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PAPER

A novel urea amperometric biosensor based on secretion of carnation petal cells modified on a graphite-epoxy composite electrode†

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A new kind of biosensor for the detection of urea with a high selectivity, sensitivity and wide detection range was designed based on the secretion of carnation petals cells paste covered over a graphite-epoxy composite basic electrode surface. The carnation petal paste from mashed fresh carnation petals was tightly fixed on the basic electrode surface with Teflon thin film to keep it in contact with the electrode surface. Urea in aqueous solution was detected by differential pulse voltammetry based on the oxidation peak current at 0.316 V (*vs.* SCE) of the secreted species of carnation petal cells during the mashing process, which interacts with urea molecules and results in the decrease of the oxidation peak current. The oxidation peak current decreases linearly with the logarithm of urea concentration in the range of 1.3×10^{-16}–4.57×10^{-8} M and 3.4×10^{-7}–1.3×10^{-1} M with a detection limit of 7.5×10^{-16} M. The biosensor was characterized by electrochemistry and fluorescent spectrometry, and applied to the determination of urea in waste water from a river around Shenyang Normal University campus with a recovery of 104.5% (RSD is 5.00%). The presence of larger amounts of ammonium ion and nitrate ion up to the molar ratio of 10^4 do not interfere with the urea detection.

1 Introduction

Biosensors[1–3] and biochips[4,5] with unique properties of higher sensitivity, higher selectivity, higher stability, lower detection limit, and longer lifetime have been developed based on whole living cells, enzymes, DNA and proteins as the bio-recognition materials. Suitable bio-recognition units are very important in biosensor development. Most of the bio-recognition materials are from animals; only a small amount of them are from plants even though plants form a very large part of bioresources,[6,7] and are easily grown to be manipulated and sensitive in response to their environments.[8,9] Plants and their tissues have been used as bio-recognition units for biosensors[10,11] and other biological systems.[12–14]

Urea as a fertilizer of plants and a metabolic product of animals is a very important molecule in biological systems, and has been determined by spectrophotometry,[15] infrared spectroscopy,[16] and enzyme biosensor methods.[17] Carnation petal has been used as a bio-recognition unit in an amperometric biosensor for the detection of urea[18,19] and has attracted much attention. In the present paper, mashed carnation petal paste was coated on a graphite-epoxy composite electrode surface to build up a secretion biosensor for the detection of urea in aqueous solution. The biosensor was characterized by electrochemistry and fluorescent spectrophotometry.[20,21] Some interesting results are reported here.

2 Experimental

2.1 Instrument and reagents

Electrochemical experiments were performed on an electrochemical analyzer (model CHI620B, Chen Hua Instrument Company, Shanghai) with a three electrode system, a mashed carnation petals modified graphite-epoxy composite electrode as the working electrode, a piece of platinum wire as the counter electrode and a saturated calomel electrode as the reference electrode—all potentials were measured and reported with respect to this reference electrode.

Fluorescence experiments were carried out on a fluorescence spectrophotometer (Eclipse, Varian Co.). The imaging of cells in petals or in petal paste was carried out by metallurgical microscopy (CK40M,Olympus Co., Japan).

Potassium chloride, urea, disodium hydrogen phosphate, potassium dihydrogen phosphate, ammonium chloride, and potassium nitrate were all analytically pure, and prepared as electrolyte solutions, standard stock solutions, and pH buffer solutions. All solutions were prepared with Ultrapure water (18.3 MΩ from MilliQ water system). All electrolyte solutions were deaerated with high purity nitrogen gas to remove oxygen prior to use.

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2.2 Fabrication of the biosensor

A graphite-epoxy composite electrode (GECE)[22] was chosen as the basic electrode due to its higher affinity for organic and biomolecules and tissues. It was prepared with a mixture of 2.00 g of graphite powder, 0.60 g of epoxy resin and 0.50 g of polyamide resins, and filled into a piece of glass tube (the inner diameter was about 4 mm) with a piece of copper wire as the electrode lead. The prepared GECE was dried in air for one week, and the distal was polished with thin sandpaper and glassy paper into a mirror-like surface.[23]

The carnation flower used in this work was bought from a common flower shop in China. The metallurgical microscopic images of cells in petal tissue and in the mashed paste are shown in the supplemental figures (see ESI†). A piece of carnation petal was excised from a fresh carnation flower, mashed into paste with the basic electrode on a clean glass plane. The petal paste was coated on the basic electrode surface, and covered tightly with a PTFE membrane[22] in order to obtain a quick response of the biosensor to the urea in aqueous solution.

