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Study of inflammatory factors' effect on the endothelial barrier using piezoelectric biosensor

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A B S T R A C T

This paper used piezoelectric sensor to study the dysfunction of endothelial cell monolayer barrier caused by inflammatory factors. The biocompatible conductive polymer membrane of pPy[pGlu]-pLys was prepared on the surface of the ITO work electrode to improve the interface between the endothelial cell and the electrode. Both the impedance analysis data and the stable plateau stage of sensor's frequency shift indicated that endothelial cells formed a good monolayer barrier on this polymer surface. The response frequency shifts of lipopolysaccharide (LPS)- and histamine-induced endothelial barrier dysfunction were different, which distinguished their different stimulation mechanism. It provided a valuable analysis method for detecting the endothelial barrier function affected by inflammatory factor, and could further promote the application of piezoelectric sensor in cell biology and toxicology research.

1. Introduction

The endothelial cells are lined to blood vessels. The integrity of vascular endothelial barrier plays an important role in maintaining blood stable circulation and the function of tissues and organs (Edens and Parkos, 2003). Under physiological conditions, the endothelial cells not only grow and proliferate, but also secrete a variety of extracellular matrix proteins, such as cadherin, occludin, elastin and fibrin, etc. These mutually inter-linked proteins constitute basal membrane, which only allows small molecules go through freely and restricts the passing of macromolecular substances. It serves as a barrier between blood and tissues. The dysfunction of endothelial cell barrier usually reflects pathological inflammatory reaction firstly, and it is also closely related to the severity of disease (Bone et al., 1997; Peters et al., 2003).

Analysis and evaluation method of the endothelial cells' barrier function is performed by the permeability measurements. It mostly used membrane filter to detect transmittance of the marker (Fluorescein isothiocyanate-dextran, FITC-dextran) through endothelial monolayer (Van Nieuw Amerongen and van Hinsbergh, 2002; Ammori et al., 1999). These detections are complicated, and can only analyze endothelial permeability at certain time point. While the physiological and pathological changes of endothelial cells are dynamic processes. Therefore, we proposed to track these processes in real-time using sensor. The piezoelectric sensor has many advantages in monitoring cell dynamic processes (Tong and Lian, 2014; Tong et al., 2015, 2016). It is label-free, and carries out quantitative and dynamic monitoring. So it is suitable for studying the barrier functions of endothelial cells.

To study the barrier function of endothelial cells by sensor method, it is necessary to lay endothelial monolayer in good growth state on the surface of the sensor's sensitive electrode. In order to simulate endothelial cells' physiological conditions in vivo, and make more accurate evaluation of the effect of inflammatory factor, biocompatible conductive material can be adopted to modify the work electrode surface. It can not only be used as the biomimetic membrane of endothelial cell basement, but also reflect the signals of cell, which is conducive to studying the effect of stimulants on the barrier function of endothelial cells. The use of pLys as the substrate film on gold piezoelectrodes for designing biosensors had been explored (Stobiecka and Hepel, 2011). The biosensors utilizing cells and organelles had also been developed to evaluate their responses to various factors. A piezoelectric sensor with immobilized mitochondria was designed for measuring ion flaxes via potassium channels in hypotonic and hypertonic solutions, as well as under the influence of valinomycin (Magdalena et al., 2017).

The conductive polypyrrole doped with long-chain biomolecule had good biological compatibility and could enhance nerve cell's adhesion to basement. It was used to lead nerve cells to grow into the micropattern as designed in advance (Kim et al., 2010). In this study we adopted the biocompatible and conductive polymer membrane to modify the electrode of piezoelectric sensor. The preparing procedures of polymer membrane and endothelial cell monolayer were

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demonstrated by electrochemical impedance spectroscopy (EIS). The charge transfer resistance and capacitance of each modified layer were consistent with the electrical properties on the same layer. Series piezoelectric sensor with modified electrodes was used to measure the influences of lipopolysaccharide (LPS), histamine and other exogenous or endogenous inflammatory substances on the barrier function of endothelial cells. The results had shown that piezoelectric sensor with electrode modified by biocompatible and conductive polymer membrane was a good method to evaluate the inflammatory damage to the barrier function of endothelial cells.

2. Experimental section

2.1. Materials and reagents

Pyrrole; poly-glutamate (pGlu, MW = 17,000 by viscosity, about 100 monomers); poly-L-lysine (pLys, MW = 70,000–150,000); 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); *N*-hydroxysuccinimide (NHS); 2-[*N*-morpholino] ethane sulfonic acid (MES) and bovine serum albumin (BSA) (Sigma-Aldrich). LPS and histamine were dissolved in PBS buffer solution and sterilized by filtration. All other reagents used in the experiment were AR grade. The water was ultra pure.

