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ORIGINAL CONTRIBUTION

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Redox reactions of nitrite ions on the surface of colloidal magnetite particles coated with chondroitin sulfate

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Abstract Colloidal magnetite particles coated with chondroitin sulfate (abbreviated as Fe/CS) were prepared under conditions of varying Fe(II) fractions at a fixed Fe concentration and a given concentration of CS. The average size of the magnetite core region was estimated as 7 nm from transmittance electron microscopy measurements, while the size of the Fe/CS particles ranged 155–175 nm, as estimated using Rayleigh scattering measurements by reference to a control size derived from the dynamic light scattering. The reaction of various Fe/CS with NO_2^- in aqueous solutions was determined by fluorometry using 2,3-diaminonaphthalene as a probe and by gas chromatography–mass spectrometry. The concentrations of NO_2^- in the reaction mixtures decreased in the presence of Fe/CS to a greater extent under Ar compared with aerobic conditions. The reactivity of Fe/CS toward NO_2^- under aerobic conditions increased with decreasing the size of Fe/CS particles or with increasing content of Fe(III) in the Fe/CS solutions, but was independent of the Fe(II) fraction in the preparation process. While CS molecules had no influence on the NO_2^- decomposition, those coated with the magnetite core may prevent the diffusion of NO_2^- to be adsorbed on the core surfaces. NO_2^- was concluded to undergo redox reactions with Fe(II) and Fe(III) located on the core surface of magnetite crystalline structures of Fe/CS.

Key words Colloidal magnetite particles · Chondroitin sulfate · Nitrite ions · Redox reactions · Core surface

Introduction

The redox reaction of nitrogen oxides (NO_x) has been extensively studied in relation not only to physiological action to cells, but also to air pollution produced by automobiles and factories, photochemical ozone formation and degradation [1], global nitrogen cycle by organisms involving a series of reductions as in (Eq. 1), etc.

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (1)$$

Particularly nitric oxide (NO) is well known to play important roles in vivo as a vasodilator, i.e., the endothelium-derived relaxation factor [2], neurotransmitter [3], inhibition of blood coagulation [4]. NO is oxidized in vivo to nitrites, which are harmful particularly to new-born babies, because their livers function insufficiently [5]. Nitrites are further oxidized to stable forms of nitrates in the liver. A large amount of NO production, however, is harmful and results in inflammation and infectious diseases, which cause cell death, apoptosis [6]. Moreover, NO and nitrites are associated with diseases such as sepsis [7, 8], Kawasaki disease to infants [9] and methemoglobinemia [10].

NO has been reported to bind with trivalent iron, (Fe(III), in nitrite reductase [11], methemoglobin [12], cytochrome P450s [13] and chelaters [14]. Recently, we prepared colloidal magnetite (Fe_3O_4) particles, which were coated with chondroitin sulfates [15]. Since magnetite in the crystal structure contains Fe(III) in the oxidized form, the reactivity of the colloidal magnetite particles toward NO is an interesting subject for investigation.

In this work, we studied the reaction with nitrite ions (NO_2^-) instead of an unstable compound, NO, for characterizing the redox properties of colloidal magnetite particles coated with chondroitin sulfate (CS). It is noted that CS is one of the naturally occurring mucopolysaccharides and may therefore be less toxic toward living matter. In view of a potential application of the particles to contrast enhancers for magnetic resonance imaging, it is important to study the reaction between nitrites and colloidal magnetite particles.

Experimental

Preparation of colloidal magnetite particles coated with CS

Magnetite was produced by a coprecipitation method from aqueous solutions in various compositions of iron(II) chloride tetrahydrate [$Fe(II)Cl_2 \cdot 4H_2O$, Wako Chemicals] and iron(III) chloride hexahydrate [$Fe(III)Cl_3 \cdot 6H_2O$, Wako Chemicals] [16]. Both Fe(II) and Fe(III) ions in aqueous solution were fed at a fixed total concentration of 14 mM with varying mole fractions of Fe(II) from 11.4 to 57.5%. The oxidation of Fe(II) was prevented by bubbling Ar throughout the aging process of the colloidal magnetite particles.

