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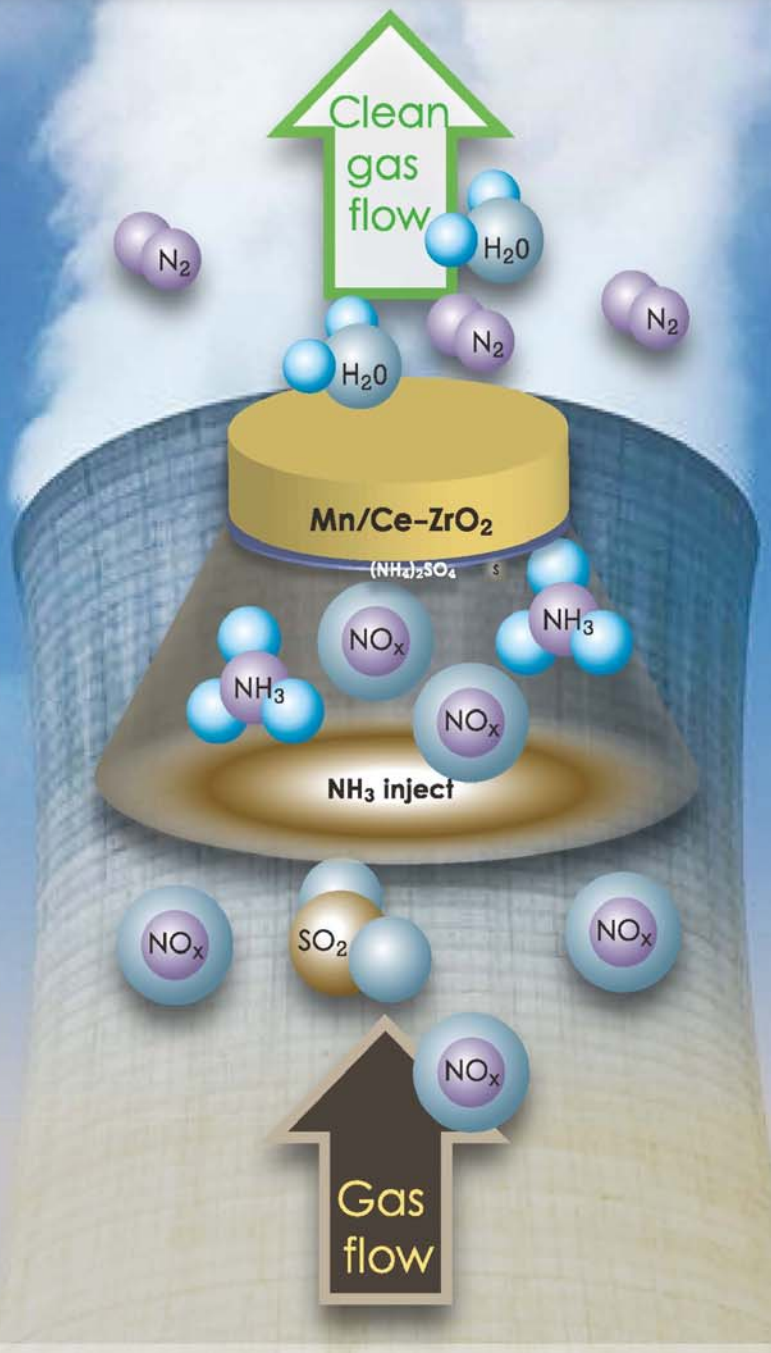

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Toxicity detection of sodium nitrite, borax and aluminum potassium sulfate using electrochemical method