2.2. Apparatus

CHI6000B electrochemical workstation (Chenhua, shanghai); HP4192A impedance analyzer (Hewlett-Packard, USA); Biological phase contrast microscope (CKX41, Olympus, Japan). Indium tin oxide (ITO) semiconductor slides with the conductive layer thickness and surface resistance of 78.5 ± 1.5 nm and 20.6 ± 2.5 Ω/cm^{-2}, respectively, were obtained from Shenzhennanbo, China. The piezoelectric biosensor was self-developed based on the principle of series piezoelectric response in our laboratory. It consisted of culturing-detection system, oscillating circuit, microprocessor and computer operating system as shown in Fig. 1.

2.3. Preparation of biologically compatible conductive polymer membrane

The biologically compatible conductive polymer membrane on electrode surface was prepared by two steps of electrodepositing and chemical cross-linking. Electrodepositing was conducted by cyclic voltammetry (CV). 400 μL pyrrole monomer (200 mM) and 200 μL pGlu solution (2 mM) were added into the sensor culturing-detection cell which basement was indium tin oxide (ITO) electrode. The supporting electrolyte was sodium para benzene sulfonate solution (pH = 7). Connect the sensor's electrode leads to CHI6000B electrochemical workstation and select three-electrode system. The ITO electrode at the bottom of detection cell worked as working electrode, the platinum wire as counter electrode, and the saturated calomel electrode (SCE) as reference electrode. The polypyrrole (pPy) film doped with pGlu was electrodeposited on the surface of the ITO electrode by scanning the potential between 0.0 and 1.0 V for 10 cycles at a rate of 100 mV/s. The temperature should be controlled at 5 ℃ in reaction process.

Poly-L-lysine chain was chemical cross-linked with pGlu chain which exposed outside of pPy film by synthesizing amide bond in EDC/NHS catalytic reaction. Carefully suck out the previous reaction's residues and then rinse the culturing-detection cells with sterile water for three times. 100 μL each of 0.5 mg/mL EDC, 0.5 mg/mL NHS, 0.1 M MES and 0.5 M NaCl solution was mixed uniformly and added into the cells. Incubate it for 30 min at room temperature to synthesize PPy [pGlu]-NHS which could activate the surfaces of polymers film. Then carefully suck out the residues, rinse the culturing-detection cells again. Add 200 μL pLys solution (100 μg/mL) and incubate it for 2 h at RT to synthesize PPy[pGlu]-pLys film which was the biologically compatible conductive polymer membrane on the surface of the sensor electrode. Sterilize it under ultraviolet light for 10 min, and then rinse the culturing-detection cell for many times. Before seeding endothelial cells, Incubate it with culture medium containing BSA for 0.5 h.

2.4. Measurement of the electrochemical impedance

The modification of the electrode by pPy[pGlu] or pPy[pGlu] and the cell growth on the surface of the polymer membrane would cause changes in the interfacial impedance. It was measured by electrochemical impedance with three electrode system. The sensor's detection panel electrode leads were connected to the HP4192A impedance analyzer. ITO electrode modified by polymer membrane worked as working electrode, platinum wire as counter electrode, SCE as reference electrode, and K3Fe(CN)6/K4Fe(CN)6 (5 mM , 1:1) as the redox probe. Scanning frequency range was in 5–13 Hz. And sinusoidal voltage amplitude was at 10 mV. The impedance spectroscopy of electrode with different modification was measured in culture solution (pH7.8). Zsimp Win was performed for fitting the data.

2.5. Preparation of endothelial cells monolayer

Human umbilical vein endothelial (HUVEC) cells were collected by trypsinization (0.25% trypsin/0.02% EDTA) and suspended in low-glucose DMEM medium containing 10% FBS. The suspended cells were seeded in the sensor's culturing-detection cell with a density of 1×10^5cell/mL and the detection electrode were connected with instruments. Then the detection plate was put in incubator at 37 ℃. The growth curve of HUVEC cells was detected and recorded by computer. After 72 h of culturing, HUVEC cells attached to the whole bottom of the culturing cells and covered the surface of electrode forming