2.3 The measurement procedure

Electrochemical experimental procedure. Three electrodes were put into a 0.1 M KCl electrolyte solution (pH 7.5, phosphate buffer system with a total concentration of 0.020 M). The cyclic voltammetry and differential pulse voltammetry experiments were performed as the basic curves of the biosensor at an initial potential of 0.0 V (*vs.* SCE) for 2 s with 0.004 V increment potential, 0.05 V amplitude, a pulse width of 0.05 s, sample width of 0.0167 s and sensitivity of 1 e^{-5} A V^{-1}. After addition of urea solution in different concentrations into the electrolyte solution, the electrochemical experiments were performed again. The obtained voltammetric curves were used for the analysis of the interactions between carnation petal paste and urea in solutions.

Fluorescent spectrophotometric experimental procedure. One piece of petal was picked off from a fresh carnation flower, and mashed into a paste. One piece of carnation petal or carnation petal paste were covered on a reflection mirror (a part of the fluorescent spectroscopy apparatus for solid materials from the instrument company) with a piece of PTFE, the reflection mirror was set up in the chamber of the fluorescent spectrophotometer, and the fluorescence experiments were performed. Fluorescence spectra of carnation petal and carnation petal paste were obtained.

The reflection mirror with carnation paste was put into a fluorescent cell with 1.0×10^{-4} M urea, and fluorescence experiments were performed again. The recorded fluorescent spectra were used to test the interactions of carnation paste with urea molecules.

3 Results and discussion

3.1 Design and characterization of the biosensor

Design of the biosensor. According to the excitable properties of plants,[6,7] the cells, tissues and organs in plants alter their internal conditions and external reactions under the influence of various environmental factors referred to as irritants, such as temperature, chemicals, mechanical wounding, insects, cutting by releasing some chemicals as secretion, and these signals are quickly transferred through the whole plant as well as to the plants nearby. In this way, the higher plants can respond to small amounts of chemicals sensitively and selectively, which is known as communication or signaling between cells or plants. There are at least two steps in this sensing process. The irritant chemicals are firstly recognized by the bio-recognition molecules with the help of enzymatic systems[8,9] in the cells of the plant tissue in contact with the chemicals and release their secretion in response to the chemical irritant. Secondly the cells transfer this signal to nearby cells through the plasmodesmata,[24] and irritate the nearby cells to secrete the same species. This signal was amplified biologically according to the quorum sensing theory.[25] In biosensor design, we pay more attention to the first step using a variety of enzymes and DNA, to recognize the target molecules, and build up several kinds of biosensors. But we have not yet found a suitable way to utilize the bioamplification function of cells in the biosensor design.[26]

In general plant tissue biosensors,[27,28] the biosensor transducers cannot respond to the communication processes between the cells, so that there is no obvious improvement in the sensitivity of the biosensor. If the petal tissue is mashed into paste, the cells are stripped from the petal. The stripped cells themselves reorganize on a suitable surface[29,30] into a pattern similar to that in the petal tissue. The metallurgical microscopic images of cells in petal tissue and the cells in the mashed paste are shown in the supplemental figures (see ESI†). The self-reorganized cells can keep the quorum sensing number[25] and transfer the biological signal to the electrode. In order to keep the petal paste and cells on the electrode surface without leaking into the solution during the experiments, a piece of PTFE membrane was used to cover the paste tightly on the electrode surface. The biosensor was fabricated based on this idea as described in Section 2.2, and characterized as in the following.

Electrochemical characterization of the biosensor. The three electrodes were set up in electrolyte solution (0.1 M KC1 and pH 7.5 phosphate buffer with total concentration of 0.02 M) with or without urea. Cyclic voltammetric (CV) and differential pulse voltammetric (DPV) experiments were performed. The CV and

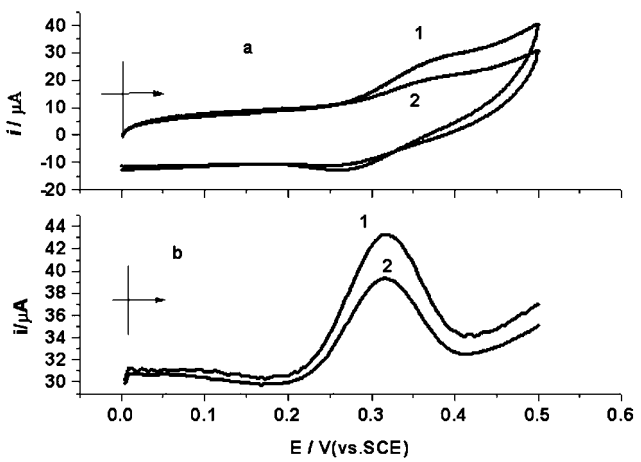

Fig. 1 The CV curves (a) and DPV curves (b) of the biosensor with urea (2); without urea (1) in 0.1 M KCl electrolyte at pH 7.5. Quiet time: 2 s scan rate: 0.05 v/s, urea concentration of 1.0×10^{-5} M.

DPV curves were obtained as shown in Fig. 1. The biosensor shows an oxidation peak at 0.37 V, and a reduction peak at 0.26 V (curve 1 in Fig. 1a). The peak-peak potential difference of 110 mV indicates that this is a quasi-reversible electrochemical process. The quasi-reversible property means the electrochemical active species can follow the potential scan slowly, and return to the initial stage after electrochemical experiments without loss in activity or amount. The two redox peaks may belong to the same redox active substances. With the addition of 1.0×10^{-5} M urea into the solution, the redox potentials were not shifted, but the redox currents were reduced (curve 2 in Fig. 1a), which indicates that the urea molecules can react with the redox active substance, form a redox inactive one, and result in the decrease of the redox current.

The oxidation DPV curves with one oxidation peak located at 0.316 V are shown in Fig. 1b. With the addition of urea, the oxidation peak current at 0.316 V decreased, which was more sensitive than that in CV curve, and can be used to monitor the concentration of urea in solution.

Under the same conditions, the CV experiments were performed at different scan rates. The oxidation peak current at 0.37 V was increased linearly with the increase of scan rate. The regression equation is as follows.

$$i_{pa} = -0.4603 + 0.08929v \qquad R = 0.9958, SD = 0.4828 \qquad (1)$$

This relationship indicates that the redox process was a surface controlled one, and the electrochemically active species was just on the electrode surface. In the biosensor design, the petal cells are located at the electrode surface, and the released electrochemically active species was also on the electrode surface, which can directly be monitored by electrochemical methods at the electrochemical transducer during the biological process. This was an important evidence that the biosensor design works well.

According to the plant secretion theory,[30,31] the higher plant can release some signaling substances to respond to the irritation from the environment such as mechanical or chemical irritants. If the released substances are electrochemically active, then the related process can be monitored by electrochemical methods.[32,33] Petals of flowers contain some polyhydroxyl phenols such as catechols,[34] which can give an oxidation peak at about 0.3 V. Polyhydroxyl phenols can also react with urea to form electrochemically inactive substances.[35] This is the working principle of the biosensor.

Fluorescent spectrophotometric characterization of the biosensor. In order to verify the process, fluorescence spectroscopy was utilized to test the system. The fluorescent spectra of carnation petal (a), of carnation petal paste (b) and of carnation petal paste after contacting with 1.0×10^{-4} M urea (c) are shown in Fig. 2.

From Fig. 2 it can be seen that carnation petal shows three obvious fluorescent peaks located at 460.00, 488.95 and 627.94 nm (Fig. 2a), but after being mashed into carnation paste, the number of fluorescent peaks increased up to 13 (Fig. 2b), however, while in contact with urea solution (1.0×10^{-4} M), the intensities of these fluorescent peaks decreased, and some of them even disapeared (Fig. 2c). These results indicate that the mashing process makes the carnation petal secrete some

