BioMetals **16:** 285–293, 2003.

Calcein as a fluorescent iron chemosensor for the determination of low molecular weight iron in biological fluids

Aktar Ali, Qi Zhang, Jisen Dai & Xi Huang*
*Department of Environmental Medicine, New York University School of Medicine, PHL Room 802, New York, NY 10016, USA; *Author for correspondence: (Tel: 212-263-6650; Fax: 212-263-6649; E-mail: xihuang@env.med.nyu.edu)*

Received 19 April 2002; Accepted 23 April 2002; published on line September 2002

Key words: calcein; iron, iron overload; Low molecular weight iron; non-transferrin bound iron

Abstract

The fluorescence quenching of calcein (CA) is not iron specific and results in a negative calibration curve. In the present study, deferoxamine (DFO), a strong iron chelator, was used to regenerate the fluorescence quenched by iron. Therefore, the differences in fluorescence reading of the same sample with or without addition of DFO are positively and specifically proportional to the amounts of iron. We found that the same iron species but different anions (e.g. ferric sulfate or ferric citrate) differed in CA fluorescence quenching, so did the same anions but different iron (e.g. ferrous or ferric sulfates). Excessive amounts of citrate competed with CA for iron and citrate could be removed by barium precipitation. After optimizing the experimental conditions, the sensitivity of the fluorescent CA assay is 0.02 μM of iron, at least 10 times more sensitive than the colorimetric assays. Sera from 6 healthy subjects were tested for low molecular weight (LMW) chelator bound iron in the filtrates of 10 kDa nominal molecular weight limit (NMWL). The LMW iron was marginally detectable in the normal sera. However, increased levels of LMW iron were obtained at higher transferrin (Tf) saturation (1.64–2.54 μM range at 80% Tf saturation, 2.77–3.15 μM range at 100% Tf saturation and 3.09–3.39 μM range at 120% Tf saturation). The application of the assay was further demonstrated in the filtrates of human liver HepG2 and human lung epithelial A549 cells treated with iron or iron-containing dusts.

Abbreviations: CA – calcein; DFO – deferoxamine; FBS – fetal bovine serum; LMW – low molecular weight; NMWL – nominal molecular weight limit; NTBI – non-transferrin bound iron; RFU – relative fluorescence units; SI – serum iron; Tf – transferrin; TIBC – total iron binding capacity; UIBC – unbound iron binding capacity.

Introduction

Iron is present in animal cells in various forms, all bound to either low molecular weight (LMW) species, such as citrate, or as an integral part of proteins, which are segregated in the various cell compartments (Jacobs 1977). Iron is long thought to be the active metal species responsible for generating reactive oxygen species through Fenton or Haber-Weiss or iron autoxidation reactions (Comporti *et al.* 2002; Toyokuni 1996; Welch *et al.* 2002). Iron stored in the proteins is tightly bound, and thus not readily bioavailable for adverse effects. The labile iron pool of cells constitutes a cytosolic fraction of LMW bound iron, which can be characterized as iron (a) exchangeable and chelatable; (b) easily bioavailable for uptake by ferritin, heme, transferrin, or chelators; (c) metabolically and catalytically reactive for oxidant formation and likely responsible for iron toxicity; (d) possibly having regulatory properties which may affect iron responsive element-binding protein activity per se (Breuer *et al.* 1996; Miller *et al.* 1991). LMW iron in human body is not well defined and levels of LMW iron in serum have never been reported or estimated. Although several methods have been introduced for the detection of non-transferrin bound iron (NTBI) (Breuer *et al.*

2000; Gosriwatana *et al.* 1999), this fraction of iron includes not only LMW iron, but also iron bound to other proteins such as albumins. Several epidemiological studies have shown that high levels of iron storage in the body or high iron intake through diet may have increased risk of developing cancer (Selby & Friedman 1988; Stevens *et al.* 1988; Wurzelmann *et al.* 1996). Possible explanations for this observation include oxidative DNA damage induced by high levels of LMW iron in the body as well as LMW iron-enhanced cancer cell proliferation. Clearly, it is important to quantify levels of LMW iron accurately in biological fluids.

The recent use of calcein (CA) as a fluorescence probe for LMW iron provides a major methodological breakthrough. The principle of the test is based on the binding of CA to LMW iron, in a stoichiometric manner, which results in a fluorescence quenching. Therefore, a lower reading in fluorescence intensity indicates a higher level of LMW iron in biological fluids. Calcein acetoxymethyl ester and Fl-DFO were used to estimate labile iron pool in cells or DFO chelatable iron, a component of serum NTBI (Breuer *et al.* 2001; Breuer *et al.* 2000; Cabantchik *et al.* 1996; Epsztejn *et al.* 1997; Petrat *et al.* 1999). However, this fluorescent CA assay has some technical disadvantages when applied to biological fluids such as serum. First, fluorescence of CA can be quenched by compounds in the serum, which are not iron-related. Such interference can greatly affect the specificity of the assay and its determination of iron levels. Second, citrate present in cell lysate or serum, if excessive, may compete with CA for LMW iron, thus leading to a decreased fluorescence quenching, big variations among assays, and sometimes, irreproducible results. Third, negative calibration curve between fluorescence reading and iron concentration could be confusing in comparison with the conventional positive standard curve. In the present study, we have improved the specificity of the assay by adding iron chelator DFO into the samples and the sensitivity of the assay by removing citrate as well as using appropriate standards. By filtering the sample through an Ultra-free membrane with nominal molecular weight limit (NMWL) of 10 kDa, the iron present in the filtrates should be LMW-bound. After optimizing the experimental conditions, we have shown that CA can detect LMW iron in biological fluids at a detection limit of 0.02 μM.

