

# Rapid Production of Nitrilase Containing Silica Nanoparticles Offers an Effective and Reusable Biocatalyst for Synthetic Nitrile Hydrolysis

Joshua D. Swartz,<sup>†</sup> Scott A. Miller,<sup>†,‡</sup> and David Wright<sup>\*,†</sup>

Vanderbilt University, Department of Chemistry, Station B 351822, Nashville, Tennessee 37235-1822, U.S.A., and University of South Alabama, Department of Chemistry, 307 University Boulevard North, Mobile, Alabama 36688, U.S.A.

## Abstract:

Rapid and efficient immobilization of nitrilase within silica nanoparticles overcomes many hurdles associated with biocatalysis. A water-miscible dendrimer catalyzes the condensation of silicic acid to silica dioxide, entrapping electrostatically bound nitrilase molecules. Michaelis–Menten kinetics shows encapsulated nitrilase functions similarly to free nitrilase in solution. Additionally, HPLC analysis demonstrates that simple benchtop separation and recycling of the biocatalyst over 10 individual reactions are achieved without significant loss of enzyme and/or function. These findings broaden the use of nitrilases in the production of fine chemicals as well as general syntheses by overcoming some of the traditional barriers associated with enzyme reagents and nitrile conversion.

## Introduction

Nitrile compounds are simple aliphatic and aromatic metabolites, cyanoglucosides, and cyanolipids serving as key compounds and intermediates in a myriad of biochemical pathways.<sup>1</sup> The common biochemical transformation of organonitrile hydrolysis to higher value amide and carboxylic acid groups is often inaccessible to organic synthetic strategies due to the harsh conditions required for hydrolysis.<sup>2–5</sup> Synthetically, organonitriles are easily produced via the addition of cyanide to alkyl halides, the Strecker reaction,<sup>6–9</sup> reaction of aryl halides with copper cyanide,<sup>10</sup> and the dehydration of amides.<sup>11</sup> However, the strong acids and bases, often in conjunction with reflux conditions required to achieve nitrile hydrolysis, prove impractical during the synthesis of sensitive and complex molecules.

Microbial organonitrile transformations are achieved biochemically in ambient, aqueous environments by either a

combination of nitrile hydratase and nitrile amidase enzymes or by members of the nitrilase superfamily.<sup>12</sup> There are at least nine identified families within the nitrilase superfamily. One well-studied gene cluster from the nitrilase superfamily is the NIT1–3 cluster from *Arabidopsis thaliana*.<sup>13</sup> These enzymes utilize an active-site cysteine residue to catalyze the direct conversion of aliphatic and aromatic nitriles into carboxylic acids coupled with ammonia liberation and accept a wide array of substrates. While nitrilase-containing cell farms have found a foothold in the commercial production of some high-value fine chemicals,<sup>14</sup> they are not commonly employed in synthetic strategies due to traditional barriers of enzymatic catalysis. In general, enzymes are more expensive than many reagents, are not easily recycled after use, and may have poor shelf lives if they are not capable of activity following lyophilization or freezing. These limitations have limited the large-scale industrial use of nitrilase to maintaining various microbes expressing high levels of nitrilase, such as with acrylamide production (~6000 tons per year).<sup>15,16</sup> Herein we report the encapsulation of a cysteine active, recombinant nitrilase inside silica nanoparticles. The reaction is performed in an aqueous buffer and mediated by a water-soluble PAMAM dendrimer to yield a nitrilase-containing silica nanocomposite capable of enzymatic conversion of nitriles, easy separation from product, and reusability.

## Results

In order to improve upon the implementation and recycling of nitrilase for large-scale production and small-scale research and development, we encased a commercially available nitrilase within silica nanospheres using a biomimetic template. Previous studies of oceanic diatoms have isolated a class of proteins, called silaffins, that catalyze the condensation of silicic acid into intricate exoskeletons.<sup>17</sup> This process is amplified by polycationic lysine modifications containing many secondary and primary amines along the Sil-1A peptide.<sup>18</sup> Subsequent reports indicated that similar condensation chemistry could be achieved using a variety of amine sources.<sup>19–23</sup> Previously, we

