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ARTICLE

A novel H_2O_2 biosensor based on three-dimensional micro/nano-biointerfaces

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To detect hydrogen peroxide (H_2O_2) that *in situ* released by cells is a challenge because of its high activity and short-life time. Here, a new type of H_2O_2 biosensor was reported based on the three-dimensional (3D) micro/nano-biointerfaces as an amicable platform to promote cells adhesion, which can directly grow living cells on manganese dioxide (MnO_2)-immobilized onto a novel hollow hornlike morphology polypyrrole (hPPy) film. The composite MnO_2/hPPy array films were characterized by scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS) and elemental mapping. Further the results of cell adhesion test indicated that the as-obtained 3D micro/nano-array films had good cells adhesion. This proposed sensing platform provided high electroactivity and excellent electron transport with a lower detection limit, a higher sensitivity and a short diffusion distance to reaction sites by electrochemical study. The new design strategy we proposed can expand the bioapplication of 3D micro/nano-biointerfaces that can guide cell fate and electrochemical detection in cell biology.

Introduction

The highly sensitive determination of hydrogen peroxide (H_2O_2) is of practical importance in the bioanalysis field that due to H_2O_2 is generated as a byproduct of biological processes. Until now, various detection techniques for H_2O_2 including cell imaging,[1,2] electrochemical methods,[3-13] fluorometry,[14] and spectrophotometry[15] have been explored. Among them, the electrochemical method has attracted increasing attention because of its great advantages include rapid response, high sensitivity and selectivity, good quantitative ability and low cost. Further, fabrication of micro/nanometer electrochemical interface plays key roles in construction of H_2O_2 electrochemical sensors that can attributed to the unique electronic and catalytic properties of the micro/nanomaterials as well as their good stability.[16-21] From recent research, H_2O_2 is considered to be involved in the biofunction and signal transduction of the cells.[22,23] So *in situ* detection of H_2O_2 concentrations that released from living cells is essential to investigate the biological roles of H_2O_2. As we know, the real cells inhabit a three dimensional (3D) space with complicated microenvironment. Extensively stimuli of the complicated microenvironments including neighboring cells and extracellular matrix (ECM) can regulate living cells fate and relate tissue development. So the cells attachment and growth on the electrochemical sensing matrix are critical for *in situ* monitoring of target biomolecules that released from living cells.[24-27]

Manganese dioxide (MnO_2) is a kind of attractive inorganic material due to its low cost, high electrochemical activity, and environmentally friendly nature.[28,29] A recent work reported the MnO_2 compounds as a few enzymeless H_2O_2 sensors were also proved to be reliable for the nonenzymatic catalysts for H_2O_2.[30] However, the measurement of H_2O_2 was performed on cell culture in a petri dish subjected to drug stimulation, where the H_2O_2 molecules released from cell needed to diffuse through the medium solution before they accessed the sensing electrode surface. So it was hard to avoid an underestimation of the H_2O_2 molecules that released by the cultured cell that attributed to many H_2O_2 molecules would decayed during the diffusion because of their extremely short life time. It would require a new way of thinking about the microenvironment of detected cells and the sensing electrode surface. If the detected cells are directly cultured onto a sensing electrode platform that loaded catalysts, the released H_2O_2 molecules will be rapidly captured by the catalysts and effectively detected by the sensing electrode, which can lead to improving the detection accuracy considerably.

Conducting polypyrrole (PPy) is considered as the electrode materials due to its electric conductivity, good chemical and thermal stability, facile synthesis, and biocompatible.[31] Moreover, since PPy films as self-supporting can be *in situ* prepared on electrodes without the existence of binder, which can simplify the preparation steps and reduce the cost of synthesis. Especially, a hollow horn-like PPy (hPPy) with micro/nano structured exhibit higher electrical conductivity