A mixture of Fe(II) and Fe(III) was added to 5% CS C (Maruha) with stirring at 80 °C under Ar. The mixture of iron and CS C was adjusted promptly to pH 11 by adding 5 N NaOH, subsequently to pH 8.0 by adding 5 N HCl, and further refluxed for 1 h under Ar for aging of the magnetite particles. The formation of magnetite is represented by

$$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O \tag{2}$$

After the aging process, precipitates of the reaction mixture were removed by centrifuging at 3,000 *g* for 30 min. Supernatants filled in a cellulose membrane (Viskase, fractionation M_r, 12,000–14,000) were dialyzed for longer than 3 days against deionized water, which was replaced several times with fresh deionized water. The resulting CS C coated magnetite (abbreviated as Fe/CS) particles were concentrated under reduced pressure. The color of the Fe/CS particles varied from brown to dark brown, depending on the composition of Fe(II) and Fe(III) in the preparation. The particles were stable in the colloidal state for longer than 2 years.

Measurement of the particle size of Fe/CS

The contents of Fe(II) in Fe/CS were determined by atomic absorption spectrometry (Shimadzu, MPS-2000). The particle size of Fe/CS (57.5%) containing 57.5% Fe(II) in the feed was estimated using a dynamic light scattering apparatus (Otsuka Electronics, DLS-7000). In addition, attempts were made to measure the sizes of the Fe/CS particles with a Coulter counter (Coulter Electronics, ZM). The core region within the Fe/CS, which appeared to consist of magnetite, was observed by transmittance electron microscopy (TEM) (Hitachi, H-500) without staining.

Fluorometric assay of NO_2^- concentrations

The concentrations of NO_2^- were measured by fluorometry using a Hitachi F-2000 fluorescence spectrophotometer, using the reaction with 2,3-diaminonaphthalene (DAN) [17–19]. Treatment of a mixture (1.0 ml) of nitrite (1.0 μM) and Fe/CS (200 μM) in 10 μM tris(hydroxymethyl)aminomethane HCl at pH 7.6 with DAN (Dojindo, 7.6 μM, 25 μl) in 6.2 N HCl formed 1-naphthotriazole as a fluorescent product. Aliquots of NaOH were added to the solution after the reaction for 10 min. The concentrations of 1-naphthotriazole thus formed were determined by fluorometry at 450 nm upon excitation at 365 nm, where unreacted DAN had no influence on the fluorescence intensity. The calibration curve of the fluorescence intensity was linear in the range 0.1–1.0 μM NO_2^-.

The effect of Fe/CS on the decomposition of NO_2^- was compared with the effect of $FeSO_4$ (Wako Chemicals), $Fe_2(SO_4)_3$ (Wako Chemicals), Fe_3O_4 (Nakarai Tesq) and Fesin {Yoshitomi Medical, $[Fe(OH)_3]_n[C_{12}H_{22}O_{11}]_m$}. CS sodium salt (Nakarai Tesq) was dialyzed through a cellulose membrane (Nakarai Tesq, fractionation M_r 8000) against deionized water, which was replaced three times every 48 h. The deionized water of 15 MΩcm used throughout the measurements was obtained from a mega-pure-water purification system (Corning, MP-190).

Gas chromatography–mass spectrometry

The gas-phase composition of the reaction system of Fe/CS and NO_2^- under an Ar atmosphere was analyzed by gas-chromatography–mass spectrometry (GC-MS) (Shimadzu, GC 14A). Aliquots (2.0 ml) were collected with a syringe from the gas phase in contact with a reaction mixture of 10 mM Fe/CS (20.2%) and 0.1 mM NO_2^- incubated for 10 min and was analyzed by an electron impact method (GC-EIMS).

UV–vis absorption spectra

Commercially available Fe_3O_4 was ground sufficiently and suspended in 2 M H_2SO_4. The Fe_3O_4 suspension was filtrated with a filter (Gelman Sciences, E031, 3 mm in diameter and 0.45 μm pore size) and measured with a UV–vis spectrophotometer (Shimadzu, MPS-2000).

