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A potentiometric biosensing system based on an isolated degrading bacterium *Klebsiella* sp. MP-6 for the determination of methyl parathion†

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A potentiometric sensing system for the sensitive and selective detection of methyl parathion (MP) is described in this paper. The system is based on a degrading bacterium *Klebsiella* sp. MP-6 as recognition element and a polymeric membrane anion-sensitive electrode as a transducer. *Klebsiella* sp. MP-6 can be isolated from long-term organophosphorus pesticide contaminated soils, which is capable of biodegrading MP to produce *p*-nitrophenol. The product can be deprotonated under basic conditions and thus detected by using the anion exchanger based membrane electrode. The bioreactor is prepared by packing the bacterial cells between two polyether sulfone membranes placed in a holder. Molecularly imprinted solid-phase extraction using the MP imprinted polymer as a sorbent enables accumulation and separation of MP from real samples. Under the optimized experimental conditions, the potential response of the biosensing system is linear with the MP concentration in the range of 5–100 nM. The detection limit is 1 nM. The electrode exhibits an excellent selectivity towards other organophosphorus pesticides. The sensing system has been evaluated with spiked water samples and shows good recovery and high accuracy. This methodology is promising to develop potentiometric sensors for detecting organophosphorus pesticides at trace levels in the environment.

1. Introduction

Organophosphate compounds (OPs), which are widely used as insecticides and herbicides in modern agriculture, represent one kind of broadly existing acutely toxic species in ecosystems.[1–3] During the past century, more than 1500 kinds of OPs were synthesized. Among these compounds, OPs with a *p*-nitrophenyl functional group rank in the most poisonous class.[4] Methyl parathion (MP) is one of the most poisonous OPs. It converts to methyl paraoxon *via* a series of photolysis and metabolic oxidation processes and leaves residues on plant surfaces, which poses a threat to human health.[4,5] Therefore, it's necessary to develop a sensitive and selective method to monitor MP in the environment.

Currently, there are several laboratory-based analytical methods for the determination of OPs, including high-performance liquid chromatography,[6] gas chromatography,[7] mass spectrometry,[8] and capillary electrophoresis.[9] These methods are generally sensitive and efficient. However, the instrumentation is expensive and large-scale. Moreover, these methods need tedious pretreatment procedures and professional persons to operate them, which limit their wide applicabilities.[10,11] Alternatively, biological approaches offer sensitive detection with portable and miniaturized devices.[12,13] Electrochemical biosensors based on the inhibition of cholinesterases such as acetylcholinesterase and butyrylcholinesterase have been widely used for the detection of OPs.[14,15] However, the fabrication of the cholinesterase-based biosensors is complicated. In addition, these sensors are not specific to OPs.[15,16] As an alternative, organophosphorus hydrolase (OPH) is a typical bacterial enzyme which could catalyze the hydrolysis of OPs by producing less toxic products, such as *p*-nitrophenol and diethyl phosphate.[10,17,18] Recently, Tang *et al.* proposed a novel electrochemical microbial biosensor for the rapid monitoring of *p*-nitrophenyl-substituted OPs using the cell surface-expressed OPH.[4] Indeed, biosensors based on catalytic reactions are superior to those with inhibition mechanisms in terms of selectivity.[4,13] However, OPH has not been commercialized since the amount of OPH in natural strains is too low to harvest and the production procedures are complicated and costly.[19] Accordingly, it's important to develop a substitute material that could selectively hydrolyze OPs.

Bioremediation is an eco-friendly technique using biological organisms to remove hazardous substances from contaminated

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areas at low cost.[20] Microorganisms, especially bacteria, are key players in the processes of bioremediation of most organic pollutants. So far, many strains with the capability to degrade MP have been isolated from the contaminated environment[20–22] and the metabolic processes of MP by different bacterial strains were also studied.[23] In this paper, *Klebsiella* sp. MP-6, a bacterium isolated from long-term OPs contaminated soils, was successfully used to biodegrade MP. Our previous research has shown that *p*-nitrophenol is the major degradation product.[24]