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than conventional cauliflower-like PPy (cPPy), which reveals a huge advantages of hPPy as biosensor.[32] Taking into account these above research situations, here, a biological-electrochemical MnO_2/hPPy hybrid film with 3D structure was proposed and used as the microenvironment of detected cells for *in situ* detection of H_2O_2 that released from the cells. The hPPy film was directly prepared by using electrochemical synthesis and further obtained by electrodeposition of MnO_2 as nonenzymatic H_2O_2 sensors, whose extremely large active area for electrochemical reaction effectively strengthened its conductive capacity and accelerated the electron conveyance speed between electrode and detective molecules. Compared with various existing enzymatic H_2O_2 biosensors based on horseradish peroxidase, hemoglobin, or cytochrome c,[33-35] nonenzymatic H_2O_2 biosensors could be reduced cost, eliminated the intrinsic problems of enzymatic sensors such as interference from electroactive species and poor stability of enzymes, and facile sensing in the latter technique.[36-38] Moreover, the 3D cells culture microenvironment and electrocatalytic platform were displayed together with MnO_2/hPPy hybrid film, and MnO_2 was used as the biomimetic superoxide dismutase for H_2O_2 sensing *in situ*. The construction of 3D electrode surface, cells adhesion and the process of detection H_2O_2 were characterized by fluorescence, scanning electron microscopy (SEM) and other research devices. This research is in line with the current and further challenges of expanding the bioapplication of 3D micro/nano-biointerfaces that can guide cell fate and electrochemical detection in cell biology.

2. Experimental

2.1. Materials

Pyrrole (Py), dopamine (DA), cysteine (Cys), ascorbic acid (AA) and uric acid (UA) were purchased from Aladdin Chemistry Co.,Ltd (Shanghai, China). Glutaraldehyde (GA), Toluene-4-sulfonic acid monohydrate (TsOH), dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2, 30%) and Sodium sulfate anhydrous (Na_2SO_4) were obtained from Sinopharm Chemical Reagent Co., Ltd. Manganese sulfate monohydrate ($MnSO_4{\cdot}H_2O$) was acquired from Xilong Chemical Reagent Co., Ltd. N-formyl-methionyl-leucyl-phenylalanine (fMLP), fetal bovine serum (FBS), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dulbecco's modified eagle medium (DMEM), and penicillin/streptomycin (P/S), 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Nanjing Heyao Biotech Co.,Ltd. (Nanjing, China).

2.2. Apparatus

Electrodeposition, all the electrochemicals data, using a three-electrode cell equipped (saturated calomel electrode (SCE), a platinum electrode and working electrode), were measured by electrochemical workstation (CHI 760D, Shanghai Chenhua, China). The SEM image was obtained using a Hitachi S4800 FE-SEM (Hitachi Co., Japan). Static water contact angles were measured using the sessile drop method with a 3 μL water droplet (Model: SL200B, provided by Kono Shanghai Instrument Co., Ltd., USA). MTT assays were measuremened by a microplate reader (Multiskan FC). Fluorescent Inverted microscope was acquired from Carl Zeiss Shanghai Co., Ltd.

2.3. Fabrication of hPPy/MnO_2 composite film coated electrodes

The hPPy/MnO_2 composite film was fabricated by using a simple two-step electrochemical reduction method according to the previous reports.[30,39] 1 mol TsOH was firstly dispersed in 50 mL of H_2O, and then 170 μL of Py monomer was added to give the above homogeneous solution. After that, the hPPy film was first fabricated in a three-electrode system (stainless steel as working electrode, Pt as counter electrode, and saturated calomel electrode as reference electrode) by scanning the potential of the electrode between -0.4 V and 0.8 V at 30 $mV{\cdot}s^{-1}$ in the above solution. Electrochemical deposition of MnO_2 on hPPy film were carried out in 0.1 M Na_2SO_4 aqueous solution containing 50 mM $MnSO_4$ in the potential range from 1.4 V to -1.5 V at a scan rate of 50 $mV{\cdot}s^{-1}$, the obtained modified electrode was denoted as MnO_2/hPPy film. The as-prepared films were washed carefully with redistilled water and then dried at room temperature. As a comparison, cPPy was prepared according to the previous report.[40] All the experiments were processed at room temperature.