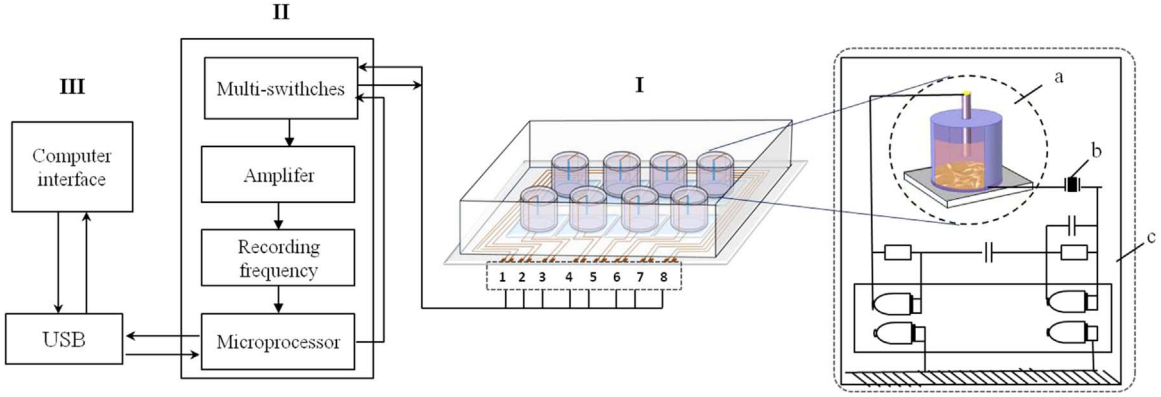


Fig. 1. Schematic diagram of the piezoelectric biosensor. (I) Cell culture-detection system (a is the culture cell; b is the 9 MHz AT-cut quartz crystal; c is the oscillating circuit); (II) Data processing system; (III) PC interface.

endothelial cell monolayer. With replacing fresh medium per 2 days, HUVEC cells monolayer maintained stably for 6–7 days.

2.6. Detection of the barrier function endothelial cells by sensor

HUVEC cells (1×10^5cell/mL) were seeded into the culturing-detection cells of sensor with electrode modified by biologically compatible conductive polymer membrane. The electrode leads were connected with the mainframe sensor, and culturing-detection plates was placed into CO_2 incubator to monitor the frequency shift in real-time. When HUVEC cells grew into monolayer form, the medium containing stimulating factor (LPS or histamine) with certain concentration was added, the frequency shift response was recorded for HUVEC cell monolayer in real-time.

3. Result and discussion

3.1. The analytical instrument of piezoelectric sensor

The schematic diagram of series piezoelectric sensor was shown in Fig. 1. It consisted of culturing-detection system (contained eight separated detection channels), data processing system and PC interface. ITO working electrode (the diameter of 1 cm) was assembled as the basement of the cell culturing-detection well. It's lead wire was serially connected with the 9 MHz AT-cut quartz crystal. Pt electrode (φ 2 mm, the length 15 mm and the distance to the substrate is 6 mm) which submerged perpendicularly into the culture medium worked as counter electrode. Each detection channel was provided with a independent oscillating circuit, therefore no interference to each other. The cells attachment and growth resulted in the electric parameter change which was transformed to change of resonant frequency (i.e. frequency shift, $^{\triangle}F$) by quartz crystal serially connected. According to the expression of frequency shift (Tong and Lian, 2014): $\Delta F = K_1 \times \Delta R_s + K_2 \times \Delta R_{cell} + K_3 \times \Delta C_{cell}$, the frequency shift of the sensor was not only affected by the medium solution resistance (R_s), but also by the adhesion capacitance (C_{cell}) and resistance (R_{cell}) of ITO to the cells. Where K_1 , K_2 , K_3 were coefficients related to the culturing-detection system. The acquisition of the response signal was controlled for every a certain time and automatically recorded by the computer software. The response curves of eight cell samples could be obtained simultaneously.

3.2. Cyclic voltammograms of electro-deposition

The Cyclic voltammograms (CVs) of electrodeposition pPy doped with pGlu was shown in Fig. 2. It could be seen that the current at oxidation peak increased when potential exceeded 0.6 V. In the tenth CVs scanning, the non-Faradaic charging current increased significantly when the voltage was between 0 and 0.6 V, and Faraday current also increased when the voltage was higher than 0.6 V. It indicated pyrrole monomers were polymerized and electro-deposited on the surface of ITO electrode which led to an increase in the effective conductivity of the electrode. The free radical's dissociation constant of pGlu was: α-COOH pKa = 2.10 ; ω-COOH pKa = 4.07 ; α-NH_2 pKa = 9.47. A large number of carboxylate radicals of pGlu was dissociated into anions at pH 7. In the process of electro-deposition, a cationic charge could make 3–5 pyrrole monomers polymerize on pPy chains (Billingham and Calvert, 1989; Diaz, 1981) which caused that the anions of pGlu flowed to pPy to keep the electric neutrality of solution. Therefore negative pGlu was doped in pPy film during the process of electro-deposition.