Materials and methods

Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), ferric sulfate pentahydrate [$Fe_2(SO_4)_3 \cdot 5H_2O$),] ferric citrate ($FeC_6H_5O_7$), sodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), calcein (CA), deferoxamine mesylate (DFO), N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), barium chloride ($BaCl_2$), and sodium phosphate were purchased from Sigma (St. Louis, MO) with the highest purity available. Kits for serum iron (SI) and total iron binding capacity (TIBC) determination were also obtained from Sigma.

Sample preparation and fluorescence reading

All solutions were prepared in HPLC grade water except CA was first dissolved in 1 N NaOH solution at 20 mM and further diluted in HPLC water. All experiments were carried out in triplicates or quadruplicates in a Costar 96-wells microplate (Fisher Scientific, Pittsburgh, PA). Each well contained 80 μl CA (2 μM), 20 μl HEPES (500 mM), pH 7.4, and 20 μl distilled H_2O. Samples or iron standards (80 μl) were then added to the well. The plate was covered and incubated in a water bath at 37 °C for 20 min. The fluorescence was then determined on a fluorescence-chemiluminescence microplate reader (SpectraMax Gemini, Molecular Devices, Sunnyvale, CA) with an emission at 515 nm and an excitation at 485 nm. To reverse iron-induced CA fluorescence quenching, 5 μl DFO (1 mM) or 5 μl H_2O were added to two of the quadruplicated samples. The reaction mixtures were incubated in a water bath at 37 °C for 10 min. The net fluorescence quenched by iron was obtained from the difference in fluorescence readings between with or without DFO ($\pm$ DFO). A plot of the difference in relative fluorescence unit (FRU) versus [Fe] can be generated, and a positive linear standard curve can be obtained for calculating levels of the LMW iron in biological fluids.

Citrate, a biologically relevant component in sera, may compete with CA for iron binding. To check this, high molar ratios of citrate/Fe^{3+} solutions (up to 500:1) were prepared by mixing ferric citrate (constant at 20 μM) with sodium citrate dissolved in HPLC H_2O. Following 30 min incubation at room temperature, the fluorescence quenching of CA by the mixtures were measured as described above. To remove the excessive citrate anions in the mixtures, $BaCl_2$ (final concentration 50 mM) was used to precip-

itate citrate out at 4 °C. After overnight incubation, the mixtures were centrifuged at 12,000 rpm for 10 min (Marathon MicroA Centrifuge), and the supernatants were assessed for CA fluorescence quenching.

Measurements of serum iron and total iron-binding capacity

Serum samples from healthy subjects were aliquoted in 1 ml in Ependorff tubes. Appropriate IBRA approval was obtained from New York University School of Medicine. Fat was removed by centrifugation. Serum samples were characterized before experiments using kits from Sigma Diagnostics. Techniques for determining both serum iron (SI) and serum unsaturated iron binding capacity (UIBC) and deriving the total iron binding capacity (TIBC) and transferrin saturation rate (%) are outlined as follows: At acidic pH (acetate buffer, pH 4.5) and in the presence of hydroxylamine (a reducing agent), transferrin-bound iron and LMW iron, if present in serum, dissociate to release ferrous ions. These react with ferrozine to form a stable magenta-colored complex (Fe^{2+}-ferrozine) with a maximum absorption at 560 nm. The difference in absorbance at 560 nm before and after ferrozine addition in serum sample is proportional to SI concentration. In contrast to SI, serum UIBC was measured at alkaline pH (TRIZMA®, pH 8.1). Ferrous ions added to the serum bind specifically with transferrin at unsaturated iron-binding sites and then remaining unbound ferrous ions are measured with the ferrozine reaction. The difference between the amount of unbound iron and the total amount added to serum is equivalent to the quantity bound to transferrin, which is the UIBC. The serum TIBC equals the SI plus the UIBC. Serum transferrin saturation rate (%) was calculated using SI divided by TIBC X 100.

Determination of LMW iron at different transferrin saturation rates

80, 100, and 120% transferrin saturation rate in serum samples were made by mixing 220 μl of serum with 220 μl of a given concentration of freshly prepared $FeSO_4$ solution. The amount of iron given was based on the data of the SI and TIBC of each individual sample. The mixtures were then incubated for overnight at 4 °C. The samples (0.4 ml) were filtered through ultra-free membrane with 10 kDa NMWL (Millipore, Bedford, MA) at 12,000 rpm for 2 h. Serum filtrates were added in quadruplicates (80 μl each) to CA (80 μl 2 μM) in Costar 96-wells plate containing 20 μl HEPES buffer (500 mM) and 20 μl distilled H_2O as previously described. After incubation at 37 °C for 20 min, the fluorescence was determined. Five μl DFO (1 mM) or H_2O were added to two of the quadruplicates and incubated at 37 °C for additional 15 min. The fluorescence was determined again and the net fluorescence quenched by iron was obtained from the data subtraction between with or without DFO addition.