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Abstract

Based on the inhibition effect on the respiratory chain activity of microorganisms by toxicants, an electrochemical method has been developed to measure the current variation of a mediator in the presence of microorganisms contacted with a toxicant. Microelectrode arrays were adopted in this study, which can accelerate the mass transfer rate of an analyte to the electrode and also increase the total current signal, resulting in an improvement in detection sensitivity. We selected *Escherichia coli* as the testee and the standard glucose-glutamic acid as an exogenous material. Under oxygen restriction, the experiments in the presence of toxicant were performed at optimum conditions (solution pH 7.0, 37°C and reaction for 3 hr). The resulting solution was then separated from the suspended microorganisms and was measured by an electrochemical method, using ferricyanide as a mediator. The current signal obtained represents the reoxidation of ferrocyanide, which was transformed to inhibiting efficiency, IC_{50}, as a quantitative measure of toxicity. The IC_{50} values measured were 410, 570 and 830 mg/L for sodium nitrite, borax and aluminum potassium sulfate, respectively. The results show that the toxicity sequence for these three food additives is consistent with the value reported by other methods. Furthermore, the order of damage degree to the microorganism was also observed to be: sodium nitrite > borax > aluminum potassium sulfate > blank, according to the atomic force microscopy images of *E. coli* after being incubated for 3 hr with the toxic compound in buffer solutions. The electrochemical method is expected to be a sensitive and simple alternative to toxicity screening for chemical food additives.

Key words: food additives; sodium nitrite; borax; aluminum potassium sulfate; microelectrode arrays

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Introduction

For centuries, food additives have been used for flavoring, coloring, and extension of the useful shelf-life of food, as well as the promotion of food safety, and have become an important impetus in scientific and technological progress and innovation in the food industry. However, food safety events have occurred frequently in recent years, and the health problems caused by the toxicity of illegally and easily abused food additives have become a matter of increasing concern. Sodium nitrite is present in a wide range of foods; however, it leads to serious toxicity and mutation in excessive doses. It can also react with secondary amines to form carcinogenic N-nitrosamines, and a correlation between nitrite-containing food consumption and the increase of cancer risk has been recently reported (Mitacek et al., 2008). Aluminum potassium sulfate is a conventional food improver and leavening agent, often used as an additive in food manufacturing of products such as fried bread sticks, vermicelli and rice flour, etc. Excessive intake of aluminum potassium sulfate will cause osteoporosis, anemia and may affect nerve cell growth due to the aluminum content. Borax is widely used in industry, but it is forbidden as a food additive by various countries due to its high toxicity.

The 50% lethal dose (LD_{50}) value is usually used for the assessment of toxicity, and also applied to food safety of food additives in national standards such as GB15193.3-2003 and others. The LD_{50}-test needs a mass of animals and it gives only semi-quantitative and ambiguous information. The toxicity is derived easily for humans by conventional national standard methods for toxicological assessment of food safety. Usually, detection methods for food additives are time-consuming, expensive and inhumane, and require a great deal of time to observe the lethal dose for a large number of cats, dogs and mice and so on.

Since conventional chemical-based methods can only

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quantify concentrations of pollutants, bio-based experiments have become very active in recent years, involving a wide range of organisms (Pasco et al., 2000) such as plants, invertebrates and fish. These techniques cannot be popularized due to their limitations of requiring specialized equipment and trained personnel. Therefore, it is a great challenge to find an effective technique which can provide rapid, simple, reliable and low-cost measurements to complement the traditional methods. The Microtox® test based on the fading of light emitted by a marine microbe (*Vibrio fischeri*) in the presence of noxious agents has been successfully used to screen the acute toxicity of a large number of chemicals (Ghirardini et al., 2009; Strigul et al., 2009); the change in light output and concentration of the toxicant produces a dose/response relationship, yielding EC_{50} (concentration producing a 50% reduction in light). The Microtox® test is thus used widely as a standard test for aquatic toxicity testing. However, it is not suitable for feculent and colored samples; furthermore, the luminous microbes must work in 3% saline solution in order to maintain the osmotic pressure of the microbes, and the solubilities of some organic chemicals are insufficient in the saline solution.

In order to remedy the defects of the luminescent test, biosensors based on microorganisms combined with electrochemical measurements have been developed (Ertl et al., 2003; Catterall et al., 2010; Chen et al., 2010), which provide a total biological toxicity based on the inhibition effect in the respiratory chain activity of microorganisms by toxicants even if the toxicity of some new compounds is not known.