2.4. Culture of cells

The living human lung adenocarcinoma cells (A549) were grown at 37 ℃in 5% CO_2 culture medium with DMEM, 10% FBS and 1% P/S. The pretreated cells, gathered by centrifugation, were added into the PBS solution including 5 $\mu g{\cdot}mL^{-1}$ fMLP and 100 mM glucose.

2.5. MTT assays

The viable cells viabilities of the hPPy film, cPPy film and hPPy/MnO_2 composite film were evaluated by MTT assays. A549 Cells, at a density of 5×10^4 cells per well, and different synthesized films were seeded in a 96-well plate for 24 h incubation period. After then, the wells were treated with 50 μL MTT (2 $mg{\cdot}mL^{-1}$) and incubated for another 4 h at 37 ℃. The medium was then displaced with 150 μL of DMSO, and a microplate reader was carried out by monitoring the absorbance at a 570 nm.

2.6. Cell Capture Experiments

1 mL of 5×10^4 $cell{\cdot}mL^{-1}$ suspension were seeded onto a 24-well plate with the different films. After 60 min incubation time at 37 ℃, the samples were rinsed with PBS at three times. The captured cells on the substrates were fixed with 2.5% GA in PBS for 4 h. The substrates were subsequently dehydrated with in a series of ethanol gradient solution with different volume fractions (30%, 50%, 70%, 90% and 100%) at each concentration for 20 min, and finally dried at room temperature to observe the morphology of the cells on the samples by SEM.

After 4 h fixed treatment with 2.5% GA, the films were covered with a DAPI solution (2 $\mu g{\cdot}mL^{-1}$) for 15 min, followed

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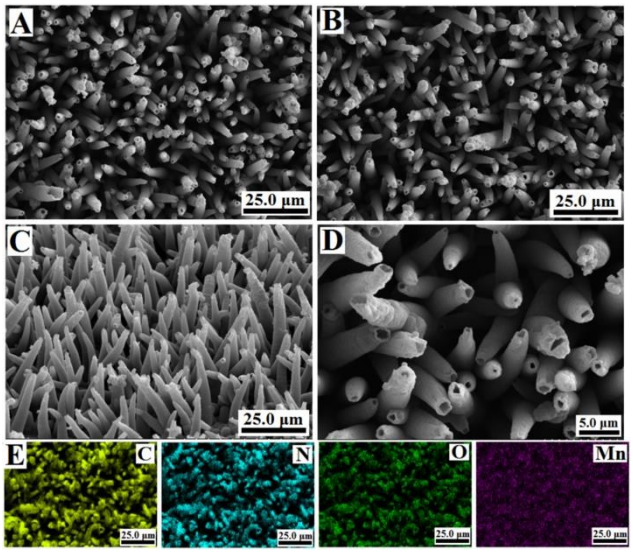

Fig. 1. SEM images of (A) hPPy array film and (B) MnO_2/hPPy array film; Cross-sectional view SEM images of (C) MnO_2/hPPy array film; (D) enlarged SEM image of MnO_2/hPPy array film; and (E) EDS mapping results from MnO_2/hPPy array film, demonstrating MnO_2 grown on the hPPy film.

by PBS washed. At last, the samples were kept at 4℃ in dark for the subsequent fluorescence microscope.

3. Results and discussion

SEM was utilized to observe the morphology and architecture of different as-obtained films. As shown in Fig. 1A, the hPPy film showed the micro/nano structure horns that vertically grown on the substrates with the dense and homogeneous arrays. Further, the growth of MnO_2 on hPPy film by template-free electrodeposition was verified from Fig. 1B,[34] which the micro/nano structure horns basically retained their morphology. The corresponding cross-sectional image of MnO_2/hPPy film was presented in Fig. 1C, implying the length of microhorns was about 25 μm. As for cPPy film, the representative texture was relatively smooth as illustrated in Fig. 1S (ESI). Compared to the hPPy film (Fig. S2, ESI), the surface of MnO_2/hPPy film (Fig. 1D) appeared coarser that due to MnO_2 coating the surface of hPPy array film. Moreover, the mapping analysis (Fig. 1E) was performed to confirm that MnO_2 decorated on the hPPy film since the elements of Mn and O were evenly spread throughout the hPPy film. In accordance with EDS spectra (Table 1S, ESI), it was also proved the existence of C, N, O and Mn, which agreed well with the mapping analysis consequences. The EDS results of the mass weight of Mn and O elements were accounted to be 1.15% and 72.13%, respectively. To check out the flexibility and stability of the MnO_2/hPPy film, its good bending and relaxing properties were further investigated, respectively (Fig. S3, ESI). It indicated the MnO_2/hPPy film has potential to fabricate some novel electrochemical biosensors with more complex structure based on the bendability and arbitrarily-cut of the

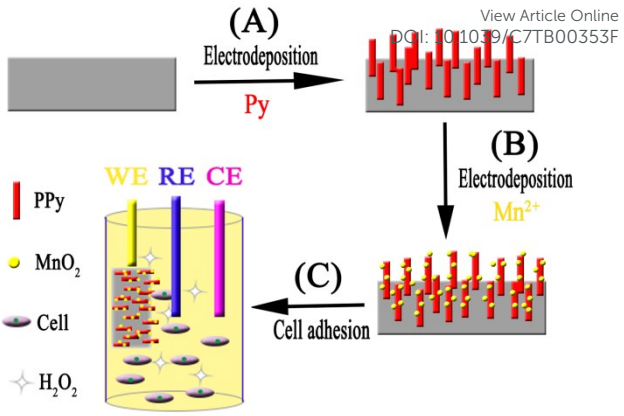

Fig. 2. Schematic illustration of the formation of MnO_2/hPPy film: (A) the formation process of hPPy film, (B) the Mn^{2+} modified procedure to obtain the MnO_2/hPPy film, and (C) the cells adhesion of MnO_2/hPPy film to estimate the outlet of H_2O_2 from living cells.

PPy film in future.

The fabricated process of the H_2O_2 biosensor we proposed based the MnO_2/hPPy film was illustrated in Fig. 2. Firstly, PPy film was deposited on stainless steel electrode by one-step of electrochemical polymerization method under a constant potential condition.[41] The prepared hPPy film possessing 3D micro/nano structure was reported that it could promptly catch cancer cells,[42] so we try to use this effect to improve the electrochemical performance of biosensor. Then, for detection of the released-H_2O_2 of cell, MnO_2 was electrodeposited on the hPPy film acted as biomimetic superoxide dismutase.[43] Finally, A549 cells were straightly cultivated on the as-prepared 3D MnO_2/hPPy film for electrochemical detection.

For the purpose of *in situ* detection of H_2O_2 that released from living cells, the attachment and growth of cells on the biosensor were particularly important. As shown in Fig. 3, fluorescence microscopy and SEM were applied to evaluate the adhesion property of A549 cells (Fig. 3A) on the sample film surface. Results of MTT assays indicated that there were no clear cytotoxic effects for the different PPy films due to their good biocompatibility in cell ambience (Fig. 3B). The fluorescence micrographs of cPPy film, hPPy film and MnO_2/hPPy film using cells adhesion treatment were obtained. Compared to the cells adhesion effect of cPPy film (Fig. 3C), obvious differences of cells attachment for hPPy film was observed and a number of cells could adhere on the surface in Fig. 3D, indicating a strong interaction between cells and the substrate.[44-47] These results were consistent with the previously reported topographical interaction between cancer cells and nanostructures.[48] In order to construct the hydrophilic surface as a more favorable platform for cell adhesion and growth,[49,50] the hPPy film was further decorated by electrodeposition of MnO_2. As shown in Fig. 3E, more cells

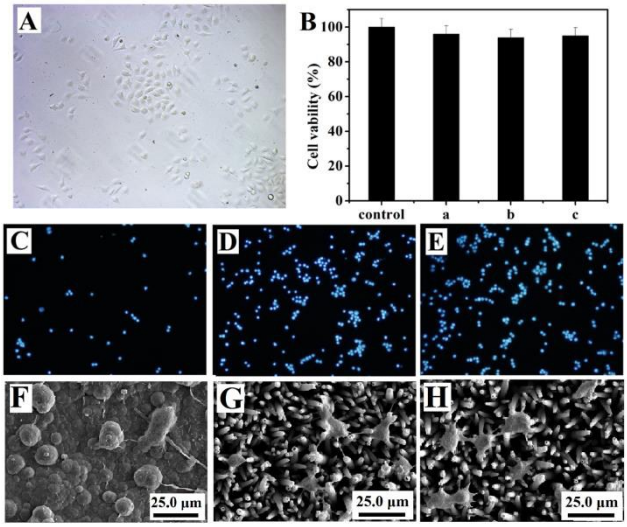

Fig. 3. (A) Microscopic images of A549 cells with cell density of 5×10^4 (magnification is 10 ×); (B) MTT tests of (a) cPPy film, (b) hPPy film and (c) MnO_2/hPPy film; Fluorescence microscope images of A549 cells cultured on (C) cPPy film, (D) hPPy array film and (E) MnO_2/hPPy array film; and SEM images of cells cultured on (F) cPPy film, (G) hPPy array film and (H) MnO_2/hPPy array film.

were adhered on the MnO_2/hPPy film, which was contributed to the hydrophilicity of MnO_2 that provided an amicable substrate for cell growth (Fig. S4, ESI).[51-53] Compared to these previous studies,[54,55] the effect of cell adhesion was achieved just by the geometrical structure of this 3D MnO_2/hPPy film we prepared, but not chemical or biochemical methods. SEM images of Fig. 3F clearly displayed few cells attaching on the surface of cPPy film, and few pseudopodia of a single cell could be found (Fig. S5A, ESI). In contrast, many cells were adhered on the surface of hPPy film (Fig. 3G) and MnO_2/hPPy film (Fig. 3H). Furthermore, the good growth status of a single cell was emerged with presentedmany stretched pseudopodia (Fig. S5B, ESI), denoting that the cell adhesion behavior was further enhanced by incorporating MnO_2 onto the surface of hPPy film. Therefore, the 3D biointerface structures of MnO_2/hPPy film exhibited good matching with cancer cells and enhanced the topographical interaction between cells and substrate, resulting in the improvement of cell-capture capability that useful for further electron transfer efficiency.

To evaluate the electrocatalytic activity of the MnO_2/hPPy film for H_2O_2 detection, the cyclic voltammetric (CV) tests of the MnO_2/hPPy film were executed with the absence (black line) and presence (red line) of H_2O_2 (Fig. 3A).[6,30] Under the absence of H_2O_2 atmosphere, a vaguely electrochemical signal was observed owing to their surface defects.[56] However, when 10.0 mM H_2O_2 was added into the solution, the oxidation-reduction peaks changed obviously, which was contributed to the catalytic performance of MnO_2 that decorated on hPPy film in the presence of H_2O_2.[57-59] From Fig. 4A, in the exsitence of H_2O_2, the apparen peaks were observed at the electrode resulting from the reduction and then the oxidation

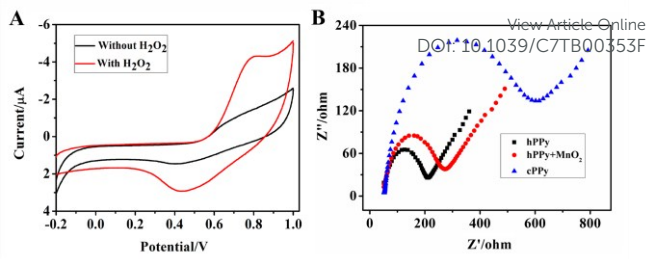

Fig. 4. (A) CVs of the MnO_2/hPPy film with and without 10.0 mM H_2O_2 in 0.1 M PBS at a scan rate of 100 mV· s^{-1}; (B) EIS of hPPy film, MnO_2/hPPy film and cPPy film.

of Mn(II) to MnO_2.[60,61] Significant information, which involved in the interfacial properties of electrodes, can be offered by electrochemical impedance spectroscopy (EIS).[62-65] Fig. 4B demonstrated the nyquist plots of hPPy film, MnO_2/hPPy film and cPPy film, respectively. While the substrate was electrodeposited by hPPy film, there existed a very small semicircular domain, suggesting that hPPy film owned outstanding conductivity.[66,67] Moreover, from EIS, it was further proved that hPPy film exhibited higher electrical conductivity than cPPy film, which was also reported by Morozan that the tubular structures exhibit higher electrical conductivity than the granular ones.[32] After decorating of MnO_2 onto the hPPy film, the semi-circular domain enlarged, which was attributed to the slightly weaker electrical conductivity of MnO_2/hPPy film and further proved the successful grafting of MnO_2.[30]

The selectivity performance of as-obtained 3D biosensor was performed with injecting 10.0 mM H_2O_2, glucose, DA, AA and Cys, sequentially. As demonstrated in Fig. 5A, the existence of varied interferences did not cause any

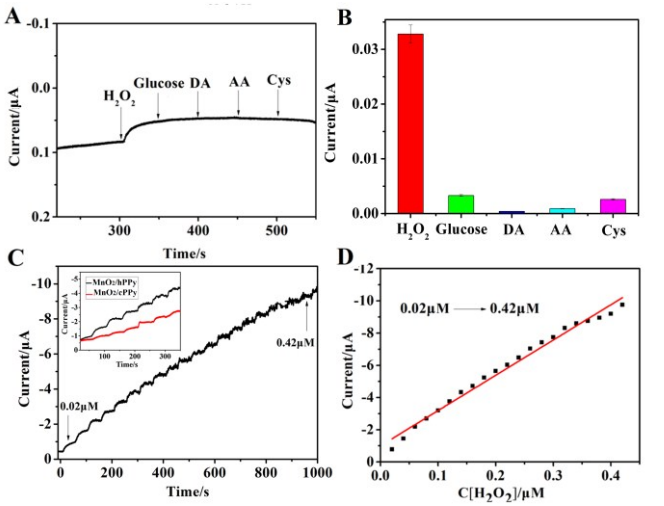

Fig. 5. (A) Interference test and (B) the relative response of MnO_2/hPPy film; (C) Current-time recordings of MnO_2/hPPy film with successive addition of 10 μL, 10.0 mM H_2O_2 solution; Inset is the typical amperometric responses of MnO_2/hPPy film (black line) and MnO_2/hPPy film (red line). (D) Corresponding calibration curves with H_2O_2 concentration.

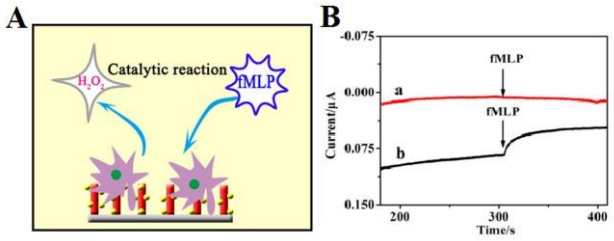

Fig. 6. (A) Schematic diagram for constructing the biosensor; and (B) Amperometric response of H_2O_2 release from the A549 cells by addition of fMLP in (a) absence, and (b) presence of cells.