Measurements of LMW iron in cells treated with $FeSO_4$ or coal dusts

Human liver HepG2 cells and human lung epithelial A549 cells were cultured in a 75-cm^2 flask (Fisher Scientific) in 10 ml of alpha Minimum Essential Medium (α-MEM) containing 10% fetal bovine serum (FBS), 1% antibiotics, 1% L-glutamine and grown in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. At approximately 70% confluence, HepG2 cells were treated with different concentrations of ferrous sulfates for 24 h. A549 cells were treated with two coal dusts without serum for 24 h. After treatment with iron or coal dusts, cells were washed with ice-cold phosphate-buffered saline and trypsinized. Cells were then counted and cell diameter measured by Coulter Particle Size and Analyzer (Beckman). Cells were finally suspended in 0.5 ml cold distilled water, and lysed by 6 cycles freeze-thaw in liquid nitrogen and in water bath at 37 °C. After centrifugation through a 10 kDa NMWL membrane at 4 °C, the filtrates were used for LMW iron determination. Levels of LMW iron were expressed as μM of iron in the filtrates or nanomoles iron per 10^6 cells or μM of iron within cells [estimated using mean cell volume (MCV)].

Results

CA fluorescence quenched by $FeSO_4$ and recovered by DFO

The quenching effect of Fe^{2+} ions ($FeSO_4$) on CA fluorescence was examined by measuring the changes in fluorescence intensity of CA after mixing various iron concentrations (0–1.6 μM range) with CA (0.8 μM final concentration) in 50 mM HEPES buffer, pH 7.4. Figure 1A shows that the CA fluorescence intensity was linearly decreased as increasing Fe^{2+} concentrations up to 0.4 μM $FeSO_4$, where the CA/Fe^{2+} molar ratio was 2:1. The fluorescence was regenerated when DFO was added. DFO alone can neither quench nor enhance the CA fluorescence intensity. Because

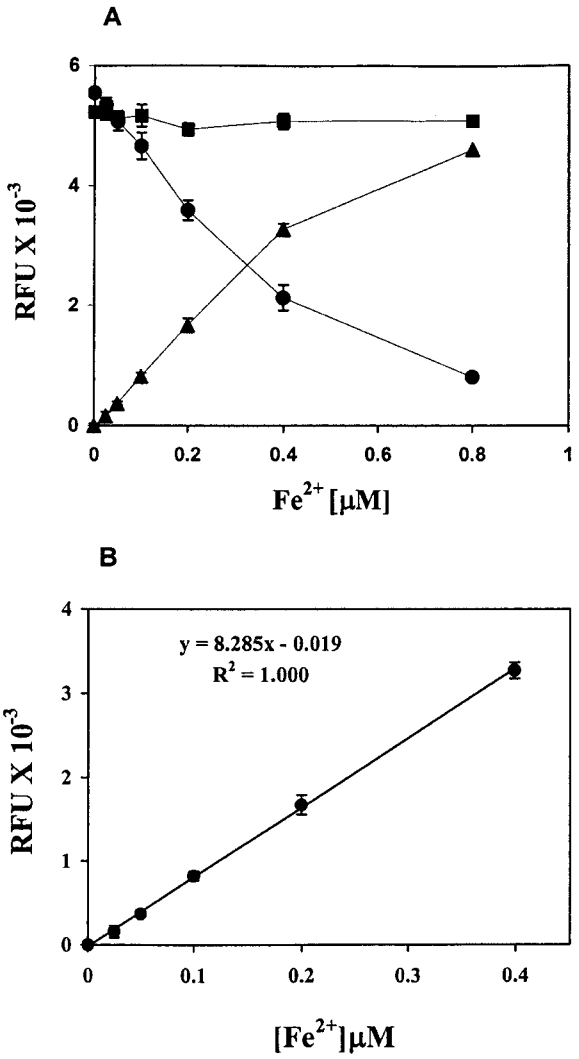

Fig. 1. CA fluorescence quenching by iron and its regeneration by DFO (A) and the differences in fluorescence reading between ± DFO as a function of iron concentrations (B). 80 μl of freshly prepared $FeSO_4$ solution were added to Costar 96-wells plate containing 80 μl CA (2 μM), 20 μl HEPES buffer (500 mM, pH 7.4), and 20 μl H_2O. The plate was incubated in a water bath at 37 °C for 20 min. The fluorescence was read on a microplate reader. 5 μl DFO (1mM) was added to each well and the fluorescence was read 10 min later. (A): ●, before DFO addition; ■, after DFO addition; ▲, differences between ± DFO addition. (B) A positive and linear correlation between iron concentrations (0–0.4 μM iron) and differences in fluorescence reading of CA (0.8 μM final) between ± DFO. Data were means ± standard deviation (SD) of 12 determinations from three independent experiments.