Potentiometry with ion-selective electrodes offers advantages of high selectivity, low cost and ease of miniaturization,[25] and has been used for analysis of pesticides and heavy metals.[26,27] In this work, a potentiometric biosensing system for detecting MP with excellent sensitivity and selectivity was developed by using *Klebsiella* sp. MP-6 as recognition element and a polymeric membrane anion-sensitive electrode as transducer. The bacterial cells are packed between two polyether sulfone membranes. Molecularly imprinted solid-phase extraction (MISPE) process was employed for sample pretreatment. The use of a molecularly imprinted polymer (MIP) in solid-phase extraction (SPE) can not only concentrate but also selectively distinguish the target analyte from complex samples.[28,29] It will be shown that the proposed system exhibits promising a potential for sensitive and selective detection of MP in the environment.

2. Experimental

2.1. Reagents and materials

High molecular weight poly(vinyl chloride) (PVC), di-*n*-octyl phthalate (DOP), *o*-nitrophenyl octylether (*o*-NPOE), bis(*o*-ethylhexyl) sebacate (DOS), tetradodecylammonium chloride (TDDACl) were purchased from Fluka AG (Buchs, Switzerland). Tetrahydrofuran (THF) was obtained from Sinopharm Chemical Reagent Co., Ltd (China) and was distilled before use. *p*-Nitrophenol was purchased from J&K Scientific Ltd. Methyl parathion (MP) was obtained from Chengdu Best Reagent Co., Ltd (China). Methacrylic acid (MAA), 2,2-azobis-isobutyronitrile (AIBN), ethylene glycol dimethacrylate (EGDMA), acetonitrile and methanol were purchased from Sigma Aldrich. 0.1 mM sodium hydroxide (NaOH) was freshly prepared. All other reagents were of analytical grade. Aqueous solutions were prepared with freshly deionized water (18.2 MΩ cm specific resistance) obtained with a Pall Cascada laboratory water system.

2.2. Cultivation and loading of the MP-degrading bacterium *Klebsiella* sp. MP-6

The MP-degrading bacterium *Klebsiella* sp. MP-6 was isolated and identified as described before.[24] It was cultured in the mineral salt medium (MSM) (1.00 g L^{-1} NaCl, 1.00 g L^{-1} NH_4NO_3, 1.50 g L^{-1} K_2HPO_4, 0.50 g L^{-1} KH_2PO_4, 0.10 g L^{-1} $MgSO_4$) at 30 °C in darkness in an orbital shaker at 180 rpm. When the bacterial cells grew to the late logarithmic phase, the cells were collected on a polyethersulfone membrane with a diameter of 25 mm and a pore size of 0.2 μm (Pall Corporation, Ann Arbor, MI, USA) *via* vacuum filtering. Then another polyethersulfone membrane was covered on the cells to form a sandwich shape. The sandwich form was fixed with an O-ring on a 25 mm membrane bracket (Pall Corporation, Ann Arbor, MI, USA). When not in use, the cells were stored at 4 °C in the MSM.[30]

2.3. Electrode preparation

The anion-exchanger based membranes contain 49.65 wt% PVC, 49.65 wt% DOP and 0.70 wt% TDDACl and were prepared by the solvent-casting technique with freshly distilled THF as the casting solvent.[31] Membrane components of 344 mg were dissolved in 4 mL THF solution. The membrane mixture were then transferred to a glass ring (5.0 cm i.d.) fixed on a glass plate and letting the solvent evaporate overnight. After the solvent evaporated overnight, a disk of 5 mm diameter was punched from the membrane and glued to a plasticized PVC tube with THF. For each electrode, 1 mM NaCl was used as the inner filling solution. All the electrodes were conditioned overnight in the solution identical to the inner filling solution.

