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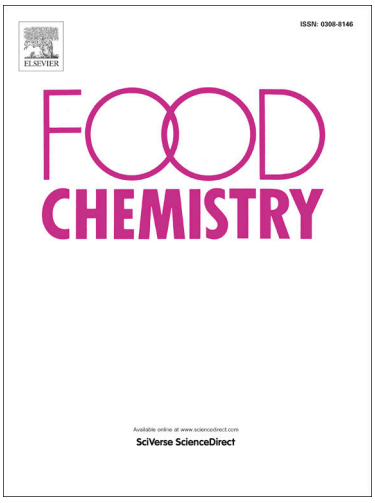

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A fluorescent biosensor based on catalytic activity of platinum nanoparticles for freshness evaluation of aquatic products

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Abstract

In this study, a fluorescence biosensor based on the peroxidase mimicking activity of platinum nanoparticles (Pt NPs) was fabricated for rapid detection of hypoxanthine (Hx), which is a sensitive indicator of the freshness of aquatic products. The fluorescence intensity of the sensing system had a linear relationship with the concentration of Hx in the range of 8-2500 μM, and the limit of detection was as low as 2.88 μM (S/N=3). Moreover, benefiting from the excellent selectivity of the biosensor, Hx content in fish, shrimp and squid samples could be quickly detected with good recovery rates (103.94-109.00%). And the Pt NPs used in the biosensor was reusable, which was proved by the recovery rate was only slightly decreased to 91% after three cycles. In addition to the advantages of facile preparation and low cost, the proposed biosensor will be a promising candidate for rapid and convenient freshness evaluation of aquatic products.

Key words:

Fluorescence biosensors; hypoxanthine; freshness of aquatic products; platinum nanoparticles

1. Introduction

The freshness of aquatic products is an important indicator for evaluating its quality (Cardenas Bonilla, Sveinsdottir, & Martinsdottir, 2007). Due to their nutrient-rich nature, aquatic products are prone to deterioration during storage and sales under the joint action of enzymes and microorganisms (Cleach, Pasdois, Marchetti, Watier, Duflos, Goffier, et al., 2019). However, freshness evaluation of aquatic products is complex because the spoilage of meat involves different microbiological, physicochemical and biochemical reactions (Ghaly, 2011; Gil, Barat, Baigts, Martínez-Máñez, Soto, Garcia-Breijo, et al., 2011). Traditionally, there have been two methods to assess the freshness of aquatic products: (1) a sensory test controlling the organoleptic features by experts, and (2) chemical and biochemical determination of the concentration of target biomarkers (Albelda, Uzunoglu, Santos, & Stanciu, 2017). Although the former method has been shown to be rapid, it is expensive and sometimes unreliable because it is difficult to assess the subtle differences in freshness of aquatic products before the initial stages of degradation (Cleach, et al., 2019). The latter method has attracted much attention and a large number of studies have attempted to evaluate the freshness of aquatic products by detecting biomarkers due to the merits of high reliability and sensitivity (Galgano, Favati, Bonadio, Lorusso, & Romano, 2009).

In recent years, various biomarkers were found to be associated with the spoilage of meat and employed as indicators of the freshness of aquatic products. For example, Chow monitored the level of total volatile base nitrogen (TVB-N) generated by the metabolic reactions of spoilage microorganisms during storage of sea bream fillets and

found the TVB-N could be used as a chemical index of freshness in fish (Castro, Padrón, Cansino, Velázquez, & Larriva, 2006; Parlapani, Mallouchos, Haroutounian, & Boziaris, 2014). In addition, pH and bacterial counts (Önal, 2007) are also important indicators for determine the freshness of aquatic products. Some novel intelligent pH-sensing systems were used in the evaluation of fish freshness (Kırca, Özkan, & Cemeroğlu, 2007; Moradi, Tajik, Almasi, Forough, & Ezati, 2019). In comparison to the above mentioned biomarkers, hypoxanthine (Hx), a metabolic intermediate of nucleotides, is much more sensitive to the spoilage of aquatic products, since it has been found that one of the most important reasons affecting the freshness of aquatic products is the formation of nucleotides and nucleoside metabolites produced by the degradation of adenosine triphosphate (ATP) (Batlle, Aristoy, & Toldra, 2001). Therefore, the freshness of aquatic products can be determined at the early stage of spoilage by detection the level of Hx (Batlle, Aristoy, & Toldra, 2001; Scheffler & Gerrard, 2007).

