Bioactive apo-ferredoxin–polycation–clay composites for iron binding

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Trace concentrations of heavy metals cause health and environmental hazards. Specifically, trace concentrations of iron induce biofilm formation which is of great concern in water systems. To remove the iron, protein–polycation–clay composites were designed based on the hypothesis that the adsorbed apo-ferredoxin (apo-mFd: mFd protein without the 2Fe–2S cluster) chelates iron. Fe^{2+} chelation by apo-mFd protein was established by optical spectroscopy and gel electrophoresis. To reduce its biodegradation, apo-mFd was adsorbed to montmorillonite and these composites were characterized by X-ray diffraction, zeta potential and adsorption isotherms. The apo-mFd–montmorillonite did not chelate iron with high efficiency; however, when a polycation–apo-mFd complex was adsorbed to the clay, the protein retained its chelating characteristics and specific interactions of iron with the biocomposite were established. Results present the innovative tailoring of bioactive protein–polycation–clay composites which may be applicable in many fields, *e.g.*, protein immobilization, drug delivery and water treatment.

Introduction

Heavy metals, even in trace concentrations, pose a threat to human health and the environment.[1–4] One example is trace concentrations of iron, which enhance bacterial (*Pseudomonas aeruginosa*) biofilm formation, causing health and mechanical problems in water systems.[5,6] Traditionally, Fe^{2+} ions are removed from the water by oxidation, crystallization and chemical sedimentation of Fe^{3+} oxides.[1,3] However, when the system comes to equilibrium trace concentrations of iron (Fe^{2+}) always remain[2,7,8] and to remove them, chemical chelators, primarily developed as pharmaceutical agents and as fertilization mediators in agriculture, are applied.[9,10] Another way to remove these trace concentrations is by their adsorption to surfaces such as activated carbon, zeolites and clay minerals.[1,10,11] However, such adsorption is non-specific and reversible because its main mechanism is cation exchange.[1,7,11–13] Nevertheless, clay minerals are widely employed as adsorbents in water treatment due to their high specific surface area and cation-exchange capacity on the one hand, and low toxicity and cost on the other.[1] To improve organic pollutant removal by clay minerals, a wide range of organically modified clays, termed organo-clays, have been designed.[14–19] A small number of studies have presented organo-clays with metal-chelating functional groups (–SH and –COOH) as possible adsorbents for heavy metals.[20–22] However, metal-chelating organo-clays remain inefficient due to a decrease in ligand activity in the adsorbed state.[21,22] Mercier and Detellier,[21] for example, reported that only 10% of the total number of thiol groups remain active upon intercalation of 3-mercapto-propyltrimethoxysilane with montmorillonite.

The modifiers of organo-clays range from small organic cations to large macromolecules such as polymers and proteins. Protein and clay–mineral interactions are dominated by hydrophobic and electrostatic interactions which are governed by the protein's internal stability and the electrical charge density of both the protein and the clay surface.[23–32] Immobilization on clay minerals has been shown to reduce biodegradation,[33] but the proteins often lose their activity in the adsorbed state due to conformational changes.[30] Therefore, immobilizing proteins on inorganic surfaces without losing partial or total activity is challenging. Patil *et al.*[34] faced this challenge and succeeded in retaining the activity of three model proteins by adsorbing them onto exfoliated aminopropyl-functionalized clay.

In this study, we report the development of an innovative biochelator, an apo-protein–polycation–clay composite which chelates trace concentrations of Fe^{2+} (Fig. 1). The protein in this composite, ferredoxin (mFd), is an iron–sulfur protein present in living organisms that contains an iron–sulfur inorganic cluster in its active site.[35] We obtained two forms of mFd from the thermophilic cyanobacterium *Mastigocladus laminosus*:[36] holo-mFd, the native protein containing a single 2Fe–2S cluster, and apo-mFd, the protein without the cluster. In the apo-mFd protein, the cysteines that normally bind the cluster in holo-mFd become functional thiol groups under reduced conditions. We hypothesized that the functionalized cysteine residues will create a chelating site for iron.[20–22,37–39] Moreover, to reduce biodegradation, apo-mFd was immobilized on montmorillonite clay as a polycation–protein complex. The challenge was to design a protein–polycation–clay composite that retains the protein's chelating activity in the adsorbed state (Fig. 1).

