AIChE Letter: Biomolecular Engineering, Bioengineering, Biochemicals, Biofuels, and Food

Biomimetic Multienzyme Complexes Based on Nanoscale Platforms

Feng Jia, Balaji Narasimhan, and Surya K. Mallapragada
Dept. of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011

DOI 10.1002/aic.13992
Published online December 27, 2012 in Wiley Online Library (wileyonlinelibrary.com)

Significance

Inspired by the widely present multienzyme complexes in nature that enable highly cooperative catalytic mechanisms, we designed a biomimetic dual-functionalized nanoparticle-based platform for colocalizing multiple enzymes. The use of nanoscale materials together with a novel sequential colocalization approach with two model enzymes [glucose oxidase (GOX) and horseradish peroxidase] resulted in a 100% increase in the overall conversion rate compared to the equivalent amount of free enzymes in solution and a physical mixture of individual immobilized enzymes on nanoparticles. GOX is an important enzyme used in glucose biosensors for diagnostics. Colocalizing GOX with peroxidase allows for colorimetric visualization of the peroxide formed that enables monitoring glucose levels in solution. This platform can be readily applicable to other multienzyme systems as well.

Keywords: functionalized nanoparticles, multienzyme colocalization, biomimetic

Introduction

Multienzyme complexes enable highly cooperative catalytic mechanisms in nature, where the reactive intermediates can be transported rapidly from one active site to the next to avoid diffusion losses.[1] By spatially colocalizing multiple enzymes on nanocarriers, the intermediates, especially the reactive and short-lived ones, can rapidly find the next active site to accelerate the reaction efficiency and to direct the overall reaction toward the desired products, particularly in comparison with free enzymes in solution.[2] Therefore, the development of nanoscale platforms for spatial control of multiple active enzymes has been an area of active interest.[1,3,4] Much of the previous work in this area has focused on adsorbing multiple enzymes on surfaces, which provides poor control of enzyme concentration and placement.[5–7] Compared to adsorption, multipoint covalent binding is more durable, stable under harsh microenvironments (i.e., extreme pH and temperature), and potentially provides higher loading efficiencies and better enzyme activity,[8–12] Although the design and fabrication of biomolecule-nanoparticle conjugates have attracted much attention for applications in biosensors, biocatalysis, and biomedicine,[13–20] these approaches have not been investigated for colocalizing multiple enzymes for biocatalytic activity.

In this study, we present a new approach based on biomimetic dual-functionalized, nanoparticle-based platforms for sequential colocalization of multiple enzymes using biospecific (biotin-streptavidin) and/or chemical conjugation strategies (Figure 1). By using different attachment strategies for each enzyme, it is possible to control the relative amounts of the enzymes on the nanoparticles, in contrast to adsorption-based methods. As proof of concept, the glucose oxidase (GOX)-horseradish peroxidase (HRP) sequential reaction cascade was chosen as a model system.[21,22]

Materials and Methods

Partial biotinylation of polystyrene nanoparticles

Polystyrene (PS) nanoparticles with carboxyl groups on the surface (C-PS; Invitrogen, Carlsbad, CA) were covalently modified using biotin hydrazide (Sigma-Aldridge, St. Louis, MO.) Briefly, 1 mL of 0.025 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.9) solution containing 20 mg/mL PS nanoparticles, 250 nmol/mL biotin hydrazide, and 10 mg/mL 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) was incubated overnight at room-temperature. The amine groups on the reagents reacted with the carboxylic groups activated by EDC to form stable amide bonds via multistep reactions.[23] The unattached reagents were subsequently separated from the functionalized particles

Correspondence concerning this article should be addressed to S. K. Mallapragada at suryakm@iastate.edu.

