Use of Silica Nanosprings in an Enzyme-Based Continuous Flow Reactor

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1 ABSTRACT

An enzyme-based continuous flow reactor (CFR) was constructed with silica Nanosprings^{TM} and β-galactosidase from Aspergillus oryzae. Preliminary results with the model substrate o-nitrophenyl-β-galactoside show the reactor to be functional, converting 71% of the substrate to product at a flow rate of $0.1ml/min$.

Keywords: Silica Nanosprings, continuous flow reactor, enzymes, immobilized

2 INTRODUCTION

The use of enzymes as industrial catalysts is a growing technology that holds great promise for the production of specialized compounds. Many pharmaceutical compounds, and other high value molecules, have complex chemical structures that make them difficult to produce by conventional chemical synthesis. An example is the chemotherapeutic compound TAXOL®, shown in figure 1, which is a low abundance natural product from the Pacific yew [1]. Because of the numerous functional groups and chiral centers in this molecule, conventional chemical synthesis is not a cost effective means of production. Another example is the antimalarial compound artemisinin, represented in figure 2, a sesquiterpene lactone from annual wormwood [2]. Like TAXOL®, the chemical complexity of artemisinin makes its complete chemical synthesis from organic reagents impractical.

These examples, and other chemically complex compounds, could potentially be produced economically by using enzymes as reagents. TAXOL® and artemisinin are both produced in their respective host organisms from common metabolites through a sequence of enzyme catalyzed reactions. If the appropriate enzymes were available, it would be possible to synthesize complex compounds like these at the laboratory bench from abundant, low cost precursors in the absence of a host organism. The sequences of enzymatic reactions involved in the natural biosynthesis of many high value natural products have been established [3], and could be used for commercial production.

Despite the promise of this technology, it is limited by the high cost of identifying and isolating the necessary enzymes. This problem is compounded by the labile nature of enzymes under industrial conditions. To be cost effective, and enzyme used as an industrial catalyst must produce more product, last longer, and function under higher product concentrations than it would under its natural conditions [4]. It has long been recognized that immobilizing enzymes on a fixed support in a continuous flow reactor (CFR) could circumvent some of these problems. Immobilization allows continuous recycling of the catalyst, mitigates product inhibition, and often increases the working life of the enzyme [5]. To be effective, however, an enzyme-based CFR must employ a high concentration of enzyme in a small area without causing a prohibitive restriction in flow.

Silica Nanosprings appear to be an excellent support material for construction of an enzyme-based CFR [6]. Nanosprings are grown as randomly oriented mats as shown in figure 3. The growth temperature can be as low as $325°C$, which allows them to be formed on a variety of substrates, including glass, aluminum, and certain plastics. Nanospring mats have a surface area of $350m^2/g$, 100% of which is accessible to reactants, and their open nature does not restrict the flow of material.

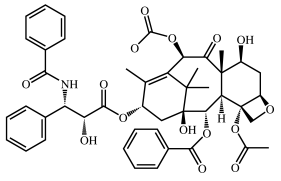

Figure 1: TAXOL®

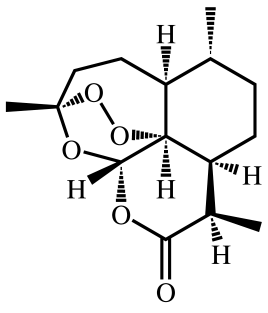

Figure 2: Artemisinin

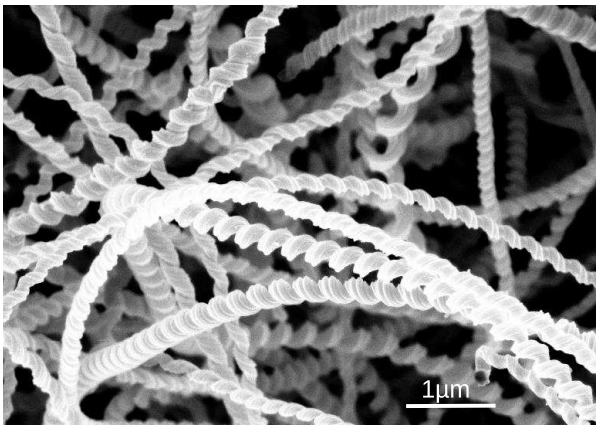

Figure 3: Secondary electron image of a silica Nanospring mat

The surface chemistry of Nanosprings is suitable for the reversible, thiol-based attachment of virtually any soluble enzyme, making them of general utility for enzyme-based CFRs.