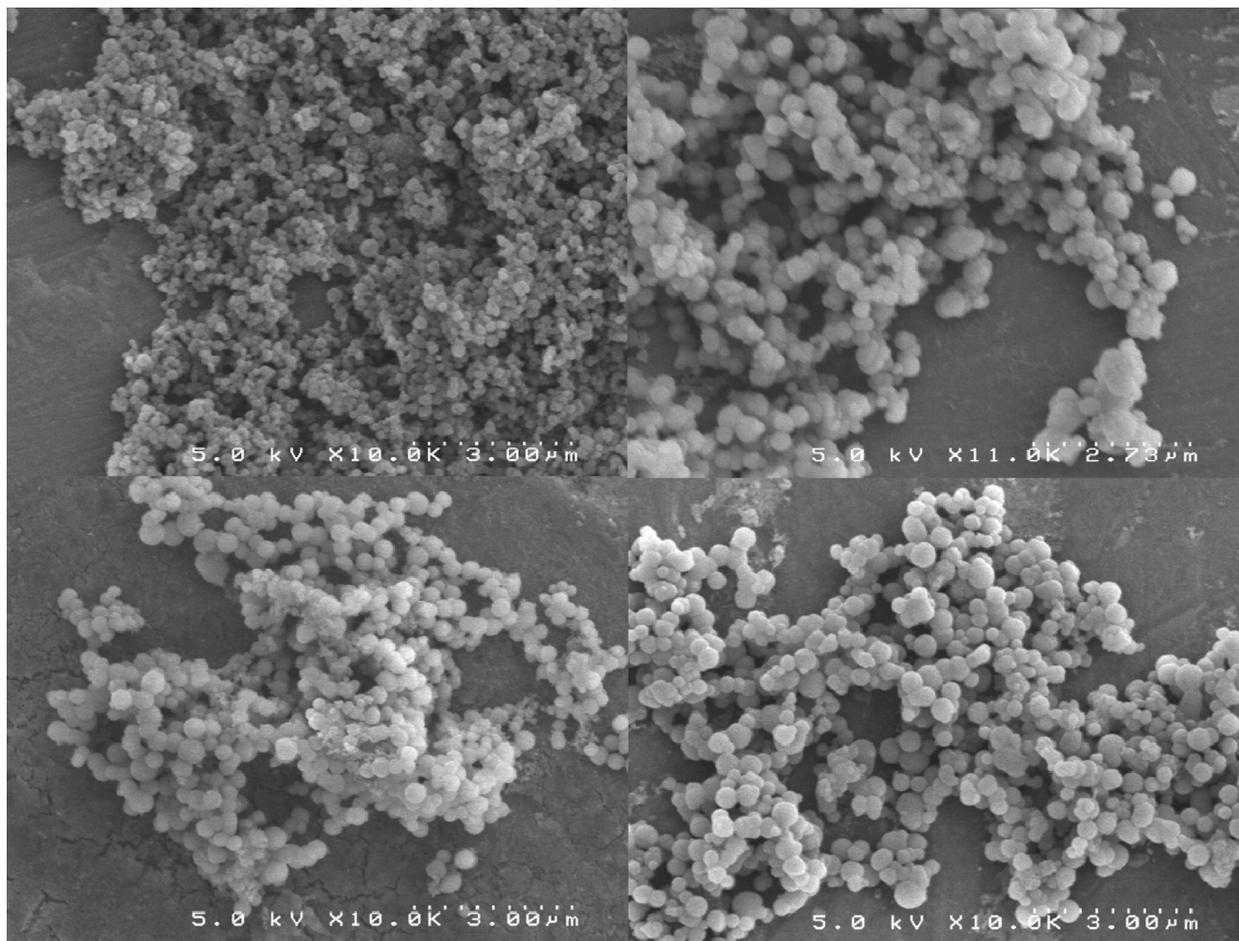
\* Author for correspondence. E-mail: David.Wright@Vanderbilt.edu. Fax: 615-343-1234. Telephone: 615-322-2636.

<sup>†</sup> Vanderbilt University.

<sup>‡</sup> University of South Alabama.

- (1) Legras, J. I.; Cguzel, G.; Arnaud, A.; Galzy, P. *World J. Microbiol. Biotechnol.* **1990**, *6*, 83–108.
- (2) Bennett, M. A.; Yoshida, T. *J. Am. Chem. Soc.* **1973**, *95*, 3030–3031.
- (3) Chin, J.; Kim, J. H. *Angew. Chem.* **1990**, *102*, 580–582.
- (4) DiBiase, S. A.; Wolak, R. P., Jr.; Dishong, D. M.; Gokel, G. W. *J. Org. Chem.* **1980**, *45*, 3630–3634.
- (5) Paraskewas, S. *Synthesis* **1974**, 574–575.
- (6) Surendra, K.; Krishnaveni, N. S.; Mahesh, A.; Rao, K. R. *J. Org. Chem.* **2006**, *71*, 2532–2534.
- (7) Yadav, J. S.; Reddy, B. V. S.; Eeshwaraiah, B.; Srinivas, M. *Tetrahedron* **2004**, *60*, 1767–1771.
- (8) Strecker, A. *Liebigs. Ann. Chem.* **1850**, *75*, 27.
- (9) Surendra, K.; Krishnaveni, N. S.; Mahesh, A.; Rao, K. R. *J. Org. Chem.* **2006**, *71*, 2532.
- (10) Clarke, H. T.; Read, R. R. *J. Am. Chem. Soc.* **1924**, *46*, 1001.
- (11) Harrison, C. R.; Hodge, P.; Rogers, W. J. *Synthesis* **1977**, 41.

