Polymeric model systems for flavoenzyme activity: towards synthetic flavoenzymes†

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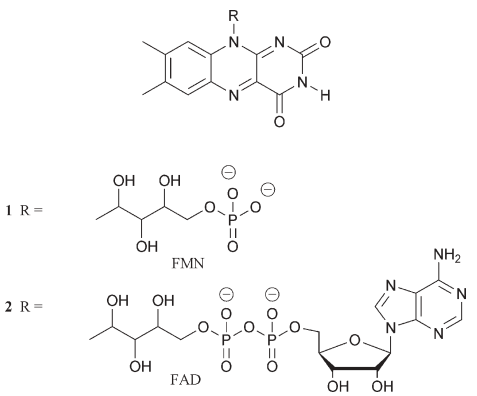
We report the synthesis of a water-soluble flavin polymer using ATRP, whereby the oligoethylene glycol backbone provides both a local hydrophobic environment and redox tuning of the flavin moiety typical of flavoenzyme prototypes.

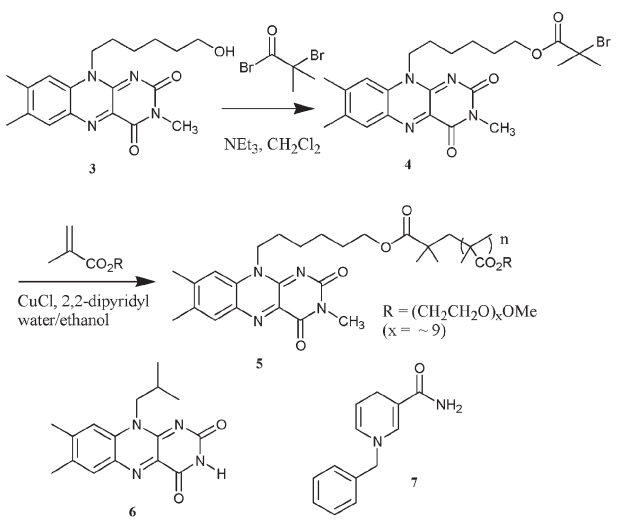
Flavoenzymes are a ubiquitous series of proteins that utilise the $FADH_2$–FAD redox cycle to catalyse a variety of biological processes such as redox transformations, signal transduction and electron-transfer.[1] In most flavoenzymes, the cofactors (*e.g.* FMN **1** or FAD **2**) are non-covalently bound to the active site of the apoenzyme *via* a range of supramolecular interactions.[2] These interactions not only fine-tune the redox properties of the cofactor to match its particular function, but also serve to create a hydrophobic binding pocket to isolate the cofactor from unwanted interactions with water and other interfering species both within and outside the enzyme.



Although a range of small molecule model systems have been prepared to investigate the role non-covalent interactions (hydrogen bonding, π-stacking and donor atom–π interactions) play in modulating the redox properties[3] and reactivity[4] of the flavin, they have generally failed to recreate the biomimetic environment provided by a typical apoenzyme. Polymeric model systems have also been prepared by grafting flavin units onto biomolecules[5] or water soluble synthetic polymers.[6] However, in these systems the nature and homogeneity of the flavin microenvironment are largely controlled by the location and number of reactive groups present in the parent macromolecule. Flavin derivatives have also been incorporated into synthetic peptides, however, this methodology usually requires lengthy synthetic protocols.[7]

The next generation of systems should aim to synergistically incorporate the important attributes of the molecular and polymeric models by constructing water-soluble polymeric systems featuring residues that can induce shielding of the flavin unit from the external environment (*via* hydrophobic interactions) and provide specific interactions to modulate the redox properties of the cofactor. Here, we report our first steps towards the goal of producing fully-synthetic flavoenzymes by using living radical polymerisation methods (in the form of atom transfer radical polymerisation (ATRP)) to synthesise a biomimetic flavin-functionalised synthetic polymer. We have exploited the well-documented ability of ATRP to synthesise polymers with well-defined composition, architecture and functionality.[8] Moreover, we have deliberately incorporated the flavin moiety into the ATRP initiator, as this will allow us to "grow" the living polymer around the flavin-based initiator, thereby allowing us to probe the effect the synthetic polymer backbone has on the properties and reactivity of a *single* flavin unit.

