

A stable biomimetic redox catalyst obtained by the enzyme catalyzed amidation of iron porphyrin†

Subhalakshmi Nagarajan,^{a,d} Ramaswamy Nagarajan,^c Ferdinando Bruno,^e Lynne A. Samuelson^e and Jayant Kumar^{b,d}

Received 8th August 2008, Accepted 13th November 2008

First published as an Advance Article on the web 18th December 2008

DOI: 10.1039/b813823k

Hematin, a hydroxyferritoporphyrin, is the more stable oxidized form of the free heme. The use of hematin as a catalyst for oxidative polymerization reactions has been restricted due to its limited aqueous solubility at low pH conditions. While there have been reports on the functionalization of hematin with poly (ethylene glycol), the esters formed are not very stable at low pH conditions. We report here the design and synthesis of hematin tethered with methoxypolyethylene glycol amine chains as a novel stable biomimetic catalyst. This one step amidation was performed under solventless conditions and catalyzed by a hydrolase (Novozyme-435). The amidation greatly improved the stability of hematin at low pH. Further, this catalyst was soluble in water and was able to catalyze the polymerization of aniline based monomers. The amide functionalized hematin serves as a robust cost-effective alternative to HRP, active even at lower pH conditions.

Introduction

In recent years, enzymatic polymerization has been investigated as a possible environmentally friendly route to the synthesis of polyphenols/polyanilines. Oxidoreductases such as Horseradish Peroxidase (HRP), Soybean Peroxidase (SBP) obtained from natural & renewable sources have been known to catalyze the polymerization of phenol^{1,2} and aniline³ based monomers under benign conditions in aqueous and mixed solvent systems. It has been reported that HRP catalyzes the oxidative coupling/polymerization of aniline and phenol based monomer derivatives to produce conjugated polymers with interesting properties. These biocatalytic reactions can be carried out in the presence of polyelectrolyte templates or in organized media such as micelles. The polyelectrolyte templates/micelles help in aligning the monomers through preferred electrostatic interactions or hydrogen bonding, and by providing a unique local environment that favors the formation of well-defined polymers. This template assisted enzymatic approach has overcome many of the limitations of traditional chemical methods of synthesis of polyaniline and polyphenol. While the advantages of

enzyme-catalyzed synthesis are numerous, there are also certain drawbacks associated with it.

The high cost of enzymes, low activity and poor stability of HRP at low pH is a major concern. In this context, metalloporphyrins have been studied both as models for cytochrome P-450 and as important catalysts for oxidative polymerization reactions.⁴

The heme prosthetic group is the active site in a number of metalloproteins and enzymes and has been reported to play a key role in electron transfer, oxygen transport and catalytic reactions.⁵ Interestingly, some metalloporphyrins have been shown to exhibit similar catalytic characteristics as that of peroxidases.⁶ Hence, there have been several reports on enhancing the activity of the porphyrins and trying to mimic naturally occurring peroxidases. The peroxidase-like activity of tetrakis(carboxyphenyl)porphyrin with different central metal ions has been evaluated.⁷ Metalloporphyrins of manganese, cobalt and iron were found to be the most efficient catalysts.⁸ Saito *et al.*⁹ reported an artificial metalloporphyrin and its ability to mimic peroxidases.

Among the iron containing metalloporphyrins, hemin has been shown to catalyze the oxidative polymerization of phenols.¹⁰ Nevertheless, the catalytic activity is still low.¹¹ Hemin has been modified by attaching pendant groups such as β -cyclodextrin.¹² The activity of the complex was still not appreciable.¹³ Wang *et al.*¹⁴ have reported the synthesis of a peroxidase mimic by modifying hemin. However the peroxidase mimic was not active below pH 9. Iron(III) tetra (p-sulfonatophenyl)porphyrin [(Fe(III)TPPS)] has been synthesized and found to oxidatively catalyze the polymerization of aniline.¹⁵ But, the synthetic route to prepare the Fe(III)TPPS is complex and involves multiple steps and purification. Hemoglobin, the major heme protein of red blood cells has by itself been shown to possess catalytic activity.¹⁶

^aDepartment of Chemistry, University of Massachusetts, Lowell, MA 01854, USA

^bDepartment of Physics, University of Massachusetts, Lowell, MA 01854, USA

^cDepartment of Plastics Engineering, University of Massachusetts, Lowell, MA 01854, USA.