Hx can be determined using classical methods such as HPLC (Fukuuchi, Yasuda, Inazawa, Ota, Yamaoka, Mawatari, et al., 2013; Luong, Pierre, & B, 1997), spectrophotometric (Amigo, Coello, & Maspoch, 2005) and chromatographic measurements (Cooper, Khosravan, Erdmann, Fiene, & Lee, 2006; Rong, Zou, Zhang, Zhang, Li, Li, et al., 2015). These methods, in general, are time consuming, expensive and require skilled persons to operate the necessary apparatus. To overcome the shortcomings of the above traditional methods, various biosensors including electrochemical fluorescence (Gorgulu, Cete, Arslan, & Yasar, 2013; Wang & Tong, 2010) and optical sensors were developed for determination of Hx, because biosensors

possesses numerous merits such as simplicity, rapid analysis, and high sensitivity. Among various biosensors, fluorescence analysis has become one of the most powerful bio-analytical and diagnostic tools due to its inherent advantages such as high sensitivity and anti-interference performance. Hu., et al. developed a 2D NH_2-Cu-MOFs fluorescent biosensor to detect Hx in fish (Hu, Yan, Huang, Guo, Lin, Luo, et al., 2018). Zhang., el.al reported a fluorescent luminogen with aggregation-induced emission feature which along with xanthine oxidize (XOD) to detect and quantify Hx (Zhang, Kwok, Yu, Tang, & Ng, 2018). However, the synthesis of the materials used in most reported methods was usually tedious and the sensor preparation was complicated. Hence, there is still a lot of scope to develop a fluorescent biosensor based on easily obtained materials for rapid and sensitive detection of Hx.

Due to their unique properties such as high surface area and small size effect, various nanomaterials including inorganic-organic hybrid (Md Rabiul Awual, Alharthi, Hasan, Karim, Islam, Znad, et al., 2017), ligand impregnated conjugate nanomaterials (Md Rabiul Awual, 2016, 2017) , silica-based porous nanomaterials (Md Rabiul Awual, Ismael, Yaita, El-Safty, Shiwaku, Okamoto, et al., 2013; M. R. Awual, Yaita, Suzuki, & Shiwaku, 2015) and noble metal nanoparticles were employed to construct biosensors. Platinum nanoparticles (Pt NPs) are such a widely used nanomaterials for their excellent stability, unique catalytic activity and facile preparation. Wu et al. prepared a biosensor based on Pt NPs for sensitive and selective detection of Hg^{2+} ions (Wu, He, Peng, Deng, Liu, Lin, et al., 2014). Deng et al. reported a biosensor for ultrasensitive colorimetric detection of silver ion by utilizing the target-triggered

inhibition of the oxidase-mimicking activity of Pt NPs (Deng, He, Lin, Yang, Lin, Chen, et al., 2019). For the advantages mentioned above, we chose Pt NPs to construct the biosensor in this study.

Herein, in order to achieve simple and rapid freshness evaluation of aquatic products, we propose a fluorescence sensor based on the peroxidase-like catalytic activity of Pt NPs to detect Hx in aquatic products. The materials used in this biosensor were commercial available or easily obtained, and the measurements were rapid, simple and convenient. Moreover, benefitting from the advantages such as good linear range, low detection limit, high selectivity and good repeatability, this fluorescence biosensor based on catalytic activity of Pt NPs could be employed to analyze real samples such as fish, shrimp and squid with good recoveries, which make it a promising biosensor for rapid evaluation of the freshness of aquatic products.