Experimental section

Materials

Wyoming Na-montmorillonite (SWy-2) clay was purchased from the Source Clays Repository of the Clay Mineral Society (Columbia, MO). Apo-mFd and holo-mFd were obtained as

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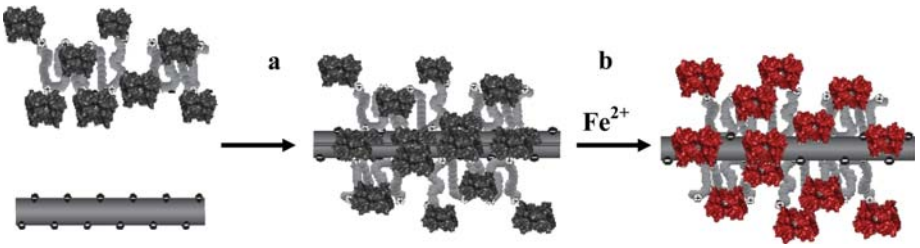

Fig. 1 The design of the protein–polycation–clay composites. (a) A complex of apo-mFd dimers (dark grey) and polycation (light grey) adsorbs on montmorillonite clay (rectangular sheet). (b) The composite (apo-mFd dimers and polycation complex on montmorillonite) chelates Fe^{2+} under reduced conditions (apo-mFd turns red upon chelation).

described previously.[35] The protein structure coordinates and sequence can be found in the PDB as 1RFK. Mercaptoethanol, Trizma base, Trizma hydrochloride, Bradford reagent and polydiallyldimethylammonium chloride (PDADMAC) (M_w 400–500 kDa) were purchased from Sigma-Aldrich (Steinheim, Germany). Iron(II) chloride was purchased from Merk (Darmstadt, Germany).

Chelation of Fe^{2+} by apo-mFd

The chelation of Fe^{2+} (4 mg L^{-1}) by apo-mFd (in 20 mM Trisma buffer pH 8.0 and 100 mM NaCl at 250 mg L^{-1}) was carried out in batch experiments in Eppendorf tubes. A solution of $FeCl_2$ (0.5 mL) was mixed with solutions (1 mL) of apo-mFd, apo-mFd with 2% v/v ME and apo-mFd with 2% ME and excess Na_2S. The optical absorption spectra of the holo-mFd (250 mg L^{-1}) and iron–apo-mFd solutions were measured by UV-Vis spectrophotometer (320–500 cm^{-1}) (Thermo Scientific, Evolution 300, Waltham, MA, USA) and subjected to electrophoresis in a native gel.

Apo-mFd and holo-mFd adsorption on montmorillonite

The protein–clay isotherms were determined in batch experiments in Eppendorf tubes. Apo-mFd and holo-mFd solutions (1 mL of 0–400 mg L^{-1} protein solution in 100 mM Trizma buffer pH 8.0 or Trizma buffer pH 4.5) were mixed with montmorillonite clay (0.5 mL of a 5 g L^{-1} suspension) under oxidized and reduced (2% ME) conditions. The solutions were kept continuously agitated for 24 h. Supernatants were separated by centrifugation at 4500 rpm for 25 min (Eppendorf centrifuge, model 5416). Protein concentration in the supernatant was determined with the Bradford reagent method (at 595 nm) by UV-Vis spectroscopic analysis.

Protein adsorption to montmorillonite with PDADMAC

PDADMAC–clay composites were prepared in Eppendorf tubes with a final concentration of 0.25 g L^{-1} PDADMAC and 1.67 g L^{-1} clay.[40] Apo-mFd (with 2% ME) or holo-mFd was added (1.5 mL of 0–400 mg L^{-1} protein) to the composite. Apo-mFd or holo-mFd (0–400 mg L^{-1}) was also premixed with 0.25 g L^{-1} PDADMAC and then added to the montmorillonite suspension. The solutions were continuously agitated for 24 h. Supernatants were separated by centrifugation at 2000 rpm for 25 min in order to allow selective sedimentation. At centrifugation speeds >4500 rpm, some sedimentation of the protein–polycation complex was observed so the centrifugation speed was lowered to an optimal separation rate. Protein concentration in the supernatant was determined by Bradford assay as described above.

Fe^{2+} binding by protein–clay composites and protein–PDADMAC–clay composites

Fe^{2+} binding by the different composites was carried out in batch experiments in Eppendorf tubes. Iron(II) chloride solutions (1.5 mL 0–8 ppm) were added to the different prepared composites and the solutions were kept under continuous agitation for 24 h. Supernatants were separated by centrifugation at 4500 rpm for 25 min and then diluted with nitric acid solution (0.1 M) and iron concentrations were determined by Side-On-Plasma (SOP) ICP-AES, model "ARCOS" (Spectro Ltd., Kleve City, Germany).