^dCenter for Advanced Materials, University of Massachusetts, Lowell, MA 01854, USA

^eU.S Army Natick Soldier Research, Development and Engineering Center, Natick, MA 01796, USA

† Electronic supplementary information (ESI) available: MALDI-TOF MS spectrum for the amidated hematin. See DOI: 10.1039/b813823k

Hematin, a hydroxy ferriprotoporphyrin, is also considered to be another promising biomimetic alternative to HRP. It is inexpensive and catalyzes the polymerization of a wide range of phenolic substrates.¹⁷ Hematin is an Fe(III) complex with a structure similar to the prosthetic iron protoporphyrin IX in HRP. One of the axial coordination sites of Fe(III) is occupied by a hydroxide ion instead of a histidine residue in HRP. Unlike peroxidases, hematin does not contain a protein environment and hence it is stable in organic solvents as well as high pH conditions. However hematin is water soluble only at high pH and ineffective for use as a catalyst at low pH aqueous conditions typically required for oxidative polymerization. To address this, we designed and synthesized a modified hematin [hematin tethered with poly (ethylene glycol) (PEG) groups].¹⁸ This PEG-hematin was soluble in water and was able to catalyze the polymerization of a wide variety of aniline and phenol monomers at a rate comparable to native HRP. However the pegylated hematin, being an ester, is also prone to hydrolysis at low pH conditions which are typically required for the oxidative polymerization of monomers like Ethylenedioxy Thiophene (EDOT). In order to overcome this limitation, we have modified hematin with Methoxypolyethylene glycol amine (MPEGNH₂). This amidation was performed in a single step using a lipase as a catalyst without the use of solvents. Novozyme-435 has been utilized as a catalyst for the amidation. The amide functionalized hematin was water-soluble, stable and catalytically active at a low pH. Initial results suggest that the amidated hematin could oxidatively catalyze the polymerization of aniline.

Results and discussion

As mentioned previously, although the PEG-hematin (ester) was able to catalyze the polymerization of a wide range of monomers, the pegylated ester by itself was not very stable at lower pH conditions. In highly acidic conditions, the ester bond between hematin and PEG chains is cleaved. As seen in Fig. 1, the pegylated hematin is not very stable at low pH. When a few drops of concentrated hydrochloric acid were added, the absorbance dropped drastically and the hematin precipitated out of the solution.

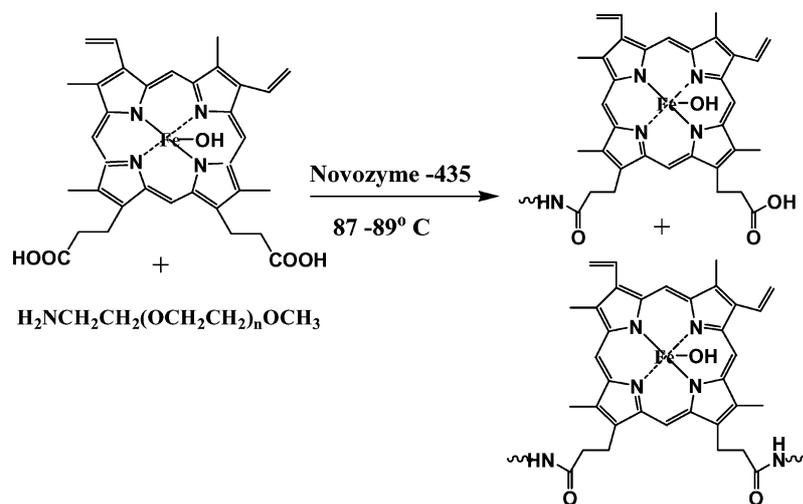


Fig. 2 Scheme for the amidation of hematin catalyzed by Novozyme-435.

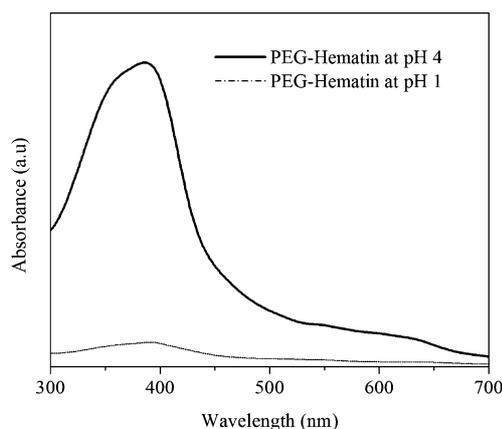


Fig. 1 Stability of the pegylated hematin at low pH conditions.