2.4. Synthesis of the MP imprinted polymer

The MP imprinted polymer was synthesized by the precipitation polymerization as described before.[32] Briefly, the template MP (0.25 mmol), MAA (1.00 mmol), were dissolved in 35 mL methanol. The solution was prepolymerized in darkness for 1 h. EGDMA (5.00 mmol) and free-radical initiator AIBN (0.07 mmol) were then added. The solution was purged with a stream of N_2 for 10 min and sealed under N_2 atmosphere.[33] Then polymerization was carried out at 60 °C for 24 h. After polymerization, the polymer particles were washed with ethanol and collected *via* centrifugation. The washing and centrifugation processes were repeated for several times until no absorption at 275 nm was observed by ultraviolet visible spectrophotometry.[32]

2.5. Apparatus

Fig. 1 shows the schematic illustration of the potentiometric microbial sensing system coupled with MISPE. The sample/test solution was delivered with a peristaltic pump (IFIS-D, Xi'an Remex Analyse Instrument Co., Ltd., Xi'an, Shaanxi, China). The bioreactor with loaded cells was placed between the pump and the detection cell. The whole flow system was assembled using Teflon tubing of 0.8 mm internal diameter. Electromotive force (EMF) values were measured at 25 °C using a PXSJ-216L digital ion analyzer (Shanghai Instruments Factory, Shanghai, China) in the galvanic cell: Ag, AgCl/3 M KCl/1 M LiOAC/sample solution/ISE membrane/inner filling solution/AgCl, Ag. Each membrane electrode was connected with an ATA-1B rotator (Model, Jiangfen Electroanalytical Instrument Co., Ltd, China) at a rotation speed of 3000 rpm.[27]

2.6. Measurements

The sample/test solution of 1 mL was driven by the peristaltic pump to fill the bioreactor for the MP biodegradation by *Klebsiella* sp. MP-6. After incubation for 30 min, 1 mL of the resulting solution with the product *p*-nitrophenol was delivered from the bioreactor into the detection cell containing 4 mL of 0.1 mM NaOH. The basic background ensures that the product

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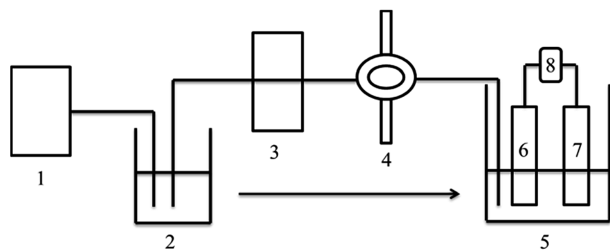

Fig. 1 Schematic illustration of the potentiometric microbial sensing system coupled with molecularly imprinted solid-phase extraction: (1) MISPE column; (2) sample/test solution; (3) peristaltic pump; (4) bioreactor; (5) detection cell; (6) indicator electrode; (7) reference electrode; (8) ion analyzer.

p-nitrophenol ($pK_a = 7.15$) exists in its anion form. The potential response was recorded by the ion analyzer. The potential difference between the baseline and the potential measured at 500 s after the addition of the biodegradation product (*i.e.*, *p*-nitrophenol) was used for quantification.

The potentiometric biosensing system was coupled with the MISPE process for detection of MP in real samples. Tap, lake and sea waters were collected for sample analysis. To remove the impurities, 0.2 μm microfiltration membranes were used to filter the samples. 100 mL samples added with 0.05 μM and 0.10 μM MP were extracted by MISPE. Empty SPE cartridges (2.5 mL) were packed with 30 mg MIP. Before use, each cartridge was conditioned with 10 mL of methanol and followed by 10 mL of ultrapure water in order to remove the potential contaminants. 100 mL sample solution was passed through the cartridge at a flow rate of 10 mL min^{-1}. After loading the sample into the SPE cartridge, 10 mL of ultrapure water was used to remove the matrix interference. The pesticide was eluted from the solid phase with 10 mL acetonitrile. The acetonitrile solution was completely dried under N_2 atmosphere. The residue obtained was redissolved in 1 mL ultrapure water to form the test solution for potentiometric detection by the biosensing system.