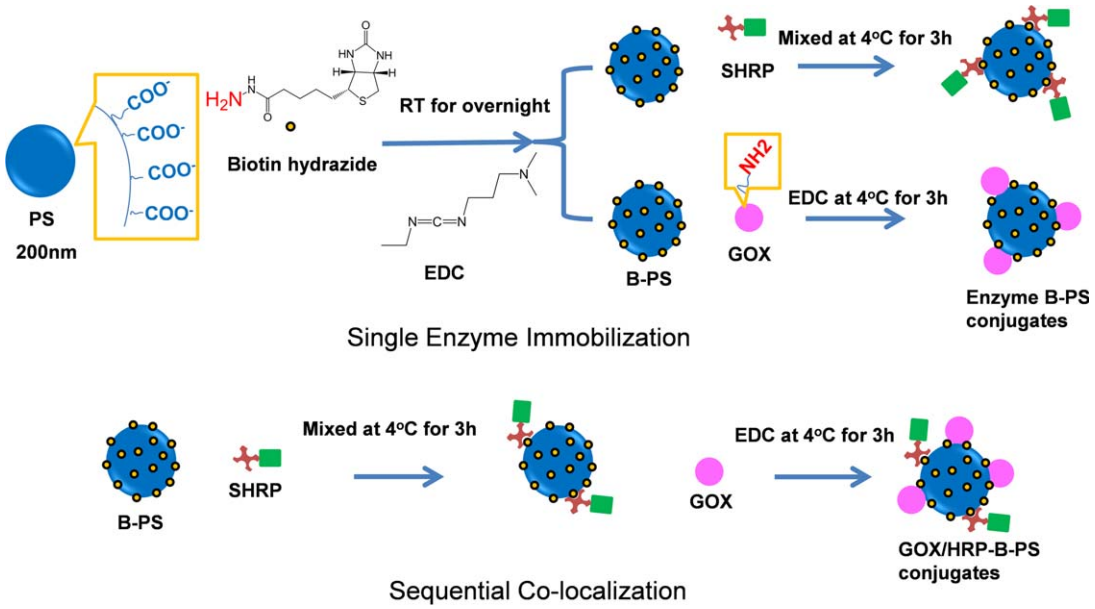

Figure 1. Schematic of single enzyme immobilization and sequential colocalization strategy.

[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

using multiple microcentrifugation cycles at 20,000*g* for 30 min. The biotin in the collected supernatant was measured using a biotin quantification assay kit. The amount of attached biotin on the particles was estimated by a mass balance, and available for attachment of streptavidin-tagged HRP (SHRP). The unmodified carboxyl groups were used to attach GOX by chemical conjugation. The zeta potential of the nanoparticles in phosphate buffered saline (PBS) buffer was measured using phase analysis light scattering (Zetasizer Nano, Malvern Instruments Ltd., Worcester, UK). The biotin-modified C-PS nanoparticles were stored at 4°C for use.

Colocalization of multiple enzymes

SHRP and GOX were colocalized onto the B/C-PS nanoparticles sequentially. First, 15 μg of the SHRP was reacted with 2.5-mg B/C-PS nanoparticles for 3 h at 4°C, followed by microcentrifugation to separate the unattached enzyme. Next, 10 μg of GOX was added to the SHRP-B/C-PS nanoparticles in the presence of 0.3 mg EDC for 3 h at 4°C. The final product was obtained by microcentrifugation. As controls, the single enzymes, GOX and SHRP, were attached to the nanoparticles using similar linkages. Unattached enzymes were separated using microcentrifugation and quantified.

GOX activity assay

In the GOX and HRP sequential reaction cascade, the H_2O_2 formed in the first reaction catalyzed by GOX is consumed by HRP in the second reaction in the presence of Amplex Red to yield a specific fluorescent product, resorufin.[22] In GOX activity assay, various concentrations (1–30 mM) of glucose substrate solution with 50-μM Amplex Red were prepared by mixing the two reagents in PBS buffer. Then, 10 μL of 10 μg/mL SHRP and 10 μL of 2 μg/mL GOX or immobilized GOX on C-PS was added in sequence into each well of a 96-well plate that was preloaded with 180 μL of substrate solution. The reaction was immediately monitored using a fluorescence plate reader (SynergyMx, Biotek, Winooski, VT) with excitation and emission wavelengths of 560 and 590 nm, respectively. The original fluorescence signal was converted into the resorufin product concentration using a calibration curve. All the enzyme activity measurements were performed in triplicate.