The primary goal of this research is to overcome this limitation by functionalizing hematin with polyethylene glycol containing a terminal amino group to form a more stable amide linkage. Lipases have been known to catalyze the amide formation reactions between acids and amines.¹⁹ We have used Novozyme-435 as a catalyst for the amidation of hematin as shown in Fig. 2. The reaction is performed in bulk at 85–87 °C and does not involve the use of solvents. Use of a mono functionalized PEG amine offers better control over the functionalization. The product formed can be separated from the starting materials by simple filtration and dialysis.

The resulting hematin functionalized with methoxypolyoxyethylene glycol amine was found to be water-soluble and is also soluble in organic solvents like DMF.

The molecular weight of the hematin tethered to poly(ethylene glycol amine) was also confirmed by Gel Permeation Chromatography (GPC) analysis. GPC confirms functionalization of the hematin with mPEG amine chains ($M_n \sim 10,711$ Da) indicating both the acid groups of the hematin have been functionalized.

GPC also indicates the small amounts of monofunctionalized hematin/unreacted PEG. However, for the intended use of this compound as a biomimetic catalyst, the presence of small

amounts of unreacted PEG will not affect the efficacy of these compounds. MALDI-TOF results also confirm the presence of a bifunctionalized hematin (see ESI†).

The spectral changes of the infrared spectra in the carbonyl region of hematin and amidated hematin are indicative of the formation of an amide linkage. In particular, a strong band at 1712 cm^{-1} observed in the case of hematin shifts to 1684 cm^{-1} indicating the conversion of the carboxylic group into an amide. We attempted to further confirm the structure of the amidated hematin using NMR. The high paramagnetic resonance field of the Fe(III) present in the amidated hematin hampered unambiguous assignment of peaks in the NMR spectrum.

In order to assess the stability of the amidated hematin, the amide was dissolved in pH 4 phosphate buffer and the pH of the solution was lowered to pH 1. The UV-visible spectra were recorded two and five days after the solution was kept at pH 1. As seen in Fig. 3, the amide functionalized hematin is very stable even after 5 days (the Soret band of the hematin remains virtually unchanged) and the amide did not precipitate out of the solution.

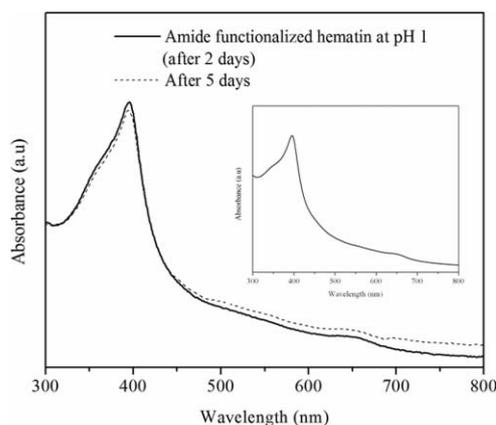


Fig. 3 UV-visible spectra for amidated hematin at pH 1 and pH 4 (inset).

The catalytic activity of peroxidases such as HRP is evaluated using the peroxidase assay originally developed by Willstaeter *et al.*^{20,21} The assay measures the rate of oxidation of pyrogallol to purpurogallin when catalysed by HRP. Specific activity of HRP is often expressed in terms of pyrogallol units. One pyrogallol unit will form 1.0 mg purpurogallin in 20 sec at pH 6.0 at 20 °C.

Since the catalytic center in HRP is an iron containing porphyrin, the peroxidase assay can also be extended to iron porphyrins like hematin. In addition, the pyrogallol assay has been used previously for studying the catalytic activity of different iron containing porphyrins.²²

Hematin was dissolved in water at pH 10 and then the pH was brought down gradually to a pH of 1 by addition of concentrated hydrochloric acid. Concentrations of hematin and the amidated hematin were chosen such that the optical densities were similar at the chosen concentration. In order to verify if the amidated hematin retained its catalytic activity at low pH, the enzymatic assay was performed at pH 1. The formation of purpurogallin was monitored at 420 nm for 8 minutes. As seen in Fig. 4, the amide functionalized hematin was active and could catalyze the transformation of pyrogallol to purpurogallin. In