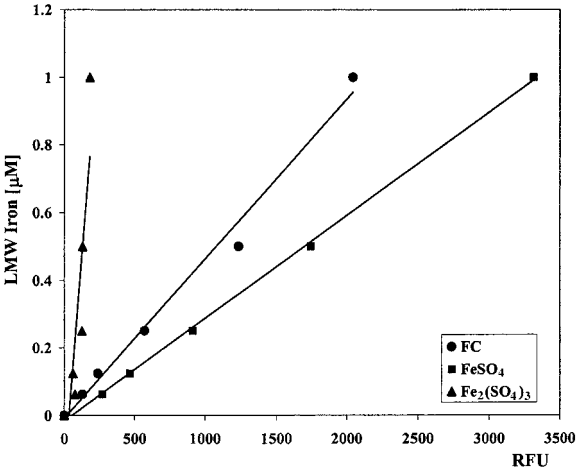

Fig. 2. Quenching effects of different iron compounds on the CA fluorescence. Data were means ± SD of three independent experiments. FC: ferric citrate. X-axis was the net fluorescence quenched by iron.

of the relative specificity of DFO for iron chelation, the differences in fluorescence reading between with and without DFO is related to the amounts of iron. Figure 1B shows a positive and linear correlation between the fluorescence differences (± DFO) and iron concentrations.

Various quenching abilities of different iron compounds

To compare the effects of Fe^{2+} and Fe^{3+} ions on CA fluorescence quenching, three iron compounds freshly prepared were used for the present study. Figure 2 shows that different iron compounds have different capacities to quench CA fluorescence. Among the three iron compounds tested, ferrous sulfate ($FeSO_4$) was the most active compound in diminishing CA fluorescence, followed by ferric citrate. Ferric sulfate had the weakest quenching effect.

Competition of citrate with CA for iron

Citrate anions are thought to be abundant in biological fluids, especially in serum (Grootveld *et al.* 1989; Jacobs 1977). Although citrate has a weaker binding affinity to iron than calcein does (Thomas *et al.* 1999), we tested a hypothesis that excessive amounts of citrate may compete with CA and thus, interfere the iron measurements. Figure 3 shows that CA fluorescence (2 μM) was quenched 80% by 2 μM Fe^{3+} when the molar ratio of citrate/ Fe^{3+} was 1:1. Increasing the concentrations of citrate while keeping Fe^{3+}

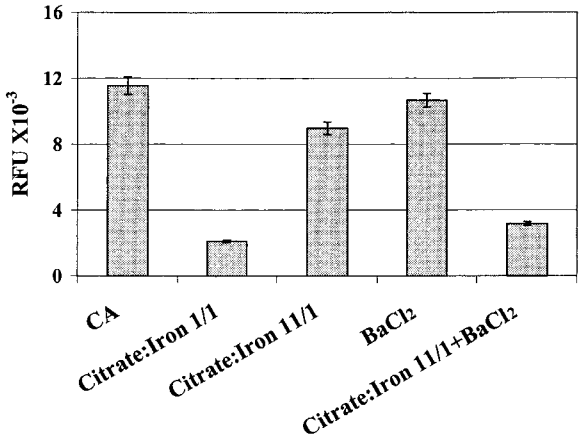

Fig. 3. Decreased CA fluorescence quenching by iron in the presence of excessive amounts of citrate and effects of $BaCl_2$ on the removal of citrate. Citrate/Fe^{3+} mixtures with molar ratios ranging from 1:1 to 501:1 were prepared for CA fluorescence quenching. The mixture (citrate: iron 11:1) was incubated with $BaCl_2$ and then re-assessed for CA fluorescence quenching after centrifugation. Data points are means ± SD of 6 determinations from two independent experiments.

constant at 2 μM, we found that a molar ratio of citrate/ Fe^{3+} of 11:1 or higher significantly diminished the CA fluorescence quenching by the same concentration of Fe^{3+} ions. This was reflected as a higher reading of fluorescence in Figure 3. These results indicate that removal of citrate is necessary to improve the sensitivity of the assay. $BaCl_2$ was used to precipitate citrate (Ba citrate: water insoluble). We found that $BaCl_2$ itself had no quenching on CA fluorescence. At a final concentration of 50 mM, $BaCl_2$ significantly removed citrate from the mixture when the citrate/Fe^{3+} ratio was less than 80:1. This was shown in Figure 3 that lower reading of CA fluorescence by the mixture treated with $BaCl_2$ as compared to the same mixture without $BaCl_2$ treatment.

Interference of fluorescence quenching by non-iron related compounds in serum

Incubation of CA (2 μM) with sera in equal volume resulted in 60–70% of CA fluorescence quenching. DFO, even at higher concentrations and longer incubation periods than previously described in the *Methods*, did not restore the fluorescence. These results indicate that large parts of the CA fluorescence quenched by sera are caused by some components, which are not iron-related. After filtering sera through the 10 kDa ultra-free membrane, the fluorescence quenching component(s) in the sera were absent in the filtrates. We found that over 99% iron (Fe^{2+} or Fe^{3+} dissolved in water or buffer) can pass through the membrane. Because of this NMWL of 10 kDa, the filtrate is termed as LMW fraction, and iron, if present in the filtrate, is termed as LMW iron.

Table 1. Levels of serum iron (SI), total iron binding capacity (TIBC), and Tf saturation (TS) in 6 healthy subjects.

Serum[a]	SI (μM)	TIBC (μM)	TS (%)
1	19.64 ± 0.38	52.70 ± 1.17	37.28 ± 1.19
2	19.01 ± 0.77	44.06 ± 0.98	43.17 ± 1.56
3	14.83 ± 1.34	48.13 ± 2.71	30.87 ± 2.03
4	15.81 ± 0.40	56.04 ± 1.69	28.20 ± 0.64
5	12.73 ± 1.31	65.29 ± 3.40	19.48 ± 1.00
6	17.57 ± 1.18	51.61 ± 4.69	34.16 ± 1.43

[a]Serum samples from 6 healthy individuals were collected on a monthly basis for three months and were combined for each individual. They were stored at −80 °C until analysis. Data presented were means ± SD of 9 determinations from three independent experiments.