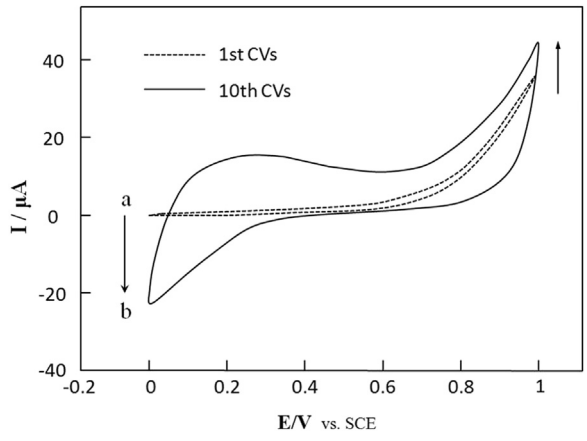


Fig. 2. Cyclic voltammograms of the pPy electro-deposition when pGlu was present in the solution. a. the 1st CVs scanning; b. the 10th CVs scanning.

3.3. Cyclic voltammograms of polymer membrane modified by pLys

PPy film doped with pGlu was coated on the surface of ITO electrode while parts of peptide chains of pGlu stretched out of the film during the electro-deposition. These peptide chains could be bonded covalently with other protein or polypeptide chains (Frey and Corn, 1996). If water-soluble EDC (the activated molecule) and NHS (the cross linking molecule) existed in the reaction system, amino groups of peptide's chain could react with carboxyl groups of other peptide's chain to form amide bond.

The free carboxylic group of pGlu was activated by EDC to form O-acyl urea derivative which was not stable in aqueous solution. The intermediate O-acyl urea derivative hydrolyzed and produced NHS- ester (Mattson et al., 1993). The latter had amino reactivity and could be linked with α-amino of pLys chain by amide bond, thus pLys covalently bound to the surface of pPy[pGlu] polymer film. The graphic of the conductive pPy polymer membrane doped with biologically compatibility molecules is shown in Fig. 3.

FTIR spectroscopy was used to confirm the formation of the amide bonds between activated carboxylic acid groups on the surface of pPy [pGlu]-NHS and lysine residues of the added pLys. As shown in Fig. 4a, Carbonyl stretching mode at 2061 cm^{-1} corresponding to the carboxylic acid groups of pGlu. Carbonyl stretching mode at 2033 cm^{-1} corresponding to the amide bonds in polypeptides. pGlu was activated by EDC, and then produced NHS- ester. The ester subsequently reacted with α-amino groups of pLys to form pPy[pGlu]-pLys. The covalent attachment (amide bond) results in an increase in the intensity of the amide peaks and the disappearance of the peaks corresponding to the succinimide ester.

Fig. 4b showed CVs of the electrode coated by pLys covalently modified pPy[pGlu] membrane in 0.25 M KCl solution. It could be seen that the CV curve of the electrode coated by pPy[pGlu] film was only different in shape with that coated by pLys covalently modified film (pPy[pGlu]-pLys). Both of them had good conductivity. The reason for the decrease capacitive current of the pPy[pGlu]-pLys was that PLys film covalently modified on pPy[pGlu] film increased the surface impedance of the electrode. It suggested that although the surface of pPy [pGlu] film was modified by non-conductive pLys, as pGlu and pLys were all long-chain molecules, and only part of pGlu chain was doped

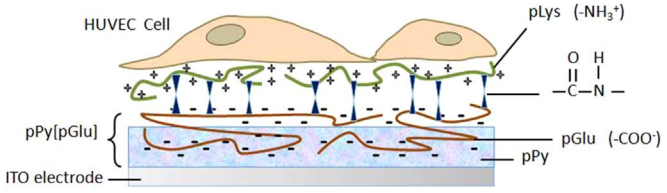


Fig. 3. The graphic of the conductive pPy polymer membrane doped with biological compatibility molecules. (pGlu was entrapped in pPy electro-deposition. The carboxyl of pGlu exposed outside the film was activated by NHS and bound with amino of pLys by EDC catalysis.).