Polymer **5** was synthesised according to Scheme 1. Flavin **3** was reacted with 2-bromoisobutyryl bromide to furnish the ATRP initiator **4** in good yield. Polymer **5** was synthesised using copper-catalysed ATRP methods as detailed in the ESI. The oligoethylene glycol units of **5** conferred good solubility in a range of polar



Scheme 1

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(*e.g.* water, DMSO) and relatively non-polar solvents (*e.g.* CH_2Cl_2), thereby facilitating its characterisation in a range of different environments. Gel permeation chromatography (GPC) performed in THF showed a polydispersity index (PDI) of 1.13 (M_n = 12165; M_w = 13700). Dynamic light scattering experiments (DLS) in water indicated the polymer forms a fairly compact structure, with a diameter of 6 nm. Molecular dynamics simulations in water corroborated the dimensions experimentally determined using DLS, and also indicated that the flavin is located in a partially hydrophobic environment that is near the enzyme surface, a feature frequently observed in electron-transfer proteins (Fig. 1).

It has been previously established that the flavin unit is a very effective system for probing the polarity of its surrounding microenvironment.[9] In particular, UV–vis spectroscopy has shown that the S_0–S_2 transition of the flavin nucleus is solvatochromic and undergoes a bathochromic shift in going from non-polar to polar media (**6** λ = 349 nm (chloroform) and **1** λ = 374 nm (aqueous)).[10] Here we have used this phenomenon to compare the microenvironment of polymer **5** in aqueous and non aqueous solvents with that of flavins **1** and **6**. Firstly, the UV–vis spectra of **5** and **1** were compared in pH = 8.0 buffered solution (Fig. 2) which showed that a 16 nm difference in wavelength was observed for the S_0–S_2 transition (λ = 374 nm for **1** and λ = 358 nm for **5**). As this blue shift was similar to the wavelength observed when **1** is recorded in a less polar solvent (*e.g.* ethanol λ = 358 nm), the data are consistent with the ethylene glycol moieties of the polymer creating a relative biomimetic hydrophobic pocket for the flavin chromophore in aqueous media. Secondly, when the UV–vis spectra of polymer **5** were compared to that of flavin **6** in chloroform solution, the S_0–S_2 transition occurred at very similar wavelengths (for **6** λ = 348 and for **5** λ = 345 nm, see ESI), consistent with the non-polar microenvironment which now surrounds the flavin.

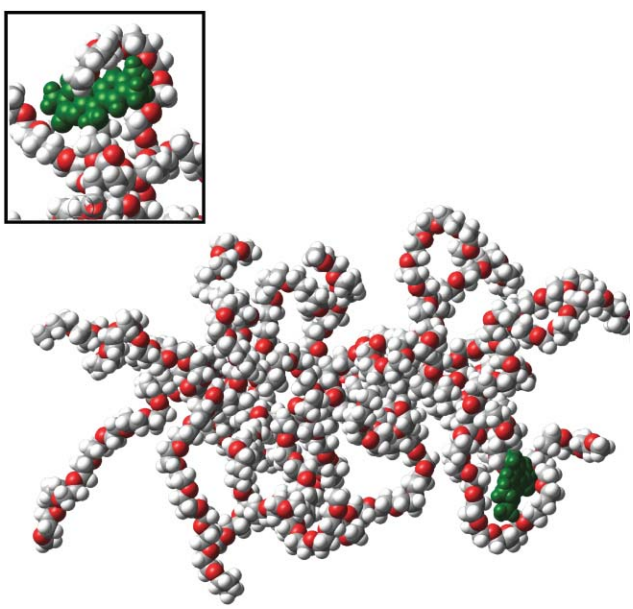

Fig. 1 Molecular dynamics simulation (Amber force field) of polymer **5** showing partial shielding of the flavin moiety (highlighted in green). The water molecules have been omitted for clarity.

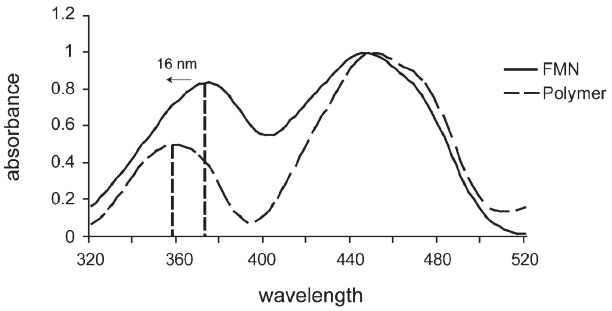

Fig. 2 Overlaid UV–vis spectra of **1** (—) and polymer **5** (- -) (51 μM [flavin] solutions in 5 mM sodium phosphate buffer, pH = 8.0).