X-Ray diffraction

The X-ray diffraction patterns of montmorillonite, apo-mFd–montmorillonite (0–400 mg L^{-1}), holo-mFd–montmorillonite (0–400 mg L^{-1}) and apo-mFd (0–400 mg L^{-1})–PDADMAC (0.25 g L^{-1})–montmorillonite were determined. A 1–2 mL aliquot of the suspension was placed on a round glass slide and left to sediment as an oriented sample for 1 day. The samples were measured with an X-ray diffractometer (Philips PW1830/3710/3020) with CuKα radiation, $\lambda = 1.526$ nm.

Zeta potential

The mobilities/zeta potentials of apo-mFd, holo-mFd, montmorillonite, apo-mFd–montmorillonite and holo-mFd–montmorillonite composites (0–100 mg protein per L) were measured using a Zetasizer Nanosystem (Malvern Instruments, Southborough, MA). The clay and clay composites were measured in a dilute suspension (~0.05% w/v clay).

Results and discussion

Chelation of Fe^{2+} by apo-mFd

Upon addition of Fe^{2+} (4 mg L^{-1}) to the apo-mFd protein (250 mg L^{-1}) under reduced conditions (2% mercaptoethanol (ME)), the colorless apo-protein solution instantly turned red, suggesting iron chelation (the holo-mFd is red due to the presence of its native 2Fe–2S cluster). Two analytical methods were used to confirm the suggested chelation: UV-Vis optical spectroscopy and native gel electrophoresis measurements (Fig. 2).

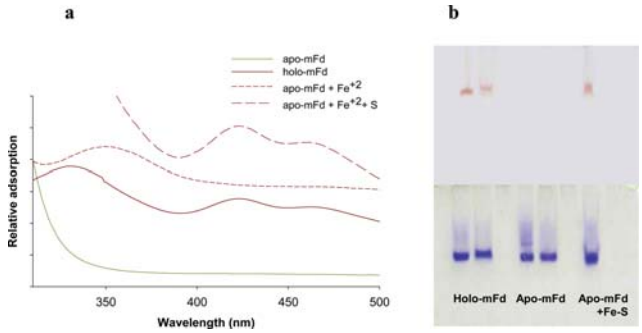

Fig. 2 Chelation of Fe^{2+} by apo-mFd. (a) UV-Vis spectrum of apo-mFd, holo-mFd, apo-mFd + $FeCl_2$, apo-mFd + $FeCl_2$ + Na_2S. (b) Native gel (upper) and denaturing polyacrylamide gel (lower) of holo-mFd (first run oxidized and second run reduced), apo-mFd (first run oxidized and second run reduced) and apo-mFd + $FeCl_2$ + Na_2S (reduced only).

While no absorption peaks were detected for apo-mFd, its absorbance spectrum (under reduced conditions) with Fe^{2+} and Na_2S was identical to that of the holo-mFd (Fig. 2a), whereas the spectrum of apo-mFd with Fe^{2+} (without sulfur) was not. However, in the latter case, an absorption band did appear at 352 nm, as reported for other iron-electron transfer protein complexes.[39] Moreover, the same electrophoretic migration distance was observed on a native gel for the holo-mFd and the apo-mFd (under reduced conditions) with Fe^{2+} (with and without Na_2S) and both protein bands were red, indicating iron chelation (Fig. 2b). The cysteine residues in the apo-mFd (which bind the Fe–S cluster in the holo-mFd) bond to each other *via* S–S bonds under oxidized conditions, but under reduced conditions, these bonds are broken and thiol groups are formed. We suggest that under reduced conditions, the apo-mFd is able to chelate the Fe^{2+} ions due to a change in its conformation (rendering it similar to the holo-protein) and the thiol groups located in its active site.

