A sensitive electrochemical approach for monitoring the effects of nano-\(\text{Al}_2\text{O}_3\) on LDH activity by differential pulse voltammetry†

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In this paper, a sensitive electrochemical approach for monitoring the effect of nano-\(\text{Al}_2\text{O}_3\) on lactate dehydrogenase (LDH) activity is established. It is based on the determination of reduction current of \(\text{NAD}^+\) involved in enzyme promoting catalytic reaction of “pyruvate + \(\text{NADH} + \text{H}^+ \rightarrow \text{Lactate} + \text{NAD}^+\)” by differential pulse voltammetry (DPV).

Various influencing factors including nano-type, nano-size, and adsorbed pollutant organics have been investigated. The experimental results show that the proposed electrochemical method is useful in monitoring and evaluating the toxic effects of nanoparticles, which might be suitable to the environmental pollutant’s toxicity analysis.

1. Introduction

With the development of industry and agriculture, nanomaterials have been widely applied in various fields.\(^{1–3}\) However, the potential toxic effects of them have received great concerns in recent years.\(^{4–8}\) Nano-\(\text{Al}_2\text{O}_3\) is one of the functional materials possessing superior structural, mechanical, and optical properties.\(^9\) It has been widespread applied in the manufacturing of ceramic-, optical-, semiconductor-, and surface protection materials, as well as catalyst carrier or catalyst. The evidence is increasing that nano-\(\text{Al}_2\text{O}_3\) may also result in similarly unique nanotoxicological effects,\(^{10}\) and its toxic effect is changed with nano-size, shape, and the surface chemistry of various nano-\(\text{Al}_2\text{O}_3\) nanomaterials.\(^{11–14}\) Due to the distinct properties of nanomaterials, there is a lack of a recognized method for evaluating their health and safety concerns, resulting in the controversial results for similar experiments carried out under different conditions. Hence, the development of effective analytical methods for evaluating the toxic effects and detecting the harmful effects of nanomaterials is important.

Biomarkers have been widely demonstrated to be able to evaluate and monitor the changes and toxic effects of environmental pollutants.\(^{15}\) Lactate dehydrogenase (LDH) is one of the most important enzymes, found in the liver, lungs, heart, and various other tissues, and plays a significant role in energy metabolism. LDH activity can be used to indicate several pathological conditions, such as gastric cancer,\(^{16}\) breast cancer,\(^{17}\) lung damage,\(^{18}\) and thrombotic thrombocytopenic purpura,\(^{19}\) etc. Thus, LDH activity also has been recognized as a useful biomarker and applied in the fields of biology, medicine, and environment.\(^{20,21}\)

Up to now, the main analytical methods used in detecting the nanotoxicological effects are: colorimetric assay, microscopy, single cell microgel electrophoresis, cell counting, fluorescent probe, and Fourier transformed infrared spectrometry, etc.\(^{22–27}\) These methods usually have some limitations in sensitivities. The electrochemical technique possesses some distinct advantages: rapidity, high sensitivity, cheap instrumentation and a simple operation procedure. On the other hand, hanging mercury drop electrode (HMDE) exhibits the prominent merit of regenerating the electrode surface easily which may avoid the serious adsorption of pollutants on the electrode surface efficiently. It could satisfy many requirements to analyze different biological systems and be widely applied to detect enzyme activity.\(^{28–31}\) In this paper, LDH activity is used as a biomarker and an electrochemical method for monitoring the toxic effect of nano-\(\text{Al}_2\text{O}_3\) on LDH activity is established.

2. Experimental

2.1 Materials and instrumentations

Most chemicals were obtained from Shanghai Chemical Reagent Factory and used as received unless otherwise noted. All chemicals were of analytical reagent grades. Bovine heart LDH (10 mg mL\(^{-1}\)) was obtained from Sigma Co. (St. Louis, MO), stored in a refrigerator of 4 °C, and diluted 100 times when used. \(\beta\)-\(\text{NAD}^+\) and NADH (purity 90%) were purchased from Shanghai Bio Life Science & Technology Co., Ltd (China). Pyruvic acid (Pyr, 98.50%) was of biological-reagent grade, purchased from Shanghai Chemical Reagent Factory. NADH (0.05 mol L\(^{-1}\)) and Pyr (0.2 mol L\(^{-1}\)) solutions were prepared with double-distilled water, and stored in a refrigerator at 4 °C. Nano-\(\text{Al}_2\text{O}_3\) was purchased from Sigma. Nano-AlN is synthesized according to ref. 32. The sizes of nano-\(\text{Al}_2\text{O}_3\) were in 20–50, 300–500, above 500 nm respectively and nano-AlN in 200–400 nm. The 0.1 mol L\(^{-1}\) pH 7.5 Tris-HCl buffer solutions were prepared as that described in ref. 33. The supporting

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electrolyte is 0.15 mol L\(^{-1}\) KCl. All dilute solutions were prepared by diluting this solution with double-distilled water.

A three-electrode system was used, which consisted of a HMDE (Jingsu Electroanalytical Instrument Factory, China), a platinum foil counter electrode, and a saturated calomel reference electrode. All electrochemical experiments were performed with a CHI660B electrochemical system (CH instruments Inc., Shanghai, China). DPV parameters were the following: scan rate 20 mV s\(^{-1}\), pulse amplitude 50 mV, pulse width 50 ms. pH measurements were carried out with a PHS-2F digital ion meter (Shanghai Precision and Scientific Instrument Company, China). The solutions were stirred with a 79-1 magnetic stirrer (Guohua Instrument Factory, China) and all the experiments were carried out with 501 superthermostatic bath maintaining 25 ± 1 °C all through the experiment (Shanghai Laboratory Instrument Company, China). The dispersions of all nanoparticles in aqueous solutions are good.

2.2 Test for the effect of nano-Al\(_2\)O\(_3\) on lactate dehydrogenase activity in LDH reaction system

Twenty-five millilitres of 0.10 mol L\(^{-1}\) Tris-HCl buffer solution and a quantitative amount of nano-Al\(_2\)O\(_3\) were transferred into the electrolytic cell. The solution was degassed for 10 min by bubbling of nitrogen gas through it and kept in the ambient of nitrogen. After injecting LDH, the solution was stirred for 5 min (with or without nanoparticles). Then 100 µL of 0.2 mol L\(^{-1}\) Pyr and 100 µL of 0.05 mol L\(^{-1}\) NAD\(^+\) were added into the 25 mL electrolyte cell, and their final concentrations were 8 × 10\(^{-4}\) mol L\(^{-1}\) and 2 × 10\(^{-4}\) mol L\(^{-1}\), respectively. The reduction currents of NAD\(^+\) (\(i_{\text{p,NAD}^+}\)) and Pyr (\(i_{\text{p,Pyr}}\)) were recorded in DPV mode at regular time intervals.

2.3 Examination of the adsorption of nano-Al\(_2\)O\(_3\) on the HMDE

Twenty-five millilitres of 0.10 mol L\(^{-1}\) Tris-HCl buffer solution was transferred into the electrolytic cell, degassed for 10 min by bubbling of nitrogen gas, then stirred quickly for 2 min and sonicated for 10 min. And the cell was sealed so that a positive pressure of nitrogen could be maintained over the surface of the samples. After adding certain amount of Pyr, the reduction peak current was recorded in DPV mode, and then certain amount of nano-Al\(_2\)O\(_3\) was added in cell. After stirring for 10 min and with an equilibration time of 1 min, DPV peak current of Pyr was recorded again. A series of similar tests for different concentrations of Pyr and in the presence of nano-Al\(_2\)O\(_3\) were carried out. Similar experiments for NAD\(^+\) in the presence and absence of nano-Al\(_2\)O\(_3\) were also performed.

2.4 Determination of Michaelis constant \(K_m\) and maximum velocity \(v_{\text{max}}\) for NADH

Twenty-five millilitres of 0.1 mol L\(^{-1}\) Tris-HCl buffer solution was treated as above. After adding 30 µL LDH (diluted 100 times), the solution was stirred for 5 min (with or without 0.25 mmol L\(^{-1}\) nanoparticles), and then 100 µL of 0.2 mol L\(^{-1}\) Pyr (0.8 mmol L\(^{-1}\)) and NADH (the concentration was varied) were added. The reduction currents of NAD\(^+\) (\(i_{\text{p,NAD}^+}\)) were recorded. The Michaelis constant \(K_m\) and maximum velocity \(v_{\text{max}}\) for NADH were calculated by using the following formula:34

\[
\frac{1}{v} = \frac{K_m}{v_{\text{max}}} + \frac{1}{v_{\text{max}}} 
\]

