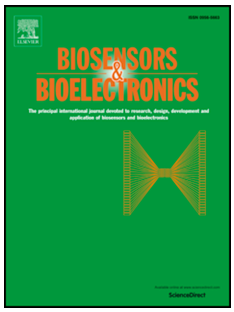


Tyrosinase nanocapsule based nano-biosensor for ultrasensitive and rapid detection of bisphenol A with excellent stability in different application scenarios

Lingxia Wu, Xianbo Lu, Kai Niu, Dhanjai, Jiping Chen

PII: S0956-5663(20)30401-2

DOI: https://doi.org/10.1016/j.bios.2020.112407

Reference: BIOS 112407

To appear in: *Biosensors and Bioelectronics*

Received Date: 6 May 2020

Revised Date: 2 June 2020

Accepted Date: 21 June 2020

Please cite this article as: Wu, L., Lu, X., Niu, K., Dhanjai, , Chen, J., Tyrosinase nanocapsule based nano-biosensor for ultrasensitive and rapid detection of bisphenol A with excellent stability in different application scenarios, *Biosensors and Bioelectronics* (2020), doi: https://doi.org/10.1016/j.bios.2020.112407.

CRediT author statement

Lingxia Wu: Data curation, Methodology, Visualization, Formal analysis, Writing- Original draft preparation.
Xianbo Lu: Conceptualization, Methodology, Writing- Reviewing and Editing, Funding acquisition, Project administration.
Kai Niu: Data curation.
Dhanjai: Methodology.
Jiping Chen: Supervision.

Tyrosinase nanocapsule based nano-biosensor for ultrasensitive and rapid detection of bisphenol A with excellent stability in different application scenarios

Lingxia Wu,[†,‡] Xianbo Lu,[*,†] Kai Niu,[†] Dhanjai,[†] Jiping Chen[†]

[†]CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, 116023, PR China

[‡]University of Chinese Academy of Sciences, Beijing 100049, PR China

Abstract: Bisphenol A (BPA), one of the most important endocrine disrupting chemicals, is a threat to human and wildlife health. Electrochemical enzyme biosensor has been regarded as ideal alternative analytical technique for ultrasensitive and rapid detection of BPA, while the unstable and easily deactivated nature of enzyme limits its development. In order to improve the stability of enzyme, tyrosinase was chosen as a model enzyme, and tyrosinase nanocapsules (nTyr) were prepared by encapsulating a single tyrosinase molecule into a thin network polymer shell through in-situ polymerization method in aqueous solution. The characterization of particle size distribution, TEM and FTIR indicated the successful formation of single tyrosinase molecule nanocapsule. And the porous network polymer shell of nTyr ensured the maintenance of tyrosinase activity and fast substrate transportation. The obtained nTyr was used to construct an electrochemical biosensor for BPA detection, exhibiting a low detection limit of 12 nmol L^{-1} and a wide linear range from 5×10^{-8} to 2×10^{-6}

* Corresponding author. E-mail: xianbolu@dicp.ac.cn.

mol L^{-1}. Compared with native tyrosinase, the nTyr based biosensor displayed dramatically enhanced stability including thermal stability, organic solvent tolerance and acid/base tolerance. The excellent performance of nTyr based biosensor was not only attributed to the protection of biocompatible rigid polymer shells, but also the multipoint covalent attachments between tyrosinase cores and polymer shells. The robust biosensor was further used for rapid detection of BPA leached from plastic products with satisfactory results. The nTyr based nano-biosensor provides a prospective solution to resolve the stability problem of enzyme biosensors in different application scenarios.

Keywords: Single enzyme molecule nanocapsules; Tyrosinase nanocapsules based biosensor; Enhanced biosensor stability; Thermal stability; Organic solvents tolerance; Acid/base tolerance

1. Introduction

Bisphenol A (2,2-bis (4-hydroxyphenyl) propane, BPA), as one of the most important endocrine disrupting chemicals, can leach from the products of polycarbonate plastics and epoxy resins, including water bottles, baby feeding bottles, beverage bottles, food containers and packaging (Huang et al., 2018; Zou et al., 2019), which can interfere with the normal function of the endocrine hormones and cause adverse effects on human and wildlife health at low doses (Fisher et al., 2019; Lee, 2018; Wu et al., 2019). Considering the highly potential exposure and health risk of BPA, it is essential to develop rapid and versatile analytical techniques for BPA detection. Compared with conventional methods, such as high performance liquid

chromatography-fluorescence detector (Xiong et al., 2018), gas chromatography tandem mass spectrometry (Fisher et al., 2019) and liquid chromatography tandem mass spectrometry (Romera-Garcia et al., 2019), the electrochemical enzyme biosensor displays excellent advantages of low cost, simple operation, fast response, high sensitivity and selectivity, which has been widely used as an ideal alternative technique for rapid detection of BPA. In the presence of dissolved oxygen, tyrosinase can specifically catalyze oxidation of BPA into corresponding o-diquinone, which can be electrochemically reduced at a low operating potential, resulting in a catalytically amplified signal (Andreescu and Sadik, 2004; Jiménez et al., 2001). Based on the above mechanism, electrochemical tyrosinase biosensors have been extensively reported for the determination of BPA with satisfactory results (Wang et al., 2015; Wu et al., 2020; Wu et al., 2019). However, tyrosinase possesses the fragile nature of enzyme, which is unstable and easily lose its activity in nonphysiological environments, such as high temperature, organic solvent, strong acid and strong base conditions, etc. The poor stability of the enzyme significantly hampers their applications and limits the development of enzyme biosensor. Therefore, exploring an effective approach to stabilize enzyme is an important way to promote the development of electrochemical enzyme biosensor.