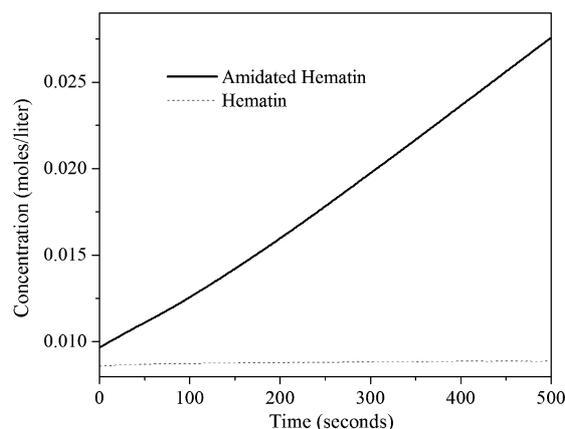


Fig. 4 Comparison of the time dependent formation of purpurogallin (moles/litre) catalyzed by hematin and amidated hematin at pH 1.

contrast, hematin by itself could not catalyze the formation of purpurogallin under identical conditions due to its poor solubility at this pH.

HRP has been modified with PEG and the pegylated HRP was found to be active in a number of organic solvents.²³ However the activity in aqueous solutions, determined using the peroxidase assay, was found to decrease with decrease in pH with optimal activity²⁴ seen at a pH around 10. Since the pegylated HRP is not active at low pH conditions, the activity of the amidated hematin was not compared with the pegylated HRP.

The amidated hematin was also effective in catalyzing the polymerization of aniline in the presence of sulfonated polystyrene (SPS). The UV-visible spectra of the polyaniline (PANI)/SPS complex formed at pH 4 after initiation of the polymerization is shown in Fig. 5. The UV-visible spectra showed the presence of polaron absorption bands at 400 nm and 800–1000 nm. This is consistent with earlier reports which have observed similar features for the PANI/SPS complex.²⁵

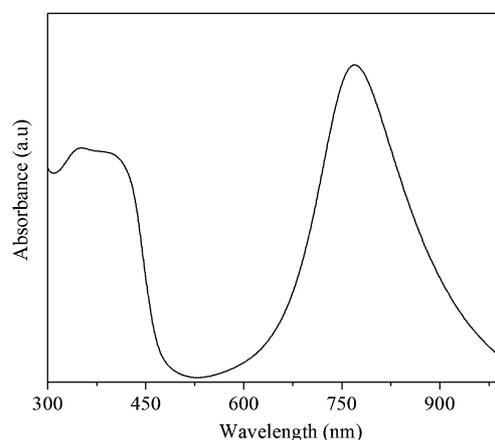


Fig. 5 UV Visible spectra for polymerization of aniline at pH 4 in the presence of SPS catalyzed by amide functionalized hematin.

The reversible redox behavior of the polyaniline was studied by dedoping and doping using base (NaOH) and acid (HCl), respectively, and the results are presented in fig. 6. The UV-visible spectra of the PANI/SPS obtained at pH 4 show some similarity to the spectra reported earlier for the SPS/PANI complex.³

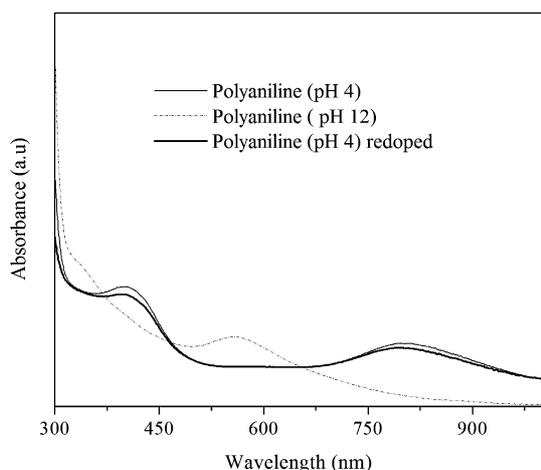


Fig. 6 UV-visible spectra of polyaniline/SPS complex during dedoping and redoping.

The polaron band at 407 nm and 800 nm confirms the presence of PANI in the doped state. When the pH of the solution is increased to 12, a strong absorption at 550–600 nm is seen due to the quinoid rings. The PANI can be redoped back to pH 4. Studies are underway to further characterize the polyaniline synthesized using amidated hematin.

The thermal stability of the catalyst was analyzed using thermal gravimetric analysis (TGA).

Fig. 7 shows the TGA for the MPEGNH₂ and the amide functionalized hematin. The free amine is known to start degrading at around 220 °C and is completely degraded by around 420 °C. In comparison, the amidated hematin is much more stable with over 20% remaining at 700 °C.