Ferredoxin adsorption to montmorillonite

Adsorption isotherms of holo- and apo-mFd on montmorillonite at pH 4.5 and 8 were obtained (Fig. 3a). Zeta potentials of the clay, holo-mFd and apo-mFd, were −41 mV, −35 mV and −27 (±5) mV, respectively (pH 8), indicating charge repulsion between the protein and the clay. Despite the lack of charge compatibility between apo-mFd (pH 8) and montmorillonite, protein adsorption was high, it did not increase with a decrease in pH and the adsorption isotherms were linear. This behavior suggests a partitioning adsorption mechanism for apo-mFd which is characteristic of "soft" proteins having an affinity to clays at pHs below and above their p*I*[31] (for mFd, the p*I* is ~3.9).[36] The electrostatic repulsion is compensated for by an entropic gain due to protein unfolding during adsorption and the consequent release of water molecules.[31] In contrast to the adsorption isotherms of apo-mFd, the adsorption of holo-mFd was low; it increased with a decrease in pH and reached a plateau. Such behavior is typical of the adsorption of "hard" proteins implying that holo-mFd has a rigid structure due to the iron–sulfur cluster (Fig. 3a).

The adsorption of apo-mFd under reduced conditions was similar to that of holo-mFd, supporting our hypothesis that

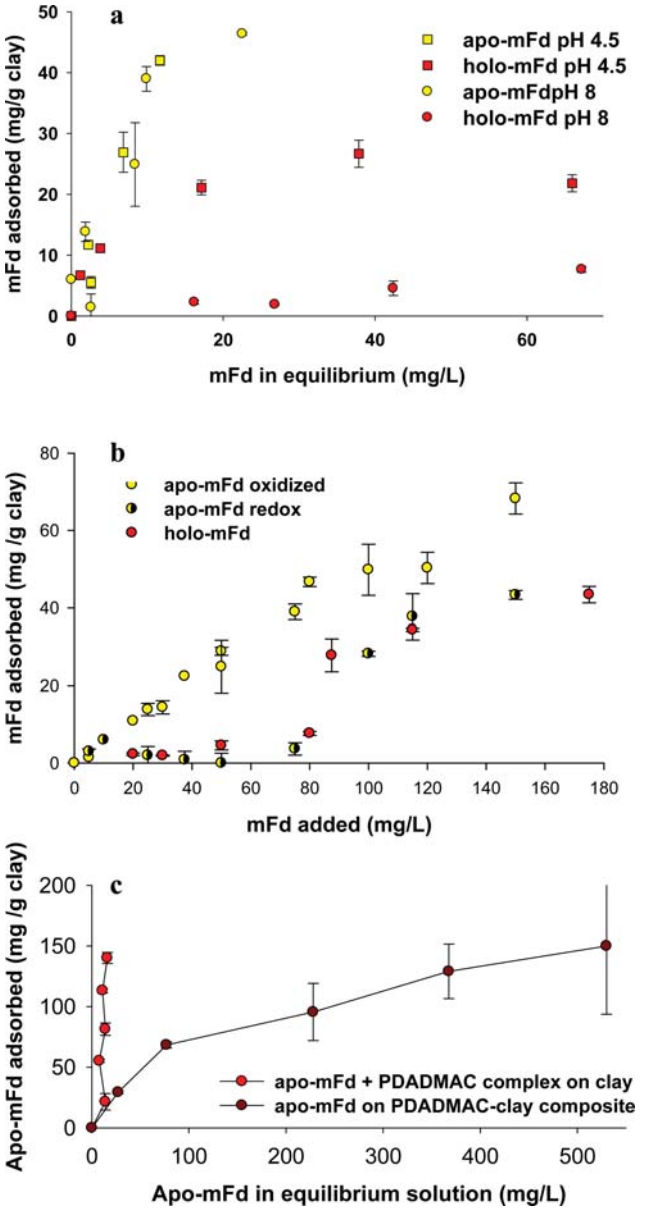

Fig. 3 Protein adsorption on montmorillonite. (a) Binding of apo-mFd (yellow) and holo-mFd (red) to montmorillonite (1.67 g L^{-1}) at pH 8.0 (circles) and pH 4.5 (squares) under oxidized conditions. (b) Binding of mFd to montmorillonite (1.67 g L^{-1}) at pH 8.0: apo-mFd under oxidized conditions (yellow), holo-mFd under oxidized conditions (red) and apo-mFd (yellow-black) under reduced conditions (2% ME). (c) Binding of apo-mFd (0–500 mg L^{-1}) to PDADMAC (0.25 g L^{-1})–montmorillonite (1.67 g L^{-1}) composites (dark red) and apo-mFd (0–500 mg L^{-1})–PDADMAC (0.25 g L^{-1}) complex adsorbed on montmorillonite (red).