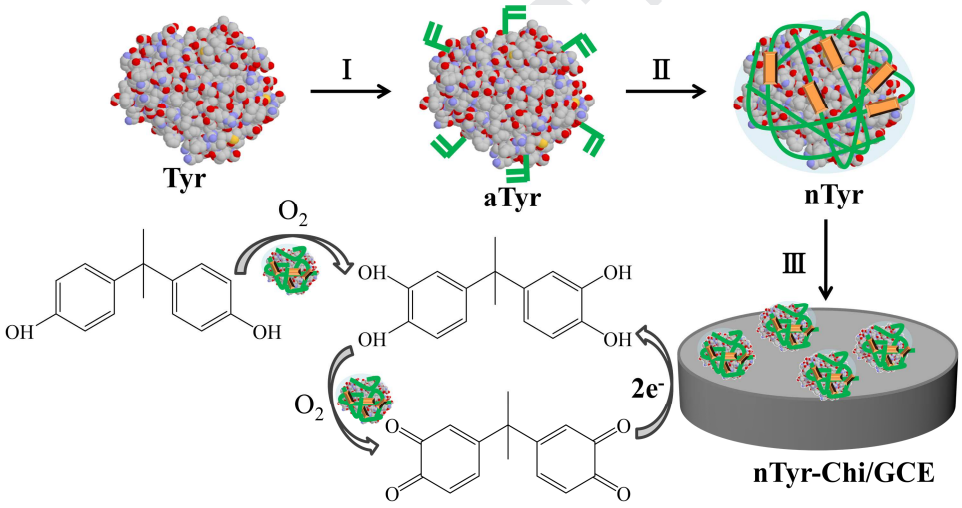
To date, there are several methods to solve the unstability problem of enzyme, such as exploring new nanomaterials or molecularly imprinted polymers as mimetic enzymes (Chen et al., 2012; Mu et al., 2014; Su et al., 2019; Yu et al., 2018), modifying enzyme through molecular evolution and protein engineering (Cai et al.,

2012; Yang et al., 2003), and using immobilization matrix to immobilize enzyme via adsorption (Govindan et al., 2014), covalent linkage (Zheng et al., 2012) and entrapment (Wu et al., 2012). However, these methods usually suffer from the disadvantages of low activity and selectivity, enzyme leakage and lacking sufficient stability. Recently, an efficient method was explored by encapsulating enzyme in silica (Zhang et al., 2015), liposomes (Jiang et al., 2014) and polymer (Liu et al., 2015b) nanocapsules. Among them, encapsulating a single enzyme molecule in a thin polymer shell is the most attractive approach for stabilizing enzyme and retaining its natural activity, which is attributed to the biocompatible microenvironment and space confinement effect for preventing the dissociation of enzyme. In addition, the porous network polymer shells are thin and permeable enabling the substrate transport freely to react with enzyme cores. In previous research, horseradish peroxidase (HRP) nanocapsule was firstly designed and prepared by a two-step procedure including surface acryloylation and in-situ aqueous polymerization, which exhibited similar biocatalytic behavior to native HRP and significantly enhanced stability at high temperature and in polar organic solvent (Yan et al., 2006). Furthermore, in order to improve the thermal stability of catalase, the catalase nanocapsules with non-degradable polymer shell were produced by encapsulating a single enzyme in a polyacrylamide shell, which could retain their catalytic activity up to 65 □ at least 60 min and efficiently scavenge 90% of free radicals in tobacco smoke (Liu et al., 2015b). Compared with native catalase, the catalase nanocapsules exhibited enhanced thermal stability and higher scavenging efficiencies even under high operational temperatures,

which opened up future application in cigarette filters. Until now, the enzyme nanocapsules of organophosphorus hydrolase, alcohol oxidase, glucose oxidase, caspase, invertase and carbonic anhydrase, have been successfully prepared and extensively applied in detoxification (Liu et al., 2013; Wei et al., 2013; Xu et al., 2018), bioluminescence imaging (Du et al., 2010), cancer therapies (Liu et al., 2015a), controlled drug release (Tian et al., 2016; Yan et al., 2010), and other biomedical fields (Gu et al., 2009). Nevertheless, there are no reports available for using enzyme nanocapsules as biosensing platform, except our previous study of glucose oxidase nanocapsule-based biosensor (Dhanjai et al., 2020). Up to date, tyrosinase nanocapsules (nTyr) have not been prepared and used in electrochemical biosensor field and other field yet.

In present report, nTyr with a single enzyme molecule core and thin polymer network shell was successfully prepared for the first time. As shown in Scheme 1, the in-situ polymerization of nTyr was synthesized with two steps: the polymerizable acryloylated groups were covalently anchored to the surface of protein by acryloylation (step □); subsequently, the polymerization in aqueous solution containing monomer and cross-linker was initiated by initiators, and a thin polymer shell was grown around each tyrosinase molecule core to form the nTyr (step □). Then we used the prepared nTyr instead of traditional native tyrosinase as biorecognition element to construct a new electrochemical nano-biosensor for the rapid detection of BPA (step □), which showed high electrocatalytic activity and dramatically enhanced stability even under high temperature, high organic solvent

content, and strong acid/base condition. Compared with traditional tyrosinase biosensor, the fabricated nTyr-based biosensor bridges the gap of activity loss of tyrosinase during practical application in varying work environment, which are attributed to the rigid polymer shell of nTyr and the multipoint covalent attachments between tyrosinase cores and polymer shells for providing structural support and biocompatible microenvironment to protect tyrosinase against dissociation even in extreme operational conditions. The nTyr based nano-biosensor demonstrates significantly improved stability in different application scenarios, which provides a prospective solution to resolve the stability problem of enzyme biosensors.



Scheme 1. Illustration for the construction of nTyr and nTyr-based biosensor for BPA detection. Step □: the polymerizable acryloylated groups were attached on the surface of tyrosinase (i.e. aTyr); Step □: a polymer shell was generated around the tyrosinase molecule by an in-situ polymerization (i.e. nTyr). Step □: the construction of nTyr-based biosensor and the biosensing mechanism for BPA.

2. Material and Methods

2.1. Material

Tyrosinase (Tyr, from mushroom, ≥ 1000 units mg^{-1}), chitosan (Chi, from shrimp shells, ≥ 75% deacetylated), 4-dimethylaminoantipyrine (DMAP) and ammonium persulfate (APS) were purchased from Sigma (USA). BPA was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). N-acryloxysuccinimide (NAS) and N,N'-methylene bisacrylamide (BIS) were purchased from J&K Chemical LTD (China). Acrylamide (AAM) was obtained from Beijing Solarbio Science & Technology Co., Ltd (China). N,N,N',N'-tetramethylethylenediamine (TEMED) was purchased from Dtrem Chemicals (USA). Sodium phosphotungstate was obtained from Aladdin (Shanghai, China). All chemicals were used as received without further purification. 50 mmol L^{-1} and 20 mmol L^{-1} phosphate buffer solution (PBS, pH 7.0) were prepared by mixing standard solutions of Na_2HPO_4 and NaH_2PO_4. Unless otherwise stated, 50 mmol L^{-1} PBS (pH 7.0) was used as the electrolyte in electrochemical experiments. Milli-Q water (>18 MΩ cm) was used throughout all experiments.