3. Results and discussion

3.1 The effects of nano-Al\(_2\)O\(_3\) on LDH activity in the LDH reaction system

In the LDH, NADH, Pyr, and nano-Al\(_2\)O\(_3\) reaction system, only the substrates Pyr and NAD\(^+\) yield electrochemical responses at HMDE.35,36 When NADH was added, the peak current of NAD\(^+\) at −0.89 V (vs. SCE, pH 7.5) was increased continually while the peak current of Pyr decreased constantly along with the time (Fig. 1). The peak current of NAD\(^+\) increased until it arrived at the plateau. Within the first 3 min of the enzyme reaction progress, there were positive linear relationships between the peak currents \(i_{\text{p,NAD}^+}\) and the time. \(i_{\text{p,NAD}^+}\) at 3 min was recorded and used to indicate LDH activity and to represent the initial velocity \(v_i\), since \(i_{\text{p,NAD}^+}\) can indicate LDH activity simply and conveniently. Thus, the effect of nano-Al\(_2\)O\(_3\) on LDH activity in the LDH reaction system can be easily inspected by adding certain amounts of nano-Al\(_2\)O\(_3\) (50 nm). Insert in Fig. 1 is the changes of \(i_{\text{p,NAD}^+}\) after adding different levels of nano-Al\(_2\)O\(_3\) (50 nm), indicating that the LDH activity decreases with the elevation of nano-Al\(_2\)O\(_3\) (50 nm).

The adsorption effect of nano-Al\(_2\)O\(_3\) on the HMDE was investigated. There are five species in enzymatic reaction system (LDH, NADH, Lac, Pyr, and NAD\(^+\)). The adsorption of NAD\(^+\), Pyr, NADH, Lac, and LDH on HMDE have been reported to be weak in a short interaction time within 15 min.37,38 Thus, only the adsorption effect of nano-Al\(_2\)O\(_3\) on the HMDE was considered. Experimental results indicate that in the absence and presence of nano-Al\(_2\)O\(_3\), the peak currents of Pyr and NAD\(^+\) almost have no

![Fig. 1 DPV responses of LDH reaction system changed with time in presence of 1 × 10\(^{-3}\) mol L\(^{-1}\) nano-Al\(_2\)O\(_3\) (50 nm). a \(\rightarrow\) i: t = 0, 1, 3, 5, 7, 9, 11, 13, 18 min. T = 25 ± 1 °C, 0.10 mol L\(^{-1}\) Tris-HCl buffer solution (pH 7.5) + 0.15 mol L\(^{-1}\) KCl, 8.0 × 10\(^{-4}\) mol L\(^{-1}\) Pyr, 2.0 × 10\(^{-4}\) mol L\(^{-1}\) NADH and 30 µL LDH. Insert: the changes of \(i_{\text{p,NAD}^+}\) after adding different levels of nano-Al\(_2\)O\(_3\) (50 nm).](image)
change in both cases (Fig. S1 in ESI†). In pH 7.5 buffer solution, both HMDE and Al2O3 possess negative charge,39 and the experimental time is also short (3 min). Thus, the adsorption of nano-Al2O3 on HMDE is weak, and nano-Al2O3 will not block the HMDE surface, and the changes of DPV responses mainly reflect the influences of nano-Al2O3 on LDH activity.

3.2 Effects of nano-Al2O3 on LDH activity with different types and sizes

As shown in Fig. 2, nano-Al2O3 inhibits the activity of LDH obviously, whereas nano-AlN shows weak inhibitory effects. It was found that under the same conditions, for the three kinds of Al species (Al(III) (data cited from ref. 38, 40), Al2O3, and AlN), low level Al(III) has activation effect on the LDH. When the Al(III) level is above 0.5 mmol L−1, the effect increased. When their concentration is 1 mmol L−1, nano-Al2O3 (50 nm) has the most serious impact on LDH activity and shows the order: nano-Al2O3 (50 nm) > nano-AlN > Al(III).

Nano-size is a key influencing factor on its toxicity.41−44 In the present investigation, the experimental results indicate that the effects of nano-Al2O3 on LDH activity increased with the decrease of its size, as shown in Fig. 2 and 3 (the Lineweaver–Burk plots). This indicates that Al2O3 at nanometre level may have more toxological effects. The Lineweaver–Burk plots and the values of $K_m$ and $v_{max}$ in the absence and presence of 2.5 × 10−4 mol L−1 nanoparticles show that the inhibitions caused by nanoparticles are all of mixed type. Furthermore, it is interesting to note that the inhibition of ZnS (6–8 nm) and TiO2 (25–30 nm), which have small size, is competitive and non-competitive character but nano-Al2O3-300 nm and AlN (200–350 nm), which have larger sizes, are non-competitive and uncompetitive type. This result might imply that the size of particle has influence on the inhibition mechanism. Table 1 indicates that the inhibiting effect of nano-Al2O3 is close to that of TiO2,43e ZnS,b,e and SWCNTs,45 but is weaker compared with that of Al(III),38e and nano-AlN.46 The trend effect is greater for smaller diameter Al2O3. In this case, the toxic mechanism may be due to some sort of steric access issue with the nanoparticles’ small size effect. Some literature contributions to tackle this issue.46 Previous studies suggest that the mechanism of nanoparticles toxicity may relate to their photosensitivity and to production of reactive oxygen species under specific wavelength high-intensity light.50,53e Nanoparticles are very small with large