SHRP activity assay

A series of hydrogen peroxide concentrations (1–80 μM) were prepared by adding respective amounts of H_2O_2 into the substrate solution containing Amplex Red. Next, 20 μL of 100 ng/mL SHRP or SHRP-B/C-PS was added into 180 μL of substrate solution so that the final reaction solution contained 1–80 μM H_2O_2 and 50-μM Amplex Red. The reaction was monitored using a fluorescence plate reader as described previously.

Colocalized GOX and SHRP

To 195 μL of glucose and Amplex red containing substrate solution, 5 μL of the colocalized sample was added so that the final solution contained 1-mM glucose, 200-μM Amplex Red, and appropriate amounts of GOX and SHRP. According to the measured quantity of each enzyme in the colocalized sample, equivalent amounts of each single enzyme bioconjugate were added to the same substrate solution as a control. Similarly, an equivalent free enzyme mixture was added into the same substrate solution for comparison. The reaction was monitored by a fluorescence plate reader as described previously.

Results and Discussion

Biotinylation of C-PS nanoparticles

Biotin hydrazide is a small molecule (254 Da, space arm length ≈16 Å) with a significantly smaller dimension

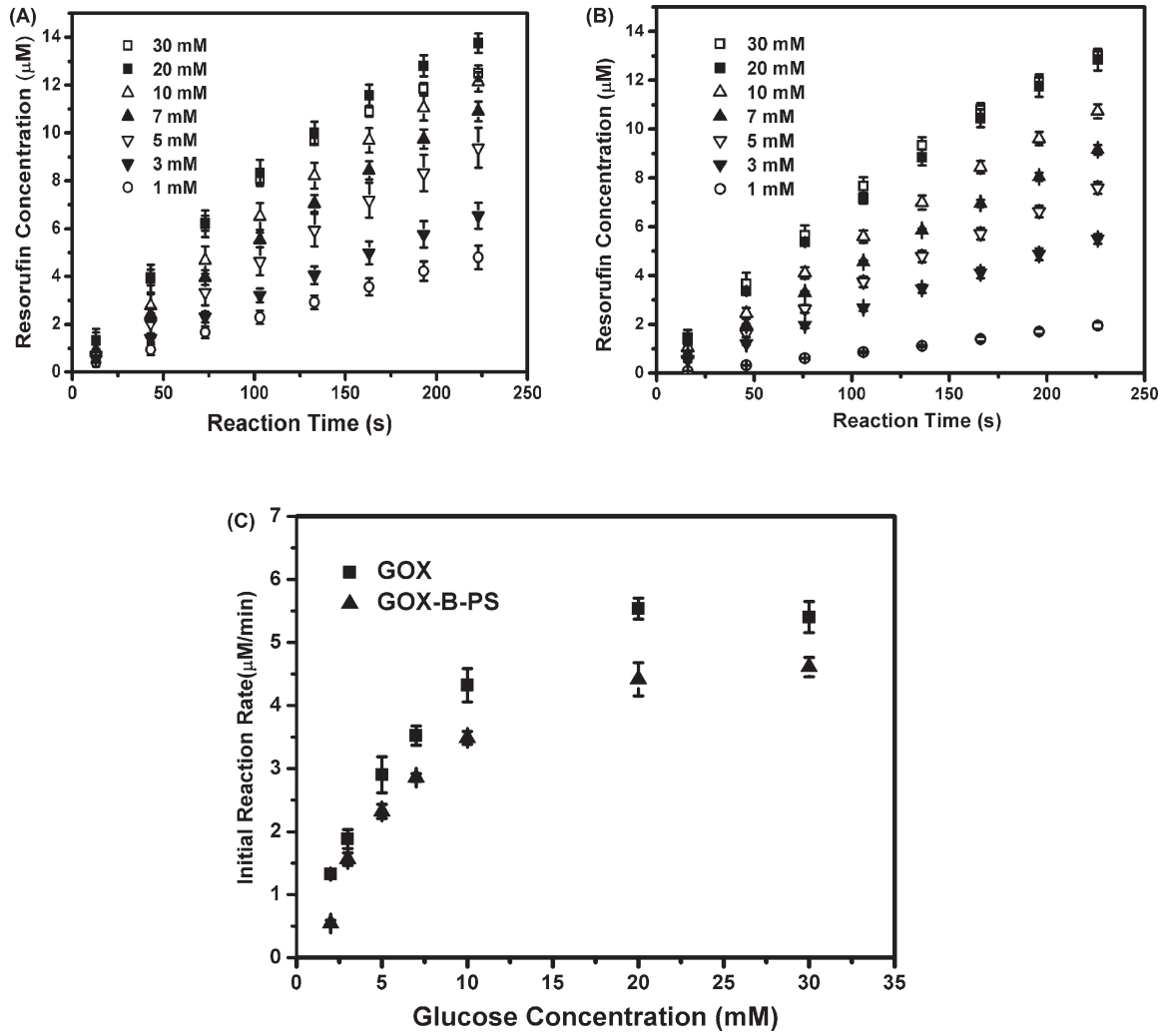

Figure 2. Comparison of the reaction kinetics catalyzed by equivalent free and immobilized GOX.

(A) Product formation vs. time for free GOX. (B) Product formation vs. time for GOX-B/C-PS (C) The initial reaction rate as a function of glucose concentration.

compared to the area occupied by the carboxylic acid moieties (~28.8 nmol/mg, where each group occupies ca.154 $Å^2$). Only up to 40% of the carboxylic acid groups were modified due to steric hindrance, thus, limiting the availability of biotin for SHRP attachment. To provide appropriate amount of carboxylic and biotin groups for attaching GOX and SHRP, respectively, to the B/C-PS surface, the biotinylation on the C-PS beads was maintained at about 40% in the single enzyme immobilization and sequential colocalization