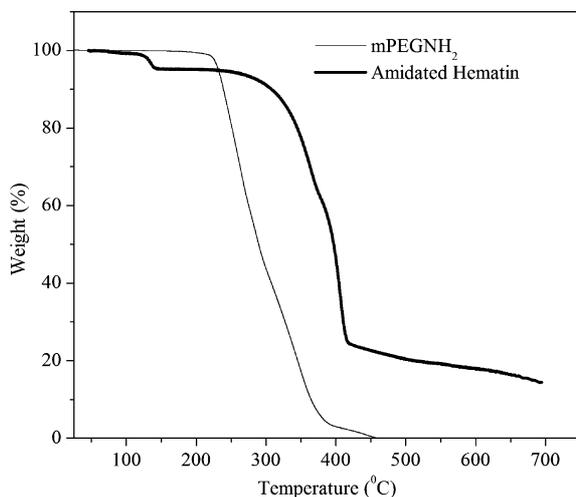


Fig. 7 TGA analysis of amidated hematin and mono-methoxy poly (ethylene glycol) amine [mPEGNH₂].

Conclusions

Hematin was modified through a one-step enzyme catalyzed reaction. The hematin functionalized amide exhibited better stability than PEG-hematin especially at low pH conditions. The modified hematin also serves as a catalyst for the polymerization of aniline. The enzymatically modified hematin can serve as a

versatile, more stable catalyst for the oxidative polymerization of a wider range of monomers like pyrrole and EDOT. This opens the possibility of using this catalyst at wider ranges of pH conditions, thus carrying out reactions that may not be possible using peroxidases. In addition, the improved stability of this amide functionalized hematin widens the scope for recycling thus making the catalyst more cost-effective.

Experimental section

Materials and methods

Hematin, aniline and sodium polystyrene sulfonate (SPS) (MW 70 kD) were purchased from Sigma Chemical Co. (St. Louis, MO) and used as received. Methoxypolyethylene glycol amine ($M_n \sim 5000$) was purchased from Aldrich, Allentown. Novozyme-435, an immobilized enzyme was a gift from Novozymes, Denmark. and was dried over P₂O₅ under vacuum prior to use. The hydrogen peroxide was diluted to 0.3% (in deionized water), and this solution was used for polymerization. All other chemicals were of reagent grade or better.

Synthesis of amidated hematin

Hematin (25mg, 0.03 mmol) and mono-methoxy poly (ethylene glycol) amine [mPEG-amine] (0.78 g, 0.15 mmol) were placed in a round-bottom flask and dried under vacuum at 87–89 °C. The enzyme (15% by weight with respect to reactants) was added to the reaction mixture and the reaction flask was placed in a constant temperature oil bath maintained at 87–89 °C under vacuum. The reaction was allowed to proceed for 48 hours. The reaction mixture was poured into water with a pH 3. The unreacted Hematin was filtered off along with the enzyme. The filtrate was dialyzed to remove unreacted [mPEG]. After the completion of dialysis, the product was obtained as a solid by freeze-drying. The gravimetric yield was between 75–78%.

Peroxidase assay

The peroxidase assay was performed based on the procedure provided by Sigma.²⁶ Concentrations of hematin and amidated hematin were chosen such that they have the same optical densities at the chosen concentration. 0.3% hydrogen peroxide was used for the assay. Absorbance was monitored at 420 nm for approximately 8 minutes.

Characterization

UV-visible near-IR spectra were obtained using a Perkin-Elmer Lambda 9 spectrophotometer. FT-IR measurements were carried out using a Perkin-Elmer 1720X series FT-IR spectrophotometer. Thermogravimetric analysis (TGA) was performed using a TA Instrument, Hi-Res 2950 thermogravimetric analyzer. The TGA of all samples was carried out under nitrogen. GPC analysis was carried out on a Agilent GPC with RI detector. GPC analysis was done on a water column calibrated with poly sodium polystyrene-sulfonate and containing 0.01% lithium bromide using a refractive index detector. 8 mg/ml of the amide functionalized with hematin was dissolved in water and a flow rate 1 ml/min was used.

MALDI experiments were done in the linear mode on a Waters MALDI-TOF MS instrument. Dithranol was used as a matrix. 10 mg/ml of the matrix was dissolved in THF: acetonitrile: water (1:1:1) ratio. A few drops of ammonium hydroxide was added to the compound before analysis. The sample and matrix solutions were mixed at 1/1 (v/v) ratio immediately before the analysis. Approximately 2 μ l of the above solution was spotted on a target plate.