2.2. Apparatus and methods

Transition electron microscopy (TEM) image was recorded by using a JEM-2100 instrument (JEOL, Japan) with an accelerating voltage of 200 kV. Dynamic light scattering (DLS) studies were measured with a Zetasizer Nano instrument (Malvern Instruments Ltd., UK). The enzyme activity assay of tyrosinase containing initiators and DMAP was performed by a UV-Vis Spectrophotometer (UV5, Mettler-Toledo Inc., Switzerland) at 475 nm. The average number of acryloylated groups conjugated onto the tyrosinase was determined by measuring the residual lysine on acryloylated

tyrosinase with a microplate fluorescence reader (Tecan Trading AG, Switzerland). Fourier transform infrared (FTIR) spectra were carried out using a spectrum GX apparatus (Perkin-Elmer Company, USA). Electrochemical measurements including amperometric current-time (I-t), were carried out by using a CHI 440B electrochemical workstation (CHI Instruments Inc., USA). The measurements were based on a conventional three-electrode system consisting of the modified glassy carbon electrode (GC, 3 mm diameter) as working electrode, an Ag/AgCl electrode (KCl concentration of 3 mol L^{-1}) as reference electrode, and a platinum wire as auxiliary electrode.

2.3. Preparation of nTyr

As illustrated in Scheme 1, the synthesis of nTyr followed a two-step procedure according to the reported method (Liu et al., 2015b) with minor revision. Considering the property of tyrosinase, DMAP was explored as stabilizer during polymerization, and the ratio of monomer, cross-linker and initiators was optimized. The preparation details are as follows:

□) Acryloylation of tyrosinase: Before polymerization, tyrosinase was firstly modified with NAS to attach acryloylated groups onto their surface. The acryloylation was achieved by adding NAS (1% in DMSO, m/v) to the tyrosinase solution (5 mg mL^{-1}, 50 mM pH 7.0 PBS) at 5:1 weight ratio (tyrosinase to NAS) and reacted for 2 h at 4 □. Then the acryloylated tyrosinase (aTyr) solution was thoroughly dialyzed against 20 mM PBS (pH 7.0) by using a dialysis tubing membrane (MWCO = 12 KDa, Sigma-Aldrich) to remove any unreacted NAS.

□) In-situ polymerization: For the radical polymerization step, the DMAP (2:5 weight ratio to tyrosinase) was used as stabilizer. Briefly, the monomer AAM and cross-linker BIS were firstly prepared as 2% and 1% (m/v) stock solution in deionized water, respectively. Then they were added into the solution containing aTyr, and the final concentration of aTyr was tuned to 1 mg mL^{-1} by diluting with PBS (50 mM, pH 7.0). Next, the radical polymerization was initiated by adding initiators APS and TEMED (TEMED/APS = 2:1, w/w). The molar ratio of aTyr: AAM: BIS: APS was 1:6000:1000:500. And the in-situ polymerization was carried out at 4□ for 2 h with the protection of DMAP. After the polymerization, the solution was dialyzed against 20 mM PBS (pH 7.0) with dialysis tubing membrane to remove unreacted monomer, cross-linker and initiators. After dialysis, the nTyr solution was vacuum lyophilized, and the nTyr powder was obtained.

2.4. Construction of electrochemical biosensor based on nTyr

The nTyr based biosensor was prepared by a simple casting method. Prior to modification, the GC electrode was polished on a polishing cloth with 1.0, 0.3 and 0.05 μm alumina powders successively, and washed with Milli-Q water followed by sonicating in ethanol and Milli-Q water respectively. Then the electrode was dried with high purity nitrogen stream. After the optimization of experimental conditions, the final mixture solution containing nTyr and chitosan were 2.5 mg L^{-1} and 1.5 mg L^{-1}, respectively. Then 5 μL of the above mixture solution was dropped onto the surface of freshly polished GC electrode carefully. The modified electrode was covered with a beaker and dried slowly at room temperature to obtain a uniform layer

193 (nTyr-Chi/GC). When not in use, the fabricated electrode was stored at 4 □. Similarly,
194 other electrodes, such as Tyr-Chi/GC and aTyr-Chi/GC electrodes, were prepared with
195 same procedures as described above. Before electrochemical measurements, all the
196 fabricated electrodes were immersed in PBS (50 mM, pH 7.0) for 30 min to remove
197 residual components.

198 **2.5. Electrochemical detection of BPA by fabricated biosensor**

199 The amperometric current-time curves for BPA were obtained to comparatively
200 investigate the performance of different biosensors and demonstrate the
201 electrocatalytic activity of different enzyme states (Tyr, aTyr and nTyr). And the
202 measurements were performed in 5 mL stirring PBS (50 mM, pH 7.0) under an
203 applied potential value of -0.04 V with successive addition of standard BPA solution
204 at appropriated time intervals.

205 **3. Results and Discussion**

206 **3.1. Structural and morphological characterization of nTyr**

207 In this work, the nTyr was synthesized using a simple two-step procedure
208 containing acryloylation and polymerization according to the reported method (Liu et
209 al., 2015b) with minor revision. And the formation of nTyr was confirmed by various
210 characterization techniques. Fig. 1A shows a representative TEM image of nTyr with
211 the polymer shell negatively stained. As shown in Fig. 1A, the nTyr with core-shell
212 structure is clearly observed with light enzyme core inside and dark polymer shell
213 outside, and displays a spherical morphology with a uniform diameter of ~ 14 nm.
214 The native tyrosinase molecule possesses an elliptic shape with a dimension of 6.5 nm

× 9.7 nm × 5.5 nm (Matoba et al., 2006), suggesting that each polymer shell contains a single tyrosinase molecule, and thus the thickness of the polymer shell is around 2.5 nm. The result was also confirmed by the size distribution of native tyrosinase and nTyr which was obtained by DLS measurements. As shown in Fig. 1B, the native tyrosinase shows a size distribution centered at 8 nm which is consistent with its molecular dimension 6.5 nm × 9.7 nm × 5.5 nm (Matoba et al., 2006). For comparison, the size distribution of nTyr is ~ 13 nm, suggesting the successful formation of the polymer shell around each tyrosinase molecule. The successful encapsulation was also characterized by FTIR spectra. As shown in Fig. 1C, almost all the characteristic adsorption peaks of tyrosinase are retained in the phase of aTyr and nTyr, indicating the native secondary structure of tyrosinase is maintained even after acryloylation and polymerization. Compared with native tyrosinase, the peak of nTyr observed at 1652 cm^{-1} (-C=O stretching) is strengthened, which is ascribed to the introduction of -C=O after polymerization. Meanwhile, new characteristic adsorption peaks of nTyr appear at 3380 cm^{-1} (N-H stretching), 1552 cm^{-1} (-NH_2 bending), 1454 cm^{-1} (C-N stretching) and 1120 cm^{-1} (C-N stretching), suggesting the formation of polymer shells.