In this article, *Escherichia coli* (*E. coli*) was used as testee in toxicity measurements under optimum conditions. Firstly, 3,5-dichlorophenol (DCP) was chosen as the reference toxicant and tested to validate the feasibility of the technique. We then applied the electrochemical method to measure toxicities of different additives in food for the first time. The atomic force microscopy (AFM) images of *E. coli* incubated in various toxic solutions were also investigated. In order to evaluate the developed method in comparison with other known measuring methods, we chose three typical substances with toxicity data available in the literature. Once the validity of the method is proven, it can be extended to measure other substances even if their toxicities are unknown. The proposed method, based on our previous works (Liu et al., 2009a, 2009b), is very simple and practical, and may be suitable for routine control of food quality.

1 Materials and methods

1.1 Reagents

Sodium nitrite and aluminum potassium sulfate were bought from North of Beijing Fine Chemicals Co., Ltd., China. Borax was purchased from Beijing Xinguang Chemical Reagent Factory, China. *E. coli* DH 5α was purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd., China. Peptone and yeast extract were purchased from OXOID Co. Ltd., UK. DCP was purchased from Aldrich, USA. Solutions of potassium ferricyanide, obtained from Sinopharm Chemical Reagent Beijing Co., Ltd., China, were made up fresh, wrapped in aluminum foil and stored in the dark until use. The Luria Bertani broth (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) was adjusted to pH 7 with 3 mol/L NaOH and then sterilized with an autoclave (SYQ-DSX-280B, Shanghai Shenan Medical Instrument, China) at 121°C for 15 min. The standard substrate solution (GGA, 150 mg/L glucose and 150 mg/L glutamic acid) was prepared according to standard procedures (APAH, 1997). All chemicals were of analytical grade and all solutions were prepared using 18 MΩ/cm deionized water.

1.2 Microbial culture

E. coli DH 5α microbes, preserved on the nutrient substrate at –80°C, were grown overnight (10 hr) at 37°C in the substrate Luria Bertani broth on a shaking incubator (220 r/min). The microbes were then harvested using a centrifuge (Eppendorf 5804R, Germany) at 6000 r/min for 10 min at room temperature, and the resultant microbes (*E. coli)* were washed twice in phosphate buffer (PBS, 0.08 mol/L KH_2PO_4/0.12 mol/L Na_2HPO_4, pH 7.0), centrifuged at 6000 r/min for 10 min at room temperature between washings, then the washed *E. coli* microbes were resuspended in the PBS. The concentration of *E .coli* solution was adjusted to an absorbance value of 24.0, measured at 600 nm using a Cary 500 Scan UV-Vis-NIR Spectrophotometer (Varian, Harbor City, USA). The *E. coli* suspensions were used for experiments on the day of harvesting.

1.3 Sample preparation

Each 3.00 mL sample was prepared according to the following procedure: 0.75 mL potassium ferricyanide (180 mmol/L), 0.75 mL GGA standard solution, 0.75 mL *E. coli* with absorbance value of 24.0 and 0.75 mL toxicant at different concentrations. Control incubations were conducted in PBS without the toxicant. Positive and negative controls refer to the presence and absence of GGA in toxic samples, respectively. Under oxygen restriction, the sealed samples were subjected to shaking incubation (220 r/min) for 3 hr at 37°C. After terminating the reaction, electrochemistry experiments were carried out with the supernatant liquid obtained from the sample solutions, which were centrifuged at 6000 r/min for 10 min.

1.4 Electrochemical measurement

Electrochemistry experiments were accomplished using a CHI 832B electrochemical analyzer (CH Instruments, USA). The applied voltage was controlled at +450 mV

versus a Ag/AgCl (saturated KCl) reference electrode. Microelectrode arrays (MEAs) (12 pieces of Pt wire with diameter 20 μm) as working electrode and a Pt gauze as auxiliary electrode were used. The MEAs were pretreated by polishing with 0.05 μm alumina slurry on a polishing cloth. The current was measured in the resulting sample solution containing the reduced mediator (ferrocyanide), which indicates the toxic effect on the respiratory chain of the microbes.