- (12) Brenner, C. *Curr. Opin. Struct. Biol.* **2002**, *12*, 775–782.
- (13) Vorwek, S.; Biernacki, S.; Hillebrand, H.; Janzik, I.; Muller, A.; Weiler, E. W.; Piotrowski, M. *Planta* **2001**, *212*, 508–516.
- (14) Breuer, M.; Ditrich, K.; Habicher, T.; Hauer, B.; KeBeler, M.; Sturmer, R.; Zelinski, T. *Angew. Chem., Int. Ed.* **2004**, *43*, 788.
- (15) Nagasawa, T.; Yamada, H. *Trends Biotechnol.* **1989**, *7*, 153–158.
- (16) Kobayashi, M.; Nagasawa, T.; Yamada, H. *TIBTECH* **1992**, *10*, 402–408.
- (17) Kröger, N.; Lehmann, G.; Rachel, R.; Sumper, M. *Eur. J. Biochem.* **1997**, *250*, 99–105.
- (18) Kröger, N.; Deutzmann, R.; Sumper, M. *Science* **1999**, *286*, 1129–1132.
- (19) Knecht, M. R.; Wright, D. W. *Langmuir* **2004**, *20*, 4728–4732.



**Figure 1.** SEM images of nitrilase-containing silica nanoparticles formed in water (top left), 8 mM (top right), 17 mM (bottom left) and 25 mM phosphate buffer (bottom right).

reported a rapid and efficient benchtop method for the immobilization of the model enzymes glucose oxidase and horseradish peroxidase.<sup>24</sup> Briefly, the enzyme is exposed to a water-miscible PAMAM dendrimer which functions as a biomimetic template providing sufficient primary amine concentrations (>20 mM) to condense silicic acid.<sup>19</sup> The resulting silica flocculates and precipitates within seconds, trapping both the dendrimer<sup>25</sup> and enzyme within the particles.<sup>21</sup> The efficiency of enzyme entrapment can be enhanced via manipulation of electrostatic attraction between the desired enzyme and cationic dendrimer surface.<sup>24</sup> Compared to other enzyme immobilization strategies such as sodium silicates,<sup>26</sup> the dendrimer-based methodology offers a significant advantage in both time and processing required to produce the immobilized biocatalysts.<sup>27,28</sup> The recombinant nitrilase (Codexis) for this

study has a theoretical *pI* between 5 and 7 and utilizes an active-site cysteine similar to the NIT1–3 cluster of *Arabidopsis thaliana*.

Nitrilase-containing silica nanoparticles were initially synthesized in water by adding 20  $\mu\text{L}$  of 1 M hydrolyzed tetramethyl orthosilicate (TMOS) to a 200  $\mu\text{L}$  solution containing nitrilase (0.400 mg) and generation-4 PAMAM dendrimer (20 mM primary amine concentration) at pH 7.8. Silica quantification of the rapidly precipitated nanocomposite (191 nm  $\pm$  44 average particle diameters (Figure 1, Table 1)) using the  $\beta$ -silicomolybdate assay method<sup>29</sup> showed 0.43 mg  $\pm$  0.1 (7.16  $\mu\text{mol}$ ) of silica produced for each reaction, consistent with previously published yields.<sup>19,24</sup> The efficiency of enzyme encapsulation quantified by the Bradford assay was rated 92.3%  $\pm$  0.5 (0.3692 mg) of the starting enzyme. The remaining unencapsulated enzyme (0.0308 mg) was found in subsequent supernatant washes. Therefore, the enzyme to silica mass ratio for these reaction conditions is 0.37:0.43, or 0.86:1 (Table 1). The specific activity of the encapsulated nitrilase was determined using a previously described fluorometric assay.<sup>30</sup> Activity of the nanocomposite toward 3-phenylpropionitrile (PPN) in 25 mM phosphate buffer (pH 7.8, 30  $^{\circ}\text{C}$ ) revealed a specific

(20) Luckarift, H. R.; Spain, J. C.; Naik, R. R.; Stone, M. O. *Nat. Biotechnol.* **2004**, *22*, 211–213.

(21) Larsen, G.; Marquez, M.; Lotero, E. *J. Phys. Chem. B* **2000**, *104*, 4840–4843.

(22) Roth, K. M.; Zhou, Y.; Yang, W.; Morse, D. E. *J. Am. Chem. Soc.* **2005**, *127*, 325–330.

(23) Jian-Jun, Y.; Ren-Hua, J. *Adv. Mater.* **2005**, *17*, 885–888.

(24) Miller, S. A.; Hong, E. D.; Wright, D. *Macromol. Biosci.* **2006**, *6*, 839–845.

(25) Knecht, M. R.; Wright, D. W. *Chem. Mater.* **2004**, *16*, 4890–4895.

(26) Bhatia, R. B.; Brinker, J. C.; Gupta, A. K.; Singh, A. K. *Chem. Mater.* **2000**, *12*, 2434–2441.

(27) Gill, I.; Ballesteros, A. *J. Am. Chem. Soc.* **1998**, *120*, 8587–8598.

(28) Avnir, D.; Braun, S.; Lev, O.; Ottolenghi, M. *Chem. Mater.* **1994**, *6*, 1605–1614.

(29) Iler, R. K. *The Chemistry of Silica*; Wiley: New York, 1979.

(30) Banerjee, A.; Sharma, R.; Banerjee, U. C. *Biotechnol. Appl. Biochem.* **2003**, *37*, 289–293.

**Table 1. Physical properties of nitrilase-containing silica nanoparticles**

encapsulation conditions	particle diameters (nm)	silica yield per batch (mg)	enzyme yield per batch (mg)	enzyme to silica ratio	enzyme yield (%)
free enzyme	n/a	n/a	n/a	n/a	n/a
water	191 ± 44	0.430 ± 0.100	0.369 ± 0.002	0.858	92.3 ± 0.5
8 mM	331 ± 83	0.442 ± 0.041	0.331 ± 0.020	0.749	82.8 ± 5.0
17 mM	353 ± 88	0.419 ± 0.026	0.290 ± 0.001	0.692	72.5 ± 0.3
25 mM	376 ± 94	0.433 ± 0.030	0.275 ± 0.017	0.635	67.8 ± 4.3

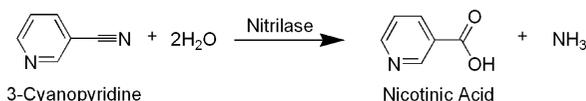
**Table 2. Kinetic parameters and specific activity of nitrilase-containing silica nanoparticles**

encapsulation conditions	$K_m$ PPN (mM)	specific activity PPN (U/mg)	$K_m$ 3-cyanopyridine (mM)	specific activity 3-cyanopyridine (U/mg)
free enzyme	2.016 ± 0.272	2.47 ± 0.15	0.569 ± 0.071	0.0514 ± 0.0036
water	3.381 ± 0.631	1.47 ± 0.19	0.901 ± 0.111	0.0489 ± 0.0045
8 mM	3.179 ± 0.691	1.81 ± 0.25	0.710 ± 0.101	0.0549 ± 0.0028
17 mM	3.370 ± 0.715	1.35 ± 0.20	0.798 ± 0.134	0.0566 ± 0.0038
25 mM	3.137 ± 0.880	1.67 ± 0.24	1.123 ± 0.162	0.0352 ± 0.0017

activity  $59.2\% \pm 5.8$  that of an equal amount of enzyme free in solution (Table 2).

Previously, we reported that the electrostatic attraction between the cationic dendrimer and surface charge of the enzyme could be tuned as a function of the ionic strength of the buffer.<sup>24</sup> When nitrilase was encapsulated over a range of phosphate buffer concentrations, the amount of total enzyme within the silica matrix increased as the buffer concentration decreased (Table 1). In addition, the particle diameter size decreased as a function of decreasing ionic strength, consistent with previously published results.<sup>24</sup> Since nitrilase has a slightly acidic  $pI$  ( $\sim 5-7$ ), it will be slightly anionic at neutral pH. Therefore, it is electrostatically attracted to the cationic dendrimer and would be in close proximity to the silica condensation event, resulting in its subsequent entrapment within the growing nanoparticle. As the ionic strength of the buffered solution is increased, the interaction between the dendrimer and enzyme is diminished, resulting in reduced encapsulation efficiency. Concurrently, the ions serve to maintain the solubility of the growing nanoparticle. As the buffer concentration is increased, the critical size for particle precipitation is increased, resulting in larger nanoparticles. It should also be noted that the absolute amount of silica produced for all of the conditions is the same regardless of the reaction conditions, suggesting that silica condensation occurs until the entire precursor is completely consumed.

Although an unbuffered solution yields optimal enzyme loading and the smallest particle diameters, it is not clear whether these conditions affect nitrilase's specific activity. Therefore, nanoparticles synthesized under each buffer condition (8, 17, and 25 mM phosphate buffer; pH 7.8) were compared to the samples prepared in water. All particles showed a similar specific activity toward PPN (Table 2), suggesting that the buffer concentration during encapsulation does not have a significant effect on the activity of nitrilase. Consequently, the increase in particle size seen in the buffered samples does not have a noticeable effect on activity, suggesting that the silica shell does not create a diffusion barrier between the enzyme and substrate.

**Scheme 1. Hydrolysis of 3-cyanopyridine with nitrilase**

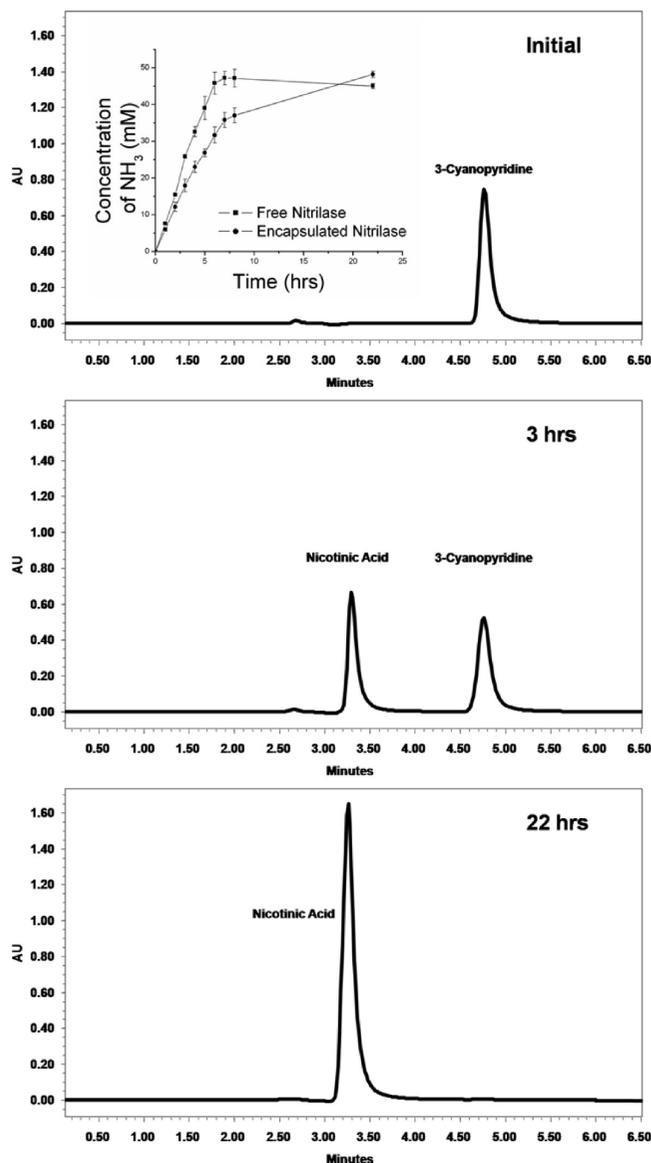
Therefore, the samples prepared in water, which had the highest enzyme yield, contain more total active enzyme per reaction batch.

To further investigate the utility of the nitrilase nanocomposite, we chose to examine nitrilase's ability to convert 3-cyanopyridine to biologically active 3-carboxypyridine (nicotinic acid; niacin) (Scheme 1). Previous reports have indicated that this niacin precursor is significantly less labile to nitrilase conversion as compared to PPN due to polarity and steric concerns, making it a more rigorous test for this system.<sup>13,31</sup> Specific activity testing of the nanocomposite with a 10 mM solution of 3-cyanopyridine revealed that the nanoparticles have nearly identical specific activity relative to the free enzyme, regardless of particle size, again suggesting that the silica matrix does not create a diffusion barrier between the substrate and enzyme.

Kinetic parameters of encapsulated nitrilase for both substrates were compared to that of free enzyme (Table 2). The apparent  $K_m$  value increased by  $\sim 60\%$  for both substrates when compared to nitrilase free in solution, regardless of the encapsulation conditions. A difference in  $K_m$  within 1-fold suggests that the enzyme is behaving kinetically similar to the free enzyme. Previous studies have reported changes of  $K_m$  values for biomimetically synthesized silica-enzyme nanoparticles anywhere from a 1-fold decrease to a 3-fold increase.<sup>24,32</sup> Changes in the  $K_m$  in these systems have been attributed to a variety of factors, including limited substrate access into the silica matrix and occlusion of the enzyme active site. Since there is little control over the orientation of the enzyme within the silica, and there is no apparent kinetic variation with different sized particles, it is likely that the active site of some of the enzyme is occluded by the silica at any given time.

(31) Mathew, C. D.; Nagasawa, T.; Kobayashi, M.; Yamada, H. *Appl. Environ. Microbiol.* **1988**, *54*, 1030-1032.

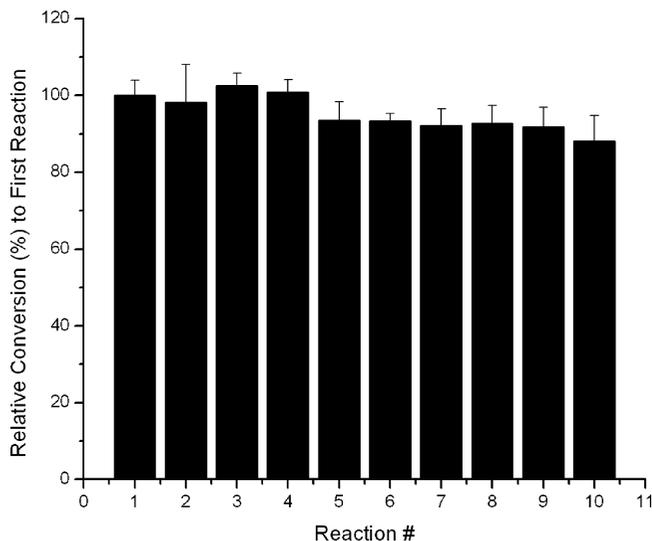
(32) Berne, C.; Betancor, L.; Luckarift, H. R.; Spain, J. C. *Biomacromolecules* **2006**, *7*, 2631-2636.



**Figure 2.** HPLC spectra following the conversion of a 50 mM solution of 3-cyanopyridine to nicotinic acid with nitrilase-containing nanoparticles. Time points are initial (top), 3 h (center), and 22 h (bottom). (Inset) Fluorometric comparison of the conversion of 50 mM 3-cyanopyridine with free and silica-encapsulated nitrilase.

To evaluate the applicability of these nanocomposites for the industrial synthesis of niacin, a single reaction batch of encapsulated nitrilase was used to drive the conversion of 3-cyanopyridine. The overall conversion rate was compared between the free and encapsulated conditions (Figure 2, inset). The conversion was verified by HPLC and characterized by NMR (Figure 2, Supporting Information). It should be noted that no enzymatic activity was observed in the supernatant, confirming that the enzyme was not simply absorbed onto the silica surface or leaching out of the silica framework. This combined with the ease of product separation from the biocatalyst makes the silica–enzyme nanocomposites attractive for large-scale use.

Since the nanocomposite can be separated from the reaction media by centrifugation, it is of interest whether the material can be used multiple times. Thus, we



**Figure 3.** Consecutive experiments performed on a single triplicate batch of nitrilase-containing silica. A 5 mM 3-cyanopyridine solution was added and incubated at RT for one hour before being removed by centrifugation. Activity of the enzyme was only diminished by 10% at reaction no. 10.

examined the biocomposites ability to convert multiple doses of 3-cyanopyridine. A single preparation of encapsulated nitrilase (same as above) was used to convert a 5 mM solution 3-cyanopyridine 10 successive times. However, loss of silica to the multiple handling procedures reduced the amount of biocatalyst present, which has a significant impact for repeated usage. Recently, a histidine-tagged R5 peptide linked to cobalt-coated agarose beads in a flow reactor was used to immobilize silica-encapsulated equine butyrylcholinesterase.<sup>33</sup> Although this process is an effective method for screening cholinesterase inhibitors, synthesizing a histidine-modified peptide can be expensive and time-consuming, especially at the large scale. Alternatively, inspired by a bed flow reactor design of PEI-templated nitrobenzene nitroreductase silica nanoparticles,<sup>32</sup> the effective, repeated separation of water-soluble nicotinic acid from the nitrilase-containing nanospheres was achieved by performing the reaction in centrifuge filters (Millipore Ultrafree-MC centrifugal filter devices with a Durapore 0.1  $\mu\text{m}$  pore size PVDF filter). Even after 10 consecutive experiments, only a 10% loss of activity was observed (Figure 3). Also, no detectable activity was observed in the supernatant after filtration. Much like the frit filters used in the nitrobenzene nitroreductase study, the centrifuge filters served as a physical barrier between the nitrilase-containing silica nanoparticles and the desired product after conversion and centrifugation, which allows for their more efficient recyclability.

A scaled preparation of niacin was produced by combining 20 batches of nitrilase-containing nanoparticles (7.4 mg of total enzyme; 8.6 mg of silica) synthesized in water. A 200 mM solution of 3-cyanopyridine was then added and allowed to convert for 12 h at 30 °C. Upon centrifugation, the supernatant

(33) Luckarift, H. R.; Johnson, G. R.; Spain, J. C. *J. Chromatogr. B* **2006**, *843*, 310–316.

was readily collected and nine additional aliquots of 3-cyanopyridine were added. After HPLC quantification of the product (10 mL total), it was determined that this system was capable of synthesizing 1.75 mg of nicotinic acid/h throughout 10 reaction cycles.

Nitrilase-containing silica particles can be utilized as a viable immobilized biocatalyst that is both rapidly produced and quickly reclaimed for use in the synthesis of nicotinic acid without loss of significant activity or need for cumbersome separation steps. Additionally, the reported long-term storage of enzymes within nanoparticles with little loss in activity adds another cost-effective benefit likely to be attractive for scale-up chemoenzymatic processes.<sup>20,24</sup> Although there may be many challenges involved with commercializing this technology, these characteristics will greatly reduce time and costs that traditionally hinder widespread use of enzymes in pharmaceutical and fine chemical production.

## Experimental Section

**Enzymes and Reagents.** Nitrilase-102 (recombinant from *Arabidopsis thaliana*) was purchased from Codexis. All other reagents were purchased from Sigma-Aldrich (U.S.A) and used as delivered.

**Nitrilase Encapsulation.** Nitrilase was suspended at 2 mg/mL in water or phosphate buffer (8, 17, and 25 mM respectively) at pH 7.8. An aliquot of 200  $\mu$ L was charged with enough generation-4 PAMAM dendrimer (Sigma; 10 wt % in MeOH) to afford a primary amine concentration of 20 mM. Tetramethylorthosilicate (Aldrich; 1 M; TMOS) hydrolyzed by 1 mM HCl (aq.) was then added (20  $\mu$ L) to achieve rapid precipitation of white silica nanoparticles. The nanoparticles were collected by centrifugation at 10,000 RPM and washed 3-fold with 25 mM phosphate buffer. General protein content was quantitated with the Bradford assay from a standard curve of nitrilase.

**PPN Activity Assay.** Encapsulated nitrilase specific activity was compared to free nitrilase by a fluorometric assay previously described.<sup>30</sup> Briefly, nitrilase was reacted with a 10 mM solution of PPN (Sigma) at 30 °C for 10 min. The reaction was stopped upon addition of an equal volume of 0.1 M HCl. Next, the amount of ammonia produced was assayed by adding a 10  $\mu$ L aliquot of the reaction solution to a 290  $\mu$ L solution containing alcoholic *o*-phthaldialdehyde (Fluka) (3.75 mM) and 2-mercaptoethanol (Sigma; 3.6 mM) in 0.2 M phosphate buffer pH 7.4. This solution stood for 35 min, followed by excitation at 407 nm and monitored emission at 460 nm (Bio-Tek Synergy HT multidetection microplate reader). A standard curve of NH<sub>3</sub> was produced for the range of 10  $\mu$ M and 1000  $\mu$ M. One unit of nitrilase activity was defined as the amount able to release 1  $\mu$ mol NH<sub>3</sub>/min per mg of nitrilase under the assay conditions. Kinetic parameters were obtained by varying the PPN concentrations and collecting several time points to acquire reaction rates.

**3-Cyanopyridine Activity Assay.** Specific activity of encapsulated and free nitrilase toward the conversion of 3-cyanopyridine (Sigma) to nicotinic acid was determined by reacting the enzyme with a 10 mM solution of 3-cyanopyridine at 30 °C for 1 h. The reaction was quenched upon addition of an equal volume of 0.1 M HCl (aq.). The amount of ammonia

released during the reaction was assayed using the same fluorometric assay used for the conversion of PPN. For kinetic experiments, 3-cyanopyridine concentrations were varied, and several time points were obtained to acquire rates.

**Nitrilase Reusability.** Reusability of the nanoparticles was assessed by resuspension in 200  $\mu$ L of phosphate buffer and transferred to centrifuge filters (Millipore Ultrafree-MC centrifugal filter devices with a Durapore 0.1  $\mu$ m pore size PVDF filter). Before the substrate was added, the extra solvent was removed by centrifugation (Beckman Coulter Allegra X-22R) at 2500 RPM for 1 min. The reaction was initiated by the addition of 200  $\mu$ L of a 5 mM 3-cyanopyridine solution in 25 mM phosphate buffer at pH 7.8 and incubated for 1 h at room temperature. The reaction was terminated by centrifugation of the supernatant at 2500 RPM for 1 min. The nitrilase-containing nanoparticles were then washed with 5 portions of 300  $\mu$ L of phosphate buffer to ensure that all substrate was removed before the next experiment. This reaction was repeated a total of 10 successive times, and the reactant and product concentrations were determined by the HPLC and the fluorometric assays.

**Batch Preparation of Nicotinic Acid.** Multiple batches of nitrilase-silica nanoparticles were prepared in water as described above. Two reaction vessels were prepared by combining reaction batches (10 per reaction vessel), and a 500  $\mu$ L solution of 200 mM 3-cyanopyridine in 25 mM phosphate buffer pH 7.8 was added and allowed to convert for 12 h at 30 °C. Additional aliquots of 3-cyanopyridine were added after the prior reaction solution was removed by centrifugation (10 mL total). The amount of synthesized nicotinic acid was quantified by HPLC.

**HPLC Quantification.** HPLC analysis was performed on the supernatant with a Waters 600E Multisolvant Delivery System equipped with a Nova Pak C18 column (reversed-phase column, 3.9 mm  $\times$  150 mm) at a flow rate of 0.5 mL/min. A 90:10 mixture of water/acetonitrile with 0.1% trifluoroacetic acid was used as the mobile phase. The  $A_{218}$  and  $A_{261}$  were measured for 3-cyanopyridine and nicotinic acid, respectively. A standard curve for 3-cyanopyridine and nicotinic acid was produced from 5  $\mu$ M to 400  $\mu$ M and 25  $\mu$ M to 500  $\mu$ M, respectively.

**Bradford Assay.** Protein mass quantification was performed by adding 160  $\mu$ L of test solution to 40  $\mu$ L of BioRad Protein Assay solution concentrate and mixing for 5 min. The change in color was monitored via UV-vis (Bio-Tek Synergy HT multidetection microplate reader) at 595 nm. A calibration curve was produced for nitrilase between the range 0.8  $\mu$ g/mL and 80  $\mu$ g/mL.

**NMR Analysis.** NMR analysis was performed on a Bruker DPX-300 at room temperature. Samples were prepared by performing encapsulation and enzymatic activity assay in deuterated 25 mM phosphate buffer. Samples tested were collected by aspirating the supernatant following centrifugation of the reaction mixture.

**Silica Quantization.** Silica nanoparticles were quantified following the  $\beta$ -silicomolybdate method described by Iler.<sup>29</sup> Samples were dissolved in 0.5 M NaOH and

incubated at 95 °C for at least 30 min. Following incubation, the liberated dendrimers were separated from solution by Centricon filtration (American Centricon filtration devices, Millipore Inc.). Molybdate reagent was added (1:4 silicic acid/molybdate) to the filtered solutions to initiate the formation of the bright-yellow product monitored by UV–vis spectrophotometry at 410 nm and quantitated from a standard curve of silicate standards.

**Scanning Electron Microscopy.** Silica samples were re-suspended in ethanol and placed on an aluminum surface SEM sample stage (Ted Pella Inc.) to dry. Samples were sputter-coated with a thin layer of gold via a Pelco model 3 Sputter Coater (Ted Pella Inc.). Thereafter, samples were imaged on a Hitachi S4200 scanning electron microscope operated at variable voltage.

### **Acknowledgment**

We thank Michelle F. Richards for her critical comments.

### **Supporting Information Available**

NMR spectroscopy of the supernatant of the synthesis of nicotinic acid with nitrilase-containing nanoparticles and additional SEM images of nitrilase-containing nanoparticles at various phosphate buffer concentrations. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Received for review January 6, 2009.

OP9000065