studies. The ζ-potential of the C-PS nanoparticles in the PBS buffer was -62.3 ± 2.3 mV, consistent with proton dissociation from the carboxylic acid. After partial biotinylation, that value increased to -32.9 ± 4.8 mV, which is reasonable, considering that more than half of the –COOH groups are unmodified.

Single enzyme immobilization and kinetics

As controls, GOX was attached to the unmodified C-PS nanoparticles and in a separate set of experiments, SHRP was attached to biotinylated C-PS nanoparticles. Typical loading quantities for the GOX and SHRP were found to be 11.0 ± 0.3 μg/mg and 1.6 ± 0.1 μg/mg, respectively, which is consistent with previous work.[5,24] Previous work has shown that covalently immobilized GOX on magnetite nanoparticles showed similar enzymatic activity compared to free GOX in solution due to favorable conformational change of the enzyme.[25] To optimize the attachment process for maximizing the enzyme activity, the performance of the immobilized single enzymes was compared with that of the equivalent amount of free enzymes using a kinetic activity assay. The GOX enzymatic activity assay was carried out by coupling the reaction catalyzed by SHRP. By adjusting the reaction time, reaction temperature, and centrifugation time, optimal conditions for GOX activity were found to be: 3 h at 4°C with 20,000*g* microcentrifugation for 6 min.

To evaluate the enzymatic performance of GOX before and after immobilization, the kinetics of each reaction was studied. The increase of the product concentration with time is shown in Figures 2A, B. A second-order polynomial was used to fit the reaction curve, and the derivatives of the

Table 1. Kinetic Parameters for Free and Immobilized Enzymes

	GOX	GOX-B-PS	SHRP	SHRP-PS
k_{cat} (s^{-1})	194.3 ± 9.6	160.1 ± 11.0	3.6 ± 0.09 × 10^4	4.0 ± 0.1 × 10^4
K_m (μM)	7.9 ± 1.2 × 10^3	7.9 ± 0.9 × 10^3	23.2 ± 1.2	20.9 ± 0.8

polynomial were used to calculate the initial reaction rate. The initial reaction rate as a function of glucose concentration is shown in Figure 2C.

Using a Michaelis–Menten model, the kinetic parameters K_m and k_{cat} were calculated using the data in Figure 2C with a linear least-squares regression method and are shown in Table 1. The K_m of the immobilized and free GOX were both 7.9 mM, which indicated that the affinity between the enzyme and the substrate did not change after the immobilization. The k_{cat} of the immobilized GOX decreased slightly (194.3 s^{-1}) in comparison to that of the free GOX (160.1 s^{-1}), indicating that the enzyme turnover number was not as efficient. However, the activity of the immobilized GOX was comparable to that of the free enzyme in solution, indicating nearly no deleterious effects of covalent immobilization on GOX affinity to the substrates.

The kinetics of the reaction catalyzed by SHRP was studied similarly. Figures 3A, B show the variation of the product concentration with time. Figure 3C shows the initial reaction rate as a function of the substrate concentration. As shown in Table 1, the K_m value of the immobilized SHRP (20.9 μM) was slightly lower than that of the free enzyme (23.2 μM) and the k_{cat} of the immobilized SHRP (3.6 × 10^4 s^{-1}) was slightly higher than that of free SHRP (4.0 × 10^4 s^{-1}). Similar to the GOX results, the immobilized SHRP retained its activity.

Colocalization of GOX and SHRP on B/C-PS nanoparticles

In this work, we designed and developed a novel colocalization strategy by use of a multifunctional nanoparticle-based platform. The SHRP was attached first because it was

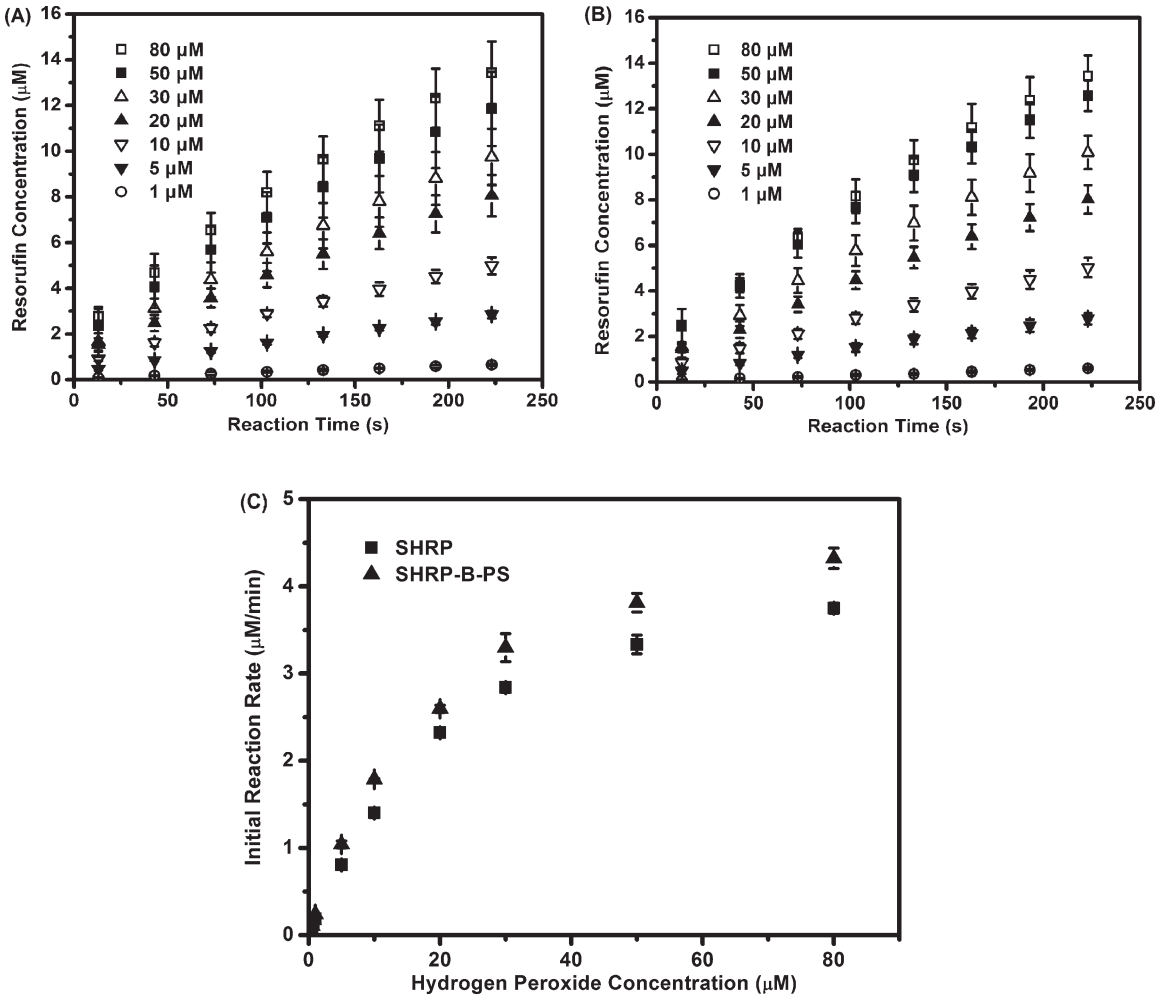

Figure 3. Comparison of the reaction kinetics catalyzed by equivalent free and immobilized SHRP.

(A) Product formation vs. time for free SHRP. (B) Product formation vs. time for SHRP-B/C-PS (C) The initial reaction rate as a function of glucose concentration.

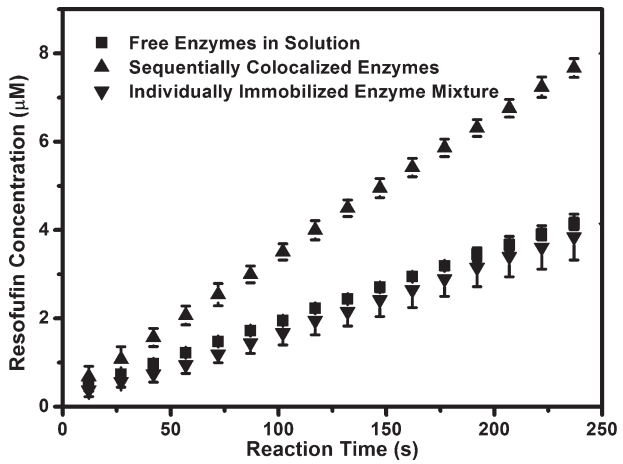

Figure 4. Comparison of the catalytic performance of sequentially colocalized enzymes with that of equivalent mixtures of single immobilized enzymes and free enzymes in solution.

Each assay contained 0.02 nmol/mL GOX and 0.06 nmol/mL SHRP on the nanoparticles or in solution.

found that if the GOX was attached first, it lost significant activity mainly due to the high-spin microcentrifugation in the second reaction step. As mentioned previously, in a typical reaction, about 40% of carboxylic acid groups were modified by biotin. In the GOX adsorption control experiment where all the experimental conditions were the same except no EDC was added in the GOX attachment step, the loading efficiency of GOX was very low (data not shown). Almost none of the GOX was adsorbed on the remaining areas, likely due to the negatively charged COO^- motifs preventing nonspecific adsorption of the negatively charged GOX enzyme in MES buffer.

The catalytic performance of the colocalized GOX and SHRP system was compared with that of equivalent amounts of the free enzymes in solution and a mixture of nanoparticles with immobilized GOX and SHRP alone. The results are shown in Figure 4, which indicate that colocalizing the two enzymes on the same nanoparticles enhanced the overall product conversion two-fold compared to the controls. The performance of the immobilized GOX and SHRP mixture was comparable to that of the free enzyme combination, which is consistent with our previous studies using single enzyme immobilization, indicating that each enzyme retained its activity after immobilization.