1.5 Morphology characterization

The harvested *E. coli* suspension with absorbance value of 24.0 was incubated for 3 hr at 37°C in the following solutions: blank, 400 mg/L of sodium nitrite, borax and aluminum potassium sulfate. Then, each sample was drawn out and washed with deionized water three times, respectively. The samples were dropped and dried on freshly cleaned glass slides. The images were obtained through a tapping mode AFM (SPI3800N-Spa 400, Seiko Instruments, Japan). A rectangular Si cantilever/tip with a spring constant of 17 N/m and a resonance frequency of 150 kHz was used. The scan speed was set at 0.7 Hz/sec and the final resolution was 256 by 256 pixels. The height scale of cells was depicted as shades of gray, with bright areas being nearer to the tip in the topography images.

2 Results and discussion

2.1 Performance of microelectrode arrays

MEAs have led to unprecedented advances in electrochemical studies since their introduction to electroanalytical chemistry over thirty years ago. Their rapid widespread application in fields including biochemical and environmental analysis (Koudelka-Hep and van der Wal, 2000; Ordeig et al., 2006) is due to their inherent features, which allow them to be used in highly resistive media, at fast scan rates, with high sensitivity (Stulik et al., 2000).

MEAs fabricated using 12 platinum wires with diameter 20 μm for this test were characterized by steady-state voltammetry, as shown in **Fig. 1**. Cyclic voltammograms of 3.3 mmol/L $K_3Fe(CN)_6$ in 0.1 mol/L KCl solution at a bare Pt MEA exhibit a sigmoidal shape, showing the feature of a reversible steady-state wave for over 20 cycles. The current can quickly reach a steady state in a short time because diffusion-controlled kinetics plays a dominant role with MEAs; furthermore, MEAs have much larger current density than that of a bulk single electrode.

2.2 Feasibility of electrochemical detection

Chronoamperometry and coulometric analysis has been widely applied in the detection of BOD (Connell et al., 2001; Fulladosa et al., 2007). We investigated the feasibility of quantifying the reduced mediator (ferrocyanide) using the MEA. A 45 mmol/L ferricyanide was used as the final concentration because *E. coli* may be damaged in

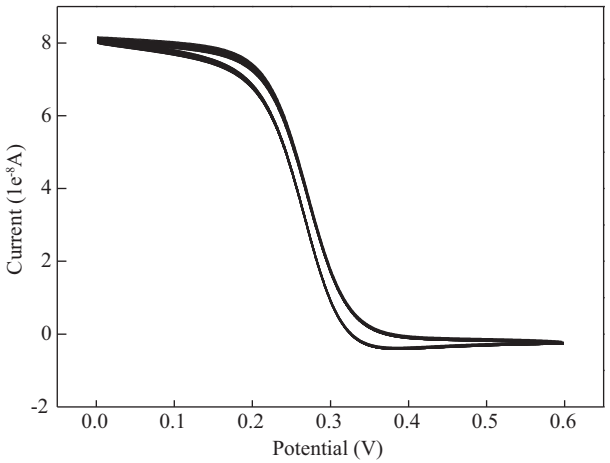

Fig. 1 Cyclic voltammograms of 3.3 mmol/L $K_3Fe(CN)_6$ in 0.1 mol/L KCl solution at a bare Pt microelectrode array (MEA) (12 platinum wires with diameter 20 μm). Scan rate: 50 mV/sec, scan number: 20 cycles.