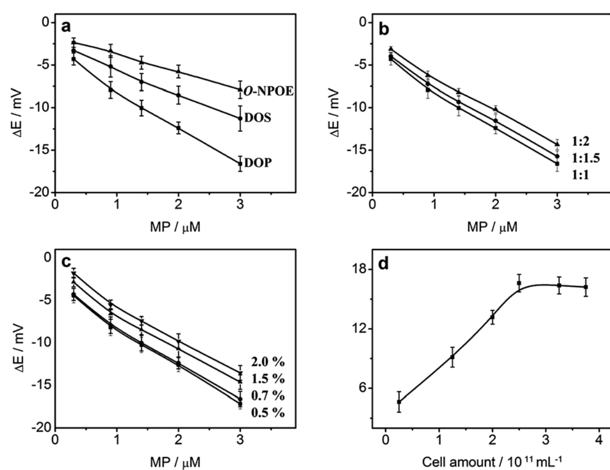

Fig. 2 Effects of the membrane composition and bacterial cells on the potentiometric microbial biosensor: (a) plasticizer; (b) weight ratio of PVC : DOP; (c) amount of TDDACl (wt%); (d) amount of cells. Unless noted otherwise, the experimental conditions were as follows: membrane composition, 49.65 wt% PVC/49.65 wt% DOP/0.70 wt% TDDACl; rotation speed, 3000 rpm; NaOH, 0.1 mM; *Klebsiella* sp. MP-6, 2.5 × 10^{11} cells. Each error bar represents one standard deviation of three measurements.

3. Results and discussion

3.1. Optimization of the potentiometric microbial biosensing system

Methyl parathion is one of the typical *p*-nitrophenyl-substituted OPs. It can be converted to less toxic *p*-nitrophenol after degradation by *Klebsiella* sp. MP-6. The deprotonated *p*-nitrophenol anion which is highly lipophilic could induce a large potential response on an anion exchanger-based membrane electrode. The influence of the membrane composition on the performance of the electrode was studied. As illustrated in Fig. 2a, the electrode prepared with DOP as a plasticizer shows a better performance than that obtained with DOS or *o*-NPOE, which is probably due to the fact that the *p*-nitrophenol anion can be effectively extracted into the membrane with the nonpolar DOP. Since lower amounts of plasticizer reduce the diffusion coefficient in the membrane phase, less plasticizer contents in the membrane could facilitate the accumulation of the *p*-nitrophenol anion at the membrane interface and thus induce higher anionic responses.[34] Therefore, a smaller weight ratio of 1 : 1 (PVC : DOP) was employed for the polymeric membrane (Fig. 2b). It's well known that the amount of ion exchanger in the membrane has a large impact on the electrode response. As shown in Fig. 2c, high anionic potential responses could be observed in the presence of TDDACl. However, the sensitivity of the electrode will decrease when the amount of TDDACl is higher than 0.70%, which is probably due to the quasi-steady-state response mechanism.[35]

The amount of the bacterial cells used is a main factor influencing the sensitivity of the potentiometric microbial biosensing system. The relationship between the potential

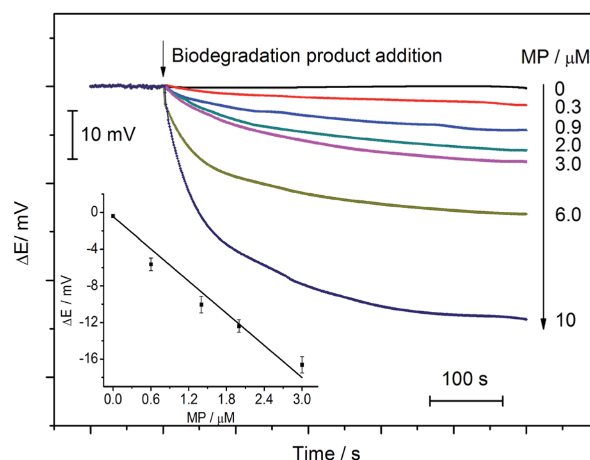

Fig. 3 Typical dynamic potential responses of the biosensing system in 0.1 mM NaOH upon additions of the product solutions after biodegradation of MP at different concentrations. Inset shows the calibration curve for MP. The other conditions are given as in Fig. 2.

