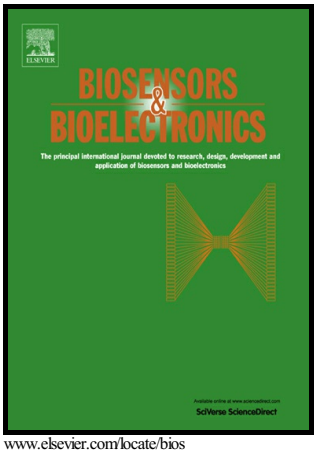

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A sensitive and simple macrophage-based electrochemical biosensor for evaluating lipopolysaccharide cytotoxicity of pathogenic bacteria

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ABSTRACT

In this study, a sensitive and simple electrochemical murine macrophage (Ana-1) cell sensor has been developed for early detection of lipopolysaccharides (LPS) to evaluate the toxicity of pathogenic bacteria. Magnetic glassy carbon electrode (MGCE), which possesses excellent reproducibility and regeneration qualities, was modified with a nanocomposite to improve electrochemical signals and enhance the sensitivity. The synthesized magnetic nanoparticles (MNPs) were internalized into murine macrophages, which completed the immobilization of macrophages onto the modified electrode for evaluating the cytotoxicity of LPS by electrochemical impedance spectroscopy (EIS). The MNPs facilitated reusability of the proposed sensor by allowing removal of the magnetic core from the electrode. Our results indicated that LPS caused a marked decrease in electrochemical impedance in a dose-dependent manner in range of 1–5 μg/ml. By SEM, we found that microvilli on the plasma membrane became scarce and the membrane became smooth on cells incubated with LPS, which lessens the absorption of cells to reduce the impedance. And biological assay indicated that EIS patterns were correlated with the calcium concentration in cells, and suggested that $[Ca^{2+}]_i$ production increased in cells incubated with LPS and its mobilization altered electrochemical signals. Compared with conventional methods, this electrochemical test is inexpensive, highly sensitive, and has a quick response, and thus provides a new avenue for evaluating the cytotoxicity of pathogens.

Keywords: MWNT-GNP/Chi/MGCE, MNPs, Ana-1 cells, LPS of pathogenic bacteria, EIS

Introduction

Pathogen detection has vital importance in the food industry, water and environmental quality control, and clinical diagnosis for health and safety reasons. Bacterial lipopolysaccharides (LPS) are the major outer surface membrane components present in almost all Gram-negative bacteria and are hazardous human pathogens. Endotoxins from Gram negative bacteria cause numerous infections/or diseases. Previous reports found that LPS extracted from the cell wall of Gram

negative bacteria is a potent inducer of the inflammatory response (Frosrt et al. 2002; Shang et al. 2015). LPS, beside being potent activators of the immune system, can also exert cytotoxic effects (Hewett and Roth 1993; Morrison and Ryan 1979). Traditionally, biological assays of cell activity and viability evaluation, such as by the CCK-8 assay, the release of the enzyme lactate dehydrogenase (LDH), and the measurement of reactive oxygen species (ROS), and mitochondrial apoptosis are often used to evaluate toxicity (Thannickal and Fanburg 2000). These methods are complex, costly, and difficult for the long-term monitoring of cells. Simple and cost-effective processes that maintain high sensitivity but reduce assay time would be very beneficial for industrial use. To simplify the test procedure, reduce costs, and enhance sensitivity, cytotoxicity tests based on the electrochemical behavior of living cells, such as electron transfer at electroactive centers in cells (Li et al. 1999), open circuit potential at the cell/sensor interface (Woolley et al. 2002), and electric cell-substrate impedance (Xiao et al. 2002), have been developed as assay methods. Electrochemical sensing to assess toxicity is inexpensive, highly sensitive, selective, fast to respond, and capable of long-term monitoring, real-time measurements, and generating reproducible results (Rahman 2011). As a noninvasive and sensitive detection technique for studying living cells, electrochemical impedance spectroscopy (EIS) measurement shows promise as a tool to monitor adhesion, viability, and environmental changes in cells (Cortes-Salazar et al. 2013). Recent studies on biosensors have demonstrated that EIS measurements produce significantly more sensitive responses than cyclic voltammograms (CV) as label-free cell-based biosensors (Asphahani and Zhang 2007). Thus, a sensitive and specific electrochemical signal generated by cells immobilized on an electrode can be detected rapidly and accurately.

As important inflammatory cells, macrophages play an important role in the development of inflammation. There have been many reports on the reactivity of macrophages in the literature with pooled macrophage samples. Some of the cellular events that occur during LPS-induced Ana-1 cell activation include an increase in intracellular calcium concentrations and the generation of reactive oxygen species (ROS) in the cytoplasm (Brubacher and Bols 2001; Jin et al. 2006; Myers and Swanson 2002). Professional phagocytes generate high levels of ROS using a superoxide-generating NADPH oxidase as part of their armory of microbicidal mechanisms, and ROS production is largely dependent on $[Ca^{2+}]_i$ mobilization (Lambeth 2004). Dense, long microvilli and filamentous pseudopods were observed

on the surface of macrophage plasma membrane. Microvilli on the plasma membrane showed spot-like distribution and became scarce in some sites and most of the membrane became smooth when macrophages were stimulated (Guo et al. 1992). Hence, murine macrophages (Ana-1) show excellent potential for biosensor applications due to their dramatic exocytotic response to bacterial lipopolysaccharides (LPS) within a short time of LPS addition. They can detect target LPS and convert biological recognition into a signal that can be recorded and quantified quite easily. The signal transduction system amplifies the input signal greatly making the system responsive to very small quantities of analyte.

Since the recognized discovery of carbon nanotubes (CNTs) (Iijima 1991)，CNTs have been the focus of intense research due to their unique structural, mechanical, electrical, thermal, magnetic and chemical properties, making them candidates for a wide range of promising applications (Grobert 2007). Metal nanoparticles, such as Au, Ag, Pt, Pd, Ni and Cu, have been coated or deposited onto the side walls of both single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs) (Day et al. 2005; Georgakilas et al. 2007; Yoon and Wai 2005). These new hybrid nanomaterials have been shown to exhibit excellent catalytic activity, electrical conductivity, and photonic properties. Of particular interest are nanocomposites containing gold nanoparticles (GNPs), due to the combination of the unique electronic properties of carbon nanotubes and their ease of surface modification, along with the biocompatibility of GNPs (Jiang et al. 2009).