ferricyanide solutions of higher concentration (55 mmol/L) (Liu et al., 2009b). DCP, which has been widely studied by other approaches (Pasco et al., 2001, 2004), was chosen as the reference for toxicity measurement. **Figure 2** shows the variation of limiting current after 3 hr incubation of the microbes suspension in a mixture of the mediator and GGA standard solution containing DCP (DCP was replaced by PBS in the positive control solution). As expected, a steady limiting current occurred at the MEA in less than 10 sec, thus toxicity could be distinguished sensitively even for a small variation of toxicant concentration. The microbe respiration was inhibited after DCP (4 mg/L) addition, as shown by a clear decrease in the ferrocyanide current compared with that of the positive control, and a small increment in the current appeared in the presence of DCP at higher concentration (16 mg/L). All results indicated the influence of the toxicant on microbe respiration, which could be estimated by chronoamperometry. The smooth current signals further indicated that no interference from other electroactive species occurred in the potential range during measurement in a Faraday cage.

2.3 Inhibitory effects of DCP on *E. coli*

In many instances, meaningful toxicity screening of environmental samples requires extensive sampling and analysis at high cost. The development of toxicity detection may offer cost-effective broad screening tools. Here, chronoamperometry was used to measure quantities of the reductive mediator produced during microbial incubation, and the limiting-current (i_{lim}) corresponding to each toxicant concentration can be converted to inhibitory percentage value (I, %) through the following equation:

$$I = \left(1 - \frac{i_{lim(toxicant)} - i_{lim(t\text{-}control)}}{i_{lim(p\text{-}control)} - i_{lim(n\text{-}control)}}\right) \times 100\%$$

where, $i_{lim(toxicant)}$ is the i_{lim} with toxicant and standard GGA solution, $i_{lim(t\text{-}control)}$ is the i_{lim} at an appropriate concentration of toxicant without GGA solution, $i_{lim(n\text{-}control)}$

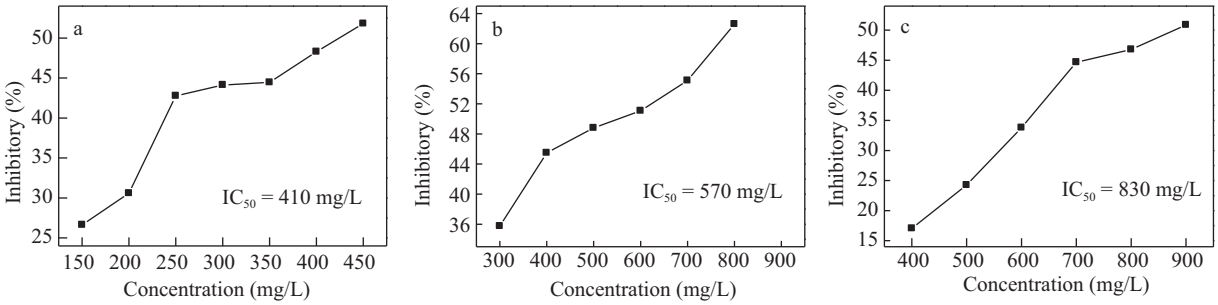

Fig. 4 Inhibitory curves of *E. coli* at different concentrations of sodium nitrite (a); sodium borate (b); aluminum potassium sulfate (c).

is the i_{lim} of the negative control without toxicant and standard GGA solution and $i_{lim(p\text{-}control)}$ is the i_{lim} of the positive control at an appropriate concentration of standard GGA solution without toxicant. The sensitive microbe *E. coli* was used in the toxicity testing, and pH 7.0 together with the GGA (Aggarwal et al., 2005) were confirmed as optimum conditions.

IC_{50} is the inhibition concentration that represents a decrease of the respiration rate by 50% measured using the present electrochemical method. **Figure 3** shows the inhibitory curves of *E. coli* at different concentrations of DCP, and the IC_{50} obtained was 5.2 mg/L. This value is in accord with the reported IC_{50} determined by other methods; therefore, the present method proposed may be applied to detect the toxicity of other chemicals.

