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Enhanced catalytic activity in organic solvents using molecularly dispersed haemoglobin–polymer surfactant constructs†

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The surface of haemoglobin (Hb) is chemically modified to produce molecular dispersions of discrete core–shell Hb–polymer surfactant bionanoconjugates in water and organic solvents. The hybrid nanoconstructs exhibit peroxidase-like catalytic activity with enhanced turnover rates compared with native Hb in water.

Enzymes have evolved to optimise their efficiency in aqueous environments, and as a consequence exhibit a strong dynamical and conformational dependence on the nature of molecular interactions with their solvent envelope. Accordingly, the catalytic efficiency of most enzymes dispersed in organic solvents is significantly retarded, as solvent exposure commonly leads to protein aggregation and denaturation.[1] These inhibitory processes can be partly rationalized by the substantially different dielectric constants of organic solvents when compared with water. The latter can be considered a poor solvent for proteins in the sense that it favours the compact structure of the globular fold. Conversely, polar organic solvents such as ethanol have a propensity to strip away tightly bound water molecules and solvate the hydrophobic core of a protein, which in turn stabilises the denatured state.[2] Proteins dispersed in highly hydrophobic solvents, however, commonly remain folded and can be extremely stable, although in many cases there is a loss of activity due to the reduction in the population of functional conformers due to molecular aggregation.[3]

Enzyme reactions in organic solvents are commonly undertaken using dispersions of freeze-dried powders, where the activity arises predominately from aggregates rather than discrete molecular entities. Accordingly, a number of approaches have been developed to increase the dispersibility and hence enzyme activity in organic reaction media. These include covalent modifications of enzyme structures using lipids[4] or surfactants,[4] entrapment of enzymes in reverse micelles or colloidosomes,[5] use of dendritic polymers[6] or copolymers,[7] and encapsulation in silica gels.[8] In particular, the covalent coupling of poly(ethylene) glycol (PEG) to the surface of enzymes (PEGylation) has been used extensively to increase the efficiency of biocatalysis in organic solvents.[9] Although the amphiphilic nature of PEG increases the range of available dielectric media, the resulting dispersions often contain large aggregates of the PEG-modified enzyme molecules that curtail intrinsic homogenous catalytic performance.

Herein, we describe a novel method using a solvent-free liquid protein[10] to produce molecular dispersions of an enzymatically active haemoglobin (Hb)–polymer surfactant conjugate in a range of organic solvents. The dispersed constructs comprise a preserved globular protein core surrounded by a compact polymer surfactant corona, and exhibit enhanced catalytic turnover rates depending on the dielectric constant of the organic solvent. The methodology involves the carbodiimide (EDC)-mediated cationization of surface accessible amino acid carboxylate side chains to produce a super-charged polycationic haemoglobin (**C**-Hb; zeta potential, +52 mV; native Hb, +7 mV), followed by electrostatic tethering of the PEG-based anionic surfactant (C_9H_{19}-C_6H_4-$(OCH_2CH_2)_{20}O(CH_2)_3SO_3^-K^+$) (**S**) to yield a charge-neutral aqueous conjugate ([**C**-Hb][**S**]) (Fig. 1), which when lyophilised and then annealed at 30 °C produced a viscous solvent-free liquid (ESI† Methods). Matrix-assisted laser deposition/ionisation time-of-flight (MALDI-TOF) mass spectroscopy (Fig. S1, ESI†) gave a cationization efficiency of *ca.* 96%, which corresponded

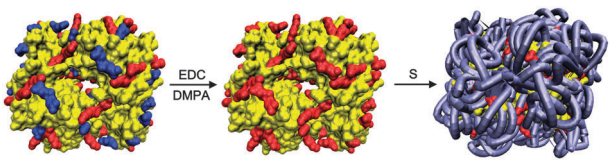

Fig. 1 Schematic illustration showing protein cationization using *N,N′*-dimethyl-1,3-propanediamine (DMPA) followed by electrostatic coupling of poly(ethylene glycol) 4-nonylphenyl 3-sulfopropyl ether, potassium salt (**S**) to yield a charge neutral stoichiometric conjugate [**C**-Hb][**S**]. Blue and red regions on the surface of the haemoglobin structures show the anionic and cationic residues respectively, and **S** molecules are represented using purple tubes.

