ChemComm

Membrane-mediated cascade reactions by enzyme-polymer proteinosomes

Journal:	*ChemComm*
Manuscript ID:	CC-COM-03-2014-002256.R1
Article Type:	Communication
Date Submitted by the Author:	17-Apr-2014
Complete List of Authors:	Mann, Stephen; University of Bristol, School of Chemistry Huang, Xin; University of Bristol, Chemistry Li, Mei; University of Bristol, Chemistry

ChemComm

RSCPublishing

COMMUNICATION

Membrane-mediated cascade reactions by enzyme-polymer proteinosomes†

Cite this: DOI: 10.1039/x0xx00000x

Xin Huang,[a] Mei Li[a] and Stephen Mann[a]*

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Proteinosomes comprising a triad of enzyme-polymer amphiphilic building blocks capable of operating together as a multi-step membrane-mediated cascade system are prepared and characterized.

Synthetic representations of biological cellularity are becoming increasingly attractive as models for understanding the chemical and physical basis of minimal cell structure and function,[1-4] exploring scenarios related to the emergence of life on the early earth,[5-7] and developing future technologies geared towards autonomously functioning microscale compartments such as self-regulating bioreactors.[8-10] In particular, considerable progress has been made recently in the design and construction of synthetic membrane-delineated compartments based on phospholipid and fatty acid vesicles,[11-13] colloidosomes[14-16] and block copolymer vesicles (polymersomes).[17-20] The latter have been exploited for the confinement of enzyme-mediated transformations, including cascade reactions in which different proteins are encapsulated within the polymersome aqueous interior, bound to the membrane, or added to the external medium.[21-26] In related studies, protein-polymer nano-constructs in the form of "giant amphiphiles" have been exploited for the spontaneous assembly of nanoscale vesicles.[22] We have recently developed this approach for the construction of a new type of compartmentalized micro-architecture (proteinosomes) based on the assembly of amphiphilic protein-polymer nano-conjugates at water droplet/oil interfaces.[27] Assembly of the protein-polymer membrane was not utilized simply as a means of emulsion stabilization,[28,29] but as a functional microscale enclosure for the design and construction of a novel type of synthetic protocell that could be transferred into water and exploited as a biomimetic micro-compartment.[27] Herein we develop this notion by preparing proteinosomes comprising a triad of enzyme-polymer amphiphilic building blocks capable of operating together as a multi-step membrane-mediated cascade system (Fig. 1). We demonstrate how the co-location or spatial separation of the three different enzyme-polymer nano-conjugates, as well as temperature-dependent changes in the conformation of the covalently attached polymer chains located on the external surface of the proteinosome membrane, can be used to regulate membrane-mediated cascade reactions.

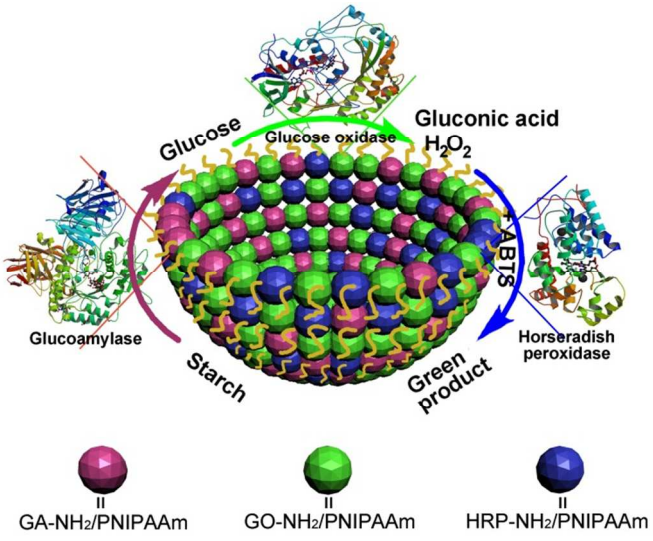

Figure 1. Schematic illustration showing a proteinosome membrane-mediated three-stage cascade reaction in water.

Glucose amylase (GA, M_w = 97 kDa), glucose oxidase (GO, M_w = 160 kDa, dimer) and horseradish peroxidase (HRP, M_w = 40 kDa) were used to prepare enzyme-PNIPAAm amphiphilic nano-conjugates by amide coupling of mercaptothiazoline-activated poly(N-isopropylacrylamide) (PNIPAAm) chains (M_w = 8,800 gmol^{-1}, PDI =1.19, monomer repeat units ≈ 75) with primary amide groups on the surface of the cationized proteins (enzyme-NH_2) (see ESI† Methods). The resulting hybrid nano-conjugates (GA-NH_2/PNIPAAm, GO-NH_2/PNIPAAm and HRP-NH_2/PNIPAAm) consisted on average of 2.9, 3.1 or 2.0 covalently attached PNIPAAm chains per enzyme molecule, respectively, and were

approximately 9 nm in size (Figs S1-S3, Tables S1-S3, ESI†). Addition of an equimolar aqueous mixture of the three enzyme-polymer nano-conjugates (2.0 mg/mL) to 2-ethyl-1-hexanol at an aqueous/oil volume fraction of 0.06 produced a dispersion of 15-30 μm-sized proteinosomes (Fig. 2a), which were stable when partially dried in air (Fig. 2b), and could be transferred into water without loss of structural integrity by cross-linking the membrane with PEG-bis(*N*-succinimidyl succinate) (M_w = 2000) (Fig. 2c). The hollow micro-architecture was clearly visible in transmission electron microscopy images, which showed collapsed but structurally intact proteinosomes (Fig. 2d). Co-location of the three enzymes in the protein-polymer membrane was confirmed by confocal fluorescence microscopy on proteinosomes prepared from enzyme-polymer nano-conjugates with different fluorescence signatures. For this, we prepared rhodamine B isothiocyanate-labeled cationized GA (RBITC-GA-NH_2, red), fluorescein isothiocyanate-labeled cationized GO (FITC-GO-NH_2, green) and DyLight 405-labeled cationized HRP (DL405-HRP-NH_2, blue) prior to attachment of the PNIPAAm chains and co-assembly of the multi-enzyme proteinosomes. The fluorescence images indicated that all three enzymes when mixed under equimolar conditions were co-assembled and integrated into the cross-linked proteinosome membrane even though they were of significantly different molecular masses and sizes (Figs 2e-g).

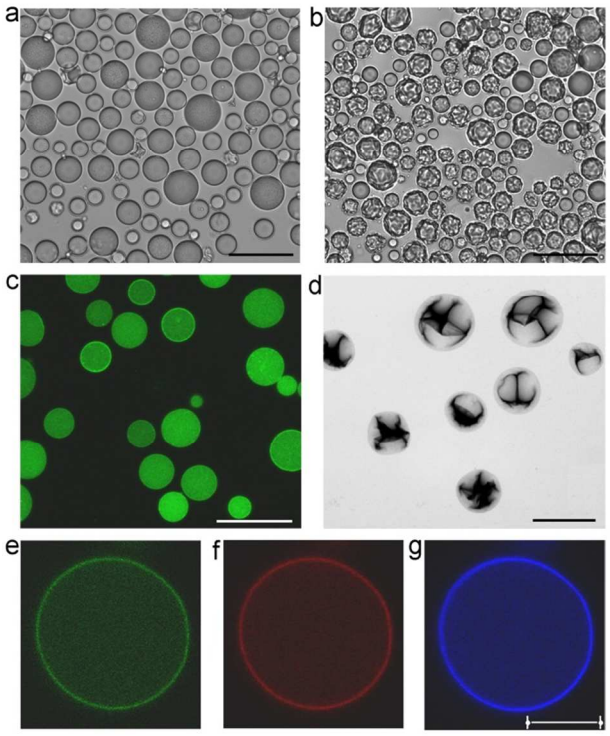

Figure 2. Optical/fluorescence microscopy images of multi-enzyme proteinosomes dispersed in oil (a), after partial drying in air (b), or after transfer into water (c); scale bars, 50 μm. The green fluorescence in (c) is associated with fluorescence dye-labelled PNIPAAm (fluorescein *O*-methacrylate (FOMA)-PNIPAAm, 0.6 wt%). (d) TEM image showing the presence of a continuous and flexible enzyme-polymer membrane in the transferred micro-compartments; 20 μm. (e-g) Confocal fluorescence microscopy images of the same multi-enzyme proteinosome in water, showing red (e), green (f) and blue (g) fluorescence associated with GA, GO and HRP, respectively, in the cross-linked membrane; scale bar, 10 μm.

Given the spatial integration of the three enzymes, we assessed the functionality of the membrane ensemble with regard to a tandem reaction involving the biomolecular-catalysed hydrolysis of starch to glucose (GA), oxidation of glucose to gluconic acid and hydrogen peroxide (GO), and hydrogen peroxide-induced peroxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate ($[ABTS]^{2-}$) by HRP. An aqueous solution of starch (M_w = 20-200 kDa) was added to a buffered ABTS-containing dispersion of cross-linked multi-enzyme proteinosomes maintained at 25°C, and the initial rate of formation of the oxidation product of $[ABTS]^{2-}$ ($[ABTS^{\cdot+}]^-$) determined by UV-vis spectroscopy. The proteinosome dispersion developed a distinct green coloration over a period of approximately 30 minutes, indicating that the cascade reaction was active in the presence of the intact cross-linked enzyme-polymer membrane. However, compared with the initial rate of $[ABTS^{\cdot+}]^-$ formation (V_0) determined for a 1 : 1 : 1 aqueous mixture of the three enzyme-polymer nano-constructs dissolved in water, the membrane-localized tandem reaction was approximately ten-times slower (Fig. 3a; respective V_0 values, 7.8 and 0.68 μM mg^{-1} s^{-1}). Although we could not rule out changes in the secondary structure of the three enzymes, we attributed the decrease in activity of the cascade reaction to the effect of cross-linking of the enzyme molecules and polymer chain entanglement in the membrane, both of which could reduce the accessibility of active sites by constraining diffusion particularly of the macromolecular starch substrate within the protein-polymer matrix.

We also investigated whether regulation of the membrane-mediated cascade reaction could be achieved by temperature-dependent changes in the conformation of the covalently attached polymer chains that adopt an extended hydrophilic or folded hydrophobic state below or above the lower critical solution temperature of PNIPAAm (*ca*. 33°C), respectively. A plot of the initial rates of product formation in the three-step enzyme cascade reaction against temperature showed maximum kinetics at the PNIPAAm transition temperature (V_0 = 0.8 μM mg^{-1} s^{-1}), after which the rate of $[ABTS^{\cdot+}]^-$ formation progressively decreased; in contrast, the tandem reaction rates for a mixture of the three enzyme-polymer nano-conjugates dissolved in aqueous solution increased linearly between temperatures of 25 and 40°C (Fig. 3a). We attributed the stimulus-responsive behavior of the multi-enzyme proteinosome membrane to loss of enzymatic activity associated with deswelling of the polymer chains above 33°C and the subsequent reduced porosity and increased hydrophobicity of the enzyme-PNIPAAm enclosed shell.

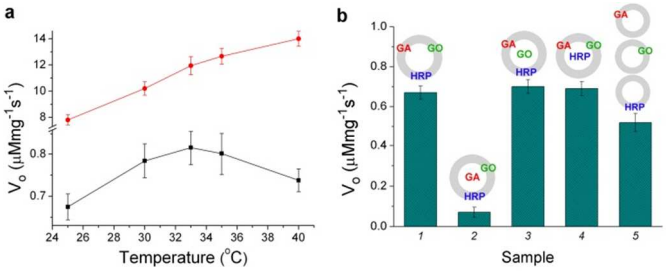

Figure 3. (a) Plots of temperature-dependent initial reaction rates (V_0) for product formation in cascade reactions associated with an aqueous mixture of GA, GO and HRP enzyme-polymer nano-conjugates (red line), or a dispersion of multi-enzyme proteinosomes comprising GA-NH_2/PNIPAAm, GO-NH_2/PNIPAAm and HRP-NH_2/PNIPAAm membrane building blocks (black line). (b) Summary chart of the catalytic activity of enzyme-polymer proteinosomes prepared with different membrane and internal spatial organizations. Values are initial reaction rates of product formation (V_0). Grey circles represent the proteinosome membrane. Error bars in (a) and (b) represent three repeat experiments.

To elucidate the importance of spatial localization on the tandem reaction, we replaced one of the three enzyme-polymer amphiphilic nano-conjugate building blocks with the corresponding hydrophilic native protein, and dispersed the aqueous mixture of all three components in 2-ethyl-1-hexanol, followed by cross-linking and transfer into a continuous water phase. The resulting proteinosomes, which consisted of a membrane and aqueous interior comprising two cross-linked enzyme-PNIPAAm conjugates and a single soluble enzyme, respectively, were then incubated with starch and $[ABTS]^{2-}$, and the initial rates of product formation determined. As summarized in Fig. 3b, the V_0 values were essentially unchanged when native GO or HRP was encapsulated in proteinosomes comprising membranes co-assembled from GA-NH_2/PNIPAAm and HRP-NH_2/PNIPAAm, or GA-NH_2/PNIPAAm and GO-NH_2/PNIPAAm building blocks, respectively. As a consequence, glucose oxidation (step 2) and $[ABTS]^{2-}$ peroxidation (step 3) were not influenced by the location of the respective enzymes, indicating that the small molecule intermediates (glucose, hydrogen peroxide) and $[ABTS]^{2-}$ substrate readily diffused into and out of the proteinosome interior. In contrast, V_0 was reduced by *ca.* 90% when native GA was encapsulated within the aqueous interior of proteinosomes comprising a membrane of cross-linked GO-NH_2/PNIPAAm and HRP-NH_2/PNIPAAm nano-conjugates, and starch macromolecules (M_w = 50-200 kDa) added to the continuous aqueous phase (Fig. 3b). As previous studies showed that macromolecules with molecular weights above *ca.* 40 kDa were retained within or excluded from the proteinosomes dispersed in water,[27] we attributed switching off of the tandem reaction when GA was relocated into the aqueous interior to diffusion-limited uptake of starch macromolecules across the proteinosome membrane.

Experiments were also undertaken at 25°C in which three different populations of proteinosomes, each comprising a single enzyme-polymer cross-linked membrane building block (GA-NH_2/, GO-NH_2/, or HRP-NH_2/PNNAAm), were mixed and the cascade reaction initiated (Fig. 3b). Under these conditions, V_0 was decreased by *ca.* 23% compared with proteinosomes comprising all three of the nano-conjugates. The results indicated that co-location within the membrane increased the catalytic efficiency of the tandem reaction, presumably by reducing diffusional constraints associated with the transfer of intermediates in the cascade process.

In conclusion, we demonstrate the design, construction and utilization of a new type of compartmentalized micro-architecture based on the interfacial co-assembly of an ensemble of functionally integrated enzyme-polymer nano-conjugates. The amphiphilic enzyme-NH_2/PNIPAAm nano-conjugates can be readily prepared by a robust synthetic methodology, and used as combined structural and functional building blocks for the assembly of a semi-permeable catalytically active proteinosome membrane. We show how the assembly and spatial separation of the triad of enzymes within the proteinosome membrane, as well as temperature-dependent changes in the conformation of the covalently attached polymer chains located on the external surface of the proteinosome membrane, can be used to regulate a three-step surface-mediated cascade reaction.

Notes and references

[a] Centre for Protolife Research and Centre for Organized Matter Chemistry, School of Chemistry, University of Bristol, Bristol BS8 1TS, UK. *Email: s.mann@bristol.ac.uk

† Electronic Supplementary Information (ESI) available: *Experimental methods, UV-Vis spectroscopy and DLS are provided*. See DOI: 10.1039/c000000x/

We thank the ERC Advanced Grant scheme (S.M.), and Marie Curie Fellowship (X.H.) scheme for financial support. We thank Jon Jones and Robert Harniman for help with electron and scanning probe microscopy, and Katy Jepson for assistance with confocal microscopy.

1 S. Mann, *Acc. Chem. Res.,* 2012, **45**, 2131.
2 P. Stano and P. L. Luisi, *Curr. Opin. Biotechnol.,* 2013, **24**, 633.
3 V. Noireaux, Y. T. Maeda and A. Libchaber, *Proc. Natl. Acad. Sci. USA,* 2011, **108**, 3473.
4 D. A. Hammer and N. P. Kamat, *FEBS Lett.,* 2012, **586**, 2882.
5 S. Rasmussen, L. Chen, D. Deamer, D. C. Krakauer, N. H. Packard, P. F. Stadler and M. A. Bedau, *Science,* 2004, **303**, 963.
6 A. Y. Mulkidjanian, A. Y. Bychkov, D. V. Dibrova, M. Y. Galperin and E. V. Koonin, *Proc. Natl. Acad. Sci. USA,* 2012, **109**, E821.
7 P. A. Monnard and D. W. Deamer, *Anat. Record.,* 2002, **268**, 196.
8 S. Mann, *Angew. Chem. Int. Ed.,* 2013, **52**, 155.
9 B. Städler, A. D. Price, R. Chandrawati, L. Hosta-Rigau, A. N. Zelikin and F. Caruso, *Nanoscale,* 2009, **1**, 68.
10 P. Walde and S. Ichikawa, *Biomol. Eng.,* 2001, **18**, 143.
11 A. J. Dzieciol and S. Mann, *Chem. Soc. Rev.,* 2012, **41**, 79.
12 P. L. Luisi, F. Ferri and P. Stano, *Naturwissenschaften,* 2006, **93**, 1.
13 S. S. Mansy, J. P. Schrum, M. Krishnamurthy, S. Tobé, D. A. Treco, J. W. Szostak, *Nature,* 2008, **454**, 122.
14 M. Li, D. C. Green, J. R. Anderson, B. P. Binks and S. Mann, *Chem. Sci.,* 2011, **2**, 1739.
15 M. Li, R. L. Harbron, J. V. Weaver, B. P. Binks and S. Mann, *Nat. Chem.,* 2013, *5*, 529.
16 R. K. Kumar, M. Li, S. N. Olof, A. J. Patil and S. Mann, *Small,* 2012, **9**, 357.
17 D. E. Discher and A. Eisenberg, *Science,* 2002, **297**, 967.
18 N. P. Kamat, J. S. Katz and D. A. Hammer, *J. Phys. Chem. Lett.,* 2011, **2**, 1612.
19 P. Tanner, P. Baumann, R. Enea, O. Onaca, C. Palivan and W. Meier, *Acc. Chem. Res.,* 2011, **44**, 1039.
20 R. J. Peters, I. Louzao and J. C. van Hest, *Chem. Sci.,* 2012, **3**, 335.
21 S. F. van Dongen, M. Nallani, J. J. Cornelissen, R. J. Nolte and J. van Hest, *Chem.-Eur. J.,* 2009, **15**, 1107.
22 D. M. Vriezema, P. M. Garcia, N. Sancho Oltra, N. S. Hatzakis, S. M. Kuiper, R. J. Nolte, A. E. Rowan and J. van Hest, *Angew. Chem. Int. Ed.,* 2007, **46**, 7378.
23 J. Gaitzsch, D. Appelhans, L. Wang, G. Battaglia and B. Voit, *Angew. Chem. Int. Ed.,* 2012, **51**, 4448.
24 Z. Fu, M. A. Ochsner, H.-P. M. de Hoog, N. Tomczak and M. Nallani, *Chem. Commun.,* 2011, **47**, 2862.
25 M. Marguet, C. Bonduelle and S. Lecommandoux, *Chem. Soc. Rev.,* 2013, **42**, 512.
26 R. J. Peters, M. Marguet, S. Marais, M. W. Fraaije, J. van Hest and S. Lecommandoux, *Angew. Chem. Int. Ed.,* 2014, **53**, 146.
27 X. Huang, M. Li, D. C. Green, D. S. Williams, A. J. Patil and S. Mann, *Nat. Commun.* 2013, **4***:2239*, doi: 10.1038/ncomms3239.
28 N. C. Mougin, P. Rijn, H. Park, A. H. E. Müller and A. Böker, *Adv. Funct. Mater.,* 2011, **21,** 2470.
29 P. Rijn, H. Park, H. K. Ö. Nazli, N. C. Mougin and A. Böker, *Langmuir,* 2013**, 29,** 276.