In the measurement of 0.2 mM Fe/CS (20.2%) in 2 M H_2SO_4, a sheet of Teflon of 0.3-mm thickness was fixed on the side of the transmitted light of two quartz cuvettes, which led to scattering of both transmitted light and scattered light. Such an opal-glass method [20] resulted in more sensitive spectra against each wavelength despite reduced light transmittance. UV–vis absorption spectra of various Fe/CS particles in deionized water were measured in a similar manner.

Results and discussion

Reaction of Fe/CS with NO_2^-

NO_2^- (1 μM) reacted with various Fe species (100 μM) in solution, colloid and suspension to decrease the concentration of NO_2^- in the reaction mixtures, as shown in Table 1. Both Fe(II) and Fe(III) appear to be responsible for the reaction with NO_2^- by both reduction and oxidation mechanisms. The reactivity was higher to some extent under anaerobic conditions than under aerobic conditions. Oxygen may regenerate NO_2^- in the suppression. Under anaerobic conditions of homogeneous solution, the reactivity of Fe(II) toward NO_2^- was distinctly higher than that of Fe(III). Nevertheless, the reactivity of Fe/CS was not correlated with the Fe(II) content.

The GC-MS for the gas phase were measured 10 min after mixing the Fe/CS (20.2%) and NO_2^- (Fig. 1). A product peak appeared at the retention time of 1.34 min with an *m*/*z* value of 44 in 1.45% relative abundance. In view of the evidence that the background value of CO_2 ($m/z = 44$) was measured as less than 0.3%, the observed MS peak is attributable largely to N_2O. Other nitrogen products, such as NO_2, NO and NH_3, could not be detected in the gas phase of the reaction mixture. Although NO is the most likely intermediate product [10] produced in the reduction of NO_2^- into N_2O, its lifetime may be too short to be detected in this work.

Table 1 Nitrite consumption (%) by the reaction with various iron compounds. *CS* represents chondroitin sulfate

Fe species	Conditions	Decreased concentrations (%)	
		Aerobic	Ar
Fe/CS (11.4%)	Colloid solution	43.1	44.8
Fe/CS (13.8%)	Colloid solution	42.4	44.0
Fe/CS (14.8%)	Colloid solution	40.7	41.9
Fe/CS (17.5%)	Colloid solution	55.3	57.8
Fe/CS (20.2%)	Colloid solution	55.6	58.2
Fe/CS (24.4%)	Colloid solution	42.5	44.1
Fe/CS (38.9%)	Colloid solution	45.0	47.3
Fe/CS (57.5%)	Colloid solution	41.1	42.9
$FeCl_2$	Solution	38.1	
$FeSO_4$	Solution	36.7	51.1
$FeCl_3$	Solution	18.6	25.9
$Fe_2(SO_4)_3$	Solution	25.8	
$FeSO_4/Fe_2(SO_4)_3$ (mixing ratio = 2:1)	Mixture solution	29.1	
Fe_3O_4	Suspension	14.4	19.8
Fesin	Solution	35.7	
CS	Solution	0.82	0.83

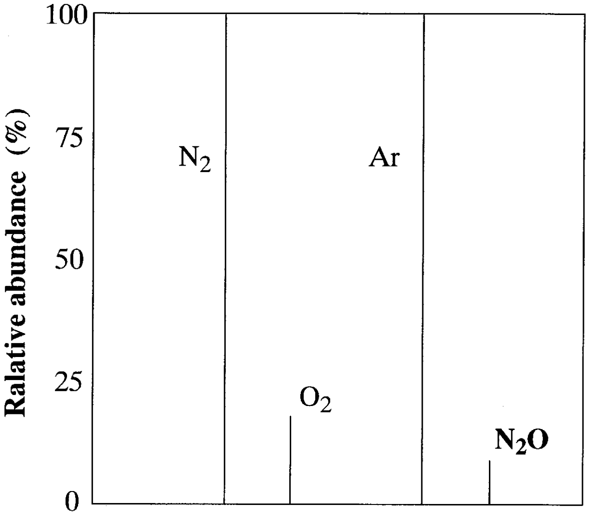

Fig. 1 Gas chromatography–mass spectrum of a gas-phase component with a retention time of 1.34 min, as obtained by the reaction between 0.05 mM NO_{2^-} and 5.0 mM Fe/chondroitin sulfate (*CS*) (20.2%) colloidal magnetite particles at 20 ° for 10 min under an Ar atomosphere