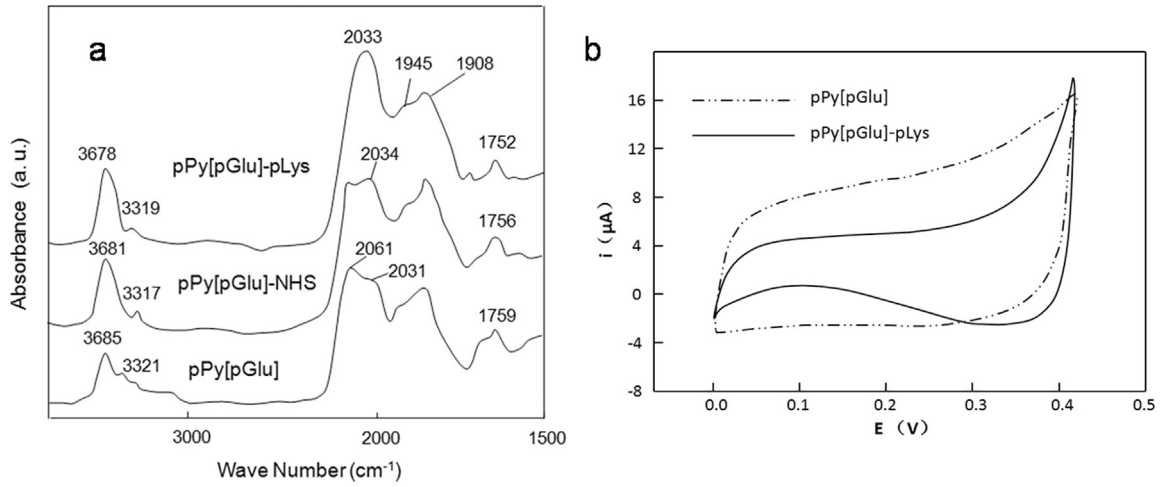


Fig. 4. (a) IR spectra of pPy[pGlu] electro-deposited onto ITO electrode, following activation by EDC/NHS and following reaction with pLys. (b) CVs of the pPy[pGlu] (the dotted line) and pPy[pGlu]-pLys (the solid line) in 0.25 M KCl solution.

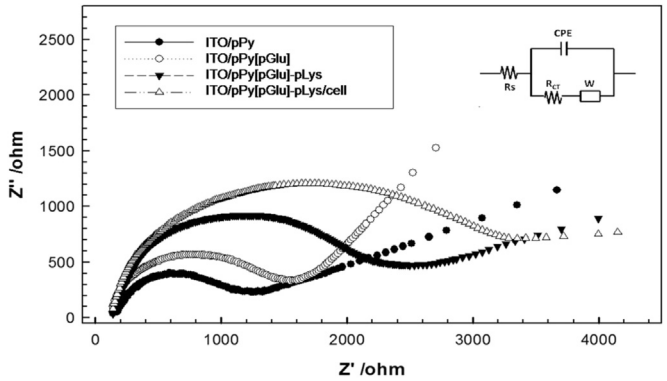


Fig. 5. The impedance spectrum of the modified electrode's measured in different step. (Insert: the fitting equivalent circuit model of the electrode modified with ITO/pPy [pGlu]-pLys/cell Nyquist curve).

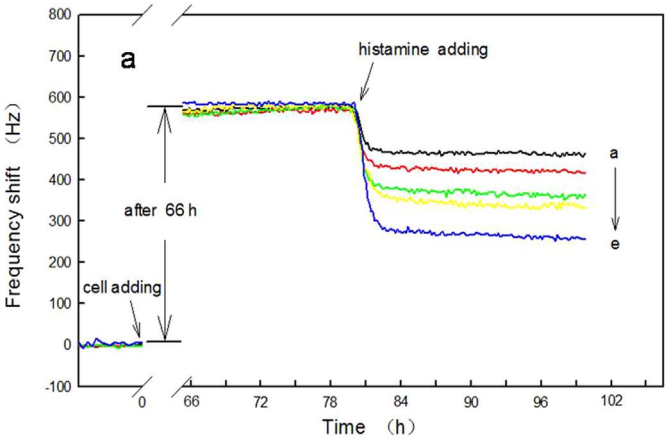
into pPy film by electro-deposited, the rest part could still stretch out of film to link with pLys. This structure could enable pPy film to contact with the ions in electrolyte solution to maintain its original conductivity.

PLys was selected to form the outermost surface of the electrode because it was a known biochemical cue that supports cell adhesion and growth (Cheng and Robert, 1999; Jordan et al., 1994). Moreover, the experimental results showed that the pLys film modified on the electrode's surface did not affect the electrode contact with electrolyte ion in the solution.

3.4. Analysis of HUVEC cell monolayer's impedance spectrum

The AC impedance spectrum detection of HUVEC cell monolayer on the electrode modified by pPy[pGlu]-pLys membrane was carried out in the culture solution (pH = 7.8) containing 5 mM $Fe(CN)_6^{4-}$/ $Fe(CN)_6^{3-}$ redox probe at room temperature. The modified electrode's impedance spectrums measured in different step were shown in Fig. 5. It could be seen that the semicircle diameter of Nyquist curve of the ITO electrode modified by pPy[pGlu] was longer than that of the ITO electrode modified only by pPy in high-frequency region. While the semicircle diameter of Nyquist curve of the ITO electrode modified by pPy[pGlu]-pLys increased. HUVEC cells were seeded on the electrode modified with pPy[pGlu]-pLys membrane and inoculated for 48 h, the semicircle diameter of its impedance spectrum increased significantly.