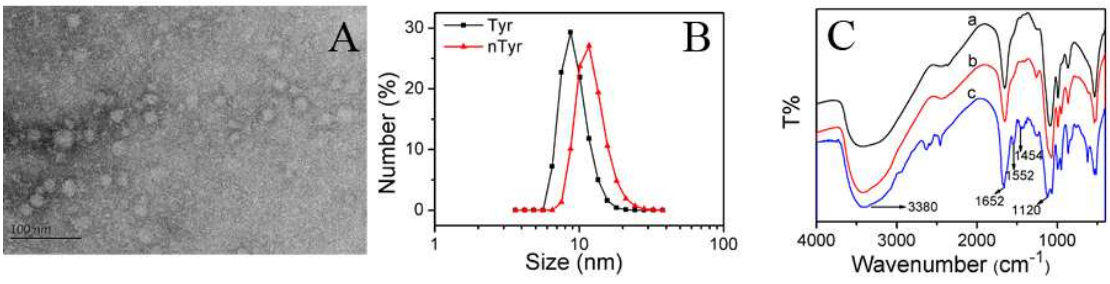


Fig. 1. (A) Representative TEM image of nTyr with spherical morphology. (B) Particle size distributions of native tyrosinase and nTyr by dynamic light scattering.

(C) FTIR spectra of native tyrosinase (a), aTyr (b) and nTyr (c).

3.2 Catalytic activity of nTyr based biosensor

In the process of acryloylation or polymerization of enzyme, the enzyme activity always decreases, which is attributed to the steric hindrance and mass transport resistance (Liu et al., 2015b). In order to ensure the activity of nTyr, we evaluated the relative activity of tyrosinase in every process of encapsulation.

Before the polymerization, acryloylated groups were attached to the surface of tyrosinase, and then we studied the effect of acryloylation on tyrosinase activity. As shown in Fig. 2A, the loss of the activity of tyrosinase is quite small after acryloylation. As the increasing molar ratio of NAS/Tyr from 50 to 200, the relative activity of aTyr are retained at above 85%, which demonstrates that the acryloylation has no significant effect on tyrosinase activity. In the procedure of polymerization, APS and TEMED were used as initiators, which not only could initiate the in-situ polymerization, but also could produce radical ions to inactivate the enzyme. In reported research (Yan et al., 2006), DMAP was employed as stabilizer for HRP, which could protect the enzyme against deactivation. In our case, we also selected DMAP as stabilizer. As shown in the supporting information of Fig. S1, the relative activity of tyrosinase decreases to 21% in the presence of initiators. After adding the stabilizer DMAP in the mixture, the relative activity of tyrosinase significantly increases. When the weight ratio of DMAP/Tyr is higher than 2:5, the relative activity of tyrosinase can be retained at above 80%, indicating that the DMAP is suitable as stabilizer to exert their protection effect during polymerization steps. As a result, there

256 are no obvious effect on tyrosinase activity in the procedure of acryloylation and
257 polymerization.

258 In our case, the nTyr based biosensor displays similar electrocatalytic activity to
259 tyrosinase based biosensor. And the electrocatalytic activity of nTyr-Chi/GC and
260 Tyr-Chi/GC biosensors were evaluated by amperometric method for BPA detection.
261 Fig. 2B illustrates and compares the typical amperometric I-t curves of Tyr-Chi/GC
262 and nTyr-Chi/GC electrodes at optimized potential of -0.04 V with successive
263 additions of BPA standard solution into PBS (50 mM, pH 7.0) under constant stirring.
264 As shown in Fig. 2B, well-defined amperometric signals are observed, indicating that
265 both tyrosinase and nTyr possess high biocatalytic activity. The response times of
266 Tyr-Chi/GC and nTyr-Chi/GC electrodes are less than 10 s (achieving 90% of
267 steady-state current), indicating the nTyr based biosensor can ensure the fast
268 transportation of substrate and electrochemical signals. Fig. 2C shows the typical
269 calibration curves of response currents of these two biosensors versus the
270 concentration of BPA. The sensitivity of nTyr-Chi/GC biosensor is 3000.7 mA cm^{-2}
271 M^{-1}, which is equivalent to that of Tyr-Chi/GC biosensor (2901.6 mA cm^{-2} M^{-1}). The
272 linear range of nTyr-Chi/GC biosensor is ranging from $5 \times 10^{-8} \sim 2 \times 10^{-6}$ mol L^{-1}
273 with a correlation coefficient of 0.996, and the limit of detection (LOD) is estimated
274 to be 12 nmol L^{-1}. The analytical performances of nTyr-based biosensor are
275 summarized and compared with other reported tyrosinase biosensors, as shown in
276 Table S1. It can be seen that the fabricated nTyr-Chi/GC biosensor without using
277 other nanomaterial for signal enhancement still exhibits a good linear range and high

sensitivity for BPA detection. Although the LOD, linear range and sensitivity of nTyr-Chi/GC biosensor are comparable to those reported native tyrosinase biosensor, the thermal stability, organic solvent tolerance and acid/base tolerance are dramatically enhanced. These results imply that the encapsulated nTyr can maintain their secondary structure and retain native biocatalytic activity, meanwhile allowing rapid substrates transportation through the porous polymer shell to react with tyrosinase.