Characteristics of serum transferrin iron from six healthy subjects

In normal healthy individuals, essentially all circulating serum iron is bound to transferrin (Tf). The data shown in Table 1 indicated that both of SI and TIBC were in normal range but were varied between 12–19 μM for SI and 44–65 μM for TIBC. Regarding Tf saturation rate (%), great individual differences were observed among the 6 individuals, ranging from 19–43% saturated. LMW iron was marginally detectable in the ultra-free filtrates of the serum samples. After subtracting the blank, levels of LMW iron varied from 0.08±0.05 to 0.19±0.07 (SD) μM. These amounts of LMW iron may be truly from the serum samples, because the differences in fluorescence reading between ± DFO in the blank (CA in Hepes buffer) was about 100, representing about 0.016 μM iron.

Determination of LMW iron in sera of healthy individuals at high Tf-saturation

According to the Table 1 of the levels of SI and TIBC in serum of each individual, a given amount of $FeSO_4$ was added to each serum in order to obtain 80, 100, or 120% Tf saturation, respectively. After filtration, LMW iron was determined using CA assay and was shown in Table 2. The LMW iron was consistently detected in all of 6 sera at 80% Tf-saturation, with concentrations ranging from 1.6 to 2.5 μM. Levels of

Table 2. Levels of LMW iron in the sera of 6 healthy subjects with different transferrin saturation (TS).

Serum[a]	LMW iron (μM)		
	80% TS	100% TS	120% TS
1	1.64 ± 0.04	2.78 ± 0.02	3.18 ± 0.11
2	1.66 ± 0.10	2.78 ± 0.11	3.18 ± 0.16
3	1.82 ± 0.39	2.77 ± 0.32	3.22 ± 0.15
4	2.26 ± 0.58	3.04 ± 0.52	3.09 ± 0.63
5	2.54 ± 0.13	3.15 ± 0.25	3.39 ± 0.13
6	1.92 ± 0.49	2.85 ± 0.05	3.26 ± 0.05

[a]The serum samples were the same as in the Table 1. According to the TIBC of each individual, transferrin were artificially saturated to the expected rate by adding $FeSO_4$ into the serum (details in the Methods). Data presented were means $\pm$ SD of 12 determinations from three independent experiments.

LMW iron were increased as increasing Tf saturation. The average levels of LMW iron among the 6 individuals were 2.02 μM for 80%, 2.92 μM for 100%, and 3.25 μM for 120% Tf saturation rate, respectively.

Addition of $BaCl_2$ to the LMW fraction of serum filtrate did not result in neither precipitation nor higher fluorescence quenching, suggesting that citrate anions may not be excessive to the extent for competing with CA. To be prudent, the calibration curve using $FeSO_4$ was constructed in the control serum filtrates where an equation of [LMW iron] $=0.0005 \times$ [RFU] $+$ B, $r = 0.99$, was obtained. RFU is the difference in relative fluorescence units (RFU) between $\pm$ DFO of the same sample. When the same iron standard tested in HPLC water, a linear calibration with equation of [LMW iron] $= 0.0003 \times$ [RFU] $+$ B, $r = 0.99$ was obtained. These results indicate that the same amounts of iron in control serum filtrates have less quenching ability than in water, probably due to some anions present in serum, which competes with CA for iron binding. The calibration curve constructed in the control serum filtrates was used to quantify the levels of LMW iron in the sera of different Tf saturations described above.

Determination of LMW iron in cell culture

Table 3 shows that LMW iron in control HepG2 cells (grown in 10% FBS) was 0.074 ± 0.032 μM (mean $\pm$ SD, n=3) within cells or 2.06 ± 0.50 nmoles/10^6 cells, levels of which cannot be measured by colorimetric methods. Levels of LMW iron were increased as increasing iron concentrations (Table 3). Higher level of LMW iron was obtained in control A549 cells grown in media without FBS during coal treatment (0.35 ± 0.02 μM within cells or 11.28 ± 0.56 nmoles/10^6 cells) (Table 4). The highest level of LMW iron was detected in the sample pre-treated with the coal dust from Pennsylvania (PA) coalmine region (2.41 ± 0.13 μM within cells or 78.59 ± 4.17 nmoles/10^6 cells at 20 μg/cm^2 treatment). Levels of LMW iron were dose-dependent in samples treated with the PA coal but not with the Utah (UT) coal (Table 4). At higher doses, the coal from UT also significantly increased levels of LWM iron. Prior to cell treatment, we have measured that levels of bioavailable Fe^{2+} and Fe^{3+} in the coals were 2786.4 parts per million (ppm) and 7244.1 ppm for the PA coal, and 19.3 ppm and 25.8 ppm for the UT coal, respectively (Huang *et al.* 1998; Zhang *et al.* 2002). Levels of LMW iron in A549 cells correlated with the levels of bioavailable iron in the coals. Interestingly, prevalence of pneumoconiosis also paralleled with the levels of LMW iron in the A549 cells (Morgan *et al.* 1973).