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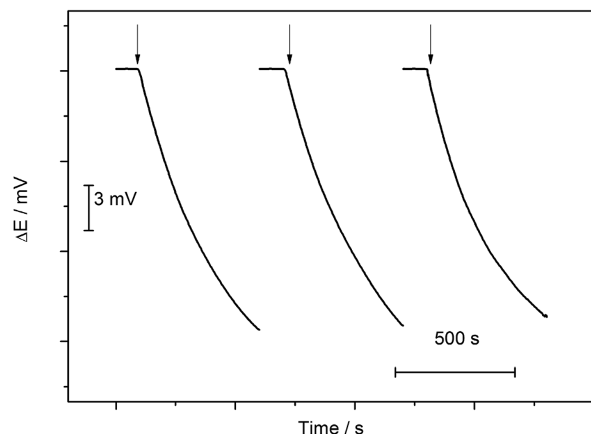

Fig. 4 Recycle potential response to 3.0 μM MP.

response and the amount of cell was investigated in the presence of 3.0 μM MP. As shown in Fig. 2d, the potential response increases with increase in the cell amount and could reach a plateau when 2.5×10^{11} cells are used. Thus, the cell amount of 2.5×10^{11} cells was chosen for *Klebsiella* sp. MP-6 packed in the bioreactor.

3.2. Characteristics of the potentiometric microbial biosensing system

Under the optimized conditions, the potential response of MP after degradation by *Klebsiella* sp. MP-6 using the proposed membrane electrode biosensing system is shown in Fig. 3. As can be seen in the inset of Fig. 3, the potential response of the proposed anion exchanger-based membrane electrode exhibits a linear relationship with the concentration of MP in the range of 0.3 to 3.0 μM. The detection limit was calculated to be 0.09 μM (3σ).

The reversibility of the proposed electrode was also investigated. After each measurement, a 5 minutes washing step with a mixture of 0.1 mM NaOH and ethanol (4/1, v/v) was employed.

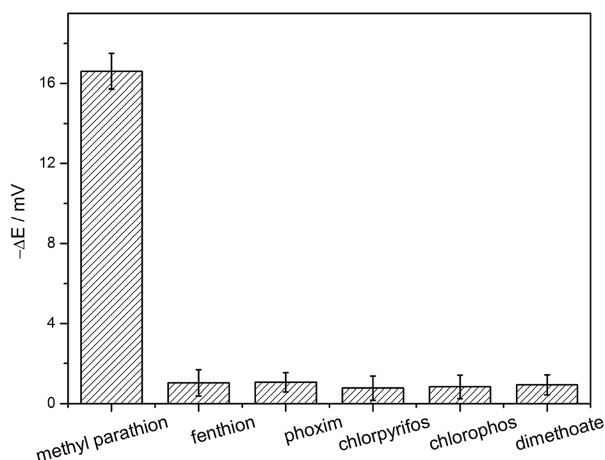

Fig. 5 Potential responses to different kinds of organophosphorus pesticides of 3.0 μM.

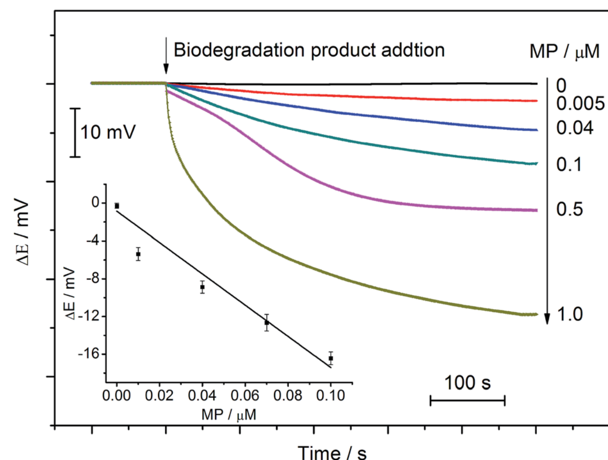

Fig. 6 Typical dynamic potential responses of the potentiometric microbial sensing system coupled with molecularly imprinted solid-phase extraction. The product solutions were added in 0.1 mM NaOH after biodegradation of MP which was extracted from samples at different concentrations. Inset shows the calibration curve for MP. The extraction conditions were as follows: sorbent, 30 mg MIP; sample volume, 100 mL; eluent, 10 mL acetonitrile. The other conditions are given as in Fig. 2.

Fig. 4 shows that the potential response of the electrode can be fully reversible with a relative standard deviation of 2.2% ($n = 3$).

The selectivity of the proposed biosensing system over other organophosphorus pesticides such as fenthion, phoxim, chlorpyrifos, chlorophos and dimethoate was investigated. As shown in Fig. 5, the biosensor exhibits a much larger potential response to MP than to the other five organophosphorus pesticides. Indeed, compared to the potential response to MP, responses to these five organophosphorus pesticides can be neglected. The high selectivity of the proposed polymeric membrane electrode biosensing system offers a promising potential for potentiometric detection of MP in real samples.

3.3. Application of the potentiometric microbial biosensing system to detection of MP in real samples

MP has been forbidden to produce and use since 2007. The residual amounts in the environment are at trace levels. Therefore, separation and enrichment pretreatment processes

Table 1 Application of the proposed biosensing system to the determination of MP in real samples

	Amount of MP (μM)		
Sample	Added	Found[a]	Recovery (%)
Tapwater 1	0.050	0.046 ± 0.011	96
Tapwater 2	0.100	0.090 ± 0.008	90
Lakewater 1	0.050	0.048 ± 0.004	96
Lakewater 2	0.100	0.102 ± 0.004	102
Seawater 1	0.050	0.053 ± 0.005	106
Seawater 2	0.100	0.104 ± 0.005	104

[a] Average value of three determinations ± standard deviation.

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Table 2 Comparation of performances of various sensors for the detection of MP

Transducer type	Linear range (M)	Detection limit (M)	Reference
Amperometry	8.0×10^{-8} to 3.0×10^{-5}	1.5×10^{-8}	4
Amperometry	1.0×10^{-8} to 7.0×10^{-6}	6.0×10^{-9}	32
Spectrophotometry	2.5×10^{-6} to 2.0×10^{-4}	1.0×10^{-6}	37
Amperometry	2.0×10^{-7} to 1.0×10^{-5}	6.7×10^{-8}	38
Amperometry	1.9×10^{-7} to 7.6×10^{-6}	1.9×10^{-8}	39
Amperometry	1.9×10^{-8} to 3.8×10^{-7}	1.2×10^{-8}	40
Potentiometry	5.0×10^{-9} to 1.0×10^{-7}	1.0×10^{-9}	This work

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are usually required before analysis. The use of MIPs in solid-phase extraction can not only concentrate but also selectively distinguish the target molecules, which is crucial for trace level detection of analytes in complex real samples.[36] As shown in Fig. 6, by coupling with MISPE, the proposed system can be used to detect of MP much more sensitively. Under the optimized conditions (see the ESI†), the potential response to MP is linear in the concentration range of 0.005 to 0.1 μM, and the detection limit is 1 nM (3σ).

To evaluate the applicability of the proposed sensing system, spiked water samples were employed. The recovery tests were investigated by the standard addition method. The results are shown in Table 1. The recoveries obtained vary from 90 to 106%, indicating that the proposed potentiometric biosensing system has a promising feasibility for sensitive and selective detection of MP.

The comparison of the analytical performance of the developed biosensor with some of other reported MP sensors is summarized in Table 2. It can be seen that the proposed biosensing system offers a lower detection limit. Compared with the tedious and complex preparation of OPH displayed on the cell surface,[37] the isolation of the MP-degrading bacteria is relatively simple and easy to operate.

4. Conclusions

In summary, a potentiometric biosensing system for the determination of methyl parathion in the environment has been developed. *p*-Nitrophenol is a product from the MP degradation by the bacterium *Klebsiella* sp. MP-6. It can be selectively preconcentrated by MISPE and sensitively detected by the polymeric membrane anion-sensitive rotating electrode. The proposed microbial biosensing system allows sensitive measurements of MP with a detection limit of 1 nM and shows good selectivity towards other organophosphorus pesticides. The system has three unique features for measuring methyl parathion in the environment. Firstly, a degrading bacterium is used as recognition element. Such a bioreceptor is superior to cholinesterases in terms of selectivity. Compared with organophosphorus hydrolase, the bacterium can be isolated from soils in a simple way with low cost. Secondly, the bacterial cells are packed between two polyether sulfone membranes placed in a holder. Therefore, the bioreceptor can be employed without leaking out of the sensing surface or losing its activities. Thirdly, molecularly imprinted solid-phase extraction is coupled to the potentiometric sensing system for real sample analysis, which offers a high-efficiency preconcentration.

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