# Hydrophobic proteins: synthesis and characterisation of organic-soluble alkylated ferritins

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**Alkylated derivatives of the iron storage protein, ferritin, have been prepared by carbodiimide-activated coupling of long chain primary amines to surface carboxylic acid residues; the proteins are soluble in dichloromethane as intact, non-aggregated biomolecules.**

Ferritin is an unusual metalloprotein in that the inorganic centre consists of a large number of ions (up to 4500 Fe atoms) present as a biomineral (ferrihydrite, $Fe_2O_3{\cdot}nH_2O$) encapsulated within a 24-subunit polypeptide shell.[1] The organic shell is remarkably stable to thermal and chemical degradation due to strong subunit–subunit interactions, a property which offers much scope in using the protein micelle as a nanoreactor for the synthesis of biocompatible nanocolloids. Recently, we have developed synthetic routes to biomimetic ferritins reconstituted with non-native inorganic nanoparticles such as $Fe_3O_4$,[2,3] MnOOH,[4] FeS[5] or CdS.[6] These routes are generally dependent on a balance between the redox chemistry, oxidation kinetics and hydrolytic behaviour of metal ions, and are limited to aqueous-based media. Further developments should be possible if ferritin could be solubilized in organic media because this would allow a wide range of organometallic precursors to be used. In particular, sol–gel reactions based on alkoxide hydrolysis could be achieved within the polypeptide cage by preparing organic-soluble ferritin molecules which have a water-filled hydrophilic inner cavity and a hydrophobically modified outer surface. Such derivatives would be analogous to the reverse micellar (water-in-oil) phase of many surfactants but would have enhanced stability owing to the absence of dynamic exchange between ferritin subunits.

Here, we describe the first step towards this goal in which ferritins soluble in dichloromethane (DCM) have been prepared by alkylation reactions using long chain primary amines ($C_n$, $n$ = 6, 9, 12, 14, 18). The method involves the carbodiimide activation of surface carboxylic acid groups[7] of the native protein or a demetallated derivative (apoferritin) followed by nucleophilic substitution in the presence of a large excess of the amine.‡ Coupling of hexyl, nonyl or lauryl chains produced clear red–orange (ferritin) or colourless (apoferritin) THF–$H_2O$ solutions which were stable for at least 12 months. Similar reactions with tetradecylamine or stearylamine gave turbid mixtures which precipitated after a few days due to the reduced solubility of the longer chain amines. Each of the alkylated proteins, except for the hexyl-derivatized ferritin, were readily extracted into DCM by addition of small amounts of NaCl to give clear red–orange (ferritin) or colourless (apoferritin) organic solutions. In contrast, control experiments involving native (apo)ferritin, or reaction mixtures of (apo)ferritin in the absence of either the amine or EDC coupling reagent, showed no evidence of phase transfer into DCM. For the alkylated proteins, UV–VIS spectra of the DCM layer showed a shouldered peak at approximately 280 nm indicative of the aromatic amino acid side chains of the transferred ferritin. The ease of transfer of the alkylated ferritins into DCM was in the order tetradecyl- > lauryl- > nonyl-amine. In general, the alkylated proteins were stable for as long as six months in the DCM layer.

TEM images of unstained samples of the alkylated ferritins transferred into DCM showed discrete, electron dense iron oxide cores with an average size of 5 nm (Fig. 1). The non-aggregated and monodisperse nature of the protein when dried onto the TEM grid indicates that the derivatized polypeptide shell is structurally intact. Unlike native ferritin, the alkylated proteins could not be stained with uranyl acetate, indicating significant changes in the surface chemistry. Staining of the samples did however show evidence of a surfactant lamellar phase, presumably formed by self-assembly of excess amine molecules during air-drying of the DCM solutions.

Polyacrylamide gel electrophoresis (PAGE) showed that the native and nonyl-derivatized proteins migrated towards the positive and negative electrodes, respectively, under non-denaturing conditions (data not shown). Migration of the alkylated ferritin terminated, however, at the interface between the stacking and resolving gels, indicating only a small net positive charge on the derivatized protein.

