Electronic Supplementary Material (ESI) for ChemComm.

Membrane-mediated cascade reactions by enzyme-polymer proteinosomes

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SUPPORTING INFORMATION

METHODS

Synthesis of enzyme-polymer nanoconjugates

Generally, a cationized enzyme (enzyme-NH_2) was synthesized by carbodiimide-activated conjugation of 1,6-diaminohexane to aspartic and glutamic acid residues on the external surface of the protein. For this, a solution of 1,6-diaminohexane (1.5 g) was adjusted to pH 6.5 using 5 M HCl, and added dropwise to a stirred solution of the enzyme (200 mg). The coupling reaction was initiated by adding N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, 100 mg) immediately, and again (50 mg) after 5 h. The pH value was maintained at 6.5 using dilute HCl, and the solution was stirred for a further 6 h. The solution was then centrifuged to remove any precipitate, and the supernatant dialysed (dialysis tubing 12–14 kDa MWCO) extensively against Milli-Q water.

End-capped mercaptothiazoline-activated PNIPAAm (M_n = 8,800 g mol^{-1}, 10 mg in 5ml of water; synthesized according to previously reported methods [Huang, X. *et al*. *Nat. Commun.* **4:2239**, doi: 10.1038/ncomms3239 (2013)] was added to a stirred solution of enzyme-NH_2 (10 mg in 5 mL of PBS buffer pH 8.0). The mixed solution was stirred for 12 h, and then purified by using a centrifugal filter (MWCO 50 kDa) to remove any unreacted PNIPAAm and salts. After freeze-drying, the obtained enzyme-NH_2/PNIPAAm conjugate was obtained. Based on UV–vis spectroscopy measurements, GA-NH_2/PNIPAAm, GO-NH_2/PNIPAAm and HRP-NH_2/PNIPAAm conjugates consisted of an average value of 2.9, 3.1 or 2.0.5 PNIPAAm chains per enzyme molecule, respectively.

Preparation of proteinosomes

The enzyme-polymer proteinosomes were prepared by mixing a 60 µL of aqueous GA-NH_2/PNIPAAm, GO-NH_2/PNIPAAm and HRP-NH_2/PNIPAAm solutions (2.0 mg/mL, 1:1:1 molar ratio, pH 8.5, 50 mM sodium carbonate buffer) with 1.0 mL of 2-ethyl-1-hexanol followed by shaking the mixture by hand for 10 s. The proteinosomes were then cross-linked in the continuous oil phase by addition of PEG-bis(N-succinimidyl succinate) (0.5 mg), which reacted with free primary amine groups on the proteins. Transfer of the cross-linked proteinosomes into water was achieved as follows. After 3 h sedimentation, the upper clear oil layer was discarded and 1mL of 65% ethanol was added and the emulsion gently shaken. The dispersion was then dialysed against 65% ethanol, and then against milli-Q water to complete the phase transfer process.

Hybrid enzyme-polymer proteinosomes comprising encapsulated native GA, GO or HRP were prepared following the above procedures except that the native proteins were added to the aqueous protein-polymer nano-conjugates solution before mixing with the oil phase.

Evaluation of enzyme activity of the proteinosomes

Studies on enzyme activity of the three-enzyme-polymer proteinosomes were carried out by using soluble starch (M_w = 20-200 kDa) that in some experiments was purified using a centrifugal filter (MWCO 50 kDa), along with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) as substrates. Generally, the enzyme reaction rate was monitored by UV-vis spectroscopy at 414 nm using a 500 µL quartz cuvette containing 100 µL of the proteinosome dispersion (0.05 mg/mL), 100 µl of ABTS aqueous solution (0.2 mg/mL), and 200 µL of PBS buffer (100 mM, pH 7.4). The reaction was initiated by addition of 100 µL of starch (2.5 mg/mL) to the cuvette. The enzyme reaction was monitored for 30 min. The initial catalytic reaction rate was calculated based on the following equation: $[V_0]=\Delta A/(\Delta t \times \varepsilon \times b \times [E_0])$, where, $\Delta A/\Delta t$ was the initial change of absorbance with time, ε the extinction coefficient of the green product (oxidized ATBS: 36000 $M^{-1}cm^{-1}$), $[E_0]$ the enzyme concentration of the proteinosome, and *b* the length of the quartz cuvette (1 cm).

Labelling proteins with fluorescence dyes

Generally, cationized enzymes (5.0 mg) were dissolved into 2.0 mL of pH 8.5 sodium carbonate buffer solution (100 mM). Then 50 µL of fluorescein isothiocyanate (FITC) rhodamine B isothiocyanate (RBITC) DMSO solution (1.0 mg/ml), or Thermo Scientific DyLight 405 (DL405) aqueous solution (1.0 mg/mL) was added dropwise. The solution was stirred at room temperature for 5 h, purified by dialyzing against Milli-Q water, and freeze-dried.

Characterization methods

Transmission electron microscopy (TEM) analysis was undertaken on a Jeol 1200 Mk2 TEM using a LaB6 filament at 120 kV in bright field mode. Samples were prepared by adding one drop of proteinosome solution (≈ 0.1 mg/mL) onto a 300 mesh, carbon film coated copper grid and the specimens were then dried in air for one day. Atomic force microscopy (AFM) images were performed on a Bruker Multimode instrument with a Quadrexed Nanoscope 3D controller. Samples were prepared by adding one drop of proteinosome solution (≈ 0.1 mg/mL) onto a clean silica wafer and dried in air for one day. Optical and fluorescence microscopies were performed on a Leica DMI3000 B manual inverted fluorescence microscope at 10x, 20x, 40x and 100x magnification. A fluorescence filter with excitation at 340-380 nm and an emission cut off at 400 nm was used. Confocal images were obtained on a Leicga SP5-II confocal laser scanning microscope attached to a Leica DMI 6000 inverted epifluorescence microscope, equipped with 150 mW Ar laser (458, 476, 488, 514 nm lines), 5 mW solid state green laser (543 nm) and 20 mW red He/Ne (633 nm) diode laser.

SI TABLES

	Concentration GA-NH_2/PNIPAAm (mg/mL)	A (280nm)	PNIPAAm Content (chains per GA-NH_2/PNIPAAm)
Test 1	0.25	0.1549	3.0
Test 2	0.50	0.2954	2.9
Test 3	1.0	0.5633	2.9

Table S1. PNIPAAm : GA stoichiometry in GA-NH_2/PNIPAAm conjugates based on UV-vis spectroscopic analysis of three different sample concentrations.

	Concentration GO-NH_2/PNIPAAm (mg/mL)	A (280nm)	PNIPAAm Content (chains per GO-NH_2/PNIPAAm)
Test 1	0.25	0.2808	3.3
Test 2	0.5	0.5490	3.0
Test 3	1.0	1.034	3.1

Table S2. PNIPAAm : GO stoichiometry in GO-NH_2/PNIPAAm conjugates based on UV-vis spectroscopic analysis of three different sample concentrations.

	Concentration HRP-NH_2/PNIPAAm (mg/mL)	A (280nm)	PNIPAAm Content (chains per HRP-NH_2/PNIPAAm)
Test 1	0.25	0.1212	2.2
Test 2	0.50	0.2412	2.0
Test 3	1.0	0.4726	1.9

Table S3. PNIPAAm : HRP stoichiometry in HRP-NH_2/PNIPAAm conjugates based on UV-vis spectroscopic analysis of three different sample concentrations.

SI FIGURES

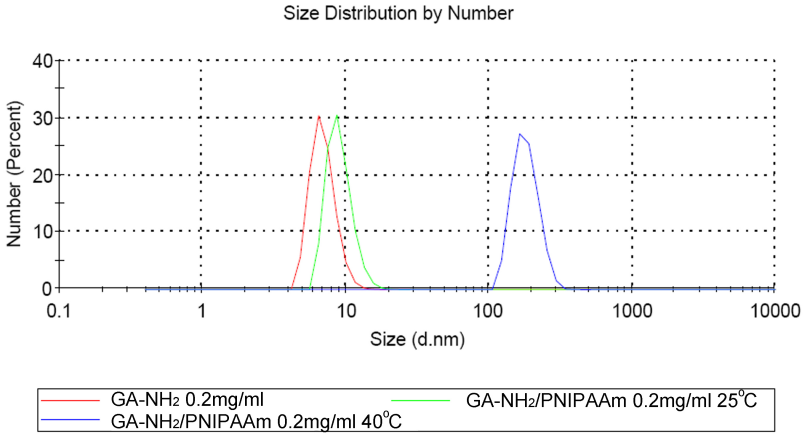

Figure S1. Dynamical light scattering (DLS) profiles for 0.2 mg/mL of GA-NH_2 (red line) and GA-NH_2/PNIPAAm conjugates (green line) recorded at 25°C, and profile for aqueous GA-NH_2/PNIPAAm at 40°C (blue line), in aqueous solution.

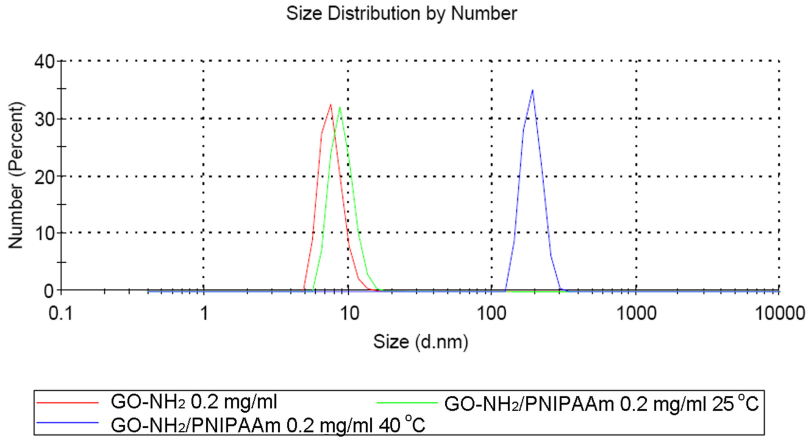

Figure S2. DLS profiles for 0.2 mg/mL of GO-NH_2 (red line) and GO-NH_2/PNIPAAm conjugates (green line) recorded at 25°C, and profile for aqueous GO-NH_2/PNIPAAm at 40°C (blue line), in aqueous solution.

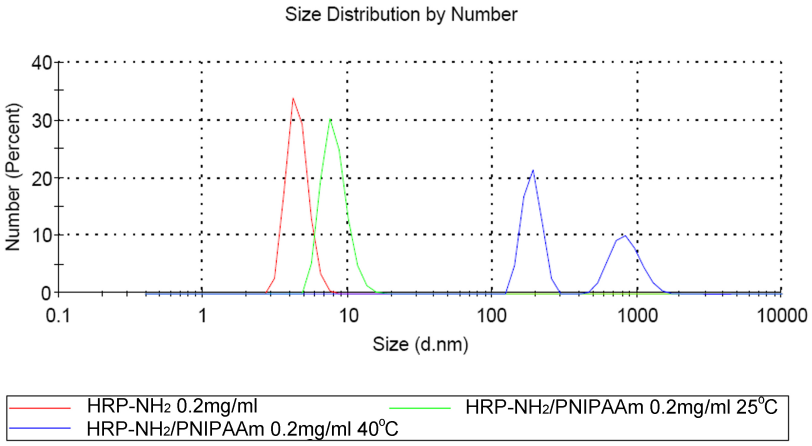

Figure S3. DLS profiles for 0.2 mg/mL of HRP-NH_2 (red line) and HRP-NH_2/PNIPAAm conjugates (green line) recorded at 25°C, and profile for aqueous HRP-NH_2/PNIPAAm at 40°C (blue line), in aqueous solution.