Representative Lineweaver–Burk plots are shown in Fig. 2 for the reactions of NO_2^- ions with Fe/CS (17.5%) and Fe/CS (57.5%), which showed almost the highest and the lowest reactivity, respectively. The kinetics constants, expressed by Eqs. (3) and (4), of the reactions of NO_2^- with Fe/CS are also listed in Table 2.

$$(v_0)^{-1} = K_M(V_{max})^{-1}([NO_2^-])^{-1} + (V_{max})^{-1} \tag{3}$$

$$k_{cat} = (V_{max})([Fe/CS])^{-1} , \tag{4}$$

where [Fe/CS] and [NO_2^-] are the concentrations of the iron in the Fe/CS particles and the NO_2^- , respectively.

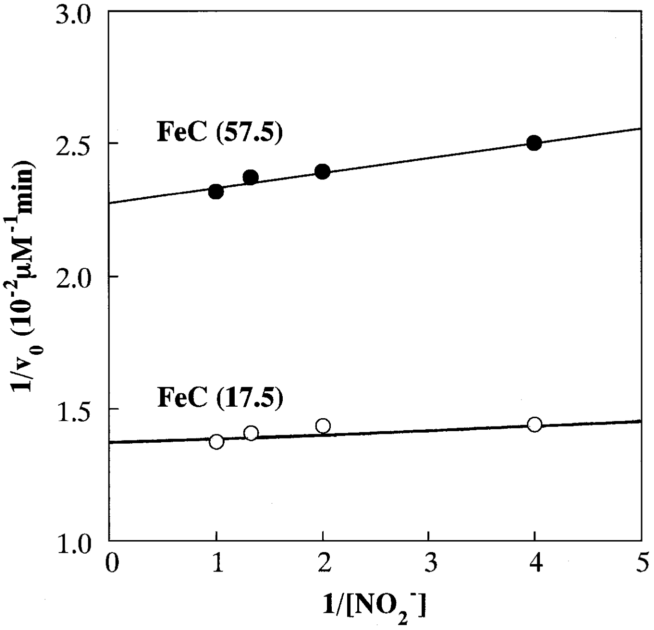

Fig. 2 Lineweaver–Burk plots for the reaction of varying concentrations of NO_{2^-} with 0.2 mM Fe/CS (17.5%) and Fe/CS (57.5%) colloidal magnetite particles at 23 °C

Table 2 Kinetics constants for the reaction of NO_2^- and colloidal magnetite particles

	K_M	V_{max} (Ms^{-1})	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}M^{-1}$)
Fe/CS (17.5%)	1.25×10^{-6}	1.21×10^{-9}	6.0×10^{-3}	4.8×10^{3}
Fe/CS (57.5%)	2.49×10^{-7}	7.32×10^{-10}	3.7×10^{-3}	1.5×10^{4}

K_M, V_{max}, v_0 and k_{cat} represent the Michaelis–Menten constant, the maximum rate, the initial rate and the specific reactivity of Fe/CS, respectively. The K_M values obtained are close to the concentration (1.0 μM) of the substrate, suggesting the formation of a relatively stable intermediate in the reaction of NO_2^- and Fe/CS. NO_2^- should coordinate to reactive sites on the Fe/CS. In view of the K_M and k_{cat}/K_M values, the affinity of NO_2^- for Fe/CS (57.5%) and the specificity on the reaction sites would be higher than those for Fe/CS (17.5%). On the other hand, the k_{cat} value of Fe/CS (17.5%) was higher than that of Fe/CS (57.5%), indicating that the number of reactive sites in Fe/CS (17.5%) is greater than that in Fe/CS (57.5%) (Table 1).