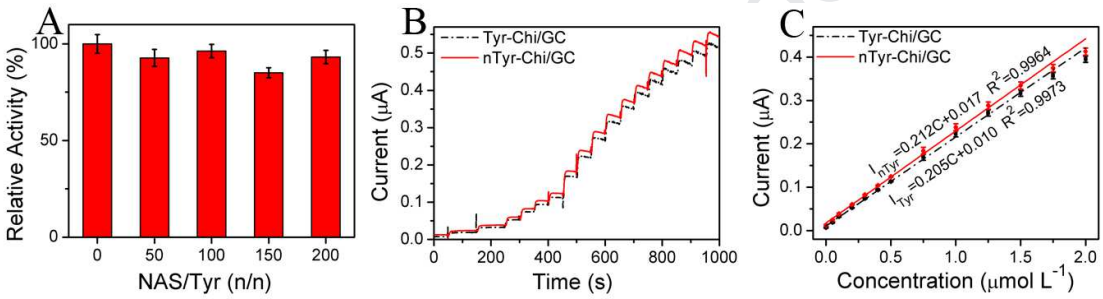


Fig. 2. (A) Relative activity of modified tyrosinase after reaction with NAS, and the different molar ratio of NAS/Tyr: 0, 50, 100, 150, 200. (B) The typical amperometric response curves of Tyr-Chi/GC and nTyr-Chi/GC biosensors with successive additions of BPA into a stirring PBS (50 mM, pH 7.0). Applied potential: -0.04 V versus Ag/AgCl. (C) The corresponding calibration curves of steady-state currents versus concentrations of BPA.

3.3 Enhanced stability of nTyr based biosensor

Besides the high electrocatalytic activity, nTyr based biosensor also exhibits significantly enhanced stability against various denaturation factors, such as high temperature, organic solvents and low/high pH. In order to investigate the stability of nTyr, the nTyr-Chi/GC biosensor for BPA detection was estimated by amperometric

297 method in different operational conditions, and then the electrocatalytic activity of
298 nTyr and native tyrosinase based biosensors were compared.

299 3.3.1 Thermal stability of nTyr based biosensor

300 In high operational temperatures, native enzymes always lose their tertiary or
301 secondary protein structure, resulting in denaturation (Daniel et al., 1996). As for
302 electrochemical enzyme biosensor, it is important to improve the thermal stability.
303 The enhanced stability of nTyr based biosensor was firstly demonstrated by testing the
304 thermal stability at elevated temperature. As shown in Fig. 3A, the relative activities
305 of nTyr-Chi/GC biosensor are compared with that of Tyr-Chi/GC biosensor after
306 incubating at different high temperature (45 □, 55 □ and 65 □) for 1 h. It can be seen
307 from Fig. 3A, the nTyr-Chi/GC biosensor exhibits significantly higher relative activity
308 than that of Tyr-Chi/GC biosensor. After 65 □ incubation for 1 h, the Tyr-Chi/GC
309 biosensor retained 31.9% of the original activity, whereas the nTyr-Chi/GC biosensor
310 retained more than 61.3%, indicating the nTyr based biosensor possesses much higher
311 thermal stability. The enhanced thermal stability of nTyr based biosensor might be
312 attributed to the nTyr with much multipoint covalent attachments between the
313 tyrosinase core and polymer shell, which could effectively protect the tyrosinase
314 against the conformation change and denaturation while they are heated.

315 In order to confirm the relationship of stabilizing effect and the numbers of
316 polymerizable groups, the nTyr with different numbers of covalent linkages between
317 the tyrosinase core and the polymer shell were formed, and the thermal endurance was
318 further determined by incubating at 65 □ for 1 h. Fig. 3B shows the relative activity

of biosensor based on nTyr prepared from tyrosinase treated with different NAS/Tyr weight ratios (0.05:1, 0.1:1, 0.2:1), and the nTyr based biosensor exhibits enhanced thermal stability with the increasing NAS/Tyr weight ratio. The number of residual lysine (Lys) was also estimated by comparing the fluorescence intensity of native tyrosinase and aTyr, and the result was listed in Table S2. From the Table S2, we can see that the number of acryloylated groups conjugated onto the tyrosinase surface increases with the increasing weight ratio of NAS/Tyr. The results indicate that the enhanced stability of nanocapsule might be contributed to the increased number of covalent linkages. And in the weight ratios of NAS/Tyr ranging from 0.05:1 ~ 0.2:1, the more covalent linkages attach the tyrosinase core and polymer shell, the higher stability of nTyr can achieve. On the basis of these results, the nTyr not only provide protective microenvironment for tyrosinase against the changes of conformation, but also retain the high catalytic activity even at very high operational temperature.

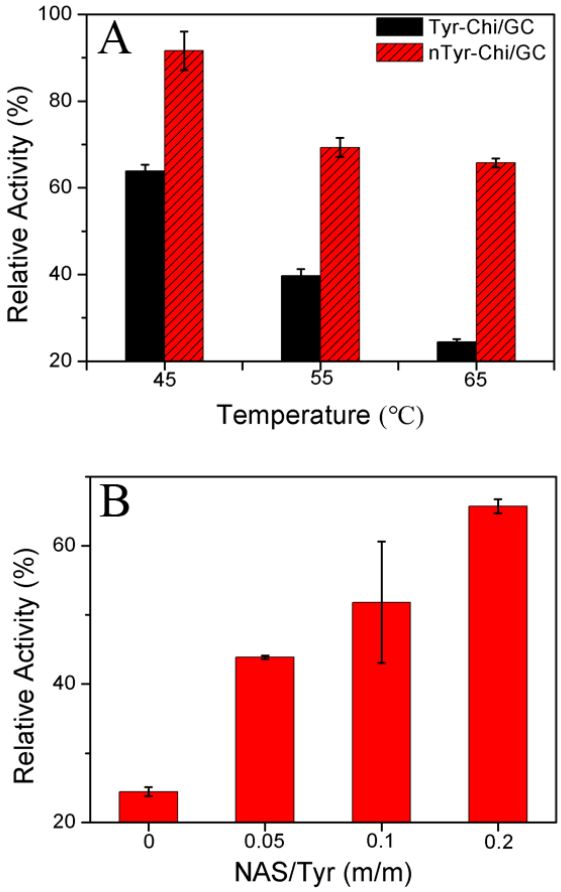


Fig. 3. nTyr based biosensor with enhanced thermal stability. (A) Relative activities of nTyr-Chi/GC and Tyr-Chi/GC biosensors incubated at 45 □, 55 □ and 65 □ for 1 h. (B) After incubated at 65 □ for 1 h, residual relative activities of biosensor based on nTyr prepared from tyrosinase treated with increasing NAS/Tyr weight ratio of 0.05:1, 0.1:1 and 0.2:1.