2. Experimental

2.1 Synthesis of Pt NPs

The Pt NPs were prepared according to a previously described method (Wu, He, Peng, Deng, Liu, Lin, et al., 2014). Briefly, 1 mL of a $H_2PtCl_6 \cdot 6H_2O$ (16 mM) aqueous solution and 1 mL of a trisodium citrate solution (40 mM) were mixed and the mixture was added dropwise to a glass flask charged with 38 mL of water under stirring. After being stirred for 30 min at room temperature, 200 μL of $NaBH_4$ solution (50 mM) was added and the resulted mixture was allowed to react at room temperature for 1 h to yield a solution of Pt NPs, which was kept at 4 ^oC for further use.

2.2 Calibration curve for Hx

Different concentrations of Hx were separately reacted with XOD solution (0.1 U/mL, 40 min, 300 K) in a 37 °C shaker for 30 minutes, followed by adding of Pt NPs and OPD (2 mM). The resulting mixtures with a final volume of 200 μL were incubated at 37 °C for 30 minutes, and then their fluorescence emission spectra were recorded. The relationship between fluorescence intensities (FL intensities) and Hx concentrations is plotted as a calibration curve.

2.3 Detection of Hx in the aquatic products

Aquatic samples (fish, shrimp or squid, 5.0 g) were mixed with 3.0 mL of 10% perchloric acid solution and incubated at 100 °C for 30 min to extract the Hx. After the mixtures were centrifuged at 8000 rpm for 20 min, the supernatants were collected and the pH was adjusted to 4.0. Subsequently, XOD (0.1 U/mL) was added into the supernatants, and the concentrations of Hx in these supernatants were measured following the same procedures described in section 2.2. In addition, the Hx concentrations were also detected by HPLC as reference.

2.4 Recycle of the Pt NPs

After the first test is completed, the fluorescing mixture was centrifuged at 10,000 r/min for 10 min, and then washed twice with ultrapure water to collect Pt NPs for reuse. Subsequently, the recovered Pt NPs were used to detect Hx following the procedures described in item 2.2 to explore the recycle of the Pt NPs. This recycle experiment was repeated for 5 times.

3. Results and discussion

3.1 Design and fabrication of biosensors for Hx detection.

Scheme 1 illustrates the sensing mechanism of Hx, the proposed biosensor was comprised of XOD, OPD and Pt NPs, in which Pt NPs were used as peroxidase mimic enzyme. Primarily, Hx reacted with oxygen in the presence of XOD and hence yielded uric acid and H_2O_2. Then under the catalysis of Pt NPs, OPD was oxidized by H_2O_2 to produce 2,3-phenylenediamine, which exhibited bright yellow fluorescence (Sun, Wang, Zhao, Li, & Yang, 2016; Yang & Wang, 2011). But with increase of the concentration of 2,3-phenylenediamine, its fluorescence will be quenched due to aggregation-caused quenching. Therefore, the FL intensity of 2,3-phenylenediamine depended on the concentration of Hx, and the content of Hx in aquatic products could be simply determined by measuring the FL intensity.

3.2 Preparation and characterization of Pt NPs

The size and morphology of the nanoparticles was analyzed by transmission electron microscopy (TEM). As shown in Figure 2A, the Pt NPs were largely monodispersed and roughly spherical with an average diameter of approximately 4.3 nm. The average hydrodynamic size measured by DLS was about 5.146 nm (Figure 2B), which was a little larger than that revealed by TEM, since the samples used for TEM observation were in a dry form. According to the N_2 adsorption-desorption isotherm, the adsorption behavior indicated a typical type I microporous material adsorption isotherm (Figure 2C). Due to the strong interaction between the adsorbate and the pore walls, the adsorption began at a very low pressure and capillary agglomeration was not observed in the micropore filling (Md Rabiul Awual, 2015; Md Rabiul Awual & Hasan, 2014; Shahat, Awual, & Naushad, 2015). On the other hand,

the specific surface area as well as the pore volume and pore size values were shown in the inset of Figure 2C. These data also confirmed the uniform geometry and porosity of Pt NPs. It has been reported that nanoparticles with a diameter of less than 6 nm and porous structure will possess high activity for combustion and selective oxidation reactions since they possess high specific surface area and can sufficiently bind with oxygen to enable the activation of O_2 (Campbell, 2013; Goodman, 1995; Valden, Lai, & Goodman, 1998). Therefore, the size and structure of the obtained Pt NPs were satisfying for its peroxidase mimicking role in this study.

3.3 Feasibility of the fluorescence sensor

To validate the feasibility of this project, consecutive assay was carried out. As shown in Figure S1A, no peak was observed in the fluorescence excitation spectrum of Pt NPs, while after adding H_2O_2 and OPD to the suspension of Pt NPs, a maximum emission peak at 580 nm was appeared. The concentration of H_2O_2 played an important role in the quenching effect. After adding H_2O_2 with different concentrations (20 μM, 5 mM), the FL intensity was significantly quenched and the quenching efficiency increased with decrement of the concentration. To further verify the response of the fluorescence sensor to H_2O_2, we also detected the FL intensities of the sensing systems after adding H_2O_2 with different concentrations. As demonstrated in Figure S1C-D, the FL intensity gradually decreased with the increase of H_2O_2 concentration, and a linear relationship between FL intensity and the concentration of H_2O_2 ranging from 5 to 10000 μM with a correlation coefficient of 0.990 was achieved, indicating that the fluorescence sensor is theoretically applicable to detect reactions involving H_2O_2.

Subsequently, we further examined the feasibility of detecting Hx by replacing the H_2O_2 with Hx and XOD, which would react to produce H_2O_2. As summarized in Figure S1B, significant change of the FL intensity was observed only when Pt NPs was incubated with Hx, XOD and OPD, which indicated that detection of Hx was feasible based on this principle.

3.4 Detection of Hx in water

Before utilizing the biosensor to detect Hx, parameters affecting the performance of the biosensor are explored to ensure the sensitivity of the fluorescence sensor. Figure S2A displayed the effect of the concentration of OPD on the FL intensity. The FL intensity gradually increases with the increment of OPD concentration, reaching a platform when the concentration of OPD was 2 mM. Similarly, the reaction time was explored. As shown in Figure S2B, after adding OPD, the FL intensity reached a plateau at 35 min. Therefore, we chose 2 mM as the appropriate concentration of OPD, 30 min as the appropriate time to oxidize the OPD.

A calibration curve was established by exploring the relationship between the concentrations of Hx and the FL intensities. Figure 3A demonstrated that the FL intensity gradually quenched with the increment of Hx concentration, and the FL intensity against the concentration of Hx was linear in the range of 8-2500 μM with a correlation coefficient of 0.990 (Figure 3B). Using this method, the limit of detection (LOD) of Hx (S/N = 3, where S represents sensitivity and N represents noise) was calculated to be 2.88 μM. According to the national standard of China, the aquatic products are considered to be fresh when the Hx concentration is lower than 529 μM.

Therefore, the proposed biosensor can be used to determine the freshness of aquatic products at a very early stage of spoilage, because the LOD is far below the concentration of Hx in degenerative aquatic products as stipulated by the national standard.

3.5 Selectivity of fluorescent biosensors

When biosensors are used in practice, there are many factors that may interfere the accuracy of the detection. Without acceptable selectivity, the biosensor will lose its application value, thus selective analysis is necessary. To investigate whether the possible interferent would affect the performance of the biosensor, Hx and other substances including glucose (Glu), lactic acid (LA), ascorbic acid (AA), uric acid (UA), K^+, Zn^{2+}, Mg^{2+}, Ca^{2+} were tested under the same conditions. After the addition of these substances (800 μM), no significant decrease in FL intensity was observed, while the FL intensity was significantly reduced after the addition of Hx compared to control (Figure 4). These results indicated that these disturbances hardly affect the performance of the biosensor, verified the good selectivity and anti-interference ability of the proposed biosensor.