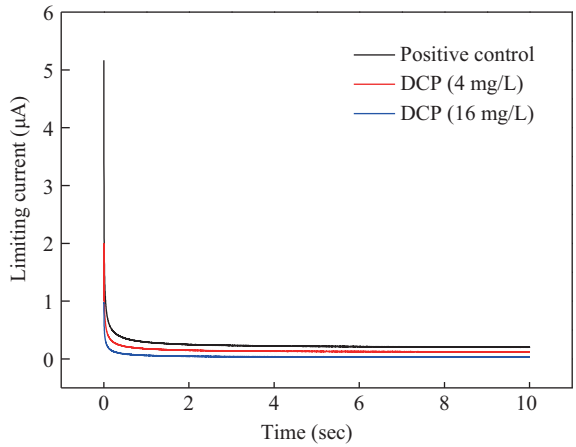

Fig. 2 Current-time curves of 3,5-dichlorophenol (DCP) on *E. coli* after 3 hr incubation for positive control, using MEA (12 platinum wires with diameter 20 μm).

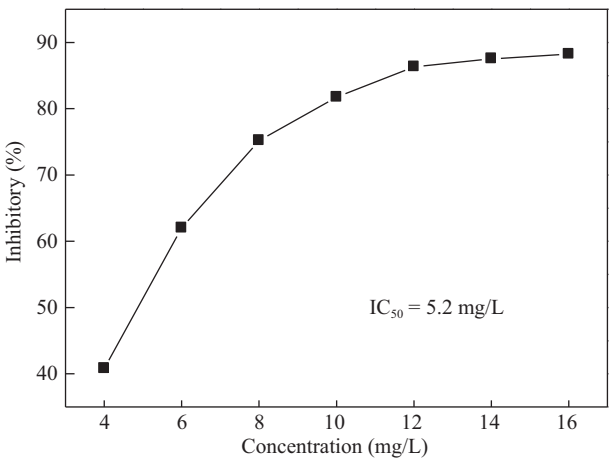

Fig. 3 Inhibitory curve of *E. coli* at different concentrations of DCP.

2.4 Inhibitory effects of unlawful and easily abused food additives on *E. coli*

Food additives have frequently been linked to acute and chronic toxicity, teratogenicity and carcinogenicity etc. International guidelines differ in the types of tests required such as the species of animals to be used and the duration of the tests. This lack of uniformity is wasteful in terms of animal use and potentially causes unnecessary additional work for the food industry. The development of efficient analytical methods for determination of the level of additives in food products is urgently needed. Such analytical methods can also provide useful information about food quality during the manufacturing process.

As displayed in **Fig. 4**, we find that there is an "S shaped plot" inhibitory curve toward *E. coli* for different concentrations of each compound. Based on the experiments, the IC_{50} values calculated were 410, 570, and 830 mg/L for sodium nitrite, borax and aluminum potassium sulfate, respectively. The results can provide a reference for comparison with other existing alternative approaches. The method is simpler than conventional methods for toxicological assessment of food additives. It can also be conducted in real time or at selected intervals following exposure to toxic food additives, and provides the basis for developing a successful toxicity-screening test.

Table 1 shows a comparison of toxicity assessments of sodium nitrite, borax and aluminum potassium sulfate using different methods (The data are quoted from http://en.wikipedia.org/wiki). Multiple internal organs will accumulate the poison if the human body takes in excessive borax: For example, the toxic dose is 1–3 g for adults, the lethal dose is 15 g for adults, the lethal dose is 2–3 g for a baby, its LD_{50} = 1200 mg/kg (rat oral), acceptable daily intake (ADI) = 10 g for adults, 5 g for a baby. Sodium nitrite: LD_{50} = 85 mg/kg (rat oral), ADI = 0–0.2 g for adults. Aluminum potassium sulfate: LD_{50} of 5000–10000 mg/kg (cat oral). These values are in accord with the order of toxicity reported by other methods.

Table 1 Comparison of toxicity assessments of sodium nitrite, borax and aluminum potassium sulfate by different methods

Compound	IC_{50} (mg/L) in present method	LD_{50} (mg/kg)	ADI	Toxic (dose/g)	Lethal (dose/g)
Sodium nitrite	410	85 (rat oral)	0–0.2 g (adult)	0.2–0.5	1–3
Borax	570	1200 (rat oral)	5 g (baby), 10 g (adult)	1–3 (adult)	2–3 (baby), 15 (adult)
Aluminum potassium sulfate	830	5000–10000 (cat oral)	–	–	–

–: not found in the literature.