reduced conditions enable a conformational change in the protein that renders it structurally similar to holo-mFd (Fig. 3b). The rise in adsorption obtained at higher added protein concentrations for the two proteins may be explained by a synergistic adsorption effect (Fig. 3b).[31]

The adsorption of reduced apo-mFd to the clay reached a maximum loading of 0.04 g protein per g clay (~50% adsorption). In an effort to improve protein loading on montmorillonite, adsorption was studied in the presence of the polycation polydiallyldimethylammonium chloride (PDADMAC) using

two approaches: adsorption of the apo-mFd to a pre-prepared polycation–clay composite (zeta potential ≈ 0 mV) and adsorption of a polycation–apo-mFd complex to the clay. Adsorption to clay of apo-mFd as a PDADMAC–apo-mFd complex was complete, reaching a high loading of 0.14 g protein per g clay (Fig. 3c, red circles). The affinity of apo-mFd to the pre-prepared polycation–clay composite was very low: to reach a similar protein loading it was necessary to add an extremely high concentration of apo-mFd (600 mg L^{-1}) and the adsorption was only ~20% (Fig. 3c).

X-Ray diffraction measurements were obtained for montmorillonite, apo-mFd–montmorillonite (reduced and oxidized), holo-mFd–montmorillonite and apo-mFd–PDADMAC–montmorillonite composites. The shifts in basal spacing ($d = 001$) were very minor, ~0.2 nm (from 1.2 nm to 1.4 nm), for all composites measured although the mFd protein measurements were approximately 2.5 nm × 3.6 nm (calculated by PyMOL on the basis of X-ray measurements). These findings indicate that the protein does not completely intercalate but is mainly located on the external clay surface with an average platelet size of 1 μm. The possible partial intercalation is in agreement with various reports in the literature on the immobilization of globular proteins on clay.[41–43]

Zeta-potential measurements were obtained for apo-mFd–montmorillonite and holo-mFd–montmorillonite composites as well as for the proteins and clay separately. All entities were similarly negatively charged. Therefore, all composites remained negative and there were no significant changes in zeta potential with the loading of the proteins on the clay (−44 mV for apo-mFd–montmorillonite and −41 mV for holo-mFd–montmorillonite, ±5.5 mV).

The effect of ionic strength on apo-mFd (reduced) adsorption to montmorillonite was also examined (0–1 M $CaCl_2$). Upon addition of 0.08 M $CaCl_2$, protein adsorption increased significantly (from 50 to 80%) due to screening of the negative charges on both the protein and the clay; however, a further increase in ionic strength had no effect on the adsorption.

Fe^{2+} binding by apo-mFd–montmorillonite and apo-mFd–PDADMAC–montmorillonite composites

Many studies have reported the high absorption of Fe^{2+} by montmorillonite due to cation exchange. In the presence of other cations, this affinity drops due to competition.[7] Fe^{2+} (4 mg L^{-1}) adsorption on montmorillonite (1.67 g L^{-1}) was ~95% under oxidized conditions. Under reduced conditions, the affinity of Fe^{2+} to the clay dropped dramatically (to ~20%), which could result from the formation of unstable iron–ME complexes involving two or more ME and iron molecules, keeping the ions in solution.[38] However, these iron–ME complexes showed affinity to the hydrophobic regions on the polycation–clay composite (zeta potential of the PDADMAC–montmorillonite composite was ~0 mV) resulting in higher Fe^{2+} binding to the polymer–clay composites (~60%).

Fe^{2+} binding to the apo-mFd–montmorillonite composite was poor (20–30%) suggesting loss of the protein's chelating ability in the adsorbed state (Fig. 4). On the other hand, apo-mFd retained its chelating ability when bound to the clay *via* its complexation with PDADMAC and as a result, iron binding was remarkably

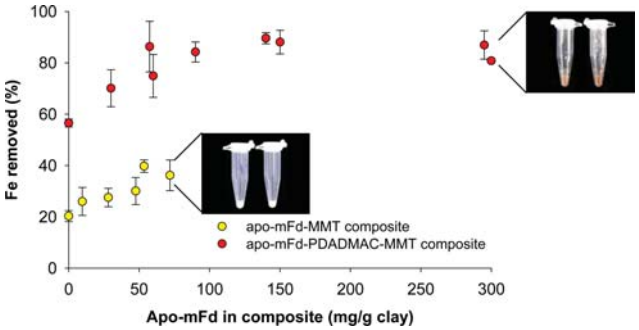

Fig. 4 Fe^{2+} (4 ppm) binding to protein–clay and protein–polymer–clay composites. Apo-mFd–montmorillonite (MMT) composites (0–75 mg protein per g clay)—yellow circles and apo-mFd–PDADMAC (complex)–MMT composite (0–300 mg protein per g clay)—red circles.