3.6 Detection of Hx in the aquatic products

To further evaluate the application prospect of the biosensor, we verified the practicability of the biosensor by detecting the content of Hx in fish, shrimp and squid (Table 1 and Table S1). Comparing the results of measured by the fluorescent biosensor with the results of HPLC measurement it was found that they show a good linear relationship (Figure S3), so we have reason to believe that the fluorescence interference

caused by the aquatic sample is relatively stable. Despite we were unable to find this interference in selective experiments, it will not affect the practicability of the proposed biosensor since the exact results can be obtained after a simple conversion using the linear equation. From the experimental results, this fluorescent biosensor based on Pt NPs can be used to detect the freshness of the aquatic products before they are spoiled, thereby judging whether the aquatic product is edible. As summarized in Table S2, the recovery rates were in the range of 103.94-109.00%. Besides, the concentration of Hx in fresh aquatic products regulated by the national standard (529 μM) is much higher than the LOD of the biosensor proposed in this study. In fact, even the Hx concentrations in live shrimp and fish (13.70 ± 1.31 μM and 12.97 ± 0.66 μM respectively) are still higher than the LOD of this biosensor. Therefore, the proposed biosensor had a good application prospect in evaluating the freshness of aquatic products thereby avoiding economic losses that caused by fish and shrimp spoilage by cumbersome testing methods.

In comparison with other methods shown in Table 2, the proposed method in this study has wider detection range than most methods, and achieves good results in the detection of freshness of aquatic products. Some reported electrochemical sensors for Hx have lower LODs than our method, but the detection range is limited and fluorescent biosensors have better reproducibility and stability than electrochemical sensors. Taking account of the facile preparation and relatively low cost, the biosensor developed in this study will have considerable superiority in practical application.

3.7 Recycle of the Pt NPs

As a biosensor based on noble metal materials, the repeatability of materials is critical to save the cost of the biosensor (Md Rabiul Awual, 2016; Shahat, Awual, Khaleque, Alam, Naushad, & Chowdhury, 2015). To explore the reuse possibility of the Pt NPs, we recovered the Pt NPs by centrifugation after a circle of detection, and the obtained Pt NPs were used in the next circle of detection. As revealed in Figure 5, the Pt NPs can be used multiple times without significantly reducing their catalytic capacity, which was proved by that the recovery rate only decreased slightly to 91% after three cycles of detection. Then in the fifth cycle, the recovery rate dropped to 80%, which may be ascribed to the loss of Pt NPs in the centrifugation and washing procedures. These results confirmed that Pt NPs has good stability and long-term reusability, thereby holding a good prospect in developing rapid and sensitive biosensors with low cost for evaluation of aquatic products freshness.

4. Conclusions

In summary, we have successfully developed a novel fluorescence sensor based on the catalytic activity of Pt NPs for detecting the Hx content in aquatic products. The quantification of Hx is highly sensitive and repeatable with a linear range of 8–2500 μM. Notably, the LOD of the proposed biosensor is 2.88 μM, which is much lower than the threshold of Hx concentration in fresh aquatic products specified by the national standard. The result showed the method had good selectivity for various potential interfering substances, and could be used for detecting the Hx contents in real samples such as fish, shrimp and squid with good recoveries. Incorporated with the merits of facile preparation and low cost as well as reusability, this fluorescence biosensor has a

good application prospect in developing applicable sensors for rapid and accurate evaluation of the freshness of aquatic products.

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Conflict of interest

The authors declare no conflict of interest with this research.

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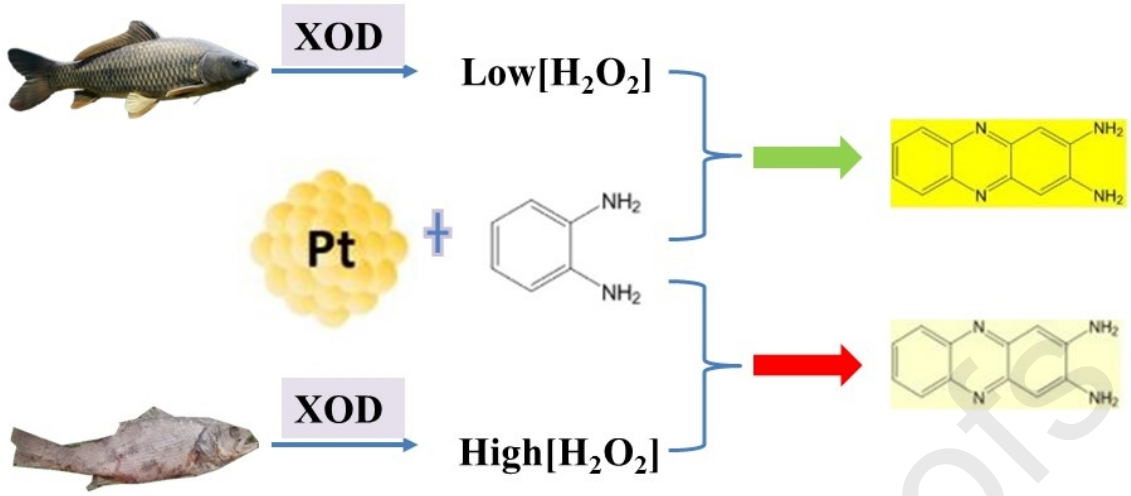

Figure 1. Principle of detecting aquatic freshness based on the fluorescence biosensor.

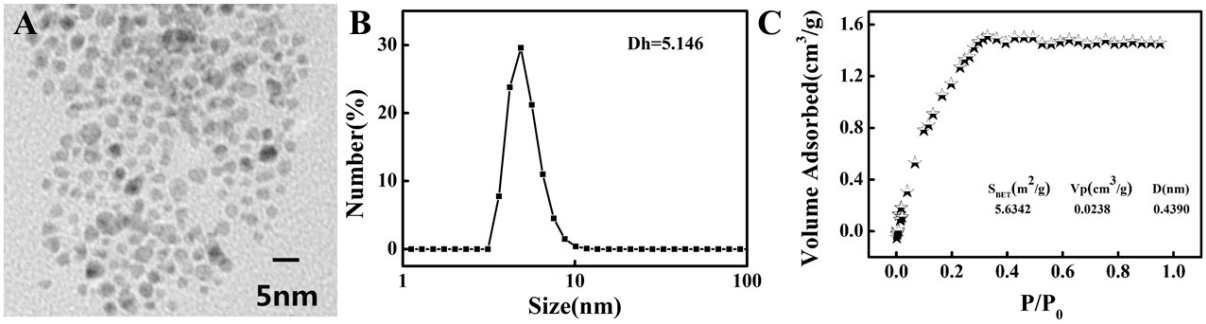

Figure 2. Characterization of Pt NPs. (A) TEM image, (B) DLS analysis, (C) N_2 adsorption-desorption isotherms.

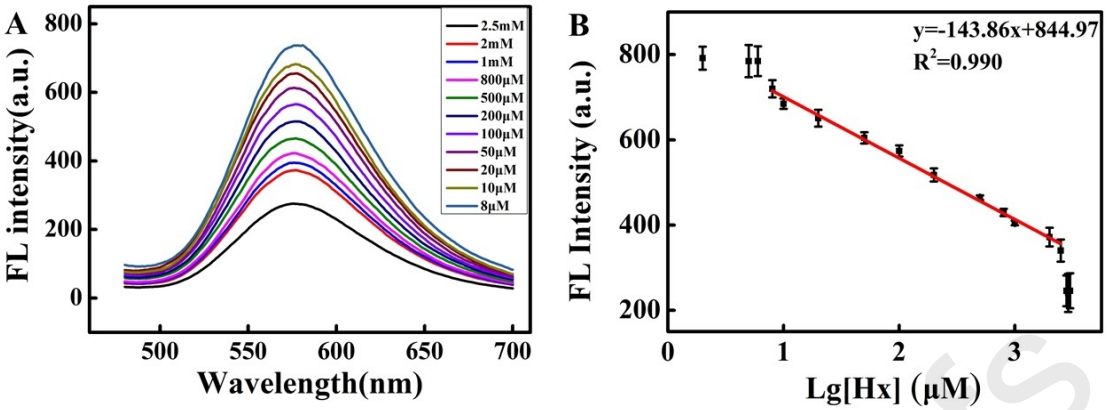

Figure 3. Quantitative detection of Hx. (A) Fluorescence spectra with varying concentrations of Hx; (B) The relationship between FL Intensity and the Hx concentration.