The impedance data of the electrode modified with ITO/pPy[pGlu]-pLys/cell was fitted by Zsimp Win. Considering the actual situation of detection system, in which the electrode interface was layered and non-homogeneous, and its capacitor and resistor are non-ideal electrical components, so it was necessary to make selection and combination to the components of equivalent circuit. Constant-phase elements (CPE) was used to replace non-linear electrical components which related to frequency, so as to establish an equivalent circuit model mostly closed to real circuit. Other components included solution resistor (R_s), charge transfer resistor of electrode interface (R_{ct}) and Warburg impedor (W). The equivalent circuit model made by computer simulation curve



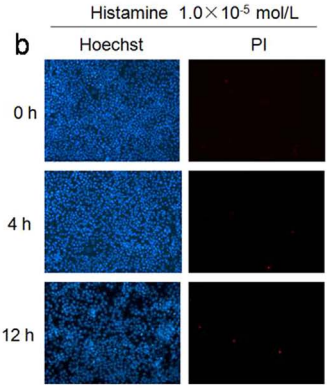


Fig. 6. (a) Detection of the effect of histamine with different concentrations on HUVEC monolayer barrier by piezoelectric sensor for real time. (a-e was the concentration of histamine respectively: 1.0×10^{-7} mol/L, 0.5×10^{-6} mol/L, 1.0×10^{-6} mol/L, 0.5×10^{-5} mol/L, 1.0×10^{-5} mol/L); (b) The fluorescence photographs of Hoechst/PI double stained HUVECs on the electrode's surface before the histamine (1.0×10^{-5} mol/L) added (0 h), at 4 h and at 12 h after the histamine added.

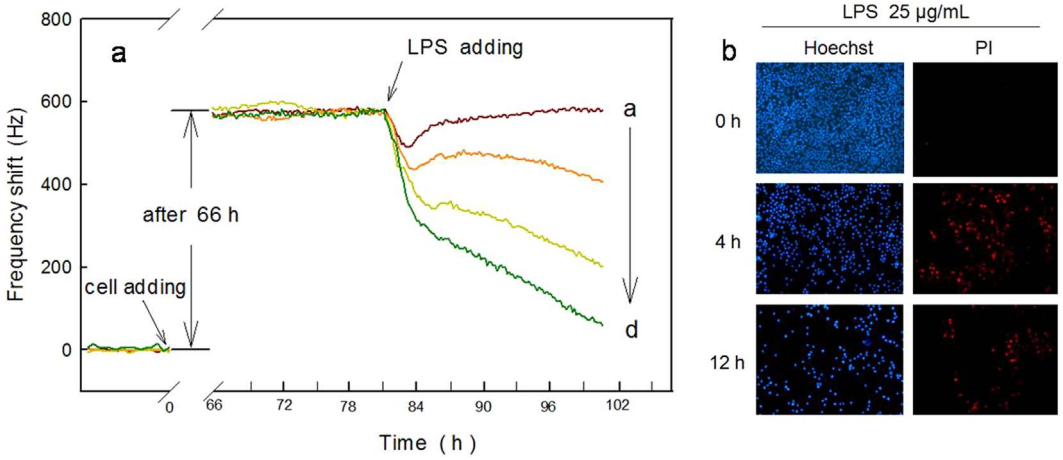


Fig. 7. (a) Detection the affect of LPS with different concentrations on HUVEC monolayer barrier by piezoelectric sensor for real time. (a-d was the concentration of histamine respectively: 0.5 μg/mL, 1 μg/mL, 5 μg/mL, 25 μg/mL); (b) The fluorescence photographs of Hoechst/PI double stained HUVECs on the electrode's surface before the histamine (1.0×10^{-5} mol/L) added (0 h), at 4 h and at 12 h after the histamine added.

fitting was shown in Fig. 5' insert. In this equivalent circuit model, CPE was reflected by the complex impedance Z. $Z = K\omega^{-\alpha}$. K and α was experimental parameter ($0 \leq \alpha \leq 1$). The value of α was related to the area of the layered structure and homogeneity of material on the modified electrode's surface. There are three different conditions about α: (1) if $\alpha \approx 0$, CPE represented an ideal resistor, then $K = R_{CPE}$; (2) if $\alpha \approx 1$, CPE represented an ideal capacitance, then $K = 1/C_{CPE}$; (3) if $\alpha \approx 0.5$, CPE represented Warburg impedor which related to diffusion process, then $K = W$. The α values extracted from the computer fitting of experimental data for the ITO/pPy[pGlu] and the ITO/pPy[pGlu]-pLys were 0.93 and 0.96, respectively. They were close to 1, which showed that CPE was essentially a specific layer capacitance. On the multilayer film modified electrode, it appeared not only the electrochemical polarization, but also concentration polarization when the alternating current signals pass through the electrode. The faraday impedance of the electrode was more complex at this time. It was capacitance arc of double electric layer in high frequency, while it was appeared as Warburg impedance in low frequency.

