Hierarchical self-assembly is used to fabricate bio-catalytically active, self-supporting protein-polymer surfactant films capable of sustaining two- or three-enzyme cascade reactions.

Although enzymes are readily employed as green catalysts in various biochemical transformations of industrial importance, their handling, separation and retention remain challenging. In this regard, covalent and non-covalent bioconjugation reactions have been immobilized using surfaces,[3–5] particles,[6–8] fibres[9] and microcapsules.[10] Co-localization of multiple enzymes for the implementation of cascade reactions[11–13] has been used to increase overall reaction rates by reducing diffusion times between active sites by substrate/intermediate channeling.[14] For example, a two-step cascade involving glucose oxidase (GOx) and horseradish peroxidase (HRP) has been employed to demonstrate proof-of-concept catalysis,[15,16] substrate channelling,[17] operation as a glucose sensor[18] and ON-OFF switching behaviour.[19] The latter is of particular interest in the development of stimuli-responsive or programmable materials exhibiting time-dependent logic functionality.[20,21]

Recently, we described a general method for the hierarchical self-assembly of protein-polymer surfactant bioconjugates to produce self-standing single-enzyme hybrid films in which the proteins serve as both architectural building blocks and functional components.[22,23] Here, we develop this approach for the fabrication of cross-linked multi-enzyme-polymer surfactant films capable of sustaining two- or three-enzyme cascade reactions involving controllable mixtures of nanobioconjugates of GOx and HRP, or β-glucosidase (BGL), GOx, and HRP, respectively (Figure 1a). We also employ the GOx/HRP tandem reaction to demonstrate how the immobilized enzyme cascade can be switched ON or OFF via simple manoeuvring of the hybrid films in and out of the substrate solution, respectively, thereby establishing temporal control of the reactions whilst simultaneously facilitating enzyme handling and recovery.

![Figure 1](https://example.com/figure1.jpg)

**Figure 1:** Multi-enzyme polymer-surfactant films: (a) Schematic showing micron-sized hierarchically self-assembled particles of $S_1$-cBGL (red), $S_2$-cGOx (green), and $S_2$-cHRP (blue) working in tandem for driving the enzymatic cascade reaction. (b) Scanning electron micrograph showing the microstructure of the $S_1$-cGOx/$S_2$-cHRP dual enzyme film comprising crosslinked micron-sized particles. (c) Photograph of a dry $S_2$-cGOx/$S_2$-cHRP film adhered to a surface of a petri dish (film diameter approximately 1 cm).
Fabrication of cross-linked multi-enzyme-polymer surfactant films was achieved by developing a previously reported three-step method (see Supplementary Information for details).[21] Cationized enzymes (cGOx, cHRP, and cBGL) were electrostatically coupled to poly(ethylene glycol) 4-nonylphenyl 3-sulfopropyl ether (C19H17O7SNa) to give three different protein–polymer surfactant nanoconjugates (S1-cGOx, S1-cHRP, and S1-cBGL), each of which spontaneously assembled into hierarchically structured nanoclusters typically less than 100 nm in size as observed using DLS (Supplementary Information, Figure S1). Aqueous mixtures of the enzyme-polymer surfactant nanoclusters were crosslinked in the presence of glutaraldehyde vapour to produce self-supporting hybrid films (Figure 1a, b and Supplementary Information, Figure S2), which when hydrated were tough and flexible, and capable of functioning as chemical platforms for two- (S1-cGOx/S1-cHRP; typical molar ratio of 1 : 3.1) or three-enzyme (S1-cBGL/S1-cGOx/S1-cHRP; typically at 0.3 : 1 : 3.1) cascade reactions (Figure 1a and Figure S3). Synchronous radiation circular dichroism (SRCD) spectra indicated that the near native secondary structure was partially retained in the crosslinked GOx/HRP films although some loss in α-helical content, typically from approximately 41 to 19% was observed, which was associated primarily with cationization of the proteins required for preparation of the S1-constructs (for details see Supplementary Information, Figure S4a and Table S1). Diffuse reflectance UV-vis (DR-UV-vis) spectra showed overlapping contributions from the heme (HRP) and FAD (GOx) peaks positioned at ~410-415 and ~450 nm, respectively, indicating that the tertiary structure of enzymes within the hybrid film remained intact (Supplementary Information, Figure S5). Thermal denaturation SRCD spectra recorded on dried S1-cGOx/S1-cHRP films over a temperature range of 25 to 215 °C indicated progressive decreases in the peak minima and maxima at 208/212 and 195 nm, which were fitted using a two-state model to give a half denaturation temperature, T_m (where ΔG=0) of ~170°C for the binary mixture (Supplementary Information, Figure S4b, c, and Table S2). This value was similar to the T_m observed for a single enzyme HRP film (Supplementary Information, Figures S6-S8, and Table S3), and significantly higher than for a mixture of the native and cationized aqueous protein mixtures (73 and 67°C, respectively (Table S2, Supplementary Information, Figures S9-S11)). This was attributed to the measured decrease in entropy associated with denaturation (ca. 774 J K⁻¹ mol⁻¹ at 73°C; ΔS = 267.7 kJ mol⁻¹; Table S2), which implied a significant restriction in the conformational freedom of the enzymes when immobilized at high concentrations (ca. 400 mg cm⁻³) into the molecular crowded environment of the crosslinked films.