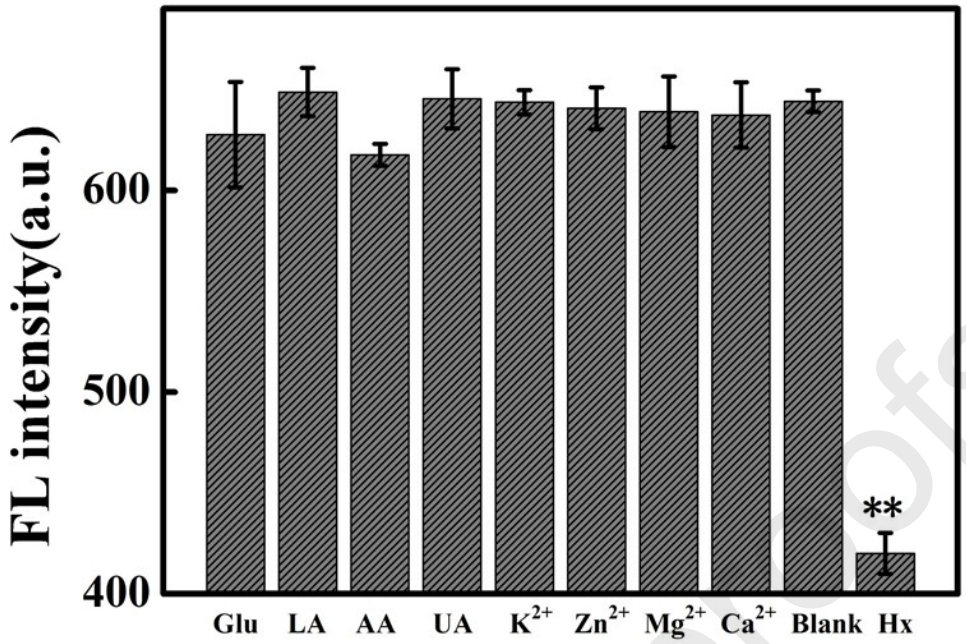

Figure 4. The FL intensity of the fluorescence senor in the presence of other substance with concentrations of 800 μM (**: $p < 0.01$, based on the Student's *t*-test).