Particle size and Fe(III) content of Fe/CS

Presumably the Fe/CS consists of the core of the magnetite region and the coated layer of CS molecules. A somewhat distorted spherical shape was observed by TEM, in which the average size of the magnetite region was estimated as 7 nm. The global sizes of the Fe/CS particles could not be measured with the Coulter counter because of the detection range being above 0.2 μm. Alternatively, the average diameter of Fe/CS (57.5%) was estimated as 173.5 nm from the measurement of dynamic light scattering [15].

UV–vis spectra of Fe/CS (17.5%) and Fe/CS (57.5%) in aqueous solution are shown in Fig. 3A. The other Fe/CS particles showed similar UV–vis spectra with absorbances varying between those of Fe/CS (17.5%) and Fe/CS (57.5%). The size of each Fe/CS particle was conveniently estimated by reference to the Fe/CS (57.5%) on the basis of Rayleigh scattering data in the wavelength region of 250–300 nm. For better characterization of Fe in the Fe/CS series, UV–vis spectra of a magnetite suspension in 2 M H_2SO_4 were measured as shown in Fig. 3B. The strong absorbance of magnetite was observed at 633 and 678 nm, besides a broad peak in the region 300–400 nm. In the spectra of Fe/CS (20.2%) in 2 M H_2SO_4, several peaks were observed at 346, 358, 370, 384 and 406 nm, as well as a strong absorbance at 636 and 677 nm corresponding to the absorbance of magnetite. Aqueous solutions of Fe(II) were almost colorless and transparent. Taking into consideration the absorbance of magnetite at the wavelengths above 350 nm and the lower intensities of light scattering at longer wavelengths, the absorbance at 406 nm in the Fe/CS solutions appears to be best related to the concentrations of Fe(III).

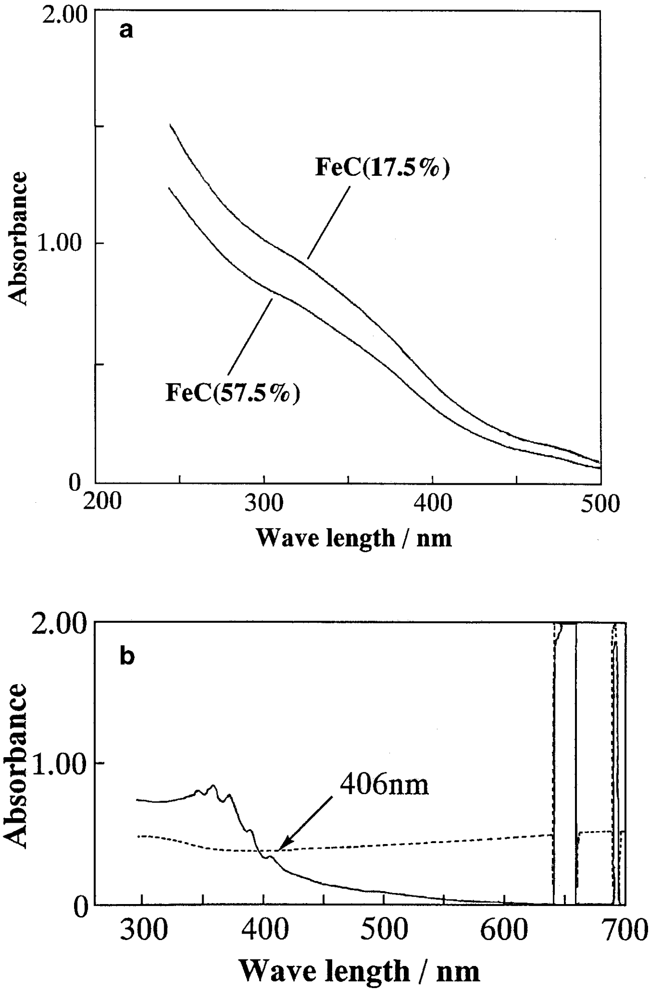

Fig. 3 UV–vis absorption spectra of **a** 0.2 mM Fe/CS (17.5%) and Fe/CS (57.5%) colloidal magnetite particles in aqueous colloidal solution and **b** magnetite suspension in 2 M H_2SO_4 (*broken line*) and 0.2 mM Fe/CS (57.5%) in 2 M H_2SO_4 (*solid line*) as measured by an opal-glass method