After being seeded into the piezoelectric sensor's detection cells with 1×10^5cell/mL and incubated for 12 h, HUVEC cells had grown and adhered to the electrode surface. Then HUVEC cells started to proliferate and almost spread to be confluent monolayer which was "paving stones" in shape after 72 h. The cells' membrane was composed of lipid bilayer, which had poor conductivity, so the current bypassed outside of cells in micro-electric field (Tiruppathi et al., 2001). Cells growing by adhering to the electrode reduced the electrode's effective conductive area, thus interfacial impedance was increased. Therefore, Nyquist curve's semicircle diameter of HUVEC cells growing on the electrode modified by pPy[pGlu]-pPys membrane increased significantly. The charge transfer resistance R_{ct} could be calculated based on the semicircle diameter of fitted curve. The R_{ct}'s value of the electrode modified by ITO/pPy[pGlu], ITO/pPy[pGlu]-pPys and ITO/pPy[pGlu]-pPys/cell was 651 Ω cm^2, 2106 Ω cm^2 and 2800 Ωcm^2 respectively. It is assumed that the total impedance was the sum of the impedance of modified electrode and the impedance of coated cells on the modified electrode's surface, it could be calculated that the resistance of HUVEC cell monolayer was 694 Ω cm^2. In the same way, the resistance of HUVEC cell monolayer growing on bare ITO electrode was 419 Ω cm^2. It was reported that the resistance value of cells monolayer was in wide range, which may be related to cell type, culture medium and the cells growth conditions (Tiruppathi et al., 2002). Rutten et al. analyzed the resistance value of endothelial cells isolated from bovine cerebral capillaries which formed cell monolayer on collagen gel substrate treated by glutaraldehyde to be 160–800 Ω cm^2.

Under the same culture conditions, the difference of the resistance of HUVEC cell monolayer growing on different substrates depended on the permeability of ion passing through cells and extracellular substrate's membranes, that was the barrier function of monolayer. HUVEC cell monolayer growing on the electrode modified by pPy[pGlu]-pPys membrane had relatively high resistance, showing that polymer materials with biological compatibility could promote the endothelial cells to adhere on it and to secret extracellular matrix proteins. This caused the cells to grow closely and well simulated the barrier function of endothelial cell in vitro. While the cell layers growing on the bare ITO electrode had relatively small resistance, it was possible that the extracellular matrix proteins connected loosely with each other, or the cell basement membrane was incomplete below the convergence cell layer which was on the bare electrode. It is an abnormal growth feature of endothelial cells.

As for the piezoelectric sensor which was constituted with electrode modified by biological compatibility conductive membrane, on the one hand, the impedance of its electrode coated by polymer membranes increased significantly compared with that of bare electrode. This low-resistance membrane could increase the density of electrical signals between biological system and electrode system, thus sensing signal more accurately. On the other hand, the long-chain biological molecules made the growth basement of endothelial cell more similar in vivo thus facilitate endothelial cell to grow to monolayer in vitro. It established a better biomimetic sensing interface for the study on the barrier function of endothelial cells.

3.5. *Analysis of the effect of inflammatory factors on the barrier function of endothelial cells by piezoelectric sensor in real time*

The barrier dysfunction of endothelial cell was one of the important inflammatory response's characteristic. It was also the main reason of severe tissue hypoxia and anasarca of critically ill patients. If there were toxic or inflammatory factors in vivo, they may stimulate endothelial cells to arise pro-inflammatory and anti-inflammatory reaction. The endothelial cell occurring pathological changes and the denaturation or rearrangement of the intercellular matrix proteins resulted in the increase of permeability of endothelial cell barrier (Selin et al., 2013). In addition, the environmental stimulating factors could also be evaluated based on the physiological or pathological changes of endothelial cell monolayer.

Bacterial lipopolysaccharide (LPS) is one of common exogenous inflammatory substances, and the most important biological factors leading to septic shock. The vascular endothelial cell is one of the major target cells which LPS tended to infect (Parrillo et al., 1990). Histamine is a kind of endogenous inflammatory mediator, which can increase

acutely general micro-vascular permeability of the whole body as well as lead to tissue edema, and even acute allergic shock (Moy et al., 1993). Our experiment had showed that both of the two stimuli could increase the permeability of HUVEC cell barrier, but their effect mechanisms have great differences.