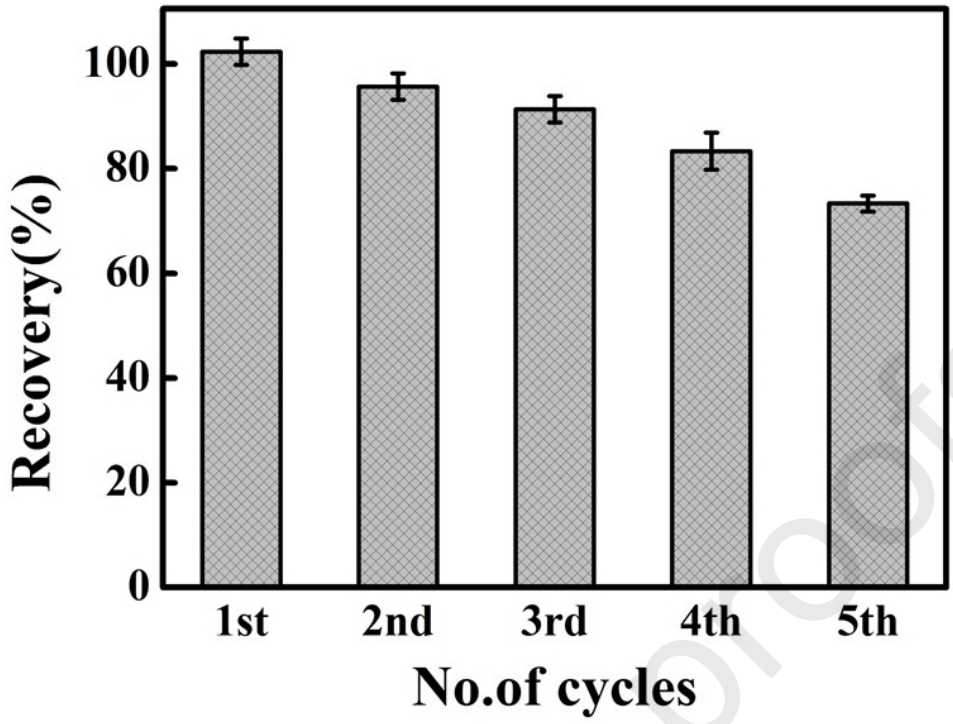

Figure.5 Recycle of the Pt NPs for five times.

Table 1 Comparison of the Hx concentrations determined by the proposed sensing system and HPLC in shrimp and fish meat.[1)]

Shrimp	C1 (μM)	C2 (μM)	Fish	C1 (μM)	C2 (μM)
0 h	5.64 ± 1.90	13.70 ± 1.31	0 h	4.85 ± 0.63	12.97 ± 0.66
0.5 h	7.25 ± 1.04	14.98 ± 0.11	0.5 h	5.75 ± 1.90	13.85 ± 0.20
1 h	7.86 ± 4.00	21.22 ± 2.49	1 h	6.45 ± 5.50	16.79 ± 1.15
2 h	16.94 ± 4.83	37.35 ± 9.07	2 h	21.77 ± 2.00	47.32 ± 14.12
3 h	28.25 ± 2.33	55.33 ± 8.47	3 h	29.84 ± 6.75	70.32±10.84
6 h	39.21 ± 1.83	77.65 ± 3.21	6 h	44.46 ± 1.58	76.37±10.20
12 h	53.74 ± 12.17	131.69 ± 2.58	12 h	57.31 ± 7.92	162.42±16.43
24 h	69.90 ± 37.68	182.02 ± 18.02	24 h	105.26±12.32	240.60 ± 15.46
36 h	94.43 ± 24.06	262.52 ±23.14	36 h	165.15 ± 5.95	381.13 ±32.77
48 h	440.04±43.18	1023.99±61.33	48 h	504.87±34.84	1038.74±75.76

[1)] 1 h represents that one hour after the death of aquatic products; C1 represents the concentration of Hx detected by HPLC; C2 represents the concentration of Hx detected by the proposed biosensor, and the aquatic product were stored at 4 ^{o}C after death. Data are shown as mean ± SD (n = 3).

Table 2 Comparison of the proposed sensing system with other reported methods for Hx detection.

	Dynamic range	LOD of Hx	Reference
Albelda., et.al	20–512 μM	9.5 μM	(Albelda, Uzunoglu, Santos, & Stanciu, 2017)
Wang., et.al	0.2–80 μM	0.12 μM	(Wang & Tong, 2010)
Hernández., et.al	15.63–127 μM	-	(Hernández-Cázares, Aristoy, & Toldrá, 2010)
A.T. Lawal., et.al	5–20 μM	4.5 μM	(Lawal & Adeloju, 2010)
Zhang., et.al	1.5–35.4 μM	0.61 μM	(Zhang, Lei, Zhang, Ding, & Ju, 2012)
This work	8-2500 μM	2.88 μM	

Highlights

1. Rapid detection of hypoxanthine to evaluate the freshness of aquatic products.
2. The LOD was lower than the threshold of hypoxanthine in fresh aquatic products.
3. The fluorescence biosensor can be used to detect real samples with good recoveries.
4. The biosensor is easy to prepare, reusable and in low cost.

Conflict of interest

The authors declare no conflict of interest with this research.