To anchor cells on the electrode, we internalized magnetic nanoparticles (MNPs) into macrophages and then utilized the magnetism to absorb onto the magnetic glass carbon electrode. Fe_3O_4 nanoparticles have become a favourite candidate for biosensing (Jiang et al. 2015), as they exhibit a high degree of biocompatibility and are environmentally safe. To acquire stable and biocompatible magnetic nanoparticles, polyethylene glycol is often added for the synthesis of magnetic nanoparticles due to its stability, biocompatibility, and low cytotoxicity (Mukhopadhyay et al. 2012; Yuan et al. 2014).

This study explored a novel method for electrochemical determination of LPS, based on magnetic bead-internalized Ana-1cells. The MNPs-internalized living cells were able to be rapidly absorbed onto modified MGCE from the medium for electrochemical investigation, improving the efficiency of the system. Different concentrations of LPS were used to stimulate Ana-1 cells to evaluate toxicity. Differing electrochemical responses caused by the stimulatory of LPS on the

macrophages are discussed below. Biological assays of cell activity and viability evaluation, such as CCK-8 assay, the measurement of reactive oxygen species (ROS), calcium concentration, and cell apoptosis are often used to evaluate toxicity. In order to rule out the toxicity of MNPs, murine macrophages were exposed to various concentrations of MNPs and the viability and apoptosis of cells were assessed using the CCK-8 assay and flow cytometry. The production of ROS and the changes of calcium concentration were detected by Widefield High-Content Analysis System and confocal laser scanning microscopy, and the fluorescence intensity was measured by using a microplate reader. Our results point out that LPS stimulation produced dose-dependent toxicity in murine macrophages. Interestingly, we found that the electrochemical signal corresponded with the changes of calcium concentration. Our results show that the proposed method is simple, rapid, inexpensive, and convenient for electrode renewal, and is recommended as a promising experimental platform for wider applications in biosensing and evaluating the cytotoxin of pathogen.

1. Experimental procedures

2.1. Materials and apparatus

Multi-walled carbon nanotubes (Purity > 95%) was obtained from Nanjing Xian Feng Nano-materials Technology Co. Ltd. (Nanjing, China). Iron chloride ($FeCl_3 \cdot 6H_2O$), ethylene glycol, sodium acetate and polyethylene glycol (PEG-6000) were from Shanghai Chemical Reagent Co. (Shanghai, China).

Chloroauric acid ($HAuCl_4 \cdot 4H_2O$), Escherichia coli lipopolysaccharide (LPS, O111:B4) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Phosphate buffered saline (PBS) and all assay kits were purchased from Beyotime Biotechnology Co.(Shanghai, China). Murine macrophages (Ana-1) cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Roswell Park Memorial Institute (RPMI) media and fetal bovine serum were obtained from Gibco Laboratories (Gaithersburg, MD, USA). All of the solutions were prepared with ultrapure water, and all of the reagents were of analytical grade.

All of the electrochemical experiments were performed with the CHI660e electrochemical workstation (Chenhua Technology Co., Ltd. , Shanghai, China) using a conventional three-electrode system in a solution containing 2.5 mM of $Fe(CN)_6^{3-/4-}$ and 1.0 M KCl. A homemade magnetic glassy carbon electrode (MGCE) (Incole Union Technology Co., Ltd., Tianjin, China) was used as a working electrode. The morphologies of magnetic nanoparticles and the modification of MWCT-GNP/Chi were characterized using transmission electron microscopy (JEM-2100, Jeol, Japan)

and scanning electron microscopy (JSM-7500F, Jeol, Japan), respectively. The morphologies of cells were characterized using scanning electron microscopy (Quanta-200, FEI, USA). Ana-1 cells were incubated in a CO_2 incubator (Thermo Scientific Forma Series II Water Jacket, Thermo Fisher Scientific Inc., Rockford, IL, USA). The CCK-8 assay and fluorescence intensity assays were performed using a microplate reader (Spectra MAX 340, Molecular Devices Co., Sunny vale, CA, USA). A confocal laser scanning microscopy (LSM710, Carl Zeiss Microscopy GmbH, Göttingen, Germany) and Widefield High-Content Analysis System (Image Xpress Micro XLS, Molecular Devices, USA) were employed for the observation of cells.

1.2. Preparation of magnetic nanoparticles

The magnetic nanoparticles (MNPs) were prepared by a solvothermal reduction method according to Deng et al (Deng et al. 2013). Briefly, 1.35 g of $FeCl_3 \cdot 6H_2O$ was dissolved in 40 mL of ethylene glycol to form a clear solution, followed by the addition of 3.6 g of sodium acetate and 1.0 g of polyethylene glycol. The mixture was stirred vigorously for 30 min and then sealed in a Teflon-lined stainless-steel autoclave. The autoclave was heated and maintained at 200℃ for 8 h, then cooled to room temperature. The black products were recovered by a magnet and washed four times with ethanol and ultrapure water and dried under vacuum.

1.3. Cells culture and magnetic nanoparticles internalization

Ana-1 cells were cultured in a flask in RPMI-1640 medium that was supplemented with 10% fetal calf serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml) at 37℃ in a humidified atmosphere containing 5% CO_2. At the growth retardation stage after 3d, the cells reached the logarithmic phase of growth, after which they were used for experiments. All cells were harvested using trypsin and were resuspended in the fresh complete medium before plating.

Briefly, 1×10^6 Ana-1 cells in growth medium containing MNPs (0.05 mg/mL) were added into plates and mixed gently, taking advantage of magnetic fields for improving the efficiency of the system. Ana-1 cells were cultured for 12 h at 37℃ in a humidified atmosphere containing 5% CO_2, then washed with sterile phosphate buffered saline (PBS) after the cells were immobilized completely under a magnetic field. The MNP-internalized living cells were then rapidly isolated from the medium for later experiments and electrochemical investigation.

1.4. Assay of MNPs cytotoxicity for cell labeling

Cytotoxicity of the prepared MNPs was evaluated by the Cell Counting Kit-8 (CCK-8) colorimetric assay. Ana-1 cells were cultured in 96-well plates with a density

of 5×10^5 cells/well and incubated for 12 h at 37°C. After the attachment of the cells, serial dilutions of different formulations containing MNPs were added to the culture medium. At the end of the incubation, the medium was removed, and 100 μl culture medium containing CCK-8 was added to each well and the cells were incubated at 37°C. After 1 h, ultraviolet absorbance at 450 nm was measured with a microplate reader. Six replicates were done for each treated group and the percent viability was normalized to the cell viability in the absence of MNPs.