Discussion

In comparison with our detailed knowledge of iron proteins and their regulations (Addess *et al.* 1997; Aisen *et al.* 1999; Theil 1990, 1998), the knowledge on the toxicological aspect of LMW iron is very limited. For example, it has been thought for a long time that tissue damage in iron overload is through LMW iron-mediated oxidative stress mechanism and iron trapped in iron proteins is not readily available for adverse effects (Huang *et al.* 2002; Kang *et al.* 1998). However, the amounts of LMW iron in healthy subjects have never been estimated, possibly due to the detection limits of the assays available. Quantification as well as clarification of the chemistry of the LMW iron will give a better insight into intracellular iron metabolism, and thereby, into a number of pathologic mechanisms induced by iron-related diseases.

The assay in the present study is based on the iron sensitive fluorescence probe CA, which fluorescence can be quenched upon binding to iron. The nature of the iron species, whether Fe^{2+} or Fe^{3+} can quench CA fluorescence, were debated and carefully studied (Thomas *et al.* 1999). Under our experimental conditions, we found that both Fe^{2+} and Fe^{3+} can quench CA fluorescence, but with different quenching ability depending upon the ligands as shown in Figure 2. Therefore, appropriate iron compound should be used for the calibration curve. Ferric citrate is likely the most appropriate iron standard for the determination

Table 3. Levels of LMW iron in human liver HepG2 cells treated with iron compound[a].

Samples	Treatments	LMW iron (μM in the filtrate)	LMW iron (nmol/10^6 cells)	LMW iron (μM within cells)[b]
1	Control[c]	0.075 ± 0.012	2.06 ± 0.50	0.074 ± 0.032
2	50 μM Fe^{2+}	0.097 ± 0.024	4.47 ± 0.60*	0.112 ± 0.044
3	100 μM Fe^{2+}	0.117 ± 0.038	5.23 ± 0.62**	0.155 ± 0.026*
4	200 μM Fe^{2+}	0.166 ± 0.056	7.49 ± 0.55**	0.215 ± 0.036**

[a]HepG2 cells were treated with $FeSO_4$ for 24 h in the presence of 10% FBS and then lysed by freeze-thaw. After ultra-free centrifugation (10 kDa), LMW iron in the filtrates was determined using differences in fluorescence reading between ± DFO of the same sample. Data presented were means ± SD of three independent experiments.
[b]Estimated using mean cell volume.
[c]Control samples were cells without any treatments.
*Significantly different from control by Student's t-test ($P < 0.05$).
**Significantly different from control by Student's t-test ($P < 0.01$).

Table 4. Levels of LMW iron in A549 cells treated with coal dusts from Pennsylvania (PA) and Utah (UT)[a].

Treatments	LMW iron (μM in the filtrate)		LMW iron (nmol/10^6 cells)		LMW iron (μM within cells)[b]	
	UT	PA	UT	PA	UT	PA
Control[c]	0.118 ± 0.005	0.118 ± 0.005	11.28 ± 0.56	11.28 ± 0.56	0.35 ± 0.02	0.35 ± 0.02
2 μg/cm^2	0.121 ± 0.010	0.184 ± 0.014	11.28 ± 0.93	26.76 ± 2.07*$^{\oslash}$	0.35 ± 0.03	0.82 ± 0.06*$^{\oslash}$
5 μg/cm^2	0.120 ± 0.012	0.235 ± 0.012	10.28 ± 1.08	27.95 ± 1.44*$^{\oslash}$	0.32 ± 0.03	0.86 ± 0.04*$^{\oslash}$
10 μg/cm^2	0.122 ± 0.009	0.270 ± 0.013	13.36 ± 0.99*	39.56 ± 1.65*$^{\oslash}$	0.41 ± 0.03*	1.21 ± 0.05*$^{\oslash}$
20 μg/cm^2	0.130 ± 0.012	0.635 ± 0.032	13.40 ± 1.23*	78.59 ± 4.17*$^{\oslash}$	0.41 ± 0.04*	2.41 ± 0.13*$^{\oslash}$

[a]A549 cells were treated with two coal dusts for 24 h, in the absence of FBS, one from PA coalmine region, and the other from UT. LMW iron was determined as described in the legend of Table 3.
[b]Estimated using mean cell volume.
[c]Control samples were cells without any treatments. Data on LMW iron were average of three independent experiments ± SD.
*:Significantly different from control by Student's t-test ($P < 0.05$)
$^{\oslash}$:Significantly different from UT by Student's t-test ($P < 0.05$)

of LMW iron because of its dominance in biological samples.

DFO was added to the filtrates, which regenerated the CA fluorescence. DFO can bind to Fe^{3+}, as well as Al^{3+} and Ga^{3+}. However, Al^{3+} and Ga^{3+} cannot quench CA fluorescence (data not shown). Moreover, neither Al^{3+} nor Ga^{3+} ions are likely to be found in serum at nanomolar or micromolar concentrations. Cu^{2+} can also efficiently quench CA fluorescence, but the quenching part cannot be regenerated by DFO. Thus, the fluorescence regenerated by DFO in the assay is solely due to iron. The differences in fluorescence reading of the same sample between ± DFO specifically quantify the LMW iron, as shown by a linear correlation in Figure 1B. Adding DFO to the filtrates showed a much faster kinetics in regenerating CA fluorescence (5–10 min incubation) than when serum was present (2 h or longer) (Grootveld *et al.* 1989).

By filtering the samples through a 10 kDa membrane, the iron present in the filtrate should be LMW bound. According to the manufacturer (Millipore), this 10 kDa membrane can eliminate 95–100% of bovine serum albumin (molecular weight limit 67 kDa) and 80–90% lysozyme (14 kDa) at 25 °C. In contrast, 90–95% of vitamin B-12 (1.3 kDa) can be recovered in the filtrate. The binding affinity of CA to iron is between those of citrate and EDTA (Thomas *et al.* 1999). Therefore, the LMW iron measured by CA fluorescence should be redox active, and thus biological relevant, particularly in the context of iron-mediated oxidative stress.

Since citrate can compete with CA, removal of citrate might be necessary to have reliable measurements of LMW iron with greater sensitivity. However, when

adding $BaCl_2$ to the serum filtrates, no precipitation was seen, suggesting that levels of citrate in the serum samples were not sufficient enough for a precipitation by 50 mM $BaCl_2$. Therefore, we would not recommend adding $BaCl_2$ into the serum filtrates, though the calibration curve in the control serum filtrates should be used to quantify the levels of LMW iron in serum.

Levels of LMW iron in the filtrates of the 6 healthy subjects ranged from 0.09 to 0.19 μM, after subtracting the blank. Differences in fluorescence reading between the blank sample (CA in Hepes buffer only) $\pm$ DFO were approximately 100 RFU, representing 0.016 μM iron in the CA solution (according to Figure 1B). Therefore, the results in the filtrates of control sera may be meaningful, although no detectable levels of LMW iron were previously reported in serum samples of healthy controls (Breuer *et al.* 2000, 2001; Breuer, Ronson *et al.* 2000; Grootveld *et al.* 1989; Porter *et al.* 1996). Increased levels of LMW iron were observed in the filtrates of sera by artificially saturating transferrin at 80, 100, and 120% rate, respectively (Table 2). Since these serum samples were iron saturated under well-controlled conditions, much lower levels of LMW iron than expected were detected at 120% Tf saturation. These results indicate that LMW iron measured by CA after filtration represents only a fraction of NTBI, which is about 30% under our experimental conditions (calculated based on the TIBC and LMW iron at 120% saturation). NTBI is probably comprised of heterogeneous forms of iron complexes. For example, we have previously shown that iron, as well as nickel, can non-specifically bind to proteins isolated from heterochromatin (Huang *et al.* 1995).

To further validate our assay, levels of LMW iron in cells treated with iron or iron containing coal dusts were measured (Tables 3 and 4). Again, increased levels of LMW iron were observed in HepG2 cells treated with pure iron compounds. The presence of 10% FBS in HepG2 cell media may chelate iron and thus, result in lower background level of LMW iron in control HepG2 cells than that in control A549 cells grown without FBS during coal treatment (Tables 3 and 4). A statistically significant increase in LMW iron was also observed in A549 cells treated with the coal from PA coalmine region. The coal from PA with a high prevalence of coal workers' pneumoconiosis (26%) released high levels of LMW iron (Attfield & Morring 1992; Huang *et al.* 1998; Morgan *et al.* 1973). The coal from UT with a low prevalence of pneumoconiosis (4%) had low levels of LMW iron. These results suggest that LMW iron released by various coal samples may contribute to the development of pneumoconiosis.

In summary, CA is a sensitive fluorescent iron chemosensor, which can be applied for the measurements of LMW iron. The assay is simple and can be iron specific. Ferric citrate is probably the most appropriate iron standard for CA fluorescence quenching. The differences in fluorescence reading between the same sample $\pm$ DFO are recommended for iron quantification. After optimizing the experimental conditions, the sensitivity of the CA assay is 0.02 μM of iron, at least 10 times more sensitive than the colorimetric assays.

Acknowledgements

This study was supported in part by a grant from the National Institute for Occupational Safety and Health (NIOSH) of the Center for Disease Control and Prevention (OH03156) and a grant from the Department of Defense (DAMD17-01-10576).

References

Addess KJ, Basilion JP, Klausner RD, Rouault TA, Pardi A. 1997 Structure and dynamics of the iron responsive element RNA: implications for binding of the RNA by iron regulatory binding proteins. *J Mol Biol* **274**, 72–83.

Aisen P, Wessling-Resnick M, Leibold EA. 1999 Iron metabolism. *Curr Opin Chem Biol* **3**, 200–206.

Attfield MD, Morring K. 1992 An investigation into the relationship between coal workers'pneumoconiosis and dust exposure in U.S. coal miners. *Am Ind Hyg Assoc J* **53**, 486–492.

Breuer W, Epsztejn S, Cabantchik ZI. 1996 Dynamics of the cytosolic chelatable iron pool of K562 cells. *FEBS Lett* **382**, 304–308.

Breuer W, Ermers MJ, Pootrakul P, Abramov A, Hershko C, Cabantchik ZI. 2000 Desferrioxamine-chelatable iron (DCI), a component of serum non-transferrin-bound iron (NTBI) used for assessing iron chelation therapy. *Transfus Sci* **23**, 241–242.

Breuer W, Ronson A, Slotki IN, Abramov A, Hershko C, Cabantchik ZI. 2000 The assessment of serum nontransferrin-bound iron in chelation therapy and iron supplementation. *Blood* **95**, 2975–2982.

Breuer W, Ermers MJ, Pootrakul P, Abramov A, Hershko C, Cabantchik ZI. 2001 Desferrioxamine-chelatable iron, a component of serum non-transferrin-bound iron, used for assessing chelation therapy. *Blood* **97**, 792–798.

Cabantchik ZI, Glickstein H, Milgram P, Breuer W. 1996 A fluorescence assay for assessing chelation of intracellular iron in a membrane model system and in mammalian cells. *Anal Biochem* **233**, 221–227.

Comporti M, Signorini C, Buonocore G, Ciccoli L. 2002 Iron release, oxidative stress and erythrocyte ageing(2) (3). *Free Radic Biol Med* **32**, 568–576.

Epsztejn S, Kakhlon O, Glickstein H, Breuer W, Cabantchik I. 1997 Fluorescence analysis of the labile iron pool of mammalian cells. *Anal Biochem* **248**, 31–40.

Gosriwatana I, Loreal O, Lu S, Brissot P, Porter J, Hider RC. 1999 Quantification of non-transferrin-bound iron in the presence of unsaturated transferrin. *Anal Biochem* **273**, 212–220.

Grootveld M, Bell JD, Halliwell B, Aruoma OI, Bomford A, Sadler PJ. 1989 Non-transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterization by high performance liquid chromatography and nuclear magnetic resonance spectroscopy. *J Biol Chem* **264**, 4417–4422.

Huang X, Kitahara J, Zhitkovich A, Dowjat K, Costa M. 1995 Heterochromatic proteins specifically enhance nickel-induced 8-oxo-dG formation. *Carcinogenesis* **16**, 1753–1759.

Huang X, Fournier J, Koenig K, Chen LC. 1998 Buffering capacity of coal and its acid-soluble Fe^{2+} content: possible role in coal workers' pneumoconiosis. *Chem Res Toxicol* **11**, 722–729.

Huang X, Dai J, Fournier J, Ali AM, Zhang Q, Frenkel K. 2002 Ferrous ion autoxidation and its chelation in iron-loaded human liver HepG2 cells. *Free Radic Biol Med* **32**, 84–92.

Jacobs A. 1977 Low molecular weight intracellular iron transport compounds. *Blood* **50**, 433–439.

Kang JO, Jones C, Brothwell B. 1998 Toxicity associated with iron overload found in hemochromatosis: possible mechanism in a rat model. *Clin Lab Sci* **11**, 350–354.

Miller LL, Miller SC, Torti SV, Tsuji Y, Torti FM. 1991 Iron-independent induction of ferritin H chain by tumor necrosis factor. *Proc Natl Acad Sci USA* **88**, 4946–4950.

Morgan WKC, Burgess DB, Jacobson G, O'Brien RJ, Pendergrass E, Reger RB, Shoub EP. 1973 The prevalence of coal workers' pneumoconiosis in US coal miners. *Arch Environ Health* **27**, 221–230.

Petrat F, Rauen U, de Groot H. 1999 Determination of the chelatable iron pool of isolated rat hepatocytes by digital fluorescence microscopy using the fluorescent probe, phen green SK. *Hepatology* **29**, 1171–1179.

Porter JB, Abeysinghe RD, Marshall L, Hider RC, Singh S. 1996 Kinetics of removal and reappearance of non-transferrin-bound plasma iron with deferoxamine therapy. *Blood* **88**, 705–713.

Selby JV, Friedman GD. 1988 Epidemiologic evidence of an association between body iron stores and risk of cancer. *Int J Cancer* **41**, 677–682.

Stevens RG, Jones DY, Micozzi MS, Taylor PR. 1988 Body iron stores and the risk of cancer. *N Engl J Med* **319**, 1047–1052.

Theil EC. 1990 Regulation of ferritin and transferrin receptor mRNAs. *J Biol Chem* **265**, 4771–4774.

Theil EC. 1998 The iron responsive element (IRE) family of mRNA regulators. Regulation of iron transport and uptake compared in animals, plants, and microorganisms. *Met Ions Biol Syst* **35**, 403–434.

Thomas F, Serratrice G, Beguin C, Aman ES, Pierre JL, Fontecave M, Laulhere JP. 1999 Calcein as a fluorescent probe for ferric iron. Application to iron nutrition in plant cells. *J Biol Chem* **274**, 13375–13383.

Toyokuni S. 1996 Iron-induced carcinogenesis: The role of redox regulation. *Free Radic Biol Med* **20**, 553–566.

Welch KD, Davis TZ, Van Eden ME, Aust SD. 2002 Deleterious iron-mediated oxidation of biomolecules(1) (6). *Free Radic Biol Med* **32**, 577–583.

Wurzelmann JI, Silver A, Schreinemachers DM, Sandler RS, Everson RB. 1996 Iron intake and the risk of colorectal cancer. *Cancer Epidemiol Biomark Prev* **5**, 503–507.

Zhang Q, Dai JS, Ali AM, Chen LC, Huang X. 2002 Roles of bioavailable iron and calcium in coal dust-induced oxidative stress: possible implications in coal workers' lung disease. *Free Radical Res* **36**, 285–294.