Table 1 The effects of nano-Al2O3 and AlN on $v_{max}$ and $K_m$ of LDH and comparisons with other known inhibitors of LDH

<table>
<thead>
<tr>
<th>Al2O3 (mmol L−1)$^b$</th>
<th>$v_{max}$ (μmol L−1 min−1)</th>
<th>$K_m$ (μmol L−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 (50 nm)</td>
<td>58</td>
<td>106</td>
</tr>
<tr>
<td>0.25 (300 nm)</td>
<td>61</td>
<td>109</td>
</tr>
<tr>
<td>0.25 (1000 nm)</td>
<td>76</td>
<td>138</td>
</tr>
</tbody>
</table>

Nanoparticles are very small with large

AlN/mmol L−1

0.25 (200–350 nm) 80 124
0.25 (200–350 nm) 66.7 156
ZnS/mmol L−1

0.25 (6–8 nm) 37.7 140
0.04 28.8 107
Nano-Al13(mg L−1)$^c$

0 38.8 85.7
60 (200–500 nm) 25 60
120 (200–500 nm) 23 61
SWCNT/mg L−1

0 (bare GCE) 110 100
35 (SWCNTs modified GCE) 185 75

$^a$ It should be pointed out that this comparison only gives a reference since their experimental conditions are different. $^b$ This paper. 0.10 mol L−1 Tris-HCl buffer solution (pH 7.5) + 0.15 mol L−1 KCl, 8.0 × 10−4 mol L−1 Pyr, 2.0 × 10−4 mol L−1 NADH, 30 μL LDH (1 : 100).

Fig. 2 The effect of different nanoparticles and size on the reaction rates of LDH reaction system. ■ nano-Al2O3-50 nm; □ nano-Al2O3-100 nm; nano-AlN-200–350 nm; ▲ Al(III). Other experimental conditions are the same as in Fig. 1.

Fig. 3 Double-reciprocal plot of $v$ vs. $C_{NADH}$. a. $C_{nanoparticles} = 0$ mol L−1; b. $C_{Al2O3 (200-350 nm)} = 0.25$ mmol L−1; c. $C_{Al2O3 (1000nm)} = 0.25$ mmol L−1; d. $C_{Al2O3 (300nm)} = 0.25$ mmol L−1; e. $C_{Al2O3 (50 nm)} = 0.25$ mmol L−1. Other experimental conditions are the same as in Fig. 1.
surface area, so that they can penetrate cell membranes and barriers. Thus, the detailed information is unclear and deserves further studies.

3.3 Effects of nanoparticles loading phenol on LDH activity

It was suggested that the toxicity of nanoparticles is not only from their own harmful nature but also from the toxic substances adsorbed by them. Studies have evidenced that nanoparticles can act as vehicles transporting toxic chemicals into the human respiratory system and the nanoparticles may be more dangerous in the presence of pollutants. Thus, the research on this subject is very valuable. In the present study, we stirred the mixture of nano-Al₂O₃ (50 nm, 1000 nm) and phenol for 30 min for full adsorption, respectively. As shown in Fig. 4, the inhibitory effects of phenol were enhanced after adsorbing by two sizes of Al₂O₃, demonstrating clearly the enhanced effect of nanoparticles on the pollutants’ toxicity. This indicates that they may have a synergistic effect.

4. Conclusions

It has been demonstrated that the electrochemical technique can be a useful tool for evaluating the toxic effects of the nanoparticles through monitoring LDH activity simply by observing the DPV reduction peak current of NAD⁺ at the HMDE. Considering the significance of the LDH in the area of medicine, biology, and biomarkers for environment, this method can be conveniently applied to evaluate the toxic effects of nanomaterials on environment. It could be anticipated to satisfy many requirements for analyzing different biological systems and be applied to study the nano-toxicity just simply through detecting enzyme activity by constructing small and portable biosensors in SECM detections.

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