These studies support our hypothesis that colocalizing multiple enzymes on multifunctional nanoparticles mimics multienzyme complexes. In many enzymatic systems, it is critical to molecularly localize these enzymes so that the intermediates can rapidly find the next active site for the reaction to proceed. This rigid synthetic C-PS platform provides nanoscale spatial control of multiple active enzymes. By appropriately modifying the surface of the C-PS nanoparticles, multiple enzymes were colocalized on these nanocarriers and the relative amounts of the attached enzymes were controlled. In situations where the intermediate product is particularly reactive, such a strategy may pay even more dividends. In comparison to the adsorption coupling strategy used in previous studies,[5–7] the sequential covalent binding/streptavidin-biotin approach outlined herein is more stable and suitable for long-term use.[26–28] The step-by-step sequential colocalization strategy was also facile in terms of controlling the amount of each enzyme and quantifying each localized enzyme on the nanoscale platform. Additionally, unlike other studies,[5,6] labeling enzymes with dyes for quantification is not needed in this approach. This strategy is broadly applicable to other sequentially coupled multienzyme reactions by appropriately tailoring the chemistry of the platform and the conjugation strategy.

Conclusions

In summary, a multifunctional nanoparticle-based platform was developed to colocalize multiple enzymes by using different attachment strategies for the enzymes to control the relative amounts of the enzymes on the nanoparticles. It was demonstrated that sequential colocalization of GOX and SHRP enhanced the overall product conversion rate by approximately two-fold compared to the equivalent amount of free enzymes in solution and a mixture of individual immobilized enzymes on nanoparticles. These studies demonstrate the clear benefits of sequentially colocalizing multiple enzymes on multifunctional nanoparticles, leading to a controllable platform for multienzyme colocalization to mimic the efficient multienzyme complex structure and function observed in Nature.

Acknowledgements

The authors are grateful to the National Science Foundation (CBET 0932517) for financial support. The authors also acknowledge support from NSF ARI-R^2 (CMMI-0963224) for funding the renovation of the research laboratories used for these studies. B.N. acknowledges the Vlasta Klima Balloun Professorship in Chemical and Biological Engineering and SKM acknowledges the Stanley Chair in Interdisciplinary Engineering.

Literature Cited

1. Schoffelen S, vanHest JCM. Multienzyme systems: bringing enzymes together in vitro. *Soft Matter*. 2012;8(6):1736–1746.
2. Garcia-Galan C, Berenguer-Murcia A, Fernandez-Lafuente R, Rodrigues RC. Potential of different enzyme immobilization strategies to improve enzyme performance. *Adv Synth Catal*. 2011;353(16):2885–2904.
3. Tran DN, Balkus KJ. Perspective of recent progress in immobilization of enzymes. *ACS Catal*. 2011;1(8):956–968.
4. Lopez-Gallego F, Schmidt-Dannert C. Multi-enzymatic synthesis. *Curr Opin Chem Biol*. 2010;14(2):174–183.
5. Keighron JD, Keating CD. Enzyme: nanoparticle bioconjugates with two sequential enzymes: stoichiometry and activity of malate dehydrogenase and citrate synthase on Au nanoparticles. *Langmuir*. 2010;26(24):18992–19000.
6. Pescador P, Katakis I, Toca-Herrera JL, Donath E. Efficiency of a bienzyme sequential reaction system immobilized on polyelectrolyte multilayer-coated colloids. *Langmuir*. 2008;24(24):14108–14114.

7. Watanabe J, Ishihara K. Sequential enzymatic reactions and stability of biomolecules immobilized onto phospholipid polymer nanoparticles. *Biomacromolecules*. 2005;7(1):171–175.
8. Gray JJ. The interaction of proteins with solid surfaces. *Curr Opin Struct Biol*. 2004;14(1):110–115.
9. Johnson AK, Zawadzka AM, Deobald LA, Crawford RL, Paszczynski AJ. Novel method for immobilization of enzymes to magnetic nanoparticles. *J Nanopart Res*. 2008;10(6):1009–1025.
10. Williams R, Blanch H. Covalent immobilization of protein monolayers for biosensor applications. *Biosens Bioelectron*. 1994;9(2):159–167.
11. Pandey P, Singh SP, Arya SK, Gupta V, Datta M, Singh S, Malhotra BD. Application of thiolated gold nanoparticles for the enhancement of glucose oxidase activity. *Langmuir*. 2007;23(6):3333–3337.
12. Matharu Z, Sumana G, Arya SK, Singh SP, Gupta V, Malhotra BD. Polyaniline Langmuir−Blodgett film based cholesterol biosensor. *Langmuir*. 2007;23(26): 13188–13192.
13. Berron BJ, Johnson LM, Ba X, McCall JD, Alvey NJ, Anseth KS, Bowman CN. Glucose oxidase-mediated polymerization as a platform for dual-mode signal amplification and biodetection. *Biotechnol Bioeng*. 2011;108(7):1521–1528.
14. Carrillo-Conde B, Garza A, Anderegg J, Narasimhan B. Protein adsorption on biodegradable polyanhydride microparticles. *J Biomed Mater Res Part A*. 2010;95A(1):40–48.
15. Christie RJ, Miyata K, Matsumoto Y, Nomoto T, Menasco D, Lai TC, Pennisi M, Osada K, Fukushima S, Nishiyama N, Yamasaki Y, Kataoka K. Effect of polymer structure on micelles formed between sirna and cationic block copolymer comprising thiols and amidines. *Biomacromolecules*. 2011;12(9):3174–3185.
16. Determan AS, Wilson JH, Kipper MJ, Wannemuehler MJ, Narasimhan B. Protein stability in the presence of polymer degradation products: Consequences for controlled release formulations. *Biomaterials*. 2006;27(17): 3312–3320.
17. Hrapovic S, Liu Y, Male KB, Luong JHT. Electrochemical biosensing platforms using platinum nanoparticles and carbon nanotubes. *Anal Chem*. 2003;76(4):1083–1088.
18. Lim SH, Wei J, Lin JY, Li QT, KuaYou J. A glucose biosensor based on electrodeposition of palladium nanoparticles and glucose oxidase onto nafion-solubilized carbon nanotube electrode. *Biosens Bioelectron*. 2005; 20(11):2341–2346.
19. Frimpong RA, Hilt JZ. Magnetic nanoparticles in biomedicine: synthesis, functionalization and applications. *Nanomedicine*. 2010;5(9):1401–1414.
20. Sundaram P, Wower J, Byrne ME. A nanoscale drug delivery carrier using nucleic acid aptamers for extended release of therapeutic. *Nanomed Nanotechnol Biol Med*. 2012:8(7):1143–1151.
21. Rupcich N, Brennan JD. Coupled enzyme reaction microarrays based on pin-printing of sol-gel derived biomaterials. *Anal Chim Acta*. 2003;500(1):3–12.
22. Logan TC, Clark DS, Stachowiak TB, Svec F, Fréchet JMJ. Photopatterning enzymes on polymer monoliths in microfluidic devices for steady-state kinetic analysis and spatially separated multi-enzyme reactions. *Anal Chem*. 2007;79(17):6592–6598.
23. Williams A, Ibrahim IT. A new mechanism involving cyclic tautomers for the reaction with nucleophiles of the water-soluble peptide coupling reagent 1-ethyl-3-(3'-(dimethylamino)propyl)carbodiimide (EDC). *J Am Chem Soc*. 1981;103(24):7090–7095.
24. Liu W, Zhang S, Wang P. Nanoparticle-supported multienzyme biocatalysis with in situ cofactor regeneration. *J Biotechnol*. 2009;139:102–107.
25. Rossi LM, Quach AD, Rosenzweig Z. Glucose oxidase-magnetite nanoparticle bioconjugate for glucose sensing. *Anal Bioanal Chem*. 2004;380:606–613.
26. Cui Y, Wei Q, Park H, Lieber CM. Nanowire nanosensors for highly sensitive and selective detection of biological and chemical species. *Science*. 2001;293(5533): 1289–1292.
27. Su S, Nutiu R, Filipe CDM, Li Y, Pelton R. Adsorption and covalent coupling of ATP-binding DNA aptamers onto cellulose. *Langmuir*. 2007;23(3):1300–1302.
28. Drechsler U, Fischer NO, Frankamp BL, Rotello VM. Highly efficient biocatalysts via covalent immobilization of Candida rugosa lipase on ethylene glycol-modified gold-silica nanocomposites. *Adv Mater* 2004;16(3):271–274.

Manuscript received July 13, 2012, and revision revised Oct. 27, 2012.