We are currently working to construct CFRs with enzymes immobilized on Nanosprings. As a means of method development, we are using β-galactosidase from Aspergillus oryzae and the model substrate o-nitrophenol-β-D-galactosylpyranoside (o-NPG). In the normal reaction catalyzed by this enzyme, lactose is hydrolyzed into glucose and galactosel as shown in figure 4. In the model reaction, o-NPG is hydrolyzed into galactose and o-nitrophenol, shown in figure 5, which can be spectrophotometrically monitored. A model CFR using Nanosprings, and this enzyme/substrate system has recently been described [8]. Once the reactor parameters have been refined with the model system, we plan to expand the focus to include other enzymes. Additionally, we are exploring the possibility of linking several different enzyme-based CFRs together in series such that the product of one reactor becomes input to the next as represented in figure 6. Such an arrangement could allow the efficient linkage of a multi-step synthetic pathway. With this concept fully developed, a low cost precursor could be applied at one end of the system and a valuable end product retrieved at the opposite end. We report here preliminary work on enzyme-based CFR construction.

3 FABRICATION

Nanosprings can be grown on a variety of substrates, including polymers such as polyimide. The only requirement is that the substrate can withstand the process temperature. In this present study, the Nanosprings were grown on 650μm thick (100) silicon wafers. A scanning electron microscope (SEM) image of an silica Nanospring mat is shown in Figure 3. McIlroy *et al.* [7] and Wang *et al.* [6] have described

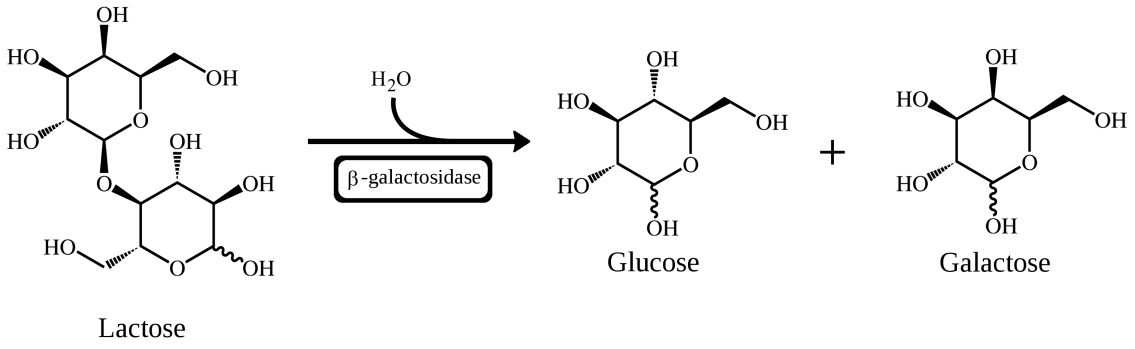

Figure 4: β-galactosidase reaction

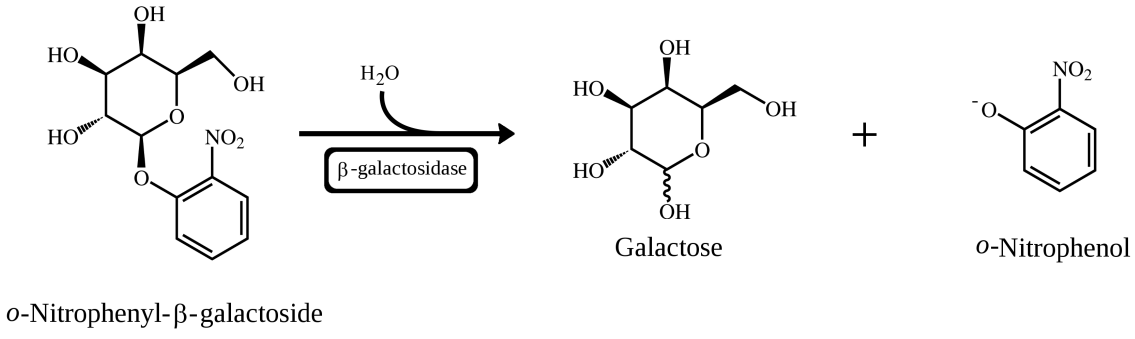

Figure 5: β-galactosidase model reaction

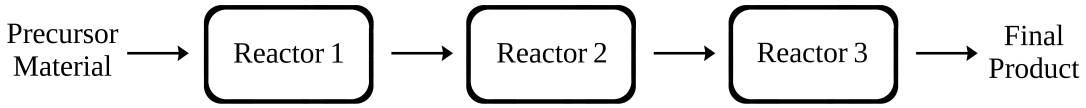

Figure 6: Connection of several enzyme-based CFRs in series to construct a synthetic pathway

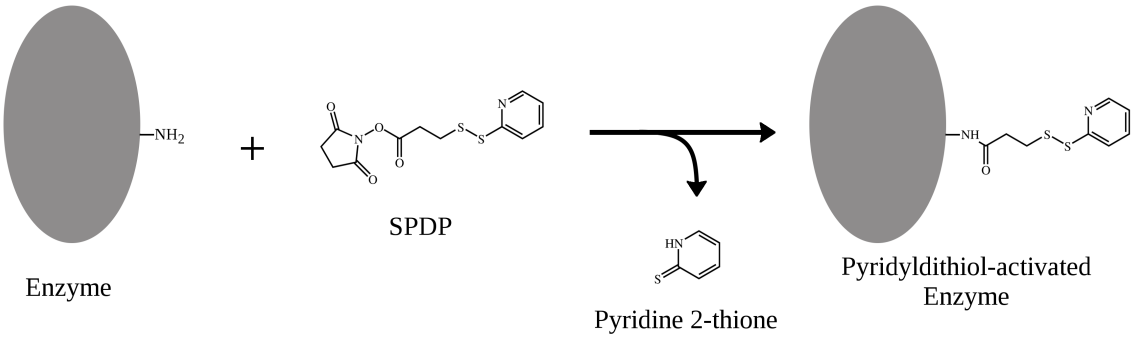

Figure 7: Modification of enzyme for Nanospring attachment

the Nanospring process in some detail. The Nanospring synthesis was performed at atmospheric pressure for 15 minutes, which correspond to an $80\mu m$ thick Nanospring mat. The general principles of this furnace were discussed in detail by McIlroy *et al.* [7]. A thin catalyst layer was sputtered on the silicon wafer prior the Nanospring synthesis.

The reactor was fabricated of Stainless Steel 316, shown in figure 8 with an internal chamber of the following dimensions: $7.5 \times 2.5cm$. The internal gap is controlled by the gasket thickness. These particular experiments were perform with a $750\mu m$ thick gasket, which generate a cross section of $2.5 \times 0.1mm$ full of Nanospring. These dimensons were choosen to enhance the amount of enzymes in contact with the fluid as well as to reduce the reactor back pressure.

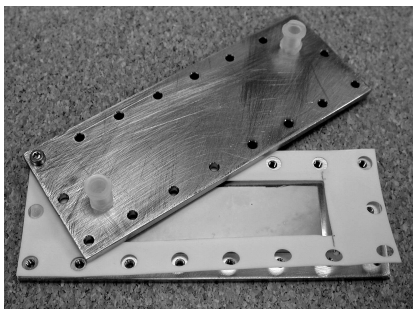

Figure 8: Prototype of a continuous flow reactor

4 EXPERIMENTAL

For attachment of β-galactosidase to the Nanospring support, we followed the procedure described in Schilke et al. [8], with some modifications. Briefly, the free amines of lysine side chains on the enzyme surface were functionalized by reacting with N-succinimidyl 3-(2-pyridyldithiol) propionate (SPDP), represented in figure 7. This was accomplished by first making a $20mg/mL$ solution of β-galactosidase in PBS with $10mM$ EDTA added. To the enzyme solution was added $50mL/mL$ of a $20mM$ solution of SPDP in DMSO. The mixture was incubated for 30 minutes at room temperature and then passed through a $6K$ MWCO polyacrylamide desalting column to remove unreacted components. The column fractions were assayed for maximum activity, and the active fractions were pooled and used to for Nanospring attachment.

Nanosprings were functionalized with thiol groups by reacting them with 3-mercaptopropyltrimethoxysilane (MPTMS), shown in figure 10. A Nanospring covered silicon wafer ($25 \times 75mm$) was incubated in a 5% MPTMS solution in acetone. After one hour, the wafer was thoroughly washed with deionized water. To covalently link the enzyme to the Nanosprings, the SPDP-modified enzyme solution was then placed on top of the thiol-functionalized wafer and incubated for one hour at room temperature, represented in figure 9. The enzyme-coated wafer was then washed with reaction buffer ($20mM$ sodium phosphate, $10mM$ sodium citrate, pH 4.5) and placed into the reactor chamber. A syringe pump was used to pump several

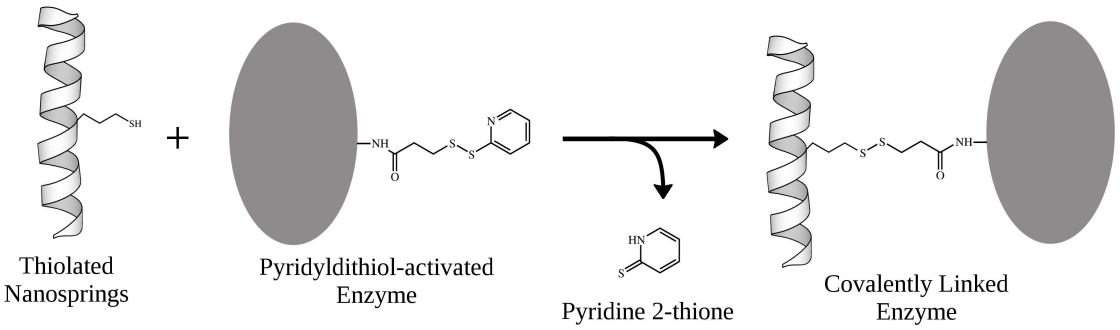

Figure 9: Covalent attachment of modified enzyme to thiolated Nanosprings

reactor volumes of reaction buffer through the reactor at $0.1mL/min$.

5 RESULTS

Activity of the reactor was measured by pumping a $5mM$ solution of substrate (o-NPG in reaction buffer) through the reactor at 0.1 or $0.2mL/min$. After several reactor volumes of substrate had been pumped through, $0.2mL$ fractions were collected into vials containing 0.6 mL of quench buffer ($100mM$ sodium borate, pH 9.8). The high pH of the quench buffer stops the reaction and converts the reaction product, o-nitrophenol, to its colored form [9]. The quantity of product was determined by measuring the absorbance at $415nm$. Product concentrations were calculated using the empirically determined extinction coefficient for o-nitrophenol of $5046M^{-1}cm^{-1}$.

6 CONCLUSIONS

Although preliminary, the procedure used here to construct an enzyme-based CFR using Nanosprings as a support material was successful. At a flow rate of $0.1mL/min$, 71% of the substrate was converted to product. There was a small decrease in efficiency (65%) when the flow rate was doubled to $0.2mL/min$. Additional work is in progress to improve reactor efficiency. Notably, the reactor configuration can be optimized to eliminate space not filled with Nanosprings. Experiments are also in progress to increase the amount of enzyme attached to Nanospring mat.

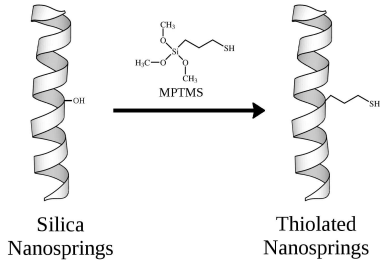

Figure 10: Thiolation of Nanosprings

REFERENCES

[1] M. C. Wan, H. L. Taylor, M. E. Wall, P. Coggon, A. T. McPhail.,J. Am. Chem. Soc., 93, 2325-2327,1971.

[2] P. Newton, N. White, Annu. Rev. Med., 50, 179-192, 1999.

[3] P. Bernhardt, S. E. OConnor, Curr. Opin. Chem. Biol., 13, 3542, 2009.

[4] D. J. Pollard, J. M. Woodley (2006) Trends Biotechnol. 25: 66-73.

[5] C. Mateo, J. M. Palomo, G. Fernández-Lorente, J. M. Guisan, R. Fernaández-Lafuente, Enzyme Microb. Technol. 40, 14511463, 2007.

[6] L. Wang, D. Major, P. Paga, D. Zhang, M.G. Norton, and D.N. McIlroy, Nanotech., 17, S298-S303, 2006.

[7] D.N. McIlroy, A. Alkhateeb, D. Zhang, D.E. Aston, A.C. Marcy, and M.G. Norton, J. Phys. Condens. Matter, 16, R415-R440, 2004.

[8] K. F. Schilke, K. L. Wilson, T. Cantrell, G. Corti, D. N. McIlroy, C. Kelly, Biotechnol. Prog., 26, 1597-1605, 2010.

[9] T. Haider, Q. Husain, Biochem Eng. J., 43, 307-314, 2009.