Analytical ultracentrifuge (AUC) analysis§ of nonyl-derivatized ferritin showed evidence of a floating (excess nonylamine) and a sedimenting component (derivatized protein) in the DCM solutions. The sedimentation coefficient distributions [$g(s^*)$] showed the presence of two well defined species corresponding to nonyl-derivatized ferritin [$s^*$ = 12.1 Sv (77.5%)] and a larger species [$s^*$ = 21.4 Sv (21.4%), Fig. 2]. The sedimentation coefficients were consistent with those expected for a monomer–dimer system of spherical molecules, according to $s_n = s_1 n^{2/3}$ with $n$ = 2. Significantly, the relative proportions of monomeric and dimeric species were similar to those previously measured for native ferritin (69.4% monomer, 19.5% dimer and 11.1% trimer) using flow-field–flow-fractionation.[8] This strongly suggests that the hydrophobic ferritin is structurally intact and that negligible degradation occurs during

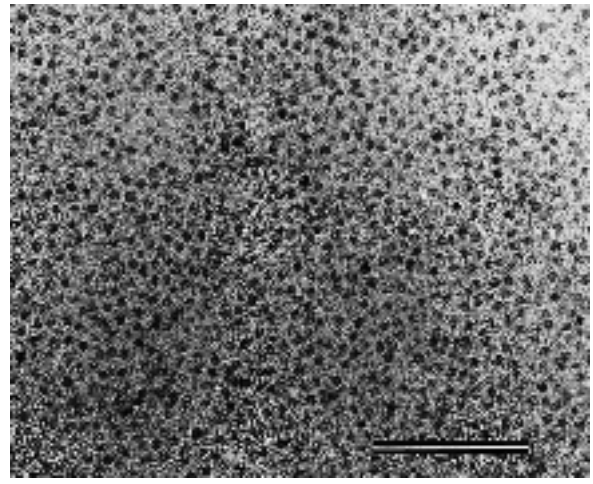

**Fig. 1** TEM image of a DCM solution of nonyl-derivatized ferritin showing discrete electron dense iron oxide cores, each of which is encapsulated within an alkylated protein shell; scale bar = 100 nm

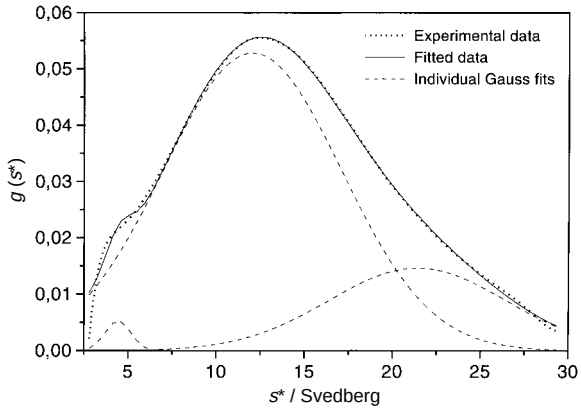


**Fig. 2** Sedimentation coefficient distribution [$g(s^*)$] for a DCM solution of nonyl-derivatized ferritin showing the presence of alkylated ferritin monomers and dimers. A minor contaminant with $s^*$ = 4.5 Sv (1.1%) is also present.

chemical functionalization in the THF–water reaction mixture.

The molar mass of nonylamine-derivatized ferritin in DCM was determined as $M_w$ = 675 kDa from sedimentation equilibrium results. This was an approximate value because the density could only be estimated from the sample composition. However, the value was consistent with results obtained from sedimentation equilibrium data for native ferritin ($M_w$ = 622.8 ± 4.3 kDa), assuming a similar monomer : dimer ratio and iron loading in the samples, and an additional mass due to the covalently attached nonylamine chains. The $M_w$ for apoferritin was determined as 497.0 ± 5.0 kDa by sedimentation equilibrium, giving molar masses, $M_w$ of 20.7 kDa and 125.8 kDa for the native subunit and FeOOH core, respectively. Assuming, $M_w$ = 675 kDa for the derivatized ferritin, gives a value of approximately 550 kDa for the molar mass of the derivatized apoferritin (22.9 kDa per subunit) which corresponds to approximately 400 covalently coupled nonylamine groups per protein molecule. This is approximately 75% of the theoretical number of amino acid carboxyl groups (*ca.* 520) in the protein molecule.[1]