The particle diameter of each Fe/CS and the reactivity with NO_2^- are plotted against the absorbance at 406 nm in Fig. 4. The lowered concentration of NO_2^- by Fe/CS increased with increasing absorbance of Fe/CS at 406 nm, indicating that the reactivity of Fe/CS toward NO_2^- is correlated to the content of Fe(III) in Fe/CS. On the other hand, the absorbance at 406 nm increased as the particle size of Fe/CS became smaller. It follows that the particle size of Fe/CS decreases with increasing Fe(III) content in Fe/CS. It is also evident that the Fe/CS with smaller particle size showed higher reactivity for the decrease in the concentration of NO_2^-. Since the size of an Fe/CS particle consisting of the same size (7 nm) of magnetite core increases with increasing thickness of the CS layer, the higher amount of the CS layer may interfere with the contact of NO_2^- with the reactive sites on the core magnetite.

The coating layer of CS was inert for the reactivity with NO_2^- (Table 1). Thus, NO_2^- is likely to undergo redox reactions on the surface of the Fe/CS core, where Fe(III) ions would be involved in the reactive site under aerobic conditions. The CS molecules with carboxyl

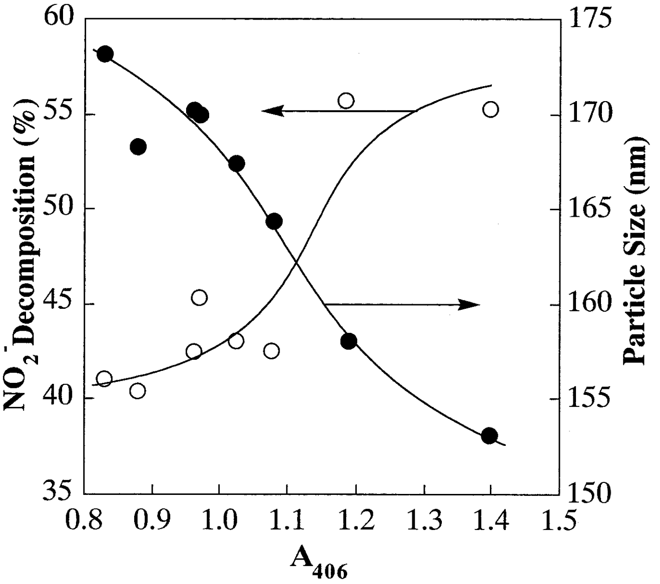

Fig. 4 Dependences of the particle size (*open circles*) and the NO_2^- decomposition (*filled circles*) on the absorbance (A_{406}) at 406 nm of colloidal magnetite particles (1 mM) in aqueous solution

groups, strong acidic sulfate groups and hydroxyl groups are considered to coordinate to Fe^{3+} and Fe^{2+} on the surfaces of the magnetite core by an electrostatic interaction. On the surface of the magnetite core in the crystalline structure, some portion of Fe(II) and Fe(III) should fail to interact with CS molecules. Probably, such Fe(II) and Fe(III) are coordinated with hydroxide ions to react with NO_2^- .

Redox reactivity of Fe/CS

The reaction of an excess amount of Fe/CS, for example, 200 times higher concentration compared with the amount of NO_2^-, may lead to a pseudo-first-order reaction, in which the rate (v) of the decrease in the NO_2^- concentration is expressed by

$$v = k[\mathrm{Fe/C}][NO_2^-] = k'[NO_2^-] \qquad (5)$$

where k is a constant and k' is a pseudo-first-order rate constant under the present experimental conditions. The k' rate constants determined are plotted against the content of Fe(II) fed in the preparation of Fe/CS (Fig. 5A) and the absorbance at 406 nm (A_{406}) as a measure of the Fe(III) content in Fe/CS (Fig. 5B). Evidently, k' increased almost linearly with the increase in the Fe(III) content in the Fe/CS colloidal solutions.