References

- 1 F. F. Bruno, R. Nagarajan, P. Stenhouse, K. Yang, J. Kumar, S. K. Tripathy and L. A. Samuelson, *J. Macromol. Sci. Part A—Pure Appl. Chem.*, 2001, **A38**(12), 1417–1426.
- 2 F. F. Bruno, S. Nagarajan, R. Nagarajan, J. Kumar and L. A. Samuelson, *J. Macromol. Sci. – Pure Appl. Chem.*, 2005, **42**(11), 1547–1554.
- 3 (a) W. Liu, J. Kumar, S. K. Tripathy, K. J. Senecal and L. A. Samuelson, *J. Am. Chem. Soc.*, 1999, **121**, 71–78; (b) F. F. Bruno, S. Fosey, S. Nagarajan, R. Nagarajan, J. Kumar and L. A. Samuelson, *Biomacromolecules*, 2006, **7**(2), 586–589.
- 4 T. G. Traylor, C. Kim, J. L. Richards, F. Xu and C. L. Perrin, *J. Am. Chem. Soc.*, 1995, **117**, 3468–3474.
- 5 (a) P. R. Ortiz de Montanallo, *Acc. Chem. Res.*, 1987, **20**, 289–294; (b) J. H. Dawson, *Science*, 1988, **240**, 433–439.
- 6 M. Sono, M. P. Roach, E. D. Coulter and J. H. Dawson, *Chem. Rev.*, 1996, **96**, 2841–2887.
- 7 Y. X. Ci, F. Wang and Fresenius, *J. Anal. Chem.*, 1991, **339**, 46–49.
- 8 Y. X. Ci and F. Wang, *Talanta*, 1990, **37**, 1133–1136.
- 9 Y. Saito, S. Nakashima, M. Mifune, J. Odo, Y. Tanaka, M. Chikuma and H. Tanaka, *Anal. Chim. Acta*, 1985, **172**, 285–287.
- 10 M. Akita, D. Tsutsumi, M. Kobayashi and H. Kise, *Biotechnology Letters*, 2001, **23**, 1827–1831.
- 11 K. Zhang, L. Mao and R. Cai, *Talanta*, 2000, **51**, 179–186.
- 12 L. Y. Mao and H. X. Shen, *Chem. J. Chin. Univ.*, 1998, **19**(Suppl.), 442.
- 13 K. Zhang, R. Cai, D. Chen and L. Mao, *Analytica Chimica Acta*, 2000, **413**, 109–113.
- 14 X. Wang, Y. Li and W. Chang, *Analytica Chimica Acta*, 1999, **400**, 135–142.
- 15 M. R. Nabid, R. Sedghi, P. R. Jamaat, N. A. Safari and A. Entezami, *J. Appl. Polym. Sci.*, 2006, **102**, 2929–34.
- 16 H. Xing, Z. Yu-Ying, T. Kai and Z. Guo-Lin, *Synth Met*, 2005, **150**, 1–7.
- 17 J. A. Akkara, J. Wang, D.-P. Yang and K. E. Gonsalves, *Macromolecules*, 2000, **33**, 2377–2382.
- 18 S. Nagarajan, R. Tyagi, R. Nagarajan, J. Kumar, A. C. Watterson, F. F. Bruno and L. A. Samuelson, *J. Macromol. Sci. Part A—Pure Appl. Chem.*, 2008, **45**(11), 952–957.
- 19 R. Irimescu and K. Kato, *Tetrahedron Letters*, 2004, **45**, 523–525.
- 20 R. Willstaetter and A. Stoll, *Liebigs Ann.*, 1918, **416**, 21.
- 21 R. Willstaetter and H. Weber, *Ann. Chem.*, 1926, **449**, 156.
- 22 Q. Wang, Z. Yang, X. Zhang, X. Xiao, C. K. Chang and B. Xu, *Angew. Chem., Int. Ed.*, 2007, **46**, 4285–4289.
- 23 P. A. Mabrouk, *J. Am. Chem. Soc.*, 1995, **117**, 2141–2146.
- 24 F.-Y. Jeng and S.-C. Lin, *Process Biochemistry*, 2006, **41**, 1566–1573.
- 25 S. Stafstrom, J. L. Breadas, A. J. Epstein, H.-S. Woo, D. B. Tanner, W.-S. Huang and A. G. MacDiarmid, *Phys. Rev. Lett.*, 1987, **59**, 1464–1467.
- 26 www.sigmaaldrich.com/img/assets/18160/Peroxidase.pdf.