The influence of MNPs internalization on cells survival and viability was examined by evaluating apoptosis via fluorescence-activated cell sorting analysis including an Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotech Co., Ltd., Shanghai, China). Briefly, Ana-1cells internalized MNPs were cultured for 24 h on 12-well plates and then were harvested for flow cytometry. Following neutralization by washing with culture media containing fetal bovine serum (FBS) and PBS, the cells were resuspended in 195 μL of AnnexinV-FITC binding buffer and stained in the dark with 5 μL Annexin V-FITC (an apoptosis marker) and 10 μL propidium iodide (PI, a necrosis marker) or 20 min at room temperature. 20,000 cells per sample were analyzed by flow cytometry and Ce1lQuest software. Fluorescent signals from FITC and PI were distinguished by 3 color detectors.

1.5. Preparation of MWNT-GNPs/Chi modified MGCE

MWNT-GNPs nanocomposites were synthesized according to Jiang et al. (Jiang et al. 2009). Basically, chitosan solution (1 wt%) was prepared by dissolving chitosan power in 1.0% (v/v) acetic acid solution with stirring for 1 h at room temperature until completely dispersed. In a typical composite material preparation, 10 mg carboxylic MWNTs were dispersed in 20 mL of chitosan solution with 2 h sonication to prepare a homogeneous dispersion of MWNT-chitosan. Subsequently, l mL of 25 mM $HAuCl_4$ was added to the MWNT-chitosan dispersion under vigorous stirring at room temperature for 10 min. The mixture was then heated to 80℃ while stirring for ca. 1 h until the color of the solutions did not change.

The magnetic glass carbon electrode (MGCE) was successively polished with 0.3 μm and 0.05 μm alumina slurry on a polishing cloth, rinsed thoroughly with doubly-distilled water, and then sonicated in ethanol and doubly-distilled water for 10 min. Then 20 μl of MWNT-GNPs nanocomposites was dropped onto the electrode to promote electron transfer rate and maintain cell viability. Before the electrochemical measurements, the modified electrode was cycled between -0.2 and 0.6 V (scan rate, 100 mV s^{-1}) several times until reproducible responses were acquired.

A 4 mL tube was prepared containing MNPs-internalized Ana-1 cells (6×10^6 cells mL^{-1}) suspension. The modification of MGCE and the immobilization of cells were carried out as described in Scheme 1.

1.6. The immobilization of Ana-1 cells and electrochemical measurements

In order to remove the MNPs that were not internalized, the cell suspension was then filtered by the Tc-s0037 Cell Strainer (1200 mesh, 11 μm, Toscience Biotechnology Co., Ltd. Shanghai, China) coupled with 50 mL centrifuge tube, and centrifuged at 1000 rpm for 5 min. Due to the different sizes of cells and MNPs (macrophages size=15μm, MNPs size=300 nm), they could be well separated through the Cell Strainer using centrifugal force. The harvested Ana-1 cells were then washed and maintained in PBS in 4 mL tube. After that, the modified MGCE was inserted into the tube and incubated at 37℃ for 10 min to immobilize cells for electrochemical analysis.

Electrochemical experiments were performed using a CHI660e electrochemical workstation (Shanghai, China) with a conventional three-electrode system comprising a platinum wire as an auxiliary, a saturated calomel electrode as a reference, and a modified magnetic electrode with cells as the working electrode. The electrochemical behavior of Ana-1 cells that were exposed to various concentrations of LPS in PBS (pH 7.4) was studied using electrochemical impedance spectroscopy (EIS) carried out in the presence of 2.5 mM $Fe(CN)_6^{3-/4-}$ and 1.0 M KCl. Cells not exposed to LPS served as the control. The impedance spectra were recorded within the frequency range of 10^{-1}-10^5 Hz. The data of EIS were captured by the ZView software (Jiang et al., 2013). All of the electrochemical experiments were carried out at room temperature. The cytotoxicity of the LPS was calculated as follows:

$$\text{Cytotoxicity}(\%)=100[(R_{cell} - R_{LPS}) / (R_{cell} - R_{modified})]$$

in which $R_{modified}$ is the impedance of the electrode modified with MWNT-GNPs/Chi, R_{cell} is the impedance of cell immobilized on the electrode, and R_{LPS} is the impedance of the cells that were treated with a certain concentrations of LPS (Gu et al. 2015).

1.7. Cell viability, intracellular Ca^{2+} and ROS measurements

The viability of cells simulated by LPS was evaluated by the Cell Counting Kit-8 (CCK-8) colorimetric assay as described above. Ana-1 cells were incubated with a certain concentrations of LPS for 3 h at 37°C. Then 10 μL CCK-8 was added to each well at 37°C for 1 h. Optical density at 450 nm was measured by a microplate reader.

The levels of Ca^{2+} were measured by Fluo-3/AM, a visible wavelength calcium probe. The dye was added to the Ana-1 cells for 1 h at 37℃ under 5 % CO_2 in the dark.

Next, the cells were washed with PBS (pH 7.4), and seeded into 96-well plates with different concentrations of LPS for 3 h and then the fluorescence was measured. The intracellular generation of ROS was investigated using 6-carboxy-2, T-diclorodihydrofluorescein diacetate, di(acetoxy ester) (DCFH-DA) (Molecular Probes, Eugene, OR). Cells preincubated in the presence of 10 μM of DCFH-DA for 1 h were treated with different concentrations of LPS for 3 h. The cells were washed three times with serum-free medium. Fluorescence was measured using a Widefield High-Content Analysis System (Image Xpress Micro XLS, Molecular Devices, USA) with an excitation of 488 nm and an emission of 525 nm. The fluorescence intensity inside the cells was determined using a microplate reader (Spectra MAX 340, Molecular Devices Co., Sunny vale, CA, USA).

1.8. Statistical analysis

Data are expressed as mean ± S.D. The statistical analysis was carried out using SPSS 17.0 programs. The significance of difference was determined by the unpaired Student's t-test. $P < 0.05$ was considered the threshold for statistical significance between the control group and the experimental groups.

2. Results and discussion

3.1. Characterization and cytotoxicity of magnetic nanoparticles

A typical Fe_3O_4 dispersion synthesized by solvothermal reduction method and polyethylene glycol was used to disperse the nanocrystals and improve biocompatibility. Transmission electron microscopy (TEM) images show that Fe_3O_4 nanoparticles prepared via high-temperature thermal decomposition were monodispersed with an average diameter of about 300 nm (Fig. 1A). CCK-8 tests investigated potential adverse effects of the MNPs, and results showed that the MNPs-internalized Ana-1 cells viability slightly decreased with the increase of the concentration of MNPs. As the concentration of MNPs increased from 0.02 to 0.5 mg/mL, cell viability decreased from 93.85% to 81.33%. When MNPs were at the concentration of 0.02 and 0.05 mg/mL, cell viability was 93.85% and 92.97% (Fig. 1B), respectively, not significantly different from the control ($P > 0.05$). Therefore, we selected 0.05 mg/mL as an adequate concentration to decrease the cytotoxicity of magnetic bread and improve the efficiency of endocytosis. Apoptosis analysis was performed by flow cytometry for the MNP-internalized cells (Fig. 1D) and control groups (Fig. 1C), using Annexin V-FITC and PI as markers for apoptosis and necrosis, respectively. There were similarly low rates of early apoptosis (0.08 ± 0.07 vs. 1.23 ± 0.55%, respectively), late apoptosis (0.04 ± 0.03 vs. 0.07 ± 0.03%) and necrosis (0.11

± 0.07 vs. 0.05 ± 0.02%, respectively). Together, the results suggested that low concentration of MNPs had no significant effect on cell viability.

3.2. Characterization of the modified glassy carbon electrode

The process of electrode modification is described in Scheme 1 and was characterized by SEM (Fig. 2A and B). The images of SEM and the absorbance peak at 525 nm in the UV-vis absorption spectra showed the synthesis of gold nanoparticles (Fig. S1A). Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were used to characterize both the process of constructing the biosensor and the cells adsorption onto the sensor surface (Fig. 3A and B). In general, MWNT-GNPs/Chi were uniformly dropped onto the surface of a clean bare MGCE. As shown in Fig. 3A, the voltammograms showed a typical, reversible redox peak at the modified MGCE surface and an increase in the anodic peak current (Ipa) was clearly observed when the electrode was modified with MWNTs and gold nanoparticles (GNPs) due to their good conductivity and the increased effective area of the working electrode. There were some similar signal changes in Fig.3B. The current increased from 374.5 μA (curve a) to 583.2 μA (curve b) and the GNPs further enhanced electronic signals, along with the biocompatibility of GNP and chitosan (curve c). Compared with bare MGCE, an increase of 59% in the peak current (from 374.5 to 911.3 μA) was clearly observed.

3.3. Electrochemical characteristics of Ana-1 cells on a modified electrode

The Ana-1 cells anchored on the modified electrode surface through the magnetic property. The characteristics of the MNPs-internalized Ana-1 cells on MGCE were also tested by cyclic voltammetry (CV), differential pulse voltammetry (DPV), and electrochemical impedance spectra (EIS) in the presence of 2.5 mM $Fe(CN)_6^{3-/4-}$ (Fig.3). The modification of Ana-1cells on the MWNT-GNP/Chi/MGCE surface resulted in a decrease in the peak current of $Fe(CN)_6^{3-/4-}$. In Fig.3A and B, a decrease in the peak current and a drastic increase in the peak separation were clearly observed (curve e) when the cells were adhered onto the sensor surface, ascribed to the blockage of electron transfer by the insulation of the cell membrane. Whereas, a slight decrease in the peak current was observed when the MNPs alone were adsorbed onto the modified electrode (curve d). It further suggested that the peak current decreased because of the adsorption of cells (curve e). We further used EIS to characterize both the process of constructing the biosensor and the process of cell adhesion onto the sensor surface. The EIS spectra of $Fe(CN)_6^{3-/4-}$ probe showed that different modified electrodes showed different electron-transfer resistance (R_{et}) values

(Fig. S2). With the bare MGCE, the R_{et} value was 102. After the modification of MWNT-GNP/Chi, the R_{et} decreased greatly to 17.45 due to its favorably high electrical transport properties. When Ana-1 cells were attached to the MWNT-GNP/Chi/MGCE, the barrier near the electrode surface hampered the redox probe and thus increased the R_{et} (Fig. 3C). The electrochemical sensing ability of the cells was measured based on the observation that living cells are highly insulating at low frequencies. Because of the adsorption of Ana-1 cells, the R_{et} value increased with the adsorption time (Fig. S2B) and cell concentration (Fig. 3C). As shown in Fig. S2B, the R_{et} value remained steady after 10 min, so we determined to absorb cells for 10 min. With increasing cell concentration ranging from 6×10^2 cells/mL to 6×10^8cells/mL, the R_{et} increased, showing increasing diameter of the semicircle on the Nyquist diagram (Fig. 3C), which implied a higher number of immobilized cells on the electrode. When the cell concentration exceed 6×10^6 cells/mL, the impedance of the modified electrode became steady. Fig.3D shows that any increase in impedance was dependent on the surface's cell coverage, which was proportional to the concentration of the cells in a certain range. The R_{et} showed a linear relationship with cell concentration from 6×10^2 to 6×10^6 cells mL^{-1}, with a correlation coefficient of 0.998. In addition, when cell numbers were more than 6×10^6, the impedance values were practically unchanged. Therefore, the appropriate cell number of 6×10^6 cells mL^{-1} was selected for LPS analysis.

These results of EIS are similar to the changes in CV and DPV responses. The current peak value changes, for MNPs internalized cells absorbed on the electrode (Fig. S3A and B), also showed increased coverage rate of MGCE, which impeded the current circulation (curve c). When exposed to LPS, the peak current dramatically increased (curve d) but was still lower than the sample with MNPs alone attached to the magnetic electrode (curve b).

3.4. Ana-1 cell sensor assays for LPS detection

The electron-transfer resistance was related to not only the amount of cells that were immobilized on the modified electrode but also to the morphology and apoptosis of the adhered cells (DePaola et al. 2001). Thus, in addition to the detection of cell immobilization, this method could be used to monitor both the morphological changes and apoptosis of cells on electrode surface in real time. In this study, to analyze the toxicity of LPS on Ana-1 cells, the modified electrode was exposed to different concentrations of LPS. Impedance changes due to cell morphological changes and

apoptosis on the electrode surface were measured three times after the treatments. The subsequent change in the electrochemical response of the Ana-1 cells indicated the cytotoxicity of the different concentrations of LPS. The electrochemical impedance signals were detected at different timepoints after the absorbed cells were exposed to LPS. The impedance value nearly kept steady for cells without LPS within 6 h (Fig. S3C), which suggested this proposed method had good stability. After 6 h, the decrease of impedance value may be ascribed to nutritional deficiency in PBS. As shown in Fig. S3D , when cells were exposed to LPS for 3 h, significant electrochemical impedance signals were detected. Fig. 4A shows the typical electrochemical impedance signals from Ana-1 cells activated by different concentrations of LPS. As the concentration of LPS increased from 1 μg/ml to 50 μg/ml, the inhibition rate of Ana-1 cells significantly increased from 14.92% to 59.57% as measured by EIS (Fig. 4B). We can clearly see from the inserted figure, LPS led to morphological changes and reduced cell survival in a dose-dependent manner at low concentrations, and showed a quasi-linear relationship ($y=-156.950x+1768.20$, $R^2=0.9970$) when LPS was between 1 and 5 μg/ml. The detection limit of LPS was 0.15 μg/ml. As the concentration of LPS was increased, the quasi-linear behavior of the EIS disappeared. The effects of the LPS may have been limited by the concentration of cells. Scanning electron microscope of Ana-1 cells showed that long microvilli and emitting filamentous pseudopods on the surface of macrophage plasma membrane (Fig. 4C). However, microvilli on the plasma membrane showed spot-like distribution and became scarce in some sites and most of the membrane became smooth when macrophages incubated with LPS (Fig. 4D). This suggests why the R_{et} decreased as LPS concentration increased, where the diameter of the semi-circle on the Nyquist diagram shrinks.

For magnetic electrode regeneration, after testing, the MGCE surface was rubbed with an alcohol pledget and washed with purified water to regenerate the magnetic electrode. Next, the MGCE was cleaned in 0.5 mol/L H_2SO_4 by performing CVs from -1.0 to 1.0 V with a scan rate of 100 mV/s for 20 cycles. After thorough rinsing with purified water and drying with high-purity nitrogen gas (99.999%), the MGCE was modified by MWNT-GNP/Chi and used to capture Ana-1 cells with MNPs for all subsequent detection experiments. Spiked cell samples at two different concentrations, 2×10^4 and 2×10^5 cells mL^{-1}, were tested by the proposed sensor. Results are shown in Table S2. The recovery values were determined by testing cell samples at different concentrations. As shown in Supplement Table S2, the results show successful

average recovery rates from 97.56% to 98.39% and coefficients of variance (CV) from 3.69% to 4.72%. These values are in accordance with the required accuracy in typical trace analysis, indicating easy reproducibility of the fabrication protocol.

3.5. Effect of LPS on the viability of Ana-1 cells

To estimate the cytotoxic interactions of LPS, five concentrations of LPS (1, 5, 10, 20 and 50 μg/ml) were used for electrochemical detection and cytotoxicity test (Fig. 5). After exposure to different amounts of LPS for 3 h, we found that LPS induced a significant impedance decrease by EIS, comparing with the detection of CCK-8 assay (Fig. 5A). This method was more sensitive than the CCK-8 assay. We also investigated the effects of LPS on $[Ca^{2+}]_i$ and ROS production (Fig. 5) in Ana-1 cells to explore inherent changes in macrophages. From Fig. 5B, we can see that the intracellular ROS production did not significantly change with 3 h LPS treatment (from 1 to 50 μg/ml) and the fluorescence was measured as Fig. S4 shown. The incubation of cells with LPS for 3 h induced an increase in intracellular Ca^{2+} level (Fig. 5C and D), which indicated that $[Ca^{2+}]_i$ firstly generated in cells incubated with LPS, then stimulated ROS release as part of the inflammatory response. Meanwhile, the inhibition rate by EIS and relative fluorescence intensity on calcium showed a nearly linear relationship ($y=0.27249x+11.77344$, $R^2=0.8426$), which indicated that it the mobility of calcium might affect electron transfer and improve the electrical signal. The proposed electrochemical method is a sufficiently sensitive test to evaluate the effect of LPS.

3. Conclusion

In this study, a highly sensitive, simple, and successful magnetic Ana-1cell-based sensor was developed by adsorbing living macrophages internalized with MNPs on MGCE, in effort to evaluating the cytotoxicity of LPS. Magnetic nanoparticles were synthesized, characterized, and internalized into Ana-1 cells. The sensor exhibited favorable correlation with cell concentration, ranging from 6×10^2 to 6×10^6 cells mL^{-1}. The electrochemical LPS sensitivity test offers potential advantages over conventional assays. Our results showed that LPS caused a marked decrease in the cell viability in a dose-dependent manner. The value of electrochemical impedance spectroscopy decreased with the concentration of LPS in range of 1-50 μg/ml with the detection limit as 0.15 μg/ml. With EIS measurements, the cell-based electrochemical biosensor can be used to evaluate the toxicity of LPS on Ana-1 cells when the morphological changes are generated on the surface of cells in the early period. EIS was correlated with calcium concentration in cells, suggesting that $[Ca^{2+}]_i$ production early took

place in cells upon incubation with LPS and the mobilization of Ca^{2+} had effects on the electrochemical signals. The proposed cell sensor is simple, rapid, inexpensive, and convenient for electrode renewal. The R.S.D of the detection results was lower than 5%, which indicated that modification electrode has good reproducibility and stability. The method is recommended for wider application in biosensing applications and to evaluate the cytotoxicity of pathogens.

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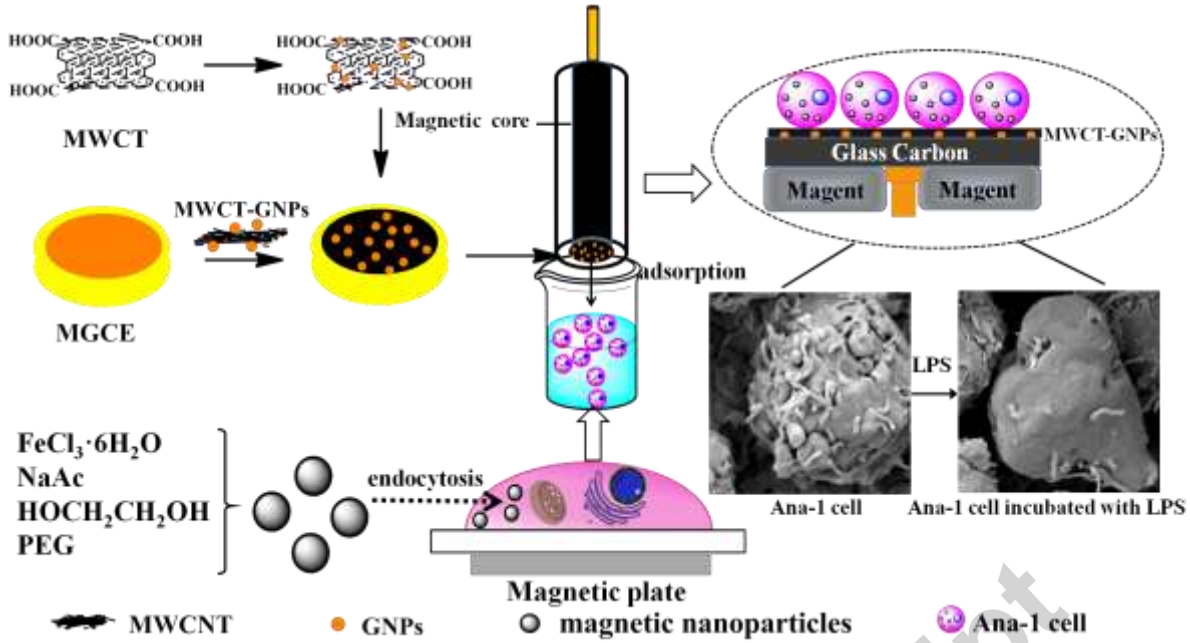

Scheme 1: Schematic illustration of the preparation of modified Ana-1 cells electrode.

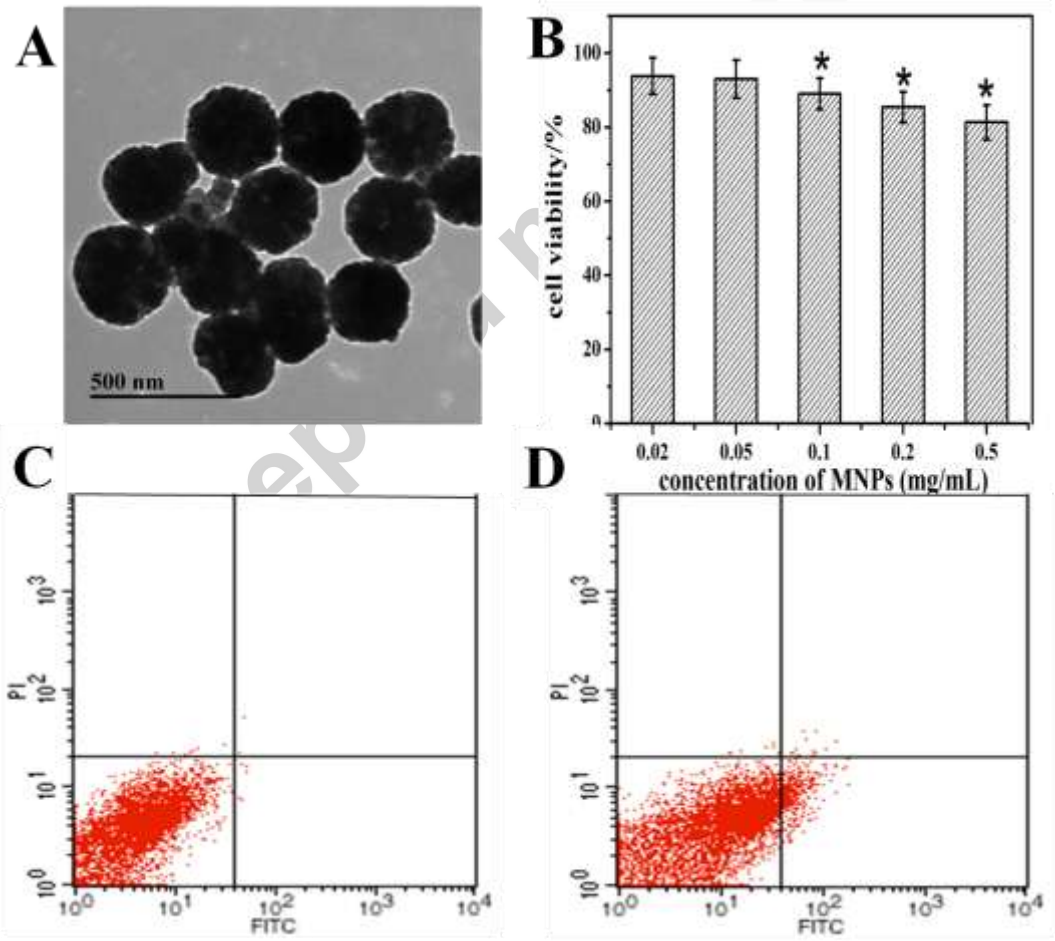

Fig.1. TEM micrography and cytotoxicity of magnetic nanoparticles. (A) TEM micrography Fe_3O_4 nanoparticles. (B) Viability of Ana-1 cells incubated with MNPs

at different concentrations for 24 h. Control experiments were carried out without MNPs. * $P < 0.05$. (C) FACS analysis of apoptosis in control and (D) MNPs internalized Ana-1 cells. The lower left quadrant contains AnnexinV-FITC (-) and PI (-) viable cells; the lower right quadrant, AnnexinV-FITC (+) and PI (-) early apoptotic cells; the upper right quadrant, AnnexinV-FITC (+) and PI (+) late apoptotic or necrotic cells; the upper left quadrant, AnnexinV-FITC (-) and PI (+) necrotic cells. Bars indicate % of apoptotic or necrotic cells in each group expressed as mean ± S.D. of data obtained from three independent experiments.

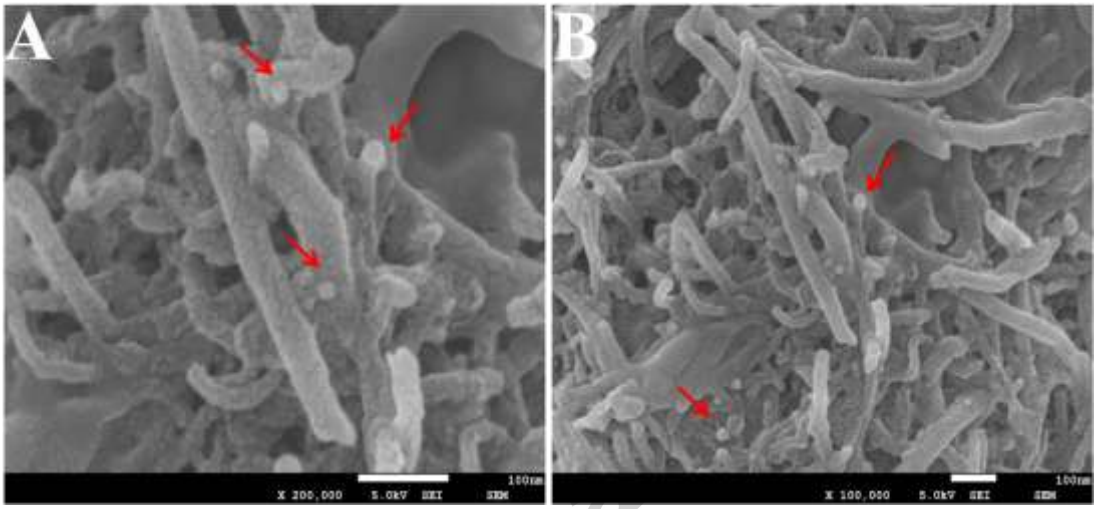

Fig.2. The SEM images of MWCT-GNP/Chi modified electrode. The arrow indicated the gold nanoparticles.

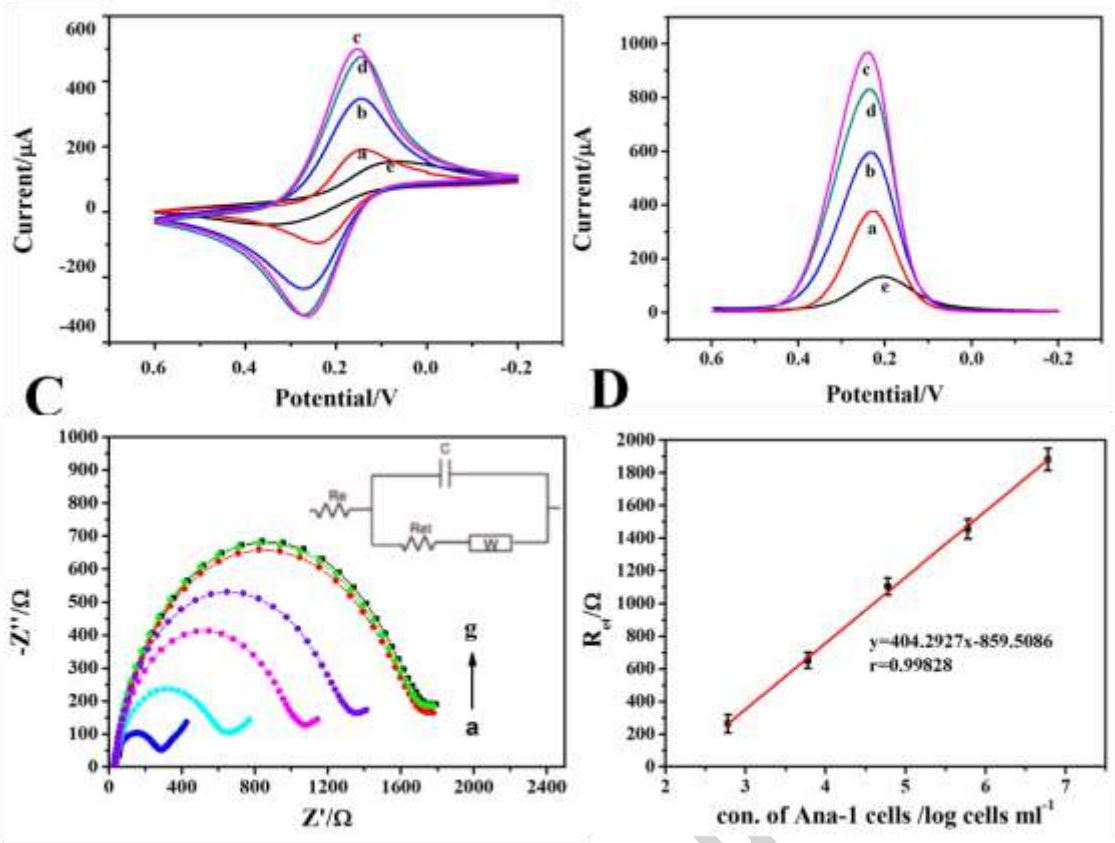

Fig.3. Electrochemical characterization of the modified magnetic glassy carbon electrode. (A) Cyclic voltammograms of (a) bare MGCE, (b) MWCT /Chi/MGCE, (c) MWCT-GNPs/Chi/MGCE, (d) MWCT-GNPs/Chi/MGCE with MNPs, and (e) MWCT-GNPs/Chi/MGCE with MNPs internalized Ana-1 cells. (B) Differential pulse voltammetry of (a) bare MGCE, (b) MWCT /Chi/MGCE, (c) MWCT-GNPs/Chi/MGCE, (d) MWCT-GNPs/Chi/MGCE with MNPs, and (e) MWCT-GNPs/Chi/MGCE with MNPs internalized Ana-1 cells in $Fe(CN)_6^{3-/4-}$, scan rate:100 mv s^{-1}. (C) Nyquist diagrams of (a) 6×10^2 cells/mL, (b) 6×10^3 cells/mL, (c) 6×10^4 cells/mL, (d) 6×10^5 cells/mL, (e) 6×10^6 cells/mL, (f) 6×10^7cells/mL, (g) 6×10^8 cells/mL Ana-1 cells immobilized on MWCT-GNPs/Chi/MGCE electrode. The inset is the equivalent circuit model, to which all of the experimental impedance curves were fitted. (D) Linear regression curve between cell concentration and R_{et} value.

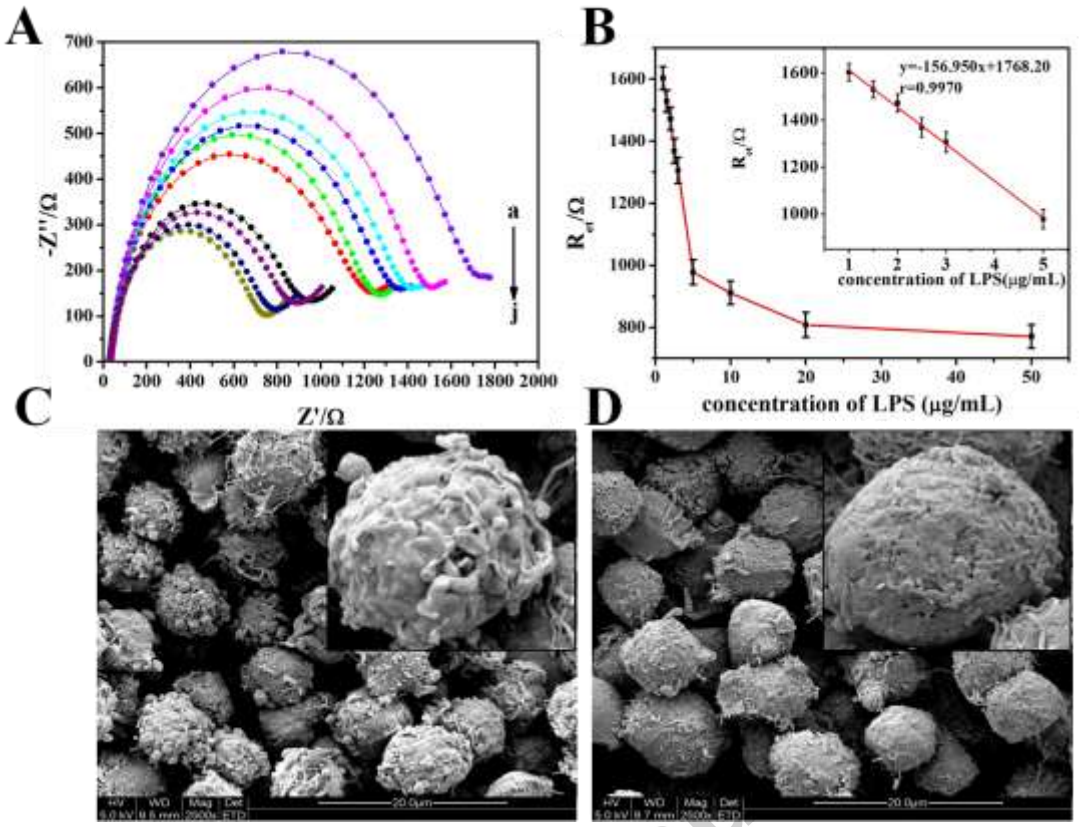

Fig.4. The changes of electrochemical impedance and morphology for Ana-1 cells treated with different doses of LPS. (A) Nyquist diagrams of electrochemical impedance spectroscopy for Ana-1 cells treated with different doses of LPS. (a⟶j) arrow indicates: 0, 1, 1.5, 2, 2.5, 3, 5, 10, 20, 50 μg/mL. (B) Calibration curve of LPS obtained with the proposed assay. The inset shows an expanded calibration curve in a low concentration range. (C) The morphology of cells without LPS and (D) cells treated with LPS. The inset shows the changes of microvilli on the plasma membrane.

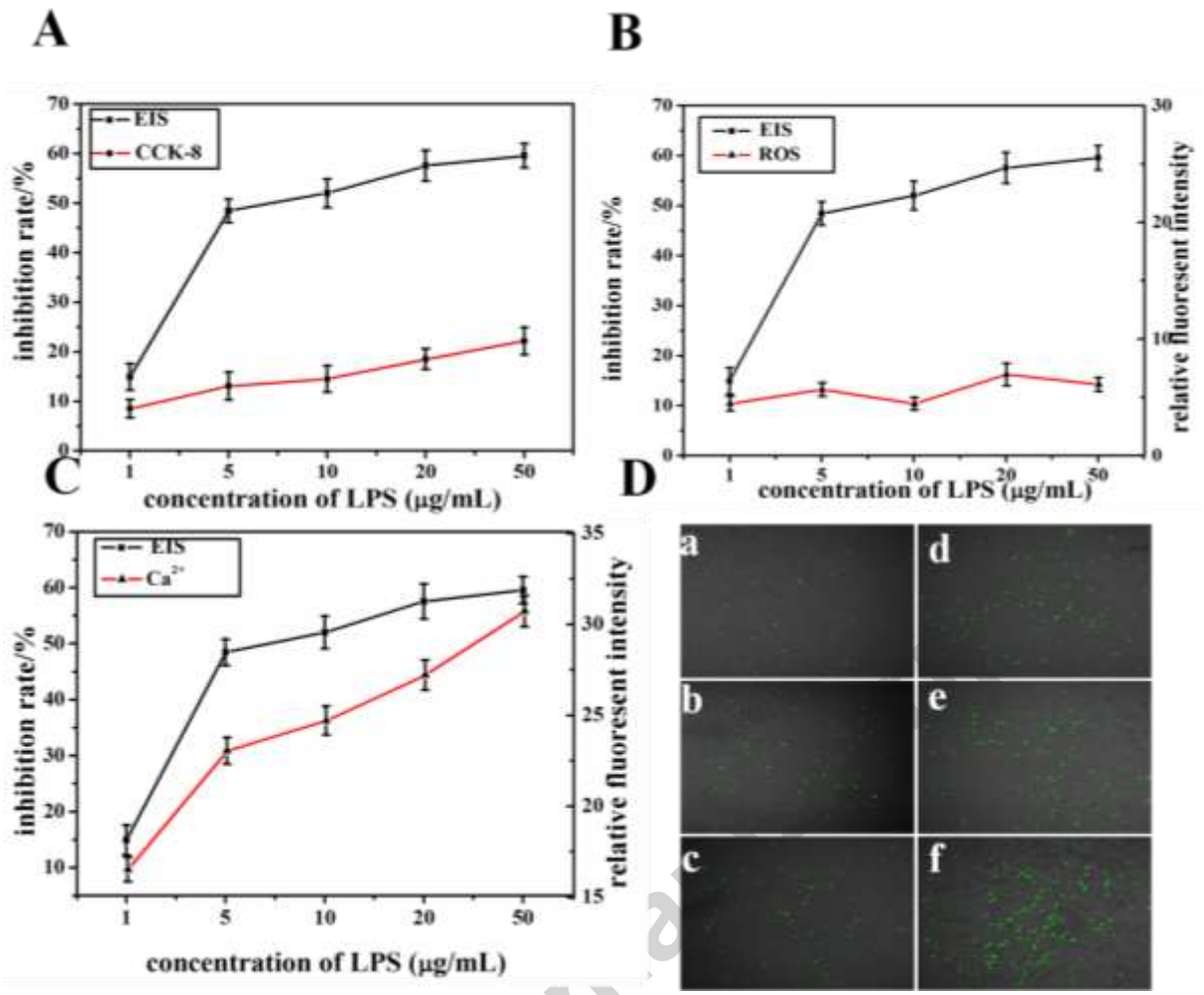

Fig.5. Effect of LPS on the viability of Ana-1 cells obtained by using the proposed electrochemical method and biological assay. (A) the EIS and the CCK-8 assay. (B) the EIS and ROS measurement. (C) the EIS and $[Ca^{2+}]_i$ measurement. (D) Fluorescent detection of calcium concentration in Ana-1 cells incubated with different concentrations of LPS (a) 0 μg/mL, (b) 1 μg/mL, (c) 5 μg/mL, (d) 10 μg/mL, (e) 20 μg/mL, (f) 50 μg/mL by Widefield High-Content Analysis System.

Highlights

- A sensitive murine macrophage cell sensor has been developed for early detection of the effect of lipopolysaccharides to evaluate the toxicity of pathogenic bacteria.
- The cell sensor utilized magnetism to immobilize cells onto modified MGCE and showed excellent regeneration and sensitivity.
- Electrochemical impedance spectroscopy (EIS) were correlated with the microvilli on the plasma membrane and the calcium concentration in cells.