NO is known to form intermediate complexes with both Fe(II) and Fe(III) ions in enzymes and iron chelators [11–14] prior to undergoing the redox reaction. The formation of intermediate complexes with Fe(II) and Fe(III) in Fe/CS is also a plausible explanation for the decrease in the concentration of NO_2^-. On the basis of the experimental results, a putative mechanism for the redox reaction of Fe/CS with NO_2^- under aerobic conditions may be represented by the following equations.

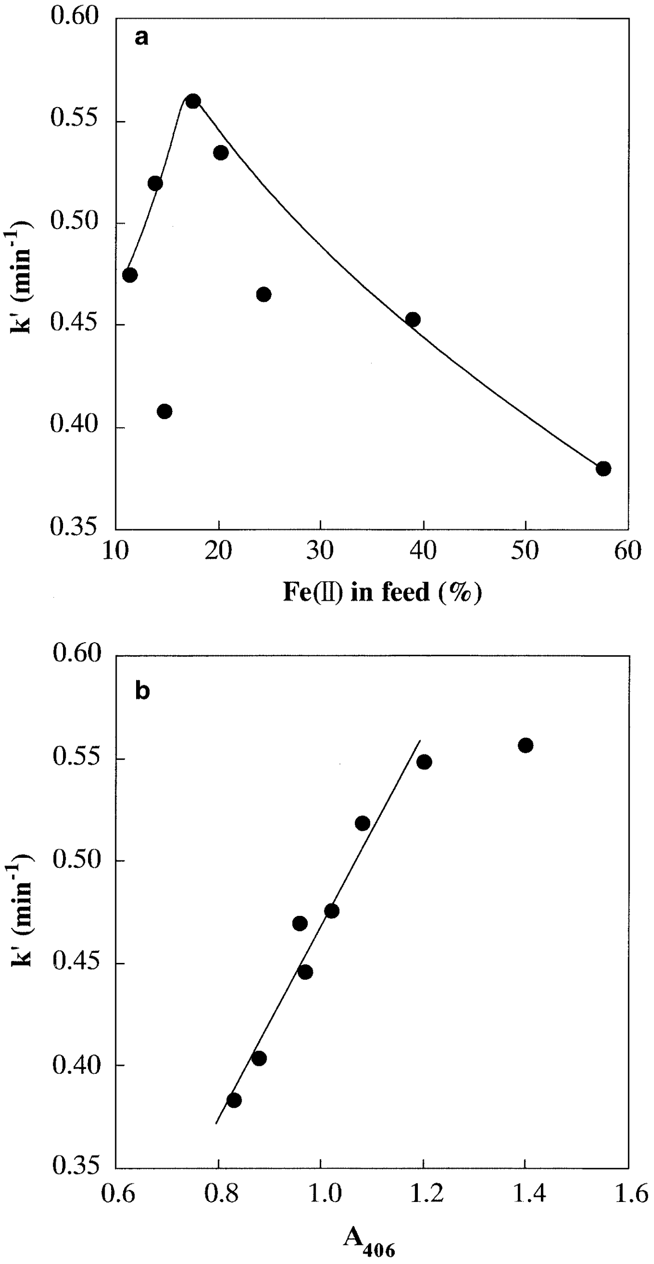

Fig. 5 Relationships of the rate constant (k', min^{-1}) for the NO_2^- decomposition with **a** Fe(II) content in the feed of colloidal magnetite particles and **b** the absorbance at 406 nm (A_{406}) as a measure of Fe(III) content on the particle surface

$$Fe^{3+} + 1/2NO_2^- \leftrightarrow \left(Fe^{3+} \cdots 1/2NO_2^-\right) \xrightarrow{\text{reduced}} Fe^{2+} + 1/2NO_3^- \qquad (6)$$

$$\mathrm{Fe}^{2+} + \mathrm{NO}_2^- \leftrightarrow \left(\mathrm{Fe}^{2+}\cdots\mathrm{NO}_2^-\right) \xrightarrow{\text{oxidized}} \mathrm{Fe}^{3+}\cdots\mathrm{NO}_3^- \leftrightarrow \mathrm{Fe}^{3+} + \mathrm{NO} \quad (7)$$