considerable impact on the 3D biosensor. The statistical data were expressed that the changes of detection current was 10%, 3.6%, 8.3% and 6.4% , respectively (Fig. 5B). At the potential of 0.42 V, the amperometric responses of MnO_2/hPPy film were measured with series of H_2O_2 concentration changes and the corresponding instant responses were presented in Fig. 5C. The biosensor based on MnO_2/hPPy film had a broad linear response over the range from 0.02 to 0.42 μM. Compared to the biosensor based on MnO_2/cPPy film, the amperometric responses of the biosensor based on MnO_2/hPPy film were more sensitive with the higher current intensity (Fig. 5C inset). Results indicated that directly living cells grown on as-prepared 3D micro/nano-array films could offer a short diffusion distance to reaction sites. As shown in Fig. 5D, there was a good linear relationship between the response current and H_2O_2 concentration. It could be expressed as i (μ A) = -1.00236-21.90442 C (μ M) with a sensitivity of 280 μ A· cm^{-2}· μ M^{-1} and low detection limit of 76.0 nM (S/N = 3, R^2 = 0.9857), which showed excellent electrochemical performance than other reported modified electrodes for H_2O_2 detection (Table S2 and Table S3, ESI)

The good stability of H_2O_2 biosensor was proved (Fig. S6, ESI). After being stored for sixty days at room temperature, the current response of the H_2O_2 biosensor was no apparent decrease, which was much longer than those obtained for enzyme-based H_2O_2 biosensors.[68-70] Results indicated that the H_2O_2 biosensor had good stability that can attributed to the stability of the hPPy/MnO_2 film without any degradation in its activity.

Detection *in situ* of released H_2O_2 from Living cells grown on the proposed 3D hybrid film was further performed. fMLP is one of the drugs that can induce H_2O_2 releasing from cell.[56] The deductive mechanisms of fMLP-induced activation on cells was illustrated as Fig. 6A. In comparison with curve a in Fig. 6B, the sensor detected in 5 × 10^4 cells· mL^{-1} suspension showed a crucially increased current response of 0.42 V (versus Hg/Hg_2Cl_2) upon injection of 5 μg· mL^{-1} fMLP (curve b), suggesting an obvious release of H_2O_2 provoked by the stimulant drug. The 0.0944 × 10^{-5} μM H_2O_2 exuded from per A549 cells was calculated according to the linear relationship in Fig. 5D.

4. Conclusions

Based on the good topographical interaction between cancer cells and micro-nanostructures that useful for cell capture, a novel 3D MnO_2/hPPy film was fabricated by two-step electrodeposition treatment and used to constructed H_2O_2 biosensor. This biosensor we proposed can be used for directly detection of H_2O_2 that released from cell in real-time with high electroactivity and selectivity. Compared to these existing methods, the proposed 3D MnO_2/hPPy array film exhibited good matching with cancer cells and enhanced the topographical interaction between cells and substrate, resulting in the improvement of cell-capture capability that useful for further electron transfer efficiency. Therefore, this work has potential to construct a novel sensing platform about living cell-based biological assays.

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Table of contents

A novel H_2O_2 biosensor based on three-dimensional micro/nano-biointerfaces

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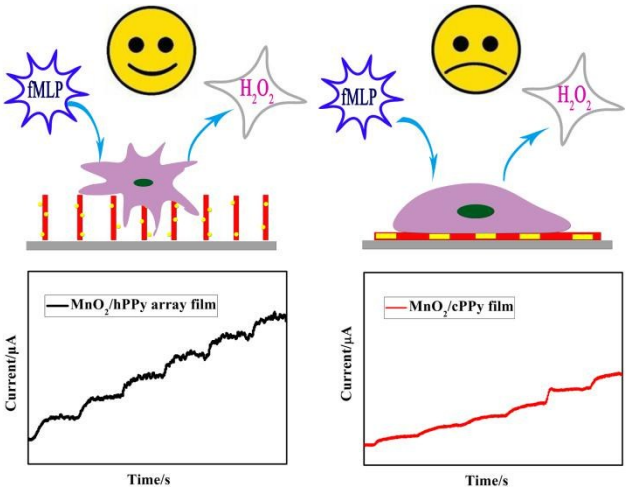

A novel H_2O_2 biosensor was firstly reported to directly grow living cells on three-dimensional micro/nano-biointerfaces to promote electrochemical performance.