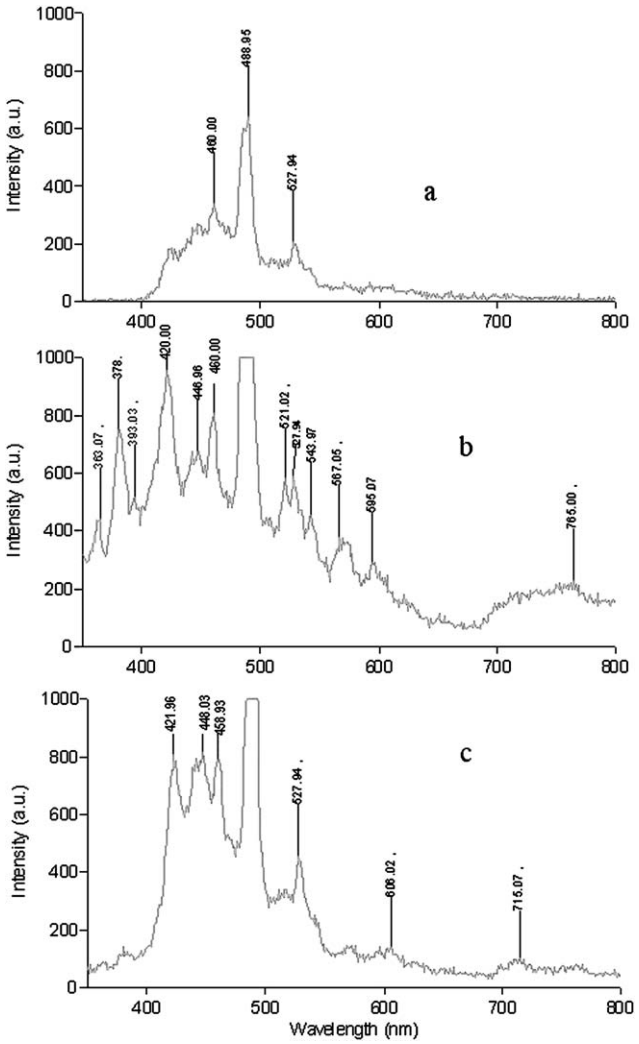

Fig. 2 The fluorescence spectra of carnation petal (a), mashed paste of carnation petals without urea (b), and with urea (c).

electrochemically active substances, which interact with urea molecules and lose their electrochemical activity and fluorescent properties. These results shown further evidence for the biosensor principle.

3.2 Optimal experimental conditions

3.2.1 Effect of solution pH. The solution pH was controlled by phosphorate buffer system (Na_2HPO_4–KH_2PO_4) in the range of pH 7.2–7.8. The DPV experiments were performed at different solution pH values. The oxidation peak current at 0.316 V was plotted against solution pH, and one peak curve was obtained with the peak point at pH 7.5 as shown in Fig. 3. The pH = 7.5 was chosen as the optimal solution pH.

3.2.2 Effect of accumulation time. Accumulation time is defined as the time period of the bio-electrode being in contact with the detected urea solution, which can be used to examine the response of the biosensor to the urea solution before the electrochemical experiments begin. The DPV experiments were performed in 0.1 M KCl electrolyte solution (pH 7.5) including

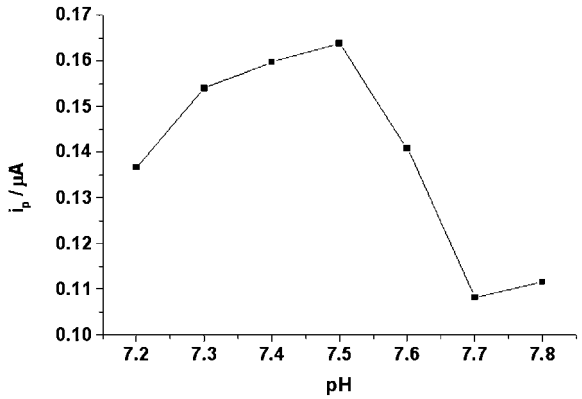

Fig. 3 The relationship between oxidation peak current and solution pH. The other experimental conditions were the same as those in Fig. 1.

1.0×10^{-5} M urea. The oxidation peak current at 0.316 V was plotted against accumulation time.

The oxidation peak current decreases linearly with an accumulation time in the range 0f 0–180 s with a regression equation of,

$$i_{pa} = 8.783 - 0.00773t$$
$$R = 0.9948,\ SD = 0.0659 \quad (2)$$

The apparent interaction rate constant was obtained as 0.00095 s^{-1}. The optimal accumulation time was chosen as 150 s, in which the DPV curve showed a better appearance with a straight basic line and normal peak shape. The oxidation current belongs to the substance released by the cell, which interacts with urea, loses its reduction activity, and results in the decrease of oxidation peak current. So the 0–150 s of the accumulation time belongs to the interaction time.

3.2.3 Effect of quiet time. The quiet time is one of the electrochemical experiment parameters, which is defined as the time period in which the initial potential was applied (0.0 V *vs.* SCE) to the working electrode before the potential scan started. The quiet time was used to test the influence of the initial potential, which can be used to adjust the redox states of the electrochemically active substance. Under the experimental conditions described earlier, the DPV experiments were performed at different quiet times. The obtained anodic peak current was plotted against quiet time as shown in Fig. 4.

With the increase of quiet time, the anodic peak current increases fast at first, reaches the maximum at 2 s, and then decreases according to an exponential function of

$$i_{pa} = 0.9478 + 0.3267 \times e^{-t/2.308}$$
$$R = 0.995, SD = 0.00015 \quad (3)$$

This result indicates that applying the initial potential to electrodes for a long time will not be of benefit to the electrochemical detection of the target substance. In practice, the optimal quiet time was chosen as 2 s. The shorter quiet time is also the result of the quasi-reversible property of the redox system.

3.2.4 The effect of urea concentration. Under the optimal conditions, the DPV experiments were performed at different urea concentrations in the range of 4.57×10^{-8}–1.3×10^{-16} M., and the DPV curves were recorded as shown in Fig. 5. The anodic peak current was plotted against the logarithm of urea concentration as a straight line as shown in the inserted plot in Fig. 5 with a regression equation of,

$$i_{pa} = 12.523 - 2.850\log(c, \mathrm{M})$$
$$R = 0.9927, SD = 1.092 \quad (4)$$

This relation indicates that the response of the biosensor to the urea concentration follows a first order reaction similar to that of the response of the enzyme to the substrate. This straight line can be seen as a part of the standard curve in the detection of urea in solution.

3.3 The methodology for urea detection

3.3.1 Standard working curves. In Section 3.2.4, a standard working curve for urea in the concentration range of 4.57×10^{-8}–1.3×10^{-16} M was obtained as shown in eqn (4), from

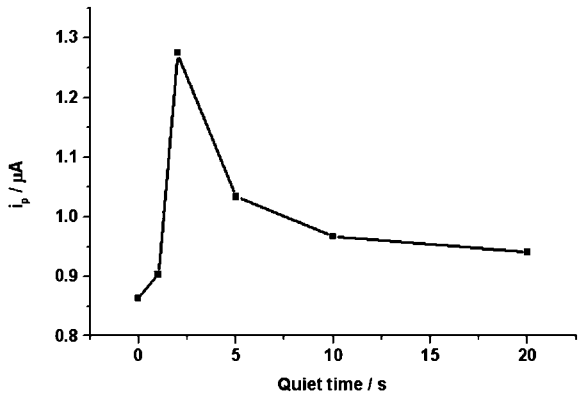

Fig. 4 The relationship between oxidation peak current and quiet times. The other experimental conditions were the same as those in Fig. 1.

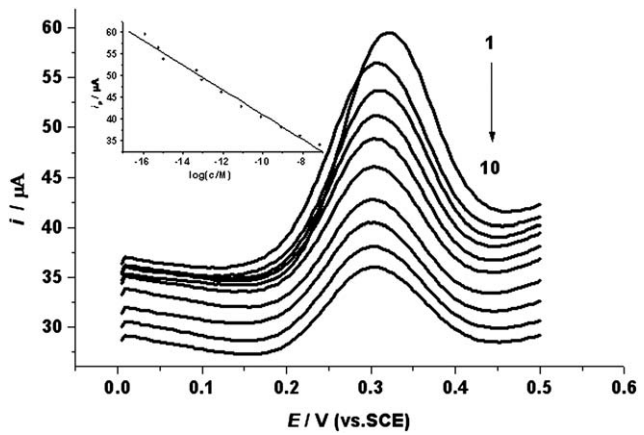

Fig. 5 The DPV curves of biosensor at different concentration of urea, and the insert shows the relationship between anodic peak current and logarithm of concentration of urea. Concentration (M): 1, 0.00; 2, 1.30×10^{-16}; 3, 6.12×10^{-16}; 4, 1.09×10^{-14}; 5, 4.83×10^{-13}; 6, 8.82×10^{-12}; 7, 8.97×10^{-11}; 8, 8.89×10^{-10}; 9, 8.74×10^{-9}; 10, 4.57×10^{-8} The other experimental conditions were the same as those in Fig. 1.

which the lowest detection was calculated as 7.5×10^{-16} M (3.3 × deviation/slope). Under the optimal experimental conditions, the DPV experiments were performed in the higher urea concentration range of 1.0×10^{-1}–3.4×10^{-7} M, and a similar regression equation to eqn (4) was obtained as,

$$i_{pa} = -0.8302 - 1.259 \times \log(c, M) \quad R = 0.992, SD = 0.3076 \tag{5}$$

Compared with eqn (4), the linear slope is decreased to 1.25 from 2.850. This means the biosensor responds less sensitively to higher concentration.

3.3.2 The deviation and recovery of the method

Deviation experiments. Under optimum experimental conditions, the DPV experiments were performed ten times at the urea concentration of 1.0×10^{-15} mol L^{-1} using same biosensor or a newly prepared biosensor with a petal from the same carnation flower. An oxidation peak current at 0.316 V was obtained with an average peak current of 53.273 μA and a relative deviation of 4.99% (the data are listed in Table 1 of the supplemental tables, see ESI†).

Recovery experiments. Under the optimal conditions, the urea concentration in waste water from a river around Shenyang Normal University campus was determined by DPV with the biosensor to be 0.175 μM. The urea standard solution calculated as 0.909 μM was added into the sample solution, and then the total concentration of urea in the sample solution was determined by the biosensor to be 1.125 μM. The addition standard recovery was calculated as 104.5% (all data are listed in Table 2 of the supplemental tables, see ESI†).

3.3.3 Interfering experiments. As the N fertilizer of plants, ammonium or nitrate ions may interfere with the detection of urea. Under the optimal conditions, the interferences were tested by adding calculated 0.10 M NH_4Cl and 0.10 M KNO_3 solutions into a solution containing 1.0×10^{-5} M urea, and the concentration of urea was determined by DPV with the biosensor. The obtained DPV curves are shown in Fig. 6. From Fig. 6a it can be seen that large amounts of NO_3^- ions slightly change the shape of the curve, but do not interfere with the detection. Large amounts of ammonium ion slightly shift the peak potential to a positive direction and change the shape of the anodic peak because ammonium is one of the products of urea metabolism, but do not influence the peak position and peak current as shown in Fig. 6b. These results indicate that a large amount of nitrate and ammonium ions do not interfere with the detection of urea even though the molar ratio of nitrite or ammonium ion to urea were up to 10^4.

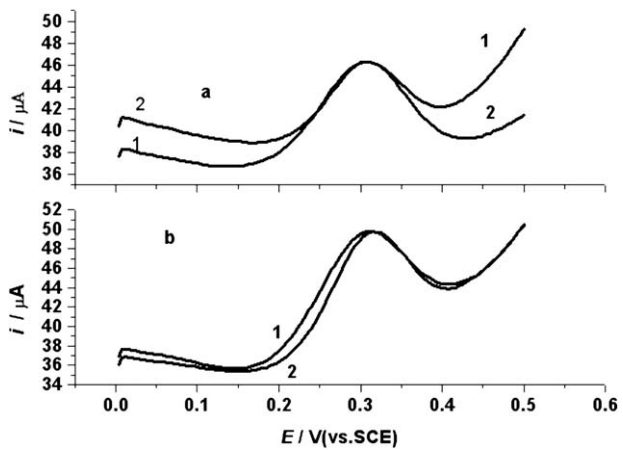

Fig. 6 DPV curves of biosensor response to urea with (1) or without (2) interferences of nitrate (A) or ammonium ion (B). The other experimental conditions were the same as those in Fig. 1.

4 Conclusions

In this paper, an amperometric biosensor was designed and fabricated based on secretion of whole living cells of carnation petals for the detection of urea. Some conclusions are as follows.

The graphite-epoxy composite electrode modified with carnation petal paste containing whole cells of carnation petal can serve as biosensor for the detection of urea with very high sensitivity and selectivity. The optimal experimental conditions were obtained as pH 7.5, contact time of 150 s, and quiet time of 2 s. The biosensor responds to urea in the concentration ranges of 4.57×10^{-8}–1.3×10^{-16} M and 1.0×10^{-1}–3.4×10^{-7} M with a relative deviation of 5.00% and recovery of 104.5%. Ammonium ion and nitrate ions do not interfere with the detection. The selectivity to urea is over 10^4.

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