3.3.2 Organic solvent tolerance of nTyr based biosensor

In organic solvents systems, native enzymes always exhibit low activities and stabilities, which limit their applications. The tertiary or secondary structure of enzyme is related to the activity, which is maintained by the intricate balance between hydrophobic interactions, electrostatic charge interactions, Van der Waals forces and hydrogen bonds (Doukyu and Ogino, 2010). The organic solvents can compete with

water to form hydrogen bonding with protein backbones, which destroy the enzyme conformation and disrupt the balance between attractive interactions leading to the loss of activity (Serdakowski and Dordick, 2008). Especially in highly polar organic solvent, the enzymes are more unstable and inactive.

The enhanced organic solvent tolerance of nTyr based biosensor was investigated by exposing the nTyr-Chi/GC and Tyr-Chi/GC biosensors to organic solvent-aqueous mixtures (V:V=50:50) for 1 h. As presented in Fig. 4A, compared with Tyr-Chi/GC biosensor, the nTyr-Chi/GC biosensor displays significantly higher relative activity and only a little of electrocatalytic activity is lost. For instance, the nTyr-Chi/GC biosensor only loses about 3% of its original activity after incubation in dimethyl sulfoxide (DMSO), while the Tyr-Chi/GC biosensor loses about 26%. And similar results are observed from the determination of the biosensors in N,N-dimethylformamide (DMF), ethanol (EtOH) and methanol (MeOH), confirming that the nTyr-Chi/GC biosensor exhibits obviously improved stability in organic solvent-aqueous mixtures.

The increased organic solvent tolerance of nTyr-Chi/GC biosensor might be attributed to the hydrophilic polymer shells of nTyr, which could provide a hydrophilic microenvironment against the loss of essential water that are necessary for retaining the tyrosinase activity and against the denaturation of tyrosinase from structure changes (Wei et al., 2013), resulting in higher electrocatalytic activity of the nTyr-Chi/GC biosensor in polar organic solvent. The enzyme nanocapsule based biosensor is proved to be a powerful technique to open up the new application fields

of biosensors in organic solvent system, and provides a great application prospect in biomedical detection, industrial and environmental analysis. For instance, when the target substrates are difficult to dissolve in aqueous solution, this kind of enzyme nanocapsule based biosensor with improved stability can be applied instead of conventional biosensor.

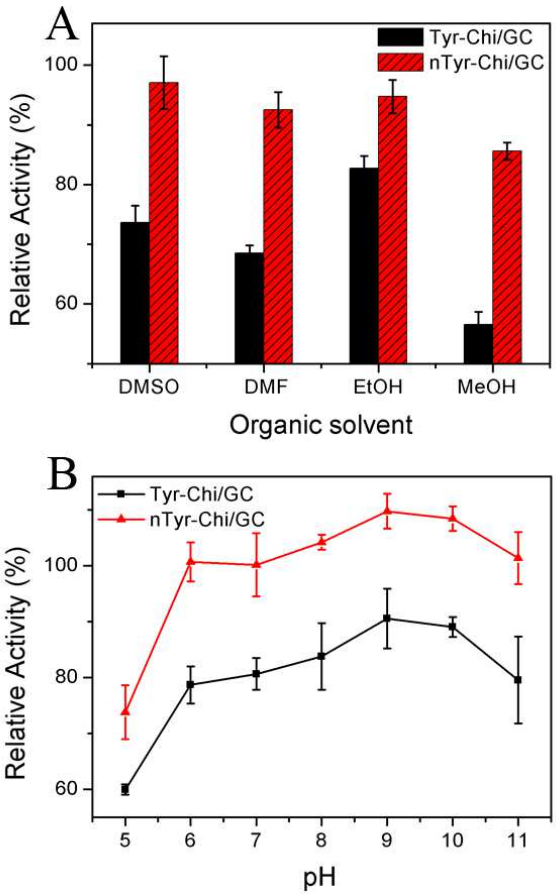


Fig. 4. The enhanced stability of nTyr based biosensor in organic solvent and acid/base condition. (A) Relative activities of nTyr-Chi/GC and Tyr-Chi/GC biosensors exposed to the mixture solution of PBS (50 mM, pH 7.0) and different kinds of organic solvents DMSO, DMF, EtOH and MeOH (V:V=50:50) for 1 h. (B) Relative activities of nTyr-Chi/GC and Tyr-Chi/GC biosensors exposed to 50 mM PBS solution with different pHs (5, 6, 7, 8, 9, 10, 11) for 1 h.

3.3.3 Acid/base tolerance of nTyr based biosensor

The enzyme dissolved in aqueous solution at optimum pH possesses highest catalytic activity, and the interactions between the side chains of the amino acids determine the structure of enzyme. Four types of attractive interactions (Doukyu and Ogino, 2010) determine the shape and stability of an enzyme, while the changes of pH affect the electrostatic charge and hydrogen bonds interactions. Electrostatic charge interactions are ionic bonds between positively and negatively charged side chains of amino acids. The increasing pH converts the $-NH_3^+$ to neutral $-NH_2$ group by adding a base, and the decreasing pH converts the $-COO^-$ to a neutral -COOH group by adding an acid. In each case, the ionic attraction disappears resulting in the enzyme shape unfolded (Sun et al., 2019). Besides, various amino acid side chains can form hydrogen bond to each other. The changing pH disrupts the hydrogen bonds, leading to the conformational changes of enzyme (Sun et al., 2019).

The improved pH tolerance of nTyr based biosensor was demonstrated by exposing the nTyr-Chi/GC or Tyr-Chi/GC biosensor to 50 mM PBS with different pHs (5, 6, 7, 8, 9, 10, 11) for 1 h. As shown in Fig. 4B, the nTyr-Chi/GC biosensor displays higher relative activity than Tyr-Chi/GC biosensor, suggesting the nTyr has increased acid and base tolerance. Clearly, the nTyr-Chi/GC biosensor did not lose any activity within the pH range of 6-11. And in the pH range from 8 to 11, the activity of nTyr-Chi/GC biosensor after incubation was even higher than that before incubation, while the Tyr-Chi/GC biosensor lost partial original activity. The results demonstrates that the nTyr-Chi/GC biosensor exhibits higher electrocatalytic activity

and pH tolerance against the pH alteration, indicating the polymer shell could protect tyrosinase to maintain its original conformation and retain natural activity. From the Fig. 4B, both the nTyr-Chi/GC and Tyr-Chi/GC biosensors lose much original activity after incubation at pH 5, which might be attributed to the film of nTyr-Chi or Tyr-Chi dropping from the electrode surface. Chitosan is a linear biopolymer for immobilizing enzymes onto the GC electrode surface, because of its excellent film-forming ability (Wang et al., 2015). And the chitosan solution is always prepared by dissolving chitosan flakes in 1.0 wt% acetic acid solution, and then adjust the pH to 4.0 ~ 5.0 using 10% NaOH solution. Therefore, the film on the electrode is easily falling off in lower pH (pH < 5), and further work should be done to solve this challenge. If a film-forming material with better pH tolerance is used for immobilizing nTyr on the GC electrode surface, the nTyr-Chi/GC biosensor can be used in lower pH range (pH < 5). The results indicates the biosensors based on nTyr can be operated in extreme wide pH range and possess much higher activity than those biosensors based native tyrosinase.