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† Electronic supplementary information (ESI): Experimental procedures, MALDI-TOF, DLS, UV-Vis and Michaelis–Menten plots are provided. See DOI: 10.1039/c3cc46101g

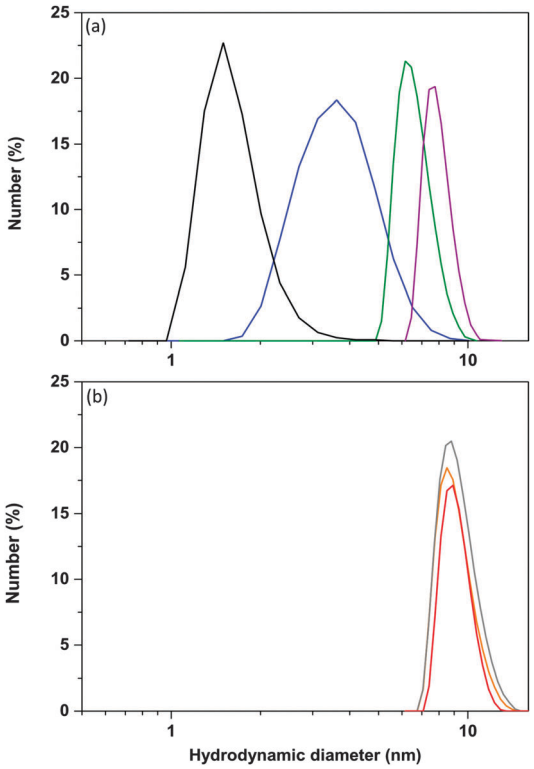

Fig. 2 DLS particle size distributions at 25 °C for (a) aqueous solutions of native Hb (blue), cationized Hb (green), polymer surfactant **S** (black) and [**C**-Hb][**S**] (purple), and (b) [**C**-Hb][**S**] in acetonitrile (orange), ethanol (grey), and isopropanol (red).

to the modification of approximately 44 carboxylates per protein, and equated to a total of 100 surfactant binding sites.

The solvent-free [**C**-Hb][**S**] liquid could be readily dispersed in water, acetonitrile, ethanol and isopropanol, and the resulting clear solutions remained stable for six months without any observable signs of aggregation (Fig. S2, ESI†). Conversely, aqueous solutions of native Hb aggregated immediately when added to any of the organic solvents (Fig. S2, ESI†). Dynamic light scattering (DLS) profiles gave average hydrodynamic diameters of 6.3 or 7.7 nm for aqueous dispersions of **C**-Hb or [**C**-Hb][**S**] (Fig. 2a), consistent with the formation of a molecularly dispersed protein–polymer surfactant construct with a densely packed polymer surfactant corona. In contrast, DLS profiles of the [**C**-Hb][**S**] conjugate dispersed in acetonitrile, ethanol or isopropanol gave particle size distributions with larger mean diameters of 8.7, 8.9 or 8.9 nm respectively (Fig. 2b). The data confirmed that the [**C**-Hb][**S**] conjugate was dispersed without significant aggregation as a discrete molecular entity, and that the polymer surfactant shell was extended in the organic solvents, presumably *via* effective solvation and extension of the hydrophobic nonylphenyl surfactant tails.