In the experiment, HUVEC cells with the density of 1×10^5cell/mL was seeded into the detection cell of piezoelectric sensor, and was incubated until HUVEC cells grew confluent to monolayer in around 80 h. When the electrode response frequency shift was stable at the plateau stage, histamine containing certain concentration was added in culture medium respectively, and continued to monitor its response of frequency shift. Fig. 6a showed the frequency shift of HUVEC cell monolayer affected by histamine with different concentrations. It could be found that after adding histamine in culture medium, the frequency shift of HUVEC cells drops rapidly, and stabilized in short period at a low frequency shift. This was because that histamine influenced the permeability of cell monolayer within a few minutes to 0.5 h.

It was reported that histamine could be bond to the receptor on endothelial cell surface, and then the cell membrane calcium channel opened due to " store-operated calcium entry " mechanism, and eventually caused the constriction of cytoskeleton to form intercellular space which damaged the barrier function of endothelial cell (Wolf et al., 1998). As to the response of piezoelectric sensor, the dysfunction of endothelial cell barrier was reflected by the drop of frequency shift. The higher concentration of histamine added in, the more severely of the HUVEC cell barrier dysfunction occurred, and the lower sensor's frequency shift was. The frequency shift doped to a certain degree, and it subsequently became stable. The result of the Hoechst/PI double fluorescence stained HUVECs on the electrode's surface were shown in Fig. 6b. HUVEC cells stained by Hoechst (all cells) before the histamine added (0 h), at 4 h and at 12 h after the histamine added were approximately the same in number. However, HUVEC cells stained by PI (dead cells) before the histamine added (0 h), at 4 h and at 12 h after the histamine added were few. It indicated that the cells did not go to apoptosis in the presence of histamine, and they were still on the surface of electrode although the histamine changed the permeability of HUVEC cell monolayer.

When HUVEC cells grew into monolayer, LPS with different concentrations was added in culture medium, the frequency shift response of piezoelectric sensor was different from that when added with histamine as shown in Fig. 7a. When low-concentration LPS (0.5 μg/mL) was added in, it stimulated HUVEC cell monolayer slightly, and the frequency shift dropped rapidly and then raised again, returning to original level gradually. But when high-concentration (5–25 μg/mL) of LPS was added in, HUVEC cell monolayer was damaged severely, the frequency shift did not decline without rebounding. The result of the Hoechst/PI double fluorescence stained HUVECs on the electrode's surface was shown in Fig. 7b. It was seen that the number of HUVEC cells stained by Hoechst (all cells) after treatment with LPS for 4 h and 12 h was significantly less than that before treatment with LPS (0 h). And the number of HUVEC cells stained by PI (dead cells) after treatment with LPS for 4 h and 12 h was greatly than that before treatment with LPS (0 h). It indicated that LPS could result in HUVEC cells apoptosis. The number of all cells stained by Hoechst or the number of dead cell stained by PI after treatment with LPS 12 h was less than that after treatment 4 h, because the dead cell fell off from the surface of electrode. The results obtained by fluorescence stain were consistent with the trend of frequency shift obtained by the sensor. The LPS caused cell to fall off from the surface of electrode, further leading to the constant drop of sensor's frequency shift. It was reported that LPS could not only change the permeability of HUVEC cell monolayer, but also affected its stress-tolerant proliferation, which resulted in cell apoptosis and necrosis was activated (Rutten et al., 1987).

4. Conclusion

The preparation steps of the polymer membrane of pPy[pGlu]-pLys were characterized by EIS. The charge transfer resistance and capacitance in each step were consistent with the electrical properties of the corresponding film. It was calculated that the resistance of HUVEC cell monolayer was $2\,k\Omega\,cm^{-2}$, showing that the endothelial monolayer had formed without big defects. The electrode modified by polymer membrane was biological compatibility and conductive. New piezoelectric sensor constructed with this kind of electrode was successfully used to detect inflammatory factor which caused dysfunction of endothelial barrier. The results showed that the response frequency shift of LPS- or histamine-induced endothelial barrier dysfunction all depended on the dosage of these stimuli. However, their frequency shifts' characteristics were quite different. It was a clue which implied the physiological mechanisms of the two kinds of stimuli' effects on HUVEC barrier function were different. This study provided a valuable analysis method for detecting the endothelial barrier function affected by inflammatory factor, and could further promote the application of piezoelectric sensor in cell biology and toxicology research.

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