3.4 Reproducibility and real sample application of the nTyr based biosensor

The electrode-to-electrode fabrication reproducibility of nTyr-Chi/GC biosensor was evaluated by amperometry for the detection of 0.25 μM BPA with three individual nTyr-Chi/GC biosensors prepared under the same conditions. The relative standard deviation (RSD) value of interelectrode is 3.4%, indicating excellent electrode-to-electrode reproducibility. Similarly, the repeatability of intraelectrode was also investigated, and the RSD value of 4.9% was obtained for 4 successive

422 determinations, revealing acceptable repeatability of the biosensing method. Besides
423 the excellent reproducibility, repeatability and significantly enhanced stability in
424 extreme conditions, the nTyr based biosensor also exhibited good long-term stability,
425 which still remained 98% of its initial activity after 18 days (stored at 4 □ when not in
426 use), providing an excellent advantage in practical applications.

427 In order to further estimate the performance of the proposed nTyr-Chi/GC
428 biosensor for real sample analysis, the biosensor was used to detect BPA leaching
429 from plastic samples. The pretreatment of plastic products (purchased from local
430 supermarket) referred to the state standard of China (GB 5009.156-2016). In brief,
431 two kind of plastic products were cut into small pieces (1 cm × 1 cm) and washed
432 thoroughly with Milli-Q water by sonicating within 3 min. Then 1.0 g of plastic
433 product pieces were immersed in 20 mL Milli-Q water and heated at 78 □ for 48 h.
434 After cooling to room temperature, the liquid phase was filtrated and collected into a
435 50 mL volumetric flask, then diluted to 50 mL with Milli-Q water. A known-amount
436 of the obtained sample solutions and the samples added BPA standard solution (0.6
437 μM) were analyzed by the fabricated nTyr-Chi/GC biosensor with amperometric
438 measurement. The obtained results were listed in Table 1. The content of BPA in water
439 bottle (PC) sample was calculated to be 7.8 μg/g, while BPA was not found in coffee
440 spoon (PP). The results revealed that BPA could leached from PC plastic into water
441 after heating, while no BPA leached from the PP material. The recoveries of PC and
442 PP plastic samples with added BPA standard solution were 94.8% and 96.0%
443 respectively, indicating that the nTyr based biosensor was a promising and reliable

444 tool for the sensitive and rapid detection of BPA leaching from PC and epoxy resins
445 products.

446 Table 1. Determination of BPA in plastic product samples.

Sample[a]	Measured (μM)[b]	Added (μM)	Found (μM)[b]	Recovery (%)
Water bottle (PC)	0.684	0.6	1.253	94.8
Coffee spoon (PP)	n.d.[c]	0.6	0.576	96.0

447 [a] PC = polycarbonate; PP= polypropylene.
448 [b] The average value of three determinations.
449 [c] n.d. means “not detectable”.

450 **4. Conclusions**

451 We have demonstrated a simple and efficient approach to prepare robust and
452 high active nTyr containing a single tyrosinase molecule core and a porous polymer
453 shell through a two-step in-situ polymerization in aqueous solution. And the prepared
454 nTyr is used to construct an electrochemical biosensor for BPA detection, which not
455 only retains the original activity of native tyrosinase, but also exhibits significantly
456 enhanced stability including excellent thermal stability, organic solvents tolerance and
457 acid/base tolerance. The multipoint covalent attachments between the tyrosinase cores
458 and the rigid polymer shells could effectively protect the enzyme against the
459 conformational change and denaturation when they are operated at high temperature
460 or exposed to organic solvents. And the polymer shells could provide biocompatible
461 microenvironment to effectively inhibit the dissociation of tyrosinase at extreme
462 conditions. In addition, the network polymer shells exhibits extraordinary substrate
463 permeability, allowing the rapid substrate transportation and resulting in fast response.

The nTyr based biosensor is further used to detect BPA leached from plastic product samples, displaying fast response and satisfactory recoveries. Such enzyme nanocapsules based biosensors provide an efficient method to dissolve unstability issues of conventional enzyme biosensors, which will absolutely promote the development of biosensors and broad their application in biomedical fields, industrial fields, environmental analysis, etc.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (Grant No. 21577139) and the Natural Science Foundation of Liaoning Province (2019-MS-317).

475 **References**

476 Andreescu, S., Sadik, O.A., 2004. Anal. Chem. 76(3), 552-560.

477 Cai, J., Liu, S., Feng, J., Kimura, S., Wada, M., Kuga, S., Zhang, L., 2012. Angew.
478 Chem. Int. Ed. 51(9), 2076-2079.

479 Chen, Y.-P., Wang, D.-N., Yin, Y.-M., Wang, L.-Y., Wang, X.-F., Xie, M.-X., 2012. J.
480 Agric. Food Chem. 60(42), 10472-10479.

481 Daniel, R.M., Dines, M., Petach, H.H., 1996. Biochem. J. 317, 1-11.

482 Dhanjai, Lu, X., Wu, L., Chen, J., Lu, Y., 2020. Anal. Chem. 92, 5830-5837.

483 Doukyu, N., Ogino, H., 2010. Biochem. Eng. J. 48(3), 270-282.

484 Du, J., Yu, C., Pan, D., Li, J., Chen, W., Yan, M., Segura, T., Lu, Y., 2010. J. Am.
485 Chem. Soc. 132(37), 12780-12781.

486 Fisher, M., Arbuckle, T.E., MacPherson, S., Braun, J.M., Feeley, M., Gaudreau, E.,
487 2019. Environ. Sci. Technol. 53(18), 10813-10826.

488 GB 5009.156-2016. National Food Safety Standard-Food contact materials and
489 products-Migration test-General rules for preprocessing.

490 Govindan, B., Madhu, R., Chen, S.-M., Veeramani, V., Balamurugan, A., Mangalaraj,
491 D., Viswanathan, C., Nagamony, P., 2014. J. Mater. Chem. B 3(7), 1360-1370.

492 Gu, Z., Yan, M., Hu, B., Joo, K.-L., Biswas, A., Huang, Y., Lu, Y., Wang, P., Tang, Y.,
493 2009. Nano Lett. 9(12), 4533-4538.

494 Huang, R.P., Liu, Z.H., Yin, H., Dang, Z., Wu, P.X., Zhu, N.W., Lin, Z., 2018. Sci.
495 Total Environ. 626, 971-981.

496 Jiang, T., Mo, R., Bellotti, A., Zhou, J., Gu, Z., 2014. Adv. Funct. Mater. 24(16),

2295-2304.

Jiménez, M., Chazarra, S., Escribano, J., Cabanes, J., García-Carmona, F., 2001. J. Agric. Food Chem. 49(8), 4060-4063.

Lee, D.H., 2018. Endocrinol. Metab. 33(1), 44-52.

Liu, C., Wen, J., Meng, Y., Zhang, K., Zhu, J., Ren, Y., Qian, X., Yuan, X., Lu, Y., Kang, C., 2015a. Adv. Mater. 27(2), 292-297.

Liu, L., Yu, W., Luo, D., Xue, Z., Qin, X., Sun, X., Zhao, J., Wang, J., Wang, T., 2015b. Adv. Funct. Mater. 25(32), 5159-5165.

Liu, Y., Du, J., Yan, M., Lau, M.Y., Hu, J., Han, H., Yang, O.O., Liang, S., Wei, W., Wang, H., Li, J., Zhu, X., Shi, L., Chen, W., Ji, C., Lu, Y., 2013. Nat. Nanotechnol. 8(3), 187-192.

Matoba, Y., Kumagai, T., Yamamoto, A., Yoshitsu, H., Sugiyama, M., 2006. J. Biol. Chem. 281(13), 8981-8990.

Mu, J., Zhang, L., Zhao, M., Wang, Y., 2014. ACS Appl. Mater. Interfaces. 6(10), 7090-7098.

Romera-Garcia, E., Caballero-Casero, N., Rubio, S., 2019. Talanta 204, 465-474.

Serdakowski, A.L., Dordick, J.S., 2008. Trends Biotechnol. 26(1), 48-54.

Su, Y., Wu, D., Chen, J., Chen, G., Hu, N., Wang, H., Wang, P., Han, H., Li, G., Wu, Y., 2019. Anal. Chem. 91(18), 11687-11695.

Sun, L.-C., Lin, Y.-C., Liu, W.-F., Qiu, X.-J., Cao, K.-Y., Liu, G.-M., Cao, M.-J., 2019. Food Hydrocoll. 93, 137-145.

Tian, H., Du, J., Wen, J., Liu, Y., Montgomery, S.R., Scott, T.P., Aghdasi, B., Xiong,

519 C., Suzuki, A., Hayashi, T., Ruangchainikom, M., Phan, K., Weintraub, G., Raed,
520 A., Murray, S.S., Daubs, M.D., Yang, X., Yuan, X.-b., Wang, J.C., Lu, Y., 2016.
521 ACS Nano 10(8), 7362-7369.
522 Wang, X., Lu, X., Wu, L., Chen, J., 2015. Biosens. Bioelectron. 65, 295-301.
523 Wei, W., Du, J., Li, J., Yan, M., Zhu, Q., Jin, X., Zhu, X., Hu, Z., Tang, Y., Lu, Y.,
524 2013. Adv. Mater. 25(15), 2212-2218.
525 Wu, L., Gao, J., Lu, X., Huang, C., Dhanjai, Chen, J., 2020. Carbon 156, 568-575.
526 Wu, L., Lu, X., Zhang, H., Chen, J., 2012. ChemSusChem 5(10), 1918-1925.
527 Wu, L., Yan, H., Wang, J., Liu, G., Xie, W., 2019. J. Electrochem. Soc. 166(8),
528 B562-B568.
529 Xiong, L., Yan, P., Chu, M., Gao, Y.Q., Li, W.H., Yang, X.L., 2018. Food Chem. 244,
530 371-377.
531 Xu, D., Han, H., He, Y., Lee, H., Wu, D., Liu, F., Liu, X., Liu, Y., Lu, Y., Ji, C., 2018.
532 Adv. Mater. 30(22), 1707443.
533 Yan, M., Du, J., Gu, Z., Liang, M., Hu, Y., Zhang, W., Priceman, S., Wu, L., Zhou,
534 Z.H., Liu, Z., Segura, T., Tang, Y., Lu, Y., 2010. Nat. Nanotechnol. 5(1), 48-53.
535 Yan, M., Ge, J., Liu, Z., Ouyang, P., 2006. J. Am. Chem. Soc. 128(34), 11008-11009.
536 Yang, H., Carr, P.D., McLoughlin, S.Y., Liu, J.W., Horne, I., Qiu, X., Jeffries, C.M.J.,
537 Russell, R.J., Oakeshott, J.G., Ollis, D.L., 2003. Protein Engineering 16(2),
538 135-145.
539 Yu, H., Han, J., An, S., Xie, G., Chen, S., 2018. Biosens. Bioelectron. 109, 63-69.
540 Zhang, C., Yan, K., Hu, C., Zhao, Y., Chen, Z., Zhu, X., Möller, M., 2015. J. Mater.

541 Chem. B 3(7), 1261-1267.

542 Zheng, D., Vashist, S.K., Al-Rubeaan, K., Luong, J., Sheu, F.-S., 2012. Analyst 137,

543 3800-3805.

544 Zou, J., Zhao, G.-Q., Teng, J., Liu, Q., Jiang, X.-Y., Jiao, F.-P., Yu, J.-G., 2019.

545 Microchem. J. 145, 693-702.

546

Highlights

- Robust single molecule enzyme nanocapsules of Tyrosinase (nTyr) were firstly synthesized.
- Polymer shell effectively stabilized the interior tyrosinase core while enabling rapid substrate transportation.
- A new class of highly stable nTyr based biosensors was constructed for detecting BPA in different application scenarios.
- The nTyr based biosensor displayed significantly enhanced stability in extreme operational conditions.
- The nTyr based biosensor provides a prospective solution to resolve the stability problem of enzyme biosensors.

Declaration of interests

☑The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: