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A kind of integrated microfluidic system for rapid pathogenic botrytis cinerea detection

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In order to overcome the poor timeliness and low automaticity of the detection of existing botrytis cinerea in fruits and vegetables, an integrated microfluidic system for rapid detection is proposed. In this system, the microelectrode is mounted on the bottom of the microfluidic chip channel and connected to a digital impedance measurement circuit. The magnetic beads whose surface is encased by mouse botrytis cinerea antibody, is controlled by an external Gauss magnetic field to capture botrytis cinerea. Then the combination of botrytis cinerea and the magnetic beads is transferred to a microelectrode array. By constructing a counting circuit based on impedance measurement at the electrode terminals, the amount of botrytis cinerea can be effectively measured. The results show the validity of the system in detecting the amount of botrytis cinerea in fruits and vegetables. In addition, the detection time is only nearly one-fiftieth compared with a traditional laboratory test.

Keywords*:* Pathogens; formatting; Integrated microfluidic; Rapid detection; Impedance

INTRODUCTION

Gray mold is one of the main diseases which damage the facilities of fruit and vegetable cultivation, mainly infecting the night shade family, cucurbit crops, onion leeks, berries and so on, leading to crop root rotting, withering away, decomposing, stem gangrening and so forth, decreasing the quality, affecting productivity and decreasing by more than 50% the productivity when this is serious. Therefore, highly efficient, rapid, portable botrytis cinerea detection is the key technology to ensure the quality of agricultural production and promotion of the intensive mode of production.

Traditional botrytis cinerea detection methods include the AGAR plate culture method [1]; the direct microscopic counting method [2]; the plate counting method [3]; the light spectrum detection method [4]; the mold rapid test paper method [5] and others. The culture medium is heated in the flame over a long period of time using the AGAR plate culture method, which is not conducive to bacteria recovery and the water is not consistent before and after loading. The plate count method has greatly influences the fungi duirng the sample treatment process, so it does not reflect the pollution situation accurately. The light spectral detection method is relatively advanced, but the process requires the help of professional laboratory personnel with complex experimental steps carried out with the aid of expensive equipment. The mold rapid test paper method is simple and easy to operate, cultivating at room temperature, the results are observable in two days, the efficiency is higher, but it is expensive. These defects limit the practical application of the technology in agricultural production it also restricts the development of a high quality and highly efficient horticultural production technology in our country.

In recent years, the micro fluidic technology is an effective method that renders, injection, mixing, reaction and detection linking integrate into a piece of the micron scale chip. The introduction of the system has greatly improved the automation and the objectivity of the fungus detection process, which has caused the wide attention of the scholars at home and abroad [6-9]. However, a complete detection method and detection system of botrytis cinerea matching in fruits and vegetables to the micro fluidic chip does not exist.

On this account, this paper presents an integrated microfluidic system for rapid pathogenic fungus detection based on immunomagnetic capture by the compound microelectrode impedance detection technique.

THE IMMUNOMAGNETIC BEAD PREPARATION AND THE ACQUISITION PRINCIPLE OF BOTRYTIS CINEREA

The pathogens' immuno magnetic capture technology uses magnetic beads as carrier for antibody immobilization, via specific binding of the

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antibody to antigen, realizing the capture of pathogens, also realizing the enrichment of the object on the solid support surface and then improving the sensitivity of the detection method [10].

The first step of the preparation of an immunomagnetic sample is $Si(OC_2H_5)$(short for TEOS) being hydrolyzed to get $Si(OH)_4$ under the catalysis of ammonia water. Then a chemical reaction occurs at some active points of the $Si(OH)_4$ and Fe_3O_4 particle surface, as the combination point on the surface film, obtaining composite magnetic micro spheres (Fe_3O_4@SiO_2). Then using triethoxyaminopropylsilane (short for APTES) as the organic precursor, an Amino modification of the silicon oxide surface of Fe_3O_4@SiO_2 takes place, obtaining Fe_3O_4@SiO_2-NH_2 composite micro beads (MMPs) in the process of preparation, the main process is shown in the Picture 1.

$$Si(OC_2H_5)+4H_2O \rightarrow Si(OH)_4+4C_2H_5OH$$

TEOS

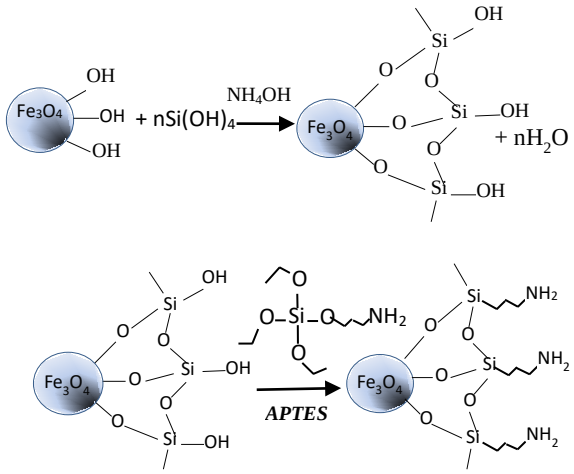


Fig. 1. Preparation principle of Fe_3O_4@SiO_2-NH_2

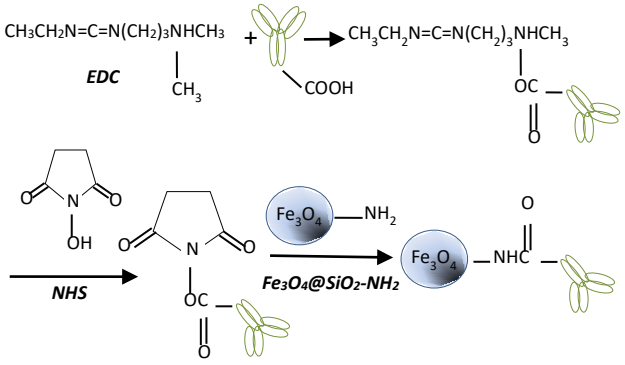


Fig. 2. The preparation principle of IMMPs.

Then activating the carboxyl group of the surface of the mouse's gray mold antibody via an EDC/NHS crosslinking agent, forming intermediate products with the active group and then after being covalently bound to the amino group on Fe_3O_4@SiO_2-NH_2, obtaining immunecomplex micro beads (IMMPs) in the process of preparation, the process is shown in the Picture 2.

CHIP OPERATING PRINCIPLE

Figure 3 is the principle diagram of an integrated microfluidic system for rapid pathogenic botrytis cinerea detection, which uses chemical crosslinking technology to make pathogens antibody to coat the micron sized magnetic beads, so as to bring beads into the microfluidic chip system, then the microfluidic chip system is placed in a Gauss magnetic field controller (as shown in Figure 4), by controlling the transformation of the magnetic field to drive the magnetic beads. In the process of beads movement, the antibody can effectively capture the pathogen antigen. Then the combination of pathogenic fungus and magnetic beads was brought to the microelectrode array at the bottom of the micro fluidic chip by the Gauss magnetic field controller. After capturing the pathogen antigen, the microelectrode array can measure the impedance change to determine the amount of pathogenic fungus in fruits and vegetables through the impedance measuring circuit.

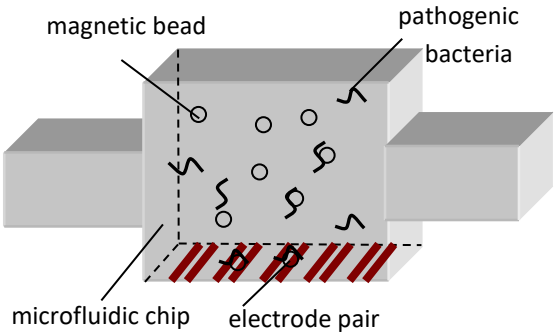
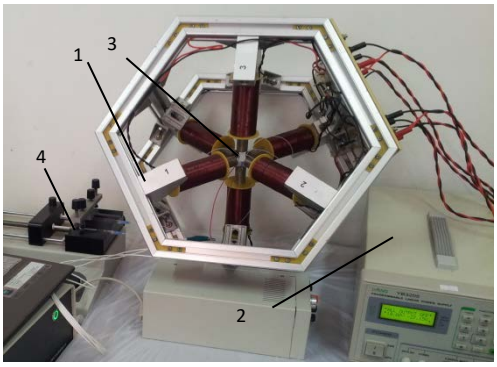


Fig. 3. The principle of rapid detection of pathogenic bacteria.



1. Gauss magnetic field controller; 2. Control power supply; 3. microfluidic chip; 4. driven pump

Fig. 4. Picture of the magnetic field controller.

In order to maximize the detection probability, the electrode array at the bottom of the microfluidic chip is arranged as dense as possible. The electrode pair is closely connected with the detection circuit, and each pair of electrodes has an independent XY address, which can be tested separately.