$$\mathrm{Fe}^{2+} + \mathrm{NO} \leftrightarrow \left(\mathrm{Fe}^{2+}\cdots\mathrm{NO}\right) \xrightarrow{\text{oxidized}} \mathrm{Fe}^{3+}\cdots\mathrm{NO}^- \xrightarrow{\mathrm{NO}^-,2\mathrm{H}^+} \mathrm{Fe}^{3+} + \mathrm{N_2O} + \mathrm{H_2O} \quad (8)$$

$$\mathrm{Fe}^{2+} + \mathrm{NO} \leftrightarrow \left(\mathrm{Fe}^{2+}\cdots\mathrm{NO}\right) \xrightarrow{\text{oxidized}} \mathrm{Fe}^{3+} + \mathrm{NO}_2^- \quad (9)$$

The concentrations of NO_2^- in the reaction mixture decreased with increasing content of Fe^{3+} in Fe/CS (Fig. 4). Some NO_2^- appear to coordinate to Fe^{3+} on the magnetite-core surface. Thus, the oxidation of NO_2^- by Fe/CS should result in the formation of a stable product of NO_3^- (Eq. 6). On the other hand, the coordination of NO_2^- to Fe^{2+} on the magnetite-core surface would result in the reduction of NO_2^- to form NO (Eq. 7). Coordination of NO to Fe^{2+} may induce further reduction to $\mathrm{N_2O}$ (Eq. 8). The apparent reactivity of Fe(II) and NO_2^- seems lower than that of Fe(III) and NO_2^- under aerobic conditions. This is because oxidation of NO to regenerate NO_2^- is also a possible reaction in the presence of Fe(III) and oxygen (Eq. 9).

Fe/CS (17.5%) has the smallest particle size among the Fe/CS species used (Fig. 4). This indicates that the core size of magnetite in Fe/CS (17.5%) may also be somewhat smaller, and thereby the amount of CS coated on the core may be more reduced compared with other Fe/CS species. Probably the highest content of the magnetite nucleus can be achieved with 17.5% Fe(II) in the feed in the process of the magnetite formation, although the stoichiometric content of Fe(II) is 33.3% as in the magnetite crystalline structure. Thus, the largest number of Fe/CS (17.5%) particles may be formed at a given Fe concentration, which results in the highest surface area of the whole magnetite core among various Fe/CS particles and in the highest reactivity (Fig. 4) with NO_2^-.

On the other hand, the precise size of the magnetite core of the present Fe/CS particles has not yet been determined. In our preparation process of the Fe/CS at pH 11 and 8 under Ar, each Fe/CS species probably grows to similar sizes of the magnetites as the core components and is coated by CS molecules. The particle size of the Fe/CS increases with increasing thickness of CS layer, assuming almost the same size of the magnetite core among various Fe/CS. Thus, the thicker CS layer may interfere with the contact of NO_2^- ions with the reactive sites on the magnetite core. Because more reactive sites of Fe(II) and Fe(III) on the surface of the core magnetite should be coordinated by CS molecules and thereby become less reactive to NO_2^- with an increasing number of CS molecules coated.

While the CS molecules in the Fe/CS are not responsible directly for the reaction with NO_2^- (Table 1), the high reactivity of the Fe/CS particles should be determined partially by the CS molecules coated on the magnetite core. Furthermore, the CS molecules coated may play a role in the adsorption of NO_2^- on the positively charged core surfaces and the stabilization of intermediate complexes between Fe/CS and NO_2^-. In other words, the CS molecules should enclose and prevent NO_2^- from diffusion into the aqueous phase.

In conclusion, NO_2^- undergoes oxidation, reduction and decomposition on the surface of the magnetite core of the Fe/CS, as shown in Eqs. 6–8. NO_2^- may have more affinity to Fe(II) rather than to Fe(III) in the Fe/CS (Table 2). The apparent reactivity of Fe(III) in the Fe/CS for the decomposition of NO_2^- is, however, higher than that of Fe(II) under aerobic conditions as a result of the NO_2^- regeneration involved in the reduction pathway (Eq. 9). The CS molecules coated are considered to stabilize the intermediate complexes of Fe(II) and Fe(III) with NO_2^- that is formed on the surfaces of the magnetite core.

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