We tested the catalytic activity of the S1-cGOx/S1-cHRP films with regard to a two-step cascade reaction involving the GOx-mediated oxidation of D-glucose to gluconic acid and H₂O₂, followed by reduction of H₂O₂ to H₂O by HRP in the presence of an electron donor (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS)) (Supplementary Information, Figure S3). For this, we immersed a known weight (ca. 0.2 mg) of a S1-cGOx/S1-cHRP film prepared at a GOx:HRP molar ratio of 1 : 3.1 into a cuvette containing a stirred solution of glucose and ABTS at 25°C (Figure 2a). The initially colourless substrate solution turned green within 5 mins, after which the colour intensified over a period of 45 minutes due to oxidation of ABTS and formation of the corresponding radical cation. Conversely, control experiments performed in the absence of the two-enzyme film or D-glucose did not show any appreciable colour change (Supplementary Information, Figure S12), indicating that the GOx/HRP cascade remained functional in the crosslinked multi-enzyme-polymer surfactant films. Steady state kinetic analyses in the presence of varying substrate concentrations yielded plots of initial rate vs [glucose] (Figure 2b), which could be fitted to the Michaelis-Menten model with values for the Michaelis constant (K_m) turnover number (k_cat) and specificity constant (k_cat/K_m) of 20.76 ± 4.7 mM, 0.245 s⁻¹, and 11.8 s⁻¹ M⁻¹, respectively. These values were similar to those obtained for a film prepared using only S1-cGOX (Supplementary Information, Figure S13; Table S4), and attributed to the excess HRP used to prepare the two-enzyme films such that the GOx-mediated conversion of glucose was rate limiting in the tandem reaction.[21]

Figure 2: Two-step cascade reaction in crosslinked S1-cGOx/S1-cHRP films. (a) Optical images showing films suspended in an aqueous mixture of glucose and ABTS at time t = 0, 5, 10, and 45 minutes. The solution turns progressively dark green as oxidized ABTS is released from the composite film at the final stage of the cascade reaction. (b) Plot of initial rate (v) against glucose concentration [S] for crosslinked two-enzyme S1-cGOx/S1-cHRP film (black squares) and crosslinked single-enzyme S1-cGOx film (blue circles). Inset show corresponding plot for single-enzyme S1-cHRP film.
absorbance at 414 nm due to formation of the ABTS oxidized product (Figure 3a; ON-1). The quasi-linear response was immediately switch off by withdrawing the film such that no appreciable increase in absorption at 414 nm occurred in the solution over a period of 10 min (Figure 3a; OFF-1). Similar ON/OFF behaviour was observed for at least five cycles (Figure 3a), with each ON period giving an approximately consistent increase in absorbance over 10 min, whereas there was no change during each OFF cycle. The results demonstrated that film integrity and associated activity of the cascade reaction were retained throughout the switching sequence, and that temporal control of the system could be achieved via physical manipulation of the materials.

As a further extension of the spatially immobilized coupled system, we incorporated a third enzyme-polymer surfactant, S₁-cBGL, into the fabrication of the S₁-cGOx/S₁-cHRP films and exposed them to aqueous solutions of the BGL-substrate p-nitrophenyl-β-D-glucopyranoside (pNPG) and ABTS at 25 or 37°C (Figure S3; Supplementary Information, Movie 1). Under these conditions, BGL-mediated breakage of the β-1,4 bond of pNPG gave rise to the in situ formation of p-nitrophenolate (pNP) and glucose, with the latter subsequently initiating the remaining two stages of the S₁-cBGL/S₁-cGOx/S₁-cHRP cascade reaction. As a consequence, the three enzymes in the film operated in tandem to generate gluconic acid, H₂O₂ and oxidized ABTS from the added pNPG substrate. The initial formation of pNP (and glucose) was detected by an increase in absorption band at 410 nm, and was followed by oxidation of ABTS that gave rise to peaks at 414 and 730 nm over a period of 14 h (Figure 3b). The onset of ABTS oxidation was determined by the presence of the absorbance peak at 730 nm, which was only observed after a lag time of ca. 200 or 90 min at 25 and 37°C, respectively. As the cascade reaction progressed, the absorbance peak centred initially at 410 nm became red-shifted towards 414 nm as the amount of oxidized ABTS product increased in the reaction solution. Increasing the temperature from 25 or 37°C gave rise to an increase in the overall rate of reaction within the first 180 min as measured by changes in intensity at 410 and 730 nm by factors of 3.4 and 2.8, respectively (Figure 3b, inset). Interestingly, increases in the 410 nm peak intensity dramatically slowed after 300 min at 37 °C (Figure 3b, inset; Supplementary Information Figure S14 and Table S5), indicative of a reduced turnover by BGL due to consumption of the pNPG substrate. In contrast, increase in the absorption at 730 nm was maintained over the same time period indicating that the HRP-catalysed final step was still operational under these conditions.

**Conclusions:**

In conclusion, our results show for the first time that self-supporting multi-enzyme films can be fabricated via hierarchical assembly of protein-polymer surfactant nanoconjugates and used to conduct two- or three-step cascade reactions. Although cationization and bioconjugation lead to some loss in enzymatic...
activity, the ability to prepare robust films containing high concentrations of functional enzymes could alleviate commonplace problems associated with handling and recovery of catalytic biomaterials. Moreover, the ease in physically manipulating the biofunctionalized films offers advantages related to the facile ON-OFF control of biochemical transformations, providing a step towards developing self-standing and manoeuvrable multifunctional biomaterials. It should also be possible to improve the enzymatic efficiency of the protein-polymer surfactant nanoconjugates by employing alternative bioconjugation techniques, or by using supercharged recombinant proteins to by-pass the cationization step. Finally, given our demonstration of an operational three-step cascade reaction in the S$_1$-cBGL/S$_1$-cGOx/S$_1$-cHRP films, we anticipate the realization of more elaborate multi-step reactions within these soft solid enzyme catalysts, leading to new approaches in biocatalysis and enzyme-assisted synthesis.

Acknowledgements:

We thank the ERC (Advanced Grant) for financial support, and Dr. Giuliano Siligardi and Dr Rohana Hussain from beamline B23 at Diamond Light Source and the EPSRC (Early Career Fellowship EP/K026720/1) for support of AWP. We thank Mr Ben Carter for contributions in drafting Figure 1a.

Notes and References: