

## **Enhancing enzymatic stability of bioactive papers by implanting enzyme-immobilized mesoporous silica nanorods into paper**

Zhengyang Zhao<sup>a</sup>, Junfei Tian<sup>b</sup>, Zhangxiong Wu<sup>a</sup>, JianLiu<sup>c</sup>, Dongyuan Zhao<sup>a,d</sup>, Wei Shen<sup>b</sup>,  
Lizhong He<sup>a\*</sup>

*<sup>a</sup>Department of Chemical Engineering, Monash University, Clayton, VIC3800, Australia*

*Email: [lizhong.he@monash.edu](mailto:lizhong.he@monash.edu)*

*<sup>b</sup>Australian Pulp and Paper Institute, Department of Chemical Engineering, Monash University, Clayton, VIC 3800, Australia*

*<sup>c</sup>Department of Chemical Engineering, Curtin University, Perth WA 6845, Australia.*

*<sup>d</sup>Department of Chemistry, Shanghai Key Laboratory of Molecular Catalysis and Innovative Materials, and Laboratory of Advanced Materials, Fudan University, Shanghai 200433, P. R. China*

## I. Experimental details

### 1. Materials

All the materials and chemicals were of analytical grade and used as received without further purification. Tetraethyl orthosilicate ( $\text{Si}(\text{OC}_2\text{H}_5)_4$ , TEOS) (> 99 %), 3-Glycidoxypropyl-trimethoxysilane (GOPS, 2530-83-8), hydrochloric acid (320331), ethanol amine (110167), Potassium Chloride (P5405), triblock copolymer Pluronic P-123 (P123), enzyme horseradish peroxidase (HRP, P8125), and phosphate buffer saline (PBS) tablets (P4417) were purchased from Sigma-Aldrich (Sydney, Australia). 3,5,3',5'-Tetramethylbenzidine (TMB) (N301) was purchased from Thermal scientific (Melbourne, Australian). Water was from a Milli Q system and had an electrical resistance of 18 M $\Omega$ ·cm.

### 2. Methods

#### 2.1 Synthesis of mesoporous silica nano-rods

Mesoporous silica nanorods were synthesised using previously reported approach as following.<sup>1</sup> 5 g of P123 and 5 g of KCl were first dissolved in 150 ml of HCl aqueous solution (2 M) at 37.5 °C under stirring (280 rpm) for 4 hours. 10.4 g of TEOS was added into the mixture and the mixture was stirred for 7 minutes. Stirring was then stopped and the mixture was kept under static conditions at 37.5 °C for 24 hours. The mixture was sealed and subjected to hydrothermal treatment at 130 °C in an autoclave for 24 hours. The mixture was filtrated and the mesoporous silica was collected and washed with water. The collected materials were dried under 60 °C overnight. The dried material was mixed with 180 ml of HCl-ethanol mixture (HCl 0.1 M) and the mixture was refluxed at 80 °C for 24 hours under 350 rpm stirring. After intensive washing with water, the materials were dried at 50 °C for overnight.

## 2.2 Surface functionalization of mesoporous silica nanorods

Mesoporous silica nanorods were functionalized using previously reported procedure.<sup>2, 3</sup> 200 mg of the mesoporous silica nanorods was dispersed in 20 ml of toluene under around 70 °C with stirring at 350 rpm. 3.2 ml of GOPS was added into dispersion and keep stirring for 24 hours. Then, the mesoporous silica nanorods were washed with 20 ml of toluene, 20 ml of methanol, 20 ml of toluene, and 20 ml of methanol sequentially. Functionalized the mesoporous silica nanorods were dried in oven under 60 °C for overnight.

## 2.3 Immobilization of HRP on functionalized mesoporous silica nanorods

22.5 mg of enzyme horseradish peroxidase (HRP) powder was dissolved into 3 ml of carbonate buffer (50 mM carbonate buffer, pH 9.5) to make 7.5 mg/ml HRP solution. 100 mg of dried functionalized mesoporous silica nanorods were mixed with 2 ml of 7.5 mg/ml HRP solution, followed by gentle vortexing. The mixture was incubated under 37 °C with shaking at 250 rpm for 24 hours. The HRP-immobilized mesoporous silica nanorods (hereafter referred to as HRP-nanorods) were settled down by centrifugation. The supernant was collected for measurement of protein concentration (see section 2.5). The silica nanorods were washed with 2 ml of water and 1 M NaCl, sequentially. Ethanolamine (1 M) was then added to block the unreacted epoxy groups, followed by washing with water. The HRP-nanorods were dispersed into 10 ml of PBS to give a particle concentration of 10 mg/ml.

## 2.4 Measurement of HRP concentration

Horseradish peroxidase (HRP, P8125) from Sigma is a salt-free product, and the concentration of HRP in the starting solution before immobilization was calculated from the weight of HRP powder. Bradford method was used to measure protein concentration in the

supernant after the immobilization step,<sup>4</sup> and the starting HRP solution was used as the standard. According to Sigma's product description, HRP contains 18% carbohydrate and there are at least seven isozymes of HRP existing in the product. The mixture nature of HRP makes it difficult to precisely determine protein concentration. Nevertheless, the Bradford method gave a good estimation of relative change of protein concentration.

### *2.5 Activity of HRP and HRP-nanorods in solution*

HRP activity was measured using TMB as a substrate.<sup>5</sup> A series of standard HRP solutions were prepared in PBS with different HRP concentration (0.25, 0.5, 0.75 and 1.0 µg/ml). 0.1 ml of HRP solution with different concentration was mixed well with 1 ml of PBS and 0.25 ml of TMB substrate by pipetting. Then, mixture was kept in static condition for 5 minutes. 0.1 ml of 1 M HCl was added into mixture to stop the reaction. The absorbance of the resulted solution at 450 nm was measured by UV spectrometer to give the standard curve of HRP activity. For activity measurement of the HRP-nanorods, 0.1 ml of 0.5 mg/ml HRP-nanorods in PBS was used instead of HRP solution. After adding HCl, the mixture was centrifuged under 16800 g for 1 minute to remove HRP-nanorods. Then, absorbance at 450 nm was measured and compared with the standard activity curve of free HRP solution.

### *2.6 Fabrication of bioactive papers*

Whatman NO. 4 filter paper was used as a paper basement. The uniform circle paper chips with 4 mm in diameter were made by hollow punch. 7.5 µl of 8 mg/ml HRP-nanorods solution was implanted into paper chips. The paper chips were exposed to air in fume hood to dry for 10 minutes. They were then transferred into a dark chamber for further drying (see section 2.8). For comparison, 7.5 µl of 6.6 µg/ml HRP solution was implanted into the paper

chips to fabricate free HRP-based bioactive papers. As a control, 7.5  $\mu$ l of PBS was implanted into the paper chips to fabricate the blank bioactive papers.

### *2.7 Stability and activity test of bioactive papers*

Following initial 10 min dry in fume hood, further drying and aging were carried out by exposing the bioactive paper chips (free HRP-based-, HRP-nanorods-based-, and controls) to air in a dark chamber at room temperature or 37 °C. Paper chips were taken out for examining of activity at different aging time ranging from 0 hr to 172 hrs. After being incubated for certain period of time, paper samples were added with 10  $\mu$ l of TMB substrate and left in static conditions for 10 minutes. Then, pictures were taken by Olympus PEN Mini E-PM1 camera in a black room under fixed white light. The distance between samples and camera lens was fixed as 11 cm. The focal distance of camera was fixed as 20 mm. Camera was set for manual operation and no flash was applied. After photographing, pictures were analysed by Adobe photo shop CS5. Under the brightness value of 150, average cyan value of whole paper chip area was read under CMYK mode. HRP enzymatic activity of bioactive papers was quantified by the difference of cyan value between the sample and the control.

### *2.8 Characterization of nanorods*

#### *2.8.1 TEM imaging*

TEM images were obtained on FEI Tecnai G2 T20 TWIN LaB6 electron microscope. The powder samples for the TEM measurements were suspended in ethanol and then dropped onto the Cu grids with holey carbon films.

#### *2.8.2 SEM imaging*

Overview SEM images were obtained by JEOL 7001F electron microscope. Ultra high resolution SEM images were obtained by FEI NOVA 450 electron microscope. The powder samples for the SEM measurements were placed on carbon tape and coated with 1 nm platinum.

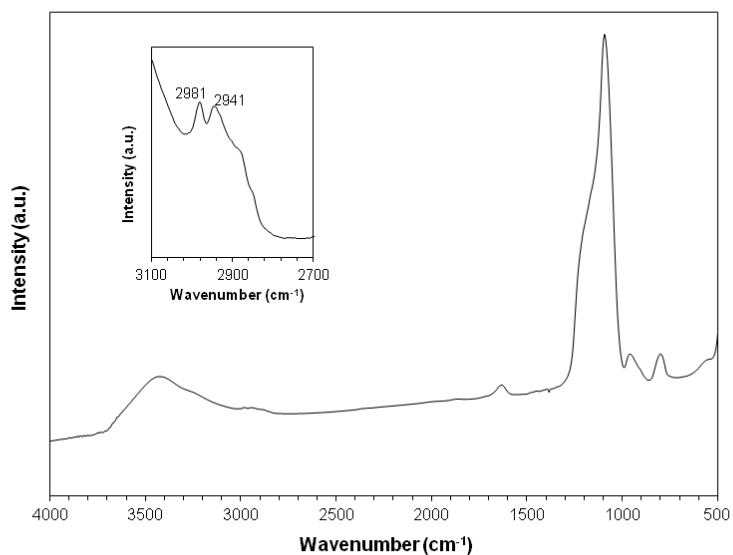
### 2.8.3 Nitrogen adsorption

Nitrogen sorption isotherms of samples were measured by a micromeritics ASAP 2020 analyser at 77 K. Prior to the measurement, the sample was degassed at 90 °C for 8 hours. The Brunauer-Emmett-Teller (BET) specific surface areas were calculated using adsorption data at a relative pressure range of  $P/P_0 = 0.05 \sim 0.3$ . Pore size distributions were derived from the adsorption branch using Barrett-Joyner-Halenda (BJH) method. The total pore volumes were estimated from the amounts adsorbed at a relative pressure ( $P/P_0$ ) of 0.99.

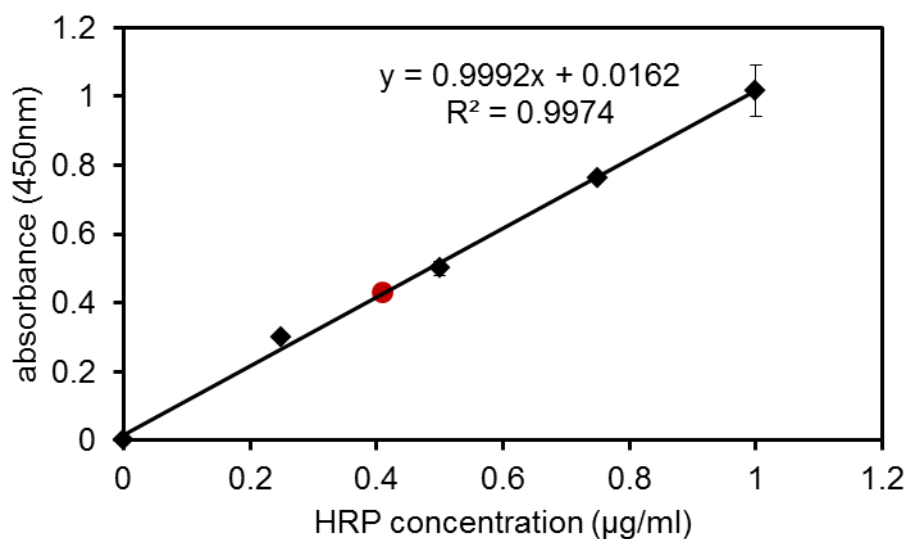
### 2.8.4 FT-IR

FT-IR measurements were carried out using PerkinElmer Spectrum 100 FT-IR spectrometer from 500 to 4000  $\text{cm}^{-1}$  by using potassium bromide as a background. Scan was repeated for 20 times.

## II. Results



**Figure S1.** FT-IR characterization of the epoxy functionalized silica nanorods



**Figure S2:** Comparison of activity of immobilized horseradish peroxidase (HRP, red) with free HRP (black). The red round point was absorbance of reaction solution catalysed by 0.5 mg/ml mesoporous silica nanorods immobilized with HRP, which activity is equivalent to 0.41 µg/ml free HRP.

**Table S1.** Activity of bioactive papers at different time\*

Incubation time (Hours)	Residual activity of bioactive papers (% of original activity)			
	Free HRP-based bioactive papers		HRP-nanorods-based bioactive papers#	
	37 °C	room temperature	37 °C	room temperature
<b>0</b>	100%	100%	100%	100%
<b>1</b>	58.8%	N.D.	N.D.	N.D.
<b>2</b>	0.72%	41.4%	N.D.	N.D.
<b>4</b>	0	9.9%	N.D.	N.D.
<b>8</b>	N.D.	10.9%	95.4%	102%
<b>16</b>	N.D.	0.77%	90.2%	98.4%
<b>24</b>	N.D.	0	95.4%	97.2%
<b>48</b>	N.D.	N.D.	95.4%	99.6%
<b>96</b>	N.D.	N.D.	97.4%	101%
<b>168</b>	N.D.	N.D.	95.1%	99.8%

\* HRP enzymatic activity of bioactive papers was quantified by the difference of cyan value between the sample and the control after reacting with TMB substrate for 10 min.

# Measurements at different hours were carried out using different samples that were fabricated under the same conditions but with different aging time. The individual samples were close to each other but not identical.

N.D. – not determined.



## References

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