We have compared the solution electrochemistry of **1** and polymer **5** in pH = 8.0 phosphate buffer using square wave and cyclic voltammetry (Fig. 3). It is clear from the electrochemical data that the polymeric backbone has a significant influence on the electrochemical properties of the flavin moiety, as the reduction potential of the flavin unit of **5** has been shifted by −61 mV compared to the reduction potential of **1**. This destabilisation of the reduced flavin is likely to result from donor atom–π interactions between the electron rich ethylene glycol side chains of the binding pocket and the flavin derivative,[11] coupled with the decreased polarity of the flavin environment displayed in the solvochromicity studies.

The true measure of a model system is the ability to replicate the behaviour of its prototype, thus we have investigated the oxidation of NADH analogue **7** by polymer **5**. The kinetics of the aerobic oxidation of **7** by **1** and **5** was monitored *via* the decrease in absorbance at 358 nm. The initial velocities (within the linear range 10 min) were recorded after addition of aliquots of flavin derivatives **1** or **5** to fresh solutions of **7** (concentration range 5–50 × 10^{-5} M). Experiments were performed in triplicate. For both flavin derivatives, plots of initial velocity *versus* concentration provide straight lines and do not follow the Michealis–Menten saturation kinetics of typical enzymes (see ESI). Instead, **5** and **1** demonstrate pseudo-first-order kinetics where only the initial portion of the saturation kinetics (which is linear) can be experimentally observed because the saturation of substrate **7** cannot be reached.[12] Double reciprocal plots afforded the catalytic constant (k_{cat}) and Michaelis–Menten constant (k_m) for **5** (k_{cat} = 830 s^{-1}, k_m = 4.48 × 10^{-3} M) and **1** (k_{cat} = 220 s^{-1}, k_m = 9.83 × 10^{-4} M), respectively. Analysis of the data reveals that both **5** and

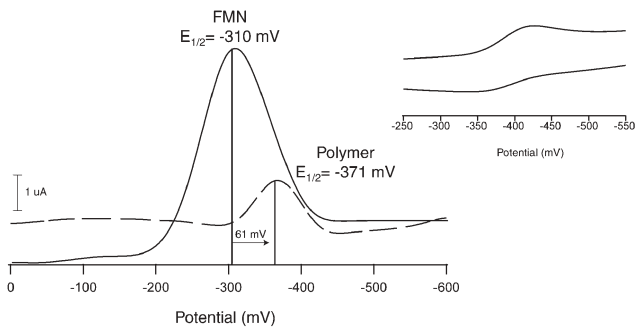

Fig. 3 Square wave voltammograms of **1** (—) and polymer **5** (- -). Inset shows cyclic voltammetry of **5** (7.5 × 10^{-3} M) recorded in sodium phosphate buffer, pH = 8.0.

1 demonstrate relatively similar catalytic behaviour within their Lineweaver–Burk plots. The fitted lines reveal comparable slopes and *y*-intercepts when error values are included in the analysis. An assessment of the Lineweaver–Burk kinetics displays slight differences between **5** and **1**. For example, the k_m^{-1} for the oxidation of **7** by **1** (k_m^{-1} = 1020) is higher than that displayed by polymer **5** (k_m^{-1} = 220). However, the k_{cat} for **5** is 2–4 fold larger than that for **1**, revealing that the hydrophobic pocket on the polymer affects the catalytic rate of the reaction.

In conclusion, we have shown that ATRP can be used to readily prepare water soluble flavin polymers. We have shown that the poly(ethylene glycol) side-chains provide both a biomimetic binding pocket and a means of tuning the redox potential of the flavin moiety in aqueous environments which is reminiscent of the apoenzyme of natural flavoenzymes. Furthermore, the oligomeric backbone does not impede reactivity of the flavin unit with nicotinamide derivatives. This study paves the way for the synthesis of more elaborate derivatives whereby functionality can be introduced to improve the degree of flavin isolation, redox tuning and reactivity. Our progress in these areas will be reported in due course.

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