high (>90%) and the complex turned red (Fig. 4). The change in color of the apo-mFd–polycation–clay composite upon iron binding strengthens our hypothesis that the protein retains its iron-chelating ability. A detailed literature survey of polycation–protein complexes indicated that such complexes create a "molecular cage" around the protein and as a result, the protein is stabilized and may retain its biological activity.[44]

According to simple stoichiometric calculations, 1 mol of protein can chelate 2 mol of iron, based on the ratio in the holo-mFd, *i.e.* 125 mg of protein can chelate 1 mg of iron. An apo-mFd–PDADMAC–clay composite loaded with 100 mg L^{-1} apo-mFd removed 3.6 mg L^{-1} iron (Fig. 4) when stoichiometrically only 0.8 mg L^{-1} iron should have been removed. The additional iron removal can be explained by non-specific electrostatic interactions between the positive Fe^{2+} ions and the negatively charged protein and clay.

To demonstrate that iron binding is not only due to these non-specific electrostatic interactions, but is also a result of specific chelation by apo-mFd adsorbed to the clay, iron binding to apo- and holo-mFd–PDADMAC–montmorillonite composites was studied at low (0 M $CaCl_2$) and high (0.1 M $CaCl_2$) ionic strength, the latter limiting electrostatic interactions. At low ionic strength (no salt added), iron (0–6 mg L^{-1}) removal by both the apo-mFd (Fig. 4) and holo-mFd polycation–clay composites (150 mg L^{-1} protein loaded) was high, reaching >90%. At high

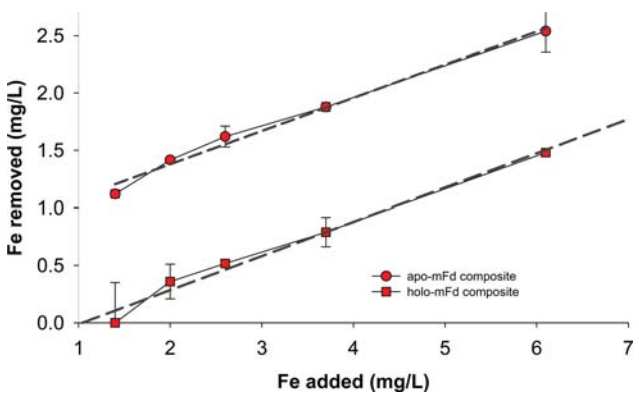

Fig. 5 Binding of iron by mFd–polycation–clay composite. Binding of Fe^{2+} (1.4–6 ppm) in the presence of 0.1 M $CaCl_2$ by apo-mFd (150 mg L^{-1})–PDADMAC–montmorillonite composites (circles) and holo-mFd (150 mg L^{-1})–PDADMAC–montmorillonite composites (squares).

ionic strength (0.1 M $CaCl_2$; Fig. 5), iron binding (1.4 mg L^{-1}) by the holo-mFd composite declined to zero whereas the apo-composite bound 1.2 mg L^{-1}, which is equivalent to the calculated stoichiometric chelating capacity of the protein loaded on the composite. Iron binding by both the apo- and holo-mFd composites increased with added iron concentration (Fig. 5). We suggest that this is due to adsorption of the above-described iron–ME complexes. Nevertheless, the difference in iron binding (1.2 ppm) between the apo- and holo-mFd composites remained constant over a range of iron concentrations (Fig. 5). These results clearly show that the protein remains active in the adsorbed state and that the binding is specific to the apo-mFd active site.

Conclusion

To conclude, tailored biomaterials lie at the interface of biology, chemistry, polymer science and engineering. The present study demonstrates that apo-mFd retains its chelating ability when bound to clay *via* its complexation with PDADMAC and as a result, iron binding is remarkably high. The chelation of Fe^{2+} was retained even at high ionic strength, suggesting the specificity of the biocomposite. These protein–polycation–clay composites may pave the way for the design of biocomposites for a wide range of applications such as immobilizing proteins, drug delivery and water treatment.

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