Dynamic light scattering for nonyl-derivatized ferritin in DCM gave an average hydrodynamic diameter of 35 nm, compared with 12 mm for aqueous solutions of native ferritin. Although further work is required, the results suggest that the derivatized protein is associated to some extent with the excess amine molecules present in the DCM solution. Reversed-phase HPLC data for native ferritin showed a sharp peak for the monomer at 15 min with a shoulder at 11.5 min corresponding to the dimer, whereas nonyl-derivatized ferritin showed a broad protein peak with a retention time centred at 24 min. Because the derivatized sample was run directly from the reaction mixture without dialysis, excess amine and coupling agent, as well as small molecule byproducts, were observed as relatively sharp peaks between 2 and 5 min. The reconstructed data from the electrospray mass spectra of the proteins showed a narrow ($m/z$ 19.9–20.5 k) and wide distribution of molecular masses ($m/z$ 20–30 k) for the native and nonyl-derivatized ferritin subunits, respectively. These values were consistent with the derivatized subunit molar mass of 22.9 kDa determined by AUC analysis.

The results indicate that alkylated ferritins with long term solubility in DCM can be readily prepared. TEM, PAGE and AUC were consistent with the preparation of intact protein molecules with significantly modified surface charge. However, HPLC and electrospray mass spectroscopy suggest that the degree of derivatization varies significantly between ferritin molecules and that a heterogeneous product is obtained. Furthermore, significant amounts of non-covalently bound amine may be strongly associated with the chemically modified protein. In this regard, it seems feasible that surfactant micellization could enhance the transfer of the alkylated ferritins into DCM, although native ferrintin could not be transferred into DCM in the presence of uncoupled amine molecules alone, indicating that alkylation is the dominant factor determining the solubility in the organic medium. Further work is in progress to expand these studies and to exploit the alkylated proteins in sol–gel reactions involving metal alkoxides.

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## Notes and References

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‡ Nonylamine (1.11 g, 7.77 mmol, 1.5 ml) was added to THF–$H_2O$ (1 : 1 v/v, 8.5 ml) and the pH adjusted to *ca.* 5.5. THF was found to be a suitable solvent for amine solubilization without protein degradation. Ferritin or apoferritin (10 mg, 2.22 × $10^{-5}$ mmol) was slowly added, and the pH readjusted to 5.5 if required. The mole ratio of nonylamine to carboxylic acid residues was *ca.* 700 : 1, assuming a total of 520 aspartic and glutamic acid residues in the 24-meric protein.[1] 1-(3-Dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC, 150 mg, 0.78 mmol) dissolved in THF–$H_2O$ (1 : 1 v/v, 1 ml), was slowly added dropwise whilst maintaining the pH at 5.5 for 2–3 h, and the reaction mixture stirred overnight at room temperature. Samples (*ca.* 1 mg) of the alkylated protein were transferred from the reaction mixture into DCM by addition of small quantitites (5–10 mg) of dry NaCl followed by shaking. Reactions were also performed at lower concentrations of nonylamine, equivalent to 0.5, 7, 70, 170 or 340 molecules per protein carboxylic acid group. In general, ratios < 1 : 170 did not result in efficient phase transfer into DCM. Other primary amines, such as hexyl, lauryl ($C_{12}$), tetradecyl ($C_{14}$) and stearyl ($C_{18}$) amine were reacted with ferritin at concentrations equivalent to 125 or 500 molecules per carboxylate group, using the above procedure.

§ The sedimentation coefficient distributions [$g(s^*)$] were evaluated from sedimentation velocity data using the time-derivative method[9] and fitted with Gaussian curves. The weight average molar mass $M_w$ was obtained from sedimentation equilibrium distributions for a concentration series by extrapolation of $M_{w,app}$ to infinite dilution. $M_{w,app}$ values were derived from direct fitting to an ideal one-component model system,[10] and also independently from the cumulative $M^*$ approach.[11]

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