There are three possible conditions between the electrodes. First, there are no free beads between the electrodes, this is the only ionization medium and the impedance level is low. Second, only the immunomagnetic beads are between the electrodes meaning that the beads do not capture the pathogenic fungus and therefore the impedance level is highest. Finally, the beads capture the free pathogen between the electrodes, under the influence of parallel micro magnetic resistance and fungal cytoplasm resistor, the impedance level reduced to an intermediate value. In order to ensure the sensitivity of the electrode detection, the voltage across the electrodes must always be greater than the bacterial cell membrane breakdown voltage, so the selection is from 0.2V to 1.5V [9]. In this way, the capacitance of the cell membrane can be neglected, which renders the bacteria into a superior conductor. Through the above working principle, a relationship can be established between the concentration and the variation of the impedance of the pathogenic fungus in fruits and vegetables. The equivalent circuit of impedance measurement of the pathogen is shown in Figure 5.

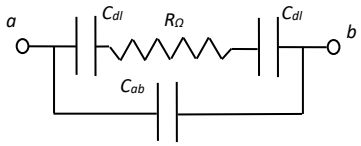


Fig. 5. The equivalent circuit for impedance measurement system

The research electrode and the auxiliary electrode are represented respectively by a and b, then the electrode impedance is calculated as follows:

$$|Z_1| = \sqrt{R_{\Omega}{}^2 + \frac{1}{(\pi f C_{dl})^2}} \quad (1)$$

$$|Z_2| = \sqrt{\frac{1}{(2\pi f C_{ab})^2}} \quad (2)$$

$$\frac{1}{Z_{total}} = \frac{1}{Z_1} + \frac{1}{Z_2} \quad (3)$$

Where, C_{ab} is the interelectrode capacitance, C_{dl} is the interface between the poles of the electric double layer capacitor, R_{Ω} is the resistance of the fungus and bead complexes, f is the frequency of impedance testing, Z_1 is the impedance of the equivalent circuit of C_{ab} + C_{dl} + R_{Ω}; Z_2 is the impedance of the equivalent circuit of C_{ab}, Z_{tatol} is the system impedance. The bacteria concentration can be assessed by determining the quantitative relationship between the systematic impedance with the concentration of pathogen.

MICROELECTRODE AND IMPEDANCE DETECTION CIRCUİT

A. The fabrication of a microchannel and microelectrode

The microfluidic chip is produced from poly dimethyl siloxane (PDMS) material. PDMS is evenly mixed with a curing agent in the proportion of 90% and the mixture is injected into the container. The significant number of bubbles produced during the mixing process can be removed by a vacuum-pumping system. The mixture stands at room temperature for about 10 hours until PDMS curing and remolding, so as to form a micro channel based on the microfluidic chip PDMS substrate. the micro channel is used to provide a place to capture the immunomagnetic beads.

The tin indium oxide conductive film is chosen as a substrate. Ultrasonic cleaning is carried out by deionized water, methylbenzene, acetone and alcohol. The water is heated to a temperature of 120℃. The negative photoresist ITO array electrode pattern can be obtained in 4 steps: whirl coating, prebaking, exposure and developing. Afterwards, the microelectrode array (20μm×20μm) is fabricated by means of hard baking, corrosion, removing of the photoresist, scouring, baking and others. Shown in Figure 6 is the microscopic image of the microelectrode array. The overall microfluidic system can be obtained by plasma bonding to the

ITO substrate with a microelectrode array and PDMS chip.

Fig. 6. The microscopic image of microelectrode array

B. Design and fabrication of the impedance detection circuit

For each pair of electrodes, there is a corresponding detection circuit which is used to detect the real part of the impedance. Figure 7 is a simplified schematic of the detection circuit. The detection circuit is used to measure the electrical level between the electrode pairs. The specific steps are the following: first the reference current is injected into one electrode and then passes through the test target and then finally it flows to earth. The test target is a parallel model of the immunomagnetic bead and the aquaculture pathogen. If K1 is closed and K2 is opened, then this state shows that the immunomagnetic bead does not capture the pathogen. If K1 and K2 are closed, then this state shows that the immunomagnetic bead captures the pathogen. The current which is exported from the current supply is converted to a voltage signal through the impedance between the electrodes. The voltage signal is compared with a reference voltage, thus an output signal that reflects the relationship between the electrodes can be obtained.

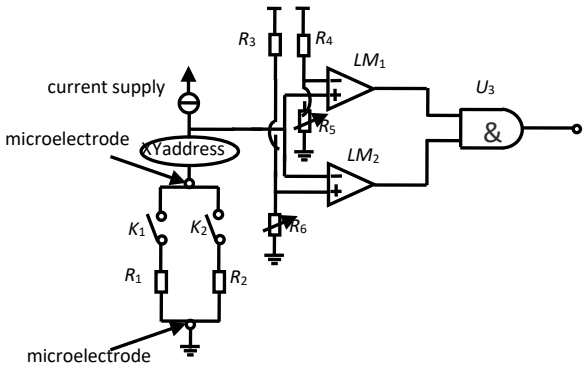


Fig. 7. Impedance detection circuit principle diagram.

