for iron(II) and their complexes into the pores (diameter 2.8 nm) of MCM-41 and APS-MCM-41 was investigated at pH 4.7 (Table 1). 1,10-Phenanthroline (phen, $pk_1 = 0.7$, $pk_2 = 4.98$)[22,23] was penetrated into MCM-41, while the accommodation of phen on APS-MCM-41 was unsuccessful. In contrast, bathophenanthrolinedisulfonate (BPS, $pk_1 = 2.83$ and $pk_2 = 5.20$)[24] was accommodated into APS-MCM-41 (Fig. S3, Supporting Information). These results suggest that the matching of charges between chromogenic reagents and nanopores are one of the important factors that control the accommodation of chromogenic reagents.

On the other hand, a positively charged iron(II) complex (diameter ~1.3 nm)[25] of phen was accommodated on MCM-41, but it did not penetrate in APS-MCM-41. An iron(II) complex (diameter ~2.4 nm)[25] of BPS was allowed to penetrate on APS-MCM-41. However, an iron(II) complex of bpy did not accommodate on both MCM-41 and APS-MCM-41, because of its small molecular size (diameter ~1.1 nm).[25] Consequently, BPS was chosen as a chromogenic agent for iron(II), because not only BPS, but also its iron(II) complex, were accommodated on APS-MCM-41.

Table 1 Accommodation of chromogenic reagents for iron(II) and their complexes on MCM-41 and APS-MCM-41

Reagent (concentration, μM)[a]	Solution[b]	Accommodation, %[c]	
		MCM-41	APS-MCM-41
Phen (32)	Acetate buffer[d]	85	n.a.[f]
BPS (46)	Acetate buffer[d]	42	70
BP (19)	Acetate buffer, 40% ethanol[e]	18	n.a.[f]
$[Fe(phen)_3)]^{2+}$ (90)	Acetate buffer[d]	100	n.a.[f]
$[Fe(BPS)_3)]^{2+}$ (21)	Acetate buffer[d]	n.a.[f]	92
$[Fe(bpy)_3]^{2+}$ (70)	Acetonitrile	n.a.[f]	n.a.[f]

a. Abbreviations: phen, 1,10-phenanthroline; BPS, bathophenanthroline-disulfonic acid, disodium salt; BP, bathophenanthroline; bpy, 2,2′-bipyridyl.
b. Solution used for accommodation of compounds.
c. Defined as accommodation (%) = $100(C_i - C_e)/C_i$, where C_i is the initial concentration of the compound in a solution and C_e the concentration of the compound remained in a solution after incubation with MCM-41 material (25 mg/mL) and washing with a buffer.
d. 0.1 M CH_3COOH/NaOH buffer (pH 4.7) containing 0.1 M NaCl.
e. 0.1 M CH_3COOH/NaOH buffer (pH 4.7) containing 0.1 M NaCl and 40% ethanol.
f. No accommodation.

Visual assay of iron(II) in a solution

Figure 1(a) shows a series of the color intensities for iron(II) at different concentrations in the presence and absence of APS-MCM-41 and Fig. 1(b) demonstrates the color intensities for iron(II) at a lower concentration range. The color intensity for APS-MCM-41(–) was obtained by the conventional solution method. One can detect the red color of the iron(II)-BPS complex by the naked eye, even at 0.5 μM iron(II). On the other hand, the color at 0.5 μM iron(II), obtained by the conventional method, was hard to be detected by the naked eye. Thus, the lower detection limit of the present assay using APS-MCM-41 is superior to that of the conventional one. In addition, the red color for the MCM-41 method was more intense than that for the conventional one. This allowed us to discriminate the color intensity more easily.

Effect of coexisting substances

The effects of the major components in human serum, *i.e.*, albumin from human serum (HSA, $pI = 4.7$[26]) and γ-globulins from human blood (γ-G, $pI = 6.0 - 8.5$),[26] bilirubin and L-ascorbic acid on the visual quantification of iron(II) were investigated. As shown in Fig. 2a, γ-G (17.1 mg/mL), L-ascorbic acid (43 μM) and bilirubin (25 μM) did not interfere with the quantification of 3.0 μM iron(II). However, in the presence of HSA (50 mg/mL), the color of an iron(II)-BPS complex did not develop, indicating that the formation of the iron(II)-BPS complex was blocked, probably because of the adsorption of HSA (molecular size $4 \times 4 \times 14$ nm)[27] on the pore inlet (diameter 2.8 nm).

Visual assay of total iron in human serum

A visual assay of two types (normal and CHV specimen) of

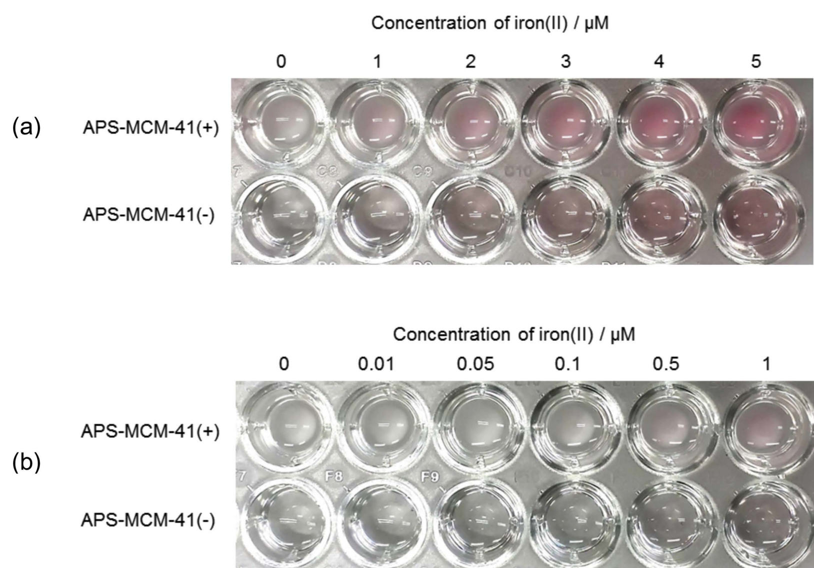

Fig. 1 Photos of a series of color intensity due to the formation of an iron(II)-BPS complex in an acetate buffer (pH 4.7) in the presence and absence of BPS-loaded APS-MCM-41. BPS-loaded APS-MCM-41: 12.5 mg/mL. Hydroxylamine concentration: 0.14 M. Concentration range: (a) 0 – 5 μM, (b) 0 – 1 μM.

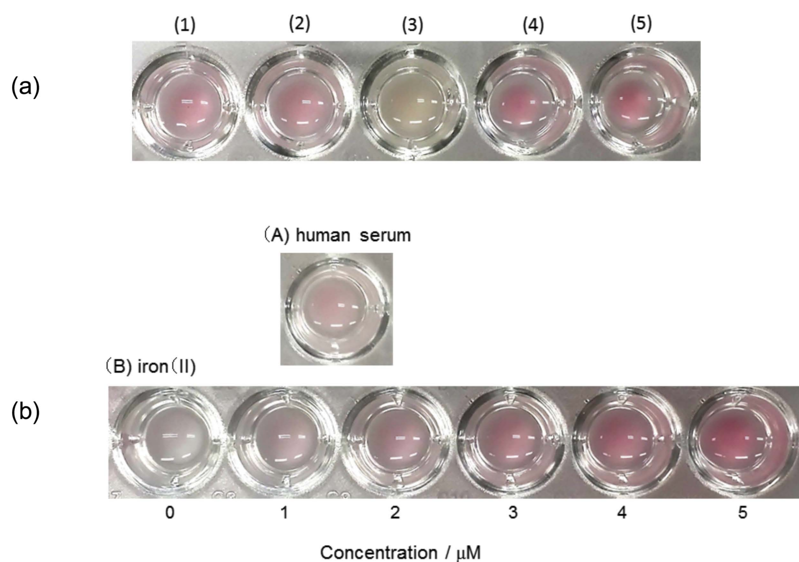

Fig. 2 (a) Effect of coexisting compounds on the color intensity for 3 μM iron (II) in an acetate buffer (pH 4.7) in the presence of BPS-loaded APS-MCM-41. Coexisting compounds: (1) None, (2) 17 mg/mL γ-globulin, (3) 50 mg/mL HSA, (4) 43 mM L-ascorbic acid, (5) 25 μM bilirubin. (b) Visual assay of total iron in normal human serum. The assay was performed after the deproteinization of human serum at 8-times dilution. BPS-loaded APS-MCM-41: 12.5 mg/mL. Hydroxylamine concentration: 0.14 M. The photo for human serum was positioned between 1 – 2 μM iron(II) in the color series of iron(II) to show that the color intensity of human serum is in the range from 1 to 2 μM iron(II).

human serum was tested with BPS-loaded APS-MCM-41. First, we tested the dilution (3 – 30 times) of human serum for a visual assay of serum iron. However, the assay using serum at 30-times dilution was unsuccessful, *i.e.*, no colors developed, seemingly because most of iron(III) in serum exists as a transferrine-bound form and/or the penetration of free iron(II) was blocked by HSA (*vide supra*). Consequently, prior to the visual assay, we carried out a deproteinization step. Figure 2b shows the photo of color intensity obtained for normal human serum at 8-times dilution. The color intensity was within the range of 1 to 2 μM iron(II), which is in agreement with the concentration (1.4 ± 0.03 μM) of iron determined by the routine spectrophotometric method. Similarly, the visual assay of the CHV specimen of human serum showed that the content of iron was between 1 and 2 μM (Fig. S4, Supporting Information), which is in agreement with that (1.5 ± 0.1 μM) obtained by the colorimetric procedure. Thus, the present assay provides a simple visual method for knowing the concentration level of total iron in human serum.

Conclusions

We have described a simple method for quantifying total iron in human serum by naked eye using BPS-loaded MCM-41. The quantification is based on observing the color intensity of the iron(II)-BPS complex formed in the pores of APS-MCM-41, which is spontaneously precipitated on the bottom of a well. Although the sedimentation of the MCM-41 material needs 2 h, the assay is simple and sensitive. The visual assay will be useful for the quantification of total iron in human serum.

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Supporting Information

Figure S1 shows the accommodation of BPS with APS-MCM-41, Fig. S2 shows effect of hydroxylamine concentration, Fig. S3 shows the extent of adsorption of BPS on the non-porous silica, and Fig. S4 shows photos that assay total iron the CHV specimen of human serum. This material is available free of charge at http://www.jsac.or.jp//analsci/.

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