Circular dichroism (CD) and UV-Vis spectroscopies were used to investigate the protein structure associated with the [**C**-Hb][**S**] conjugate after dissolution in aqueous or organic media. CD spectra from all the [**C**-Hb][**S**] dispersions showed characteristic α-helical features at approximately 195, 208 and 222 nm, originating from the amide π–π* and n–π* transitions (Fig. 3a). There was only minimal variation between the CD traces, and deconvolution of the spectra showed no significant reduction in the

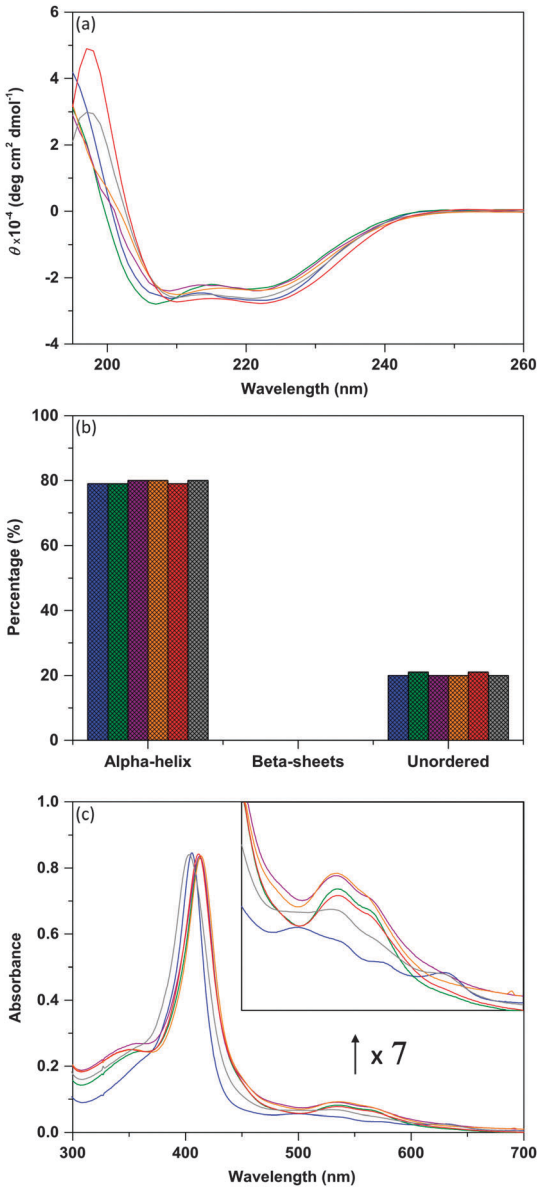

Fig. 3 (a) CD spectra, (b) protein secondary structure content, and (c) UV-Vis spectra for native Hb in water (blue), **C**-Hb in water (green), and [**C**-Hb][**S**] in water (purple), acetonitrile (orange), ethanol (grey) and isopropanol (red).

alpha helical content (*ca.* 80%) after protein cationization or surfactant conjugation (Fig. 3b), indicating that the polymer coronal layer effectively screened the globular protein core from adverse interactions with the organic solvents. UV-Vis spectra of aqueous solutions of native Hb or **C**-Mb showed Soret bands at 406 or 412 nm, and Q bands at 503, 541, 578, 631 or 532 and 562 nm, respectively (Fig. 3c). The data were consistent with a mixture of a five-coordinate high spin and six-coordinate hydroxyl-bound low spin states for native Hb,[11] and a six-coordinate low spin configuration with a strongly associated water molecule or bis-ligated distal histidine for the cationized protein.[11] In contrast, UV-Vis spectra of the [**C**-Hb][**S**] conjugate were consistent with a six-coordinate low spin state when dispersed in water, or in organic solvents such as isopropanol or acetonitrile. However, when dispersed in ethanol, the protein–polymer surfactant

conjugate displayed a UV-Vis spectrum similar to that of aqueous Hb, albeit with a slightly blue-shifted Soret band (403 nm) (Fig. 3c). UV-Vis spectra from free hemin dispersed in isopropanol, ethanol or acetonitrile (Fig. S3, ESI†) gave Soret band peak positions at 401, 400, and 379 nm respectively, indicating that exposure of [**C**-Hb][**S**] to the organic solvents did not result in haem removal.