In order to enable the detection circuit to accurately measure the amount of the pathogens, the impedance threshold of the pathogens that have fallen into the microelectrode should be measured accurately. The impedance signals that can be obtained through microelectrode measurement are introduced to the logic circuit by ITO lead wire. To ensure the tight joint between the ITO conductive glass and the electronic circuit, this paper adopts silver paste for the welding procedure. The distribution of pathogenic fungi can be observed under a microscope, the different impedance values which correspond to the magnetic beads that carry pathogenic fungai and the magnetic beads that don't carry pathogenic fungai stand idle respectively in accordance with the distribution conditions. The paper uses the Botrytis cinerea in strawberries as an example and the average impedance measured by the microelectrode load conditions is 65 kΩ. When magnetic beads don't capture the pathogenic bacteria, the average impedance is 800kΩ. On the other hand when the magnetic beads capture the pathogenic bacteria, it is 200kΩ. According to the values for the impedance, R_5 and R_6 are set effectively, when the magnetic beads capture the pathogenic fungi, the comparator LM_1 and LM_2 output at high levels at the same time so that the gate circuit output digital signal is "1" and its output digital signal is "0" under different conditions.

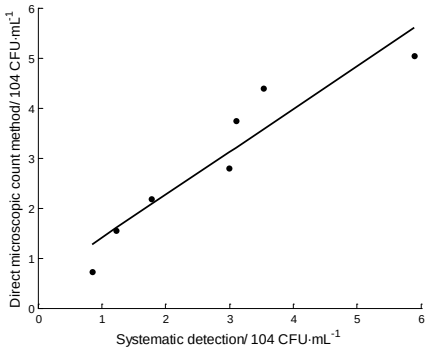


Fig. 8. Regression analysis for the two methods.

The results show that the average relative error of the two detection methods is less than 8%. Compared with the most widely used plate count method, the integrated microfluidic system for aquaculture pathogenic fungus achieved the daily detection accuracy and the detection time was shortened to about 55 minutes, the detection efficiency was raised about 50 times. In addition, the human input and complexity is far lower than for the traditional plate count method during the whole detection process, it greatly improves the

automation level in the detection of the pathogenic fungi in fruits and vegetables.

Table 1. Error comparison of the two detection methods

Sample	Microscopic Count Method CFU·mL^{-1}	Microfluidic System CFU·mL^{-1}	Relative Error
1	1.23×10^4	1.56×10^4	-2.68%
2	2.99×10^4	2.79×10^4	6.69%
3	5.89×10^4	5.04×10^4	1.44%

CONCLUSION

A rapid integrated microfluidic detection system is proposed in this paper. The system puts the microelectrode at the bottom of the microfluidic chip channel and it connects with the digital circuit for impedance measurement. The paper uses chemical crosslinking technology to make a pathogenic fungus antibody and coat the magnetic beads also using an external Gauss magnetic field to control the capture of pathogen fungi by magnetic beads. Then the combination of magnetic beads and pathogen fungi is taken to the microelectrode array and the impedance measurement circuit between the microelectrodes will detect the content of the pathogenic fungi. The research results show that the detection system has basically reached the daily detection accuracy and the detection time is shortened to about 55 minutes, the detection efficiency is raised about 50 times and the human input and complexity is far lower than the traditional plate count method during the whole detection process, this greatly improves the process of automation level detection of pathogenic fungi in fruits and vegetables. Thus, research experience was gained in the development of a portable horticultural plants pathogen detection system.

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ИНТЕГРИРАНА МИКРОФЛУИДНА СИСТЕМА ЗА БЪРЗО ОТКРИВАНЕ НА ПАТОГЕНИТЕ *Botrytis cinerea*

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(Резюме)

Предложена е микро-флуидна система за бързо откриване на патогените *Botrytis cinerea* в плодове и зеленчуци. В схемата един микроелектрод се монтира на дъното на канал на микро-флуиден чип и се свързва с цифров контур за импедансно измерване. Системата се зарежда с магнитни сфери с имобилизирани антитела от *Botrytis cinerea* (изолирани от мишки) и контролирани от външно магнитно поле за задържането на антителата. Имобилизираните антитела се прехвърлят в микроелектродното пространство. Количеството на *Botrytis cinerea* се определя чрез броящ контур, базиран на импедансно измерване. Резултатите показват приложимостта на системата. Времето за определяне е петдесет пъти по-кратко от необходимото за традиционния лабораторен тест.