2.5 Morphological characterization of damage to *E. coli*

Because detection methods for traditional food additives using mice etc. are time-consuming, expensive and inhumane, we selected *E. coli*, which is representative and universal, as the testee; the damage observed to live cells can reflect toxic effects *in vivo*. As these compounds enter into the human body and accumulate in human organs, the cells of human organs are harmed and lesions are observed. Similarly, damage of *E. coli* cells can be expected according to toxicological principles.

AFM provides the possibility of nanoscale visualization and characterization of the cells *in situ*, which makes it a powerful and very useful technique for the investigation of damage to the outer membrane of microbes. The sample preparation of AFM is also simpler than other nanoscale determination methods (Li et al., 2008).

In order to investigate the toxic damage to microbes, we used AFM to characterize the effects on the membranes of *E. coli* in the following solutions: blank, 400 mg/L of sodium nitrite, borax and aluminum potassium sulfate. **Figure 5** shows the AFM images of *E. coli* after being incubated for 3 hr in the above solutions. We found notable differences among the four images. **Figure 5a** shows no particular change when the cells were incubated in the blank solution for 3 hr, and the unharmed cells had a typical rod-shape and very smooth surface. **Figure 5b, c** and **d** show morphology changes, with some obscure patches and formation of collapse appearing on the microbe surfaces. The patches, we think, were over-

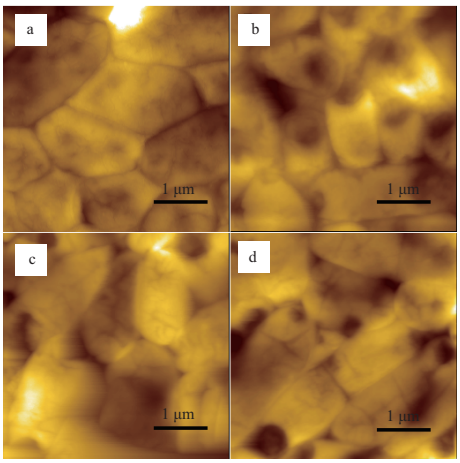

Fig. 5 AFM images of *E. coli* incubated for 3 hr in the following solutions: blank (a), 400 mg/L of sodium nitrite (b), borax (c) and aluminum potassium sulfate (d).

expression of biocomponents caused by the stimulation of the toxicant, and a concave hole appeared if a patch fell from the microbe's surface. Yang et al. (2007) proposed that these condensed patches could prevent the toxicant from attacking the cell membrane. We also found flattened empty cells with rough surfaces, which meant that most of the intracellular content had been discharged, and some *E. coli* showed incomplete morphology, with both its ends degenerated. As we observed above, there were significant changes in microbe surfaces with increasing toxicity of the three chemicals. The AFM revealed that the damages to the microbes' morphology were consistent with the toxicity order of the compounds.

3 Conclusions

The proposed method is a sensitive and rapid alternative to toxicity screening of food additives, and it can be applied to other kinds of illegal and abused food additives. The IC_{50} values, under oxygen restriction, were 410, 570 and 830 mg/L for sodium nitrite, borax and aluminum potassium sulfate, respectively, and the toxicity sequence for these three food additives is in agreement with the reported values obtained using other methods. The extent of damage observed was: sodium nitrite > borax > aluminum potassium sulfate > blank from the AFM images of *E. coli* incubated for 3 hr in various solutions. The method is rapid, cheap, simple and humane for toxicological assessment of food additives. In future works, we will utilize different microbes and various methods such as the immobilization of microbes, avoiding the need to culture fresh cells prior to each trial. We also expect the coefficient of variation will be improved when using microbial film immobilization methods.

Acknowledgments

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