Native Hb can be used to spontaneously cleave H_2O_2 to catalyse the oxidation of organic pollutants such as phenols and polycyclic aromatic hydrocarbons,[12] and this incipient peroxidase-like activity was probed by monitoring the oxidation of *o*-phenylenediamine (OPD) to phenazine by Hb and [**C**-Hb][**S**] under aqueous and non-aqueous conditions at a H_2O_2 concentration of 15 mM. The catalytic turnover rates (k_{cat}) and Michaelis–Menten constants (K_m) were determined by fitting the initial rates using the Michaelis–Menten equation (Fig. S4 and S5, ESI†), and the resulting kinetic parameters of native Hb and [**C**-Hb][**S**] are listed in Table S1 (ESI†). Significantly, an increase in k_{cat} was evident for the [**C**-Hb][**S**] conjugate when dispersed in water or a range of organic solvents compared with the peroxidase-like activity of unmodified Hb. For the organic solvents, the k_{cat} values increased in the sequence isopropanol < ethanol < acetonitrile, with a k_{cat} value approximately six-fold higher in acetonitrile when compared with the native protein. The positive correlation with increasing solvent polarity was consistent with associated changes in the dielectric constant of the active site resulting in a reduction of the energy of the activated substrate–enzyme complex. Moreover, given the similarity in the secondary structure and absorption band structure of the [**C**-Hb][**S**] conjugates in the organic solvents, it is possible that the dependence on increased solvent polarity was driven by an increase in the distribution of functional motions in the protein structure.[13] In contrast, an approximately 30% decrease in the k_{cat} values compared with native Hb in water was observed when unmodified Hb was dispersed in the above organic solvents. We attribute this to deactivation of a proportion of the protein molecules due to the inaccessibility of the active sites accompanying aggregation, as well as distortions in the surface hydration layer and denaturation of the enzyme in the presence of apolar solvents.[14]

The peroxidase-like activity of native Hb and [**C**-Hb][**S**] in water gave K_m values of 0.34 ± 0.03 and 0.056 ± 0.002 mM respectively (Table S1, ESI†). The increase in substrate affinity for [**C**-Hb][**S**] was attributed to an increase in steric restriction by the densely packed polymer surfactant corona. Under aqueous conditions, the PEG domain of the polymer surfactant is expected to form rigid hydrogen bonded networks with water molecules, and the hydrophobic nonylphenol moiety to be buried within the surrounding coronal layer. Considerably smaller K_m values were also obtained for assays performed on [**C**-Hb][**S**] in ethanol (0.049 ± 0.004 mM) and isopropanol (0.19 ± 0.03 mM), indicating high affinity for the substrate molecules under these conditions. Conversely, a significant increase in K_m (0.30 ± 0.02 mM) was observed for the peroxidase-like activity of [**C**-Hb][**S**] in acetonitrile. As the [**C**-Hb][**S**] construct showed a similar expansion in the polymer surfactant corona when dispersed in the above organic solvents (Fig. 2b), we attributed the reduced substrate affinity in acetonitrile to the absence of hydrogen bonding in this reaction medium. An increase in the propensity for hydrogen bonding would also explain the smaller K_m value for ethanol compared with isopropanol.

In conclusion, we have demonstrated that nanoscale engineering of the surface of Hb can be used to prepare a protein–polymer surfactant conjugate that can be molecularly dispersed in water or a range of organic solvents without loss of structure or enzymatic function. Significantly, the presence of a condensed amphiphilic polymer-surfactant corona not only inhibited protein aggregation in organic solvents, but also increased the peroxidase-like activity of the [**C**-Hb][**S**] construct in water, acetonitrile, ethanol or isopropanol. Although we have used haemoglobin as an archetype of a globular protein with enzymatic activity, we anticipate that our methodology could be successfully applied to industrially relevant enzymes, such as lipases, amylases or proteases to yield molecularly dispersed functional bioconjugates for application in a wide range of reaction media.

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