

Electrochemically prepared poly(L-lysine) and 3-hydroxyphenylboronic acid composite as a conventional adhesion material for rice suspension cells



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ABSTRACT

Poly(L-lysine) (PLL)-3-hydroxyphenylboronic acid (3-PBA) composite was prepared onto an Au electrode via cyclic voltammetry (CV), which was characterized by electrochemical and quartz crystal microbalance (QCM) techniques, and used to promote adhesion of plant cells. 3-PBA can interact with glycosyl compounds on the cell wall to form negatively charged covalent compounds while PLL increases the number of positively charged sites, which enhances the electrostatic interactions with the negatively charged cell wall, both promoting cell adhesion. The PLL-poly(3-PBA) composite was successfully modified on the Au electrode surface, as demonstrated by QCM and Fourier transform infrared spectrophotometry (FTIR). Moreover, we confirmed the synergistic effect of PLL and 3-PBA to adhere rice suspension cells using CV, electrochemical impedance spectroscopy (EIS), QCM and optical microscope observations. Finally, the dynamic process of rice cells' adhesion to PLL-poly(3-PBA)/Au followed by salt stress treatment of NaCl was monitored in real-time by QCM. The cells softened at first then hardened under the action of 40 mM NaCl. This work presents a novel and conventional way to immobilize plant cells onto sensors, and has importance in the study of the structure, function and behavior of plants under various stresses at the cellular level.

1. Introduction:

Cells are the basic unit of an organism's life. Cells need to adjust their internal structures and shapes while responding to the effects of various external stimuli, which result in changes in the cells' mechanical properties [1,2]. Various physiological activities of living cells involve changes in charge, and the electrochemical reaction of the cells can be considered as the important basis of life's activities. Two interface sensing techniques, QCM and EIS, have demonstrated their potential in studying cellular structures through viscoelastic measurements [3] and electrochemical activities [4] of living cells respectively. To make these interface sensing techniques useful for cellular assays, the following two problems need to be solved urgently. One is the weak adhesion of cells on the sensing interface, which may be easily desorbed. Second, the sensor interface has not been precisely controlled at the molecular level, and the response of the sensor is not necessarily caused by the living cells. Therefore, the search for adhesion molecules that can effectively adhere to cells without affecting the cells' activity has become the key to cellular analysis, in particular for plant cells which so far are rarely measured by interface sensing techniques. The

cell wall is an important feature that distinguishes plant cells from animal cells. The plant cell wall is a complex network structure polymerized by polysaccharides including cellulose, hemicellulose and pectin. Therefore, to adhere plant cells, it is desirable to construct or design some type of molecules or materials that can interact specifically with wall saccharides. Moreover, pectin, a structural acidic heteropolysaccharide, is rich in galacturonic acid with negatively charged carboxyl groups, and another conventional strategy to adhere plant cells would be based on electrostatic interactions.

Phenylboronic acid (PBA) and its derivatives can combine with dihydroxyl compounds to form negatively charged covalent cyclic esters [5–7], so PBA modified copolymers are used in a variety of applications, including saccharide sensors [8–10] and cell surface interactions [11–13]. The polymer film has the characteristics of a high concentration of active groups, large electrochemical response signal, and good chemical, mechanical and electrochemical stability [14]. Ma et al. [15] synthesized poly(aminophenylboronic acid) film on the surface of an Au electrode by electrochemical polymerization. Due to their stability and ease of control, the polymers formed via electrochemical polymerizations of amino acids, especially PLL, have received

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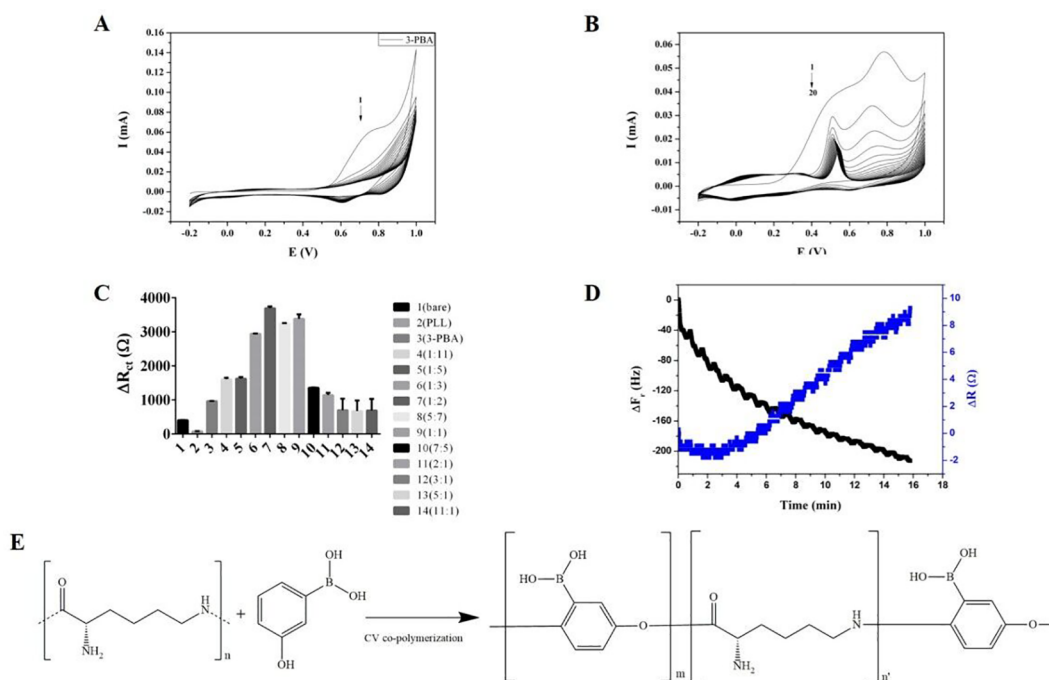


Fig. 1. (A,B) Repetitive CV polymerization of 0.5 mM 3-PBA (A) and co-polymerization of 0.33 mM 3-PBA and 0.033% (w/v) PLL (B) in pH 7.4 PBS on Au at scan rate of 50 mV/s. (C) Changes in charge transfer resistance induced by adhesions of rice suspension cells to PLL-poly(3-PBA) composites created via CV co-polymerizations of different volume ratios of 0.1% PLL and 0.5 mM 3-PBA in pH 7.4 PBS at scan rate of 50 mV/s. EIS experiments done in solution containing 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6 \cdot 3H_2O$ and 0.1 M KCl. AC frequency range 100 KHz \sim 1 MHz, amplitude 10 mV, DC bias 0.2 V vs. Ag/AgCl. (D) QCM real-time characterization during the CV co-polymerization of 0.33 mM 3-PBA and 0.033% (w/v) PLL in pH 7.4 PBS at scan rate of 50 mV/s. (E) Proposed CV co-polymerization mechanism for PLL-poly(3-PBA).

increasing attention in electrochemical sensors [16–18]. In physiological pH solution, each lysine group in PLL has a positive charge and can bind to a negatively charged cell membrane or cell wall. It has been reported [19] that PLL can be successfully modified on a glassy carbon electrode and applied to the immobilization of Jurkat cells, providing a non-toxic environment for cell immobilization. At the same time, it was confirmed by EQCM, XPS and FTIR that no obvious polymer film was found during the polymerization of L-lysine on the Au electrode due to lack of C–N link. More recently, copolymers functionalized with PBA and catechols were created as supramolecular adhesives for carbohydrates [20]. In the present work, we created PLL-poly(3-PBA)/Au by CV to adhere rice cells by taking the synergetic adhesion effects of 3-PBA and PLL. The related reaction mechanism in the preparation of PLL-poly(3-PBA)/Au was analyzed by coupling techniques of QCM, CV, EIS and FTIR. The immobilization of rice cells on PLL-poly(3-PBA)/Au was confirmed by CV scanning, EIS measurement and microscope observation. The kinetic processes of rice cells' adhesion to the PLL-poly(3-PBA)/Au followed by the treatment of salt stress were monitored by QCM.

2. Experimental

2.1. Materials and instruments

Mature seeds of *Oryza sativa* L. *japonica* cv. Nipponbare were provided by Hunan Academy of Agricultural Sciences; PLL solution (0.1% (w/v) in H_2O , mol wt:150,000–300,000) was purchased from Sigma-Aldrich; 3-hydroxyphenylboronic acid (98%) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (China); $K_3Fe(CN)_6$, $K_4Fe(CN)_6 \cdot 3H_2O$ and KCl were purchased from Sinopharm Chemical Reagent Co., Ltd (China). AT-cut 8 MHz Au-coated piezoelectric quartz crystals were purchased from RenLux Crystal (China). The water used in the experiment was Millipore Milli-Q ultrapure water.

A SP-300 electrochemical workstation, 250B-2 network analyzer, and Climacell 111 plant climatic chamber were purchased from Bio-Logic (France), Saunders & Associates (USA) and MMM (Germany) respectively. A Spectrum 65 FTIR Spectrometer and Quest ATR were purchased from PerkinElmer and Specac respectively; silver chloride and platinum wire electrodes were purchased from Gauss Union (China).

2.2. Experimental methods

A drop of Piranha solution ($V_{H_2SO_4}:V_{H_2O_2} = 3:1$) was added onto the surface of 8 MHz Au electrode at 80 °C. After about 30 s, it was washed with Millipore water and dried with N_2 , and repeated for three times followed by adding anhydrous ethanol for 3 min, rinsed with sterilized water and dried with N_2 . After drying, the Au electrode was assembled to the Teflon well, and a mixed solution of 200 μ L 0.1% PLL + 400 μ L 0.5 mM 3-PBA (pH = 7.4 PBS) was added to the well.

Using the three-electrode system, QCM Au electrode as the working electrode, Ag/AgCl as the reference electrode, platinum wire electrode as the counter electrode, CV polymerization was carried out at a scan rate of 50 mV/s with a SP-300 electrochemical workstation for 20 cycles in the potential range of -0.2 – 1.2 V. The PLL-poly(3-PBA)/Au was prepared by sucking out the solution and drying with N_2 .

1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6 \cdot 3H_2O$ and 0.1 M KCl were used as electron probe solution to characterize the modified QCM electrodes using CV and EIS. CV was run in the potential range of -0.1 – 0.6 V at 100 mV/s speed and scanned for three times. EIS was done at 0.2 V DC bias in the AC frequency range of 100 KHz–1 MHz, with 10 mV amplitude.

Before the adhesion experiment, the rice suspension cells with good growth condition were selected and filtered with 200 and 400 mesh sieves subsequently. The cells on the 400 mesh sieve were washed and resuspended with MS media. 600 μ L MS media was added to the Teflon

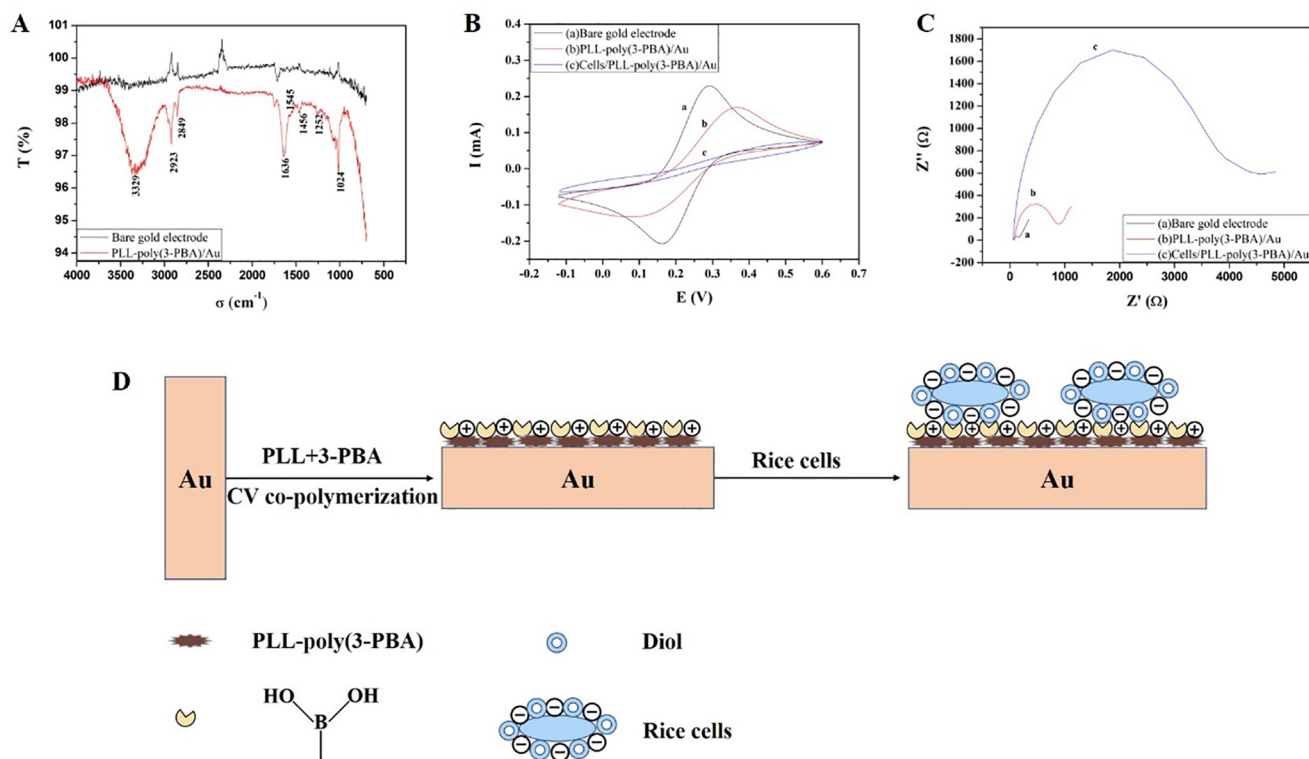


Fig. 2. (A) FTIR absorption spectra of PLL-poly(3-PBA)/Au (red curve) and bare Au (black curve). (B, C) Electrochemical characterizations of (a) Au, (b) PLL-poly(3-PBA)/Au, and (c) cells/PLL-poly(3-PBA)/Au in solution containing 1 mM $K_3Fe(CN)_6$, 1 mM 1 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 0.1 M KCl. (B) CV: scan rate 100 mV/s; (C) EIS: AC frequency range 100 KHz \sim 1 mHz, amplitude 10 mV, DC bias 0.2 V vs. Ag/AgCl. (D) Scheme showing adhesion of rice cells to PLL-poly(3-PBA)/Au. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

well with the modified Au electrode situated inside the Climacell 111 controlled at 25 °C and 60% RH, and the 250B test channel was connected to begin the resonant frequency (F_r) and motional resistance (R) measurements. After QCM responses became stable, 300 μ L media was removed and 300 μ L of rice cells (about 50,000 cells) was added. After adhesion of the cells induced QCM responses became stable, 40 mM NaCl solution was added.

3. Results and discussion

3.1. Preparation of PLL-poly(3-PBA)/Au

Fig. 1A shows that when 3-PBA was polymerized alone, a very obvious monomer oxidation peak appeared at the potential of about 0.7 V during the first scan, then it almost disappeared in the second scan. Fig. 1B shows the dynamic preparation of PLL-poly(3-PBA)/Au by CV. As shown in the figure, during the first forward scan, the first high monomer oxidation peak appeared at the potential of about 0.5 V, and the second monomer oxidation peak appeared at the potential of about 0.7 V. In the second scan, the first peak potential shifted positively and the peak current decreased obviously, and with the increase of the number of polymerization cycle (n), the peak current decreased gradually, while the second peak potential shifted negatively and the peak current decreased obviously, and eventually the oxidation peaks almost disappeared as n further increased to 20. Compared to Fig. 1A of pure 3-PBA polymerization, there is one more oxidation peak occurring at the potential of about 0.5 V during the co-polymerization of PLL and 3-PBA, and the peak currents of both peaks decreased gradually, which is in sharp contrast to the almost disappearance of the second cycle in the process of 3-PBA polymerization alone. We can infer that this is the result of the joint action of PLL. The results show that with the increase of n , a polymer film was formed on the Au electrode, which reduces the

oxidation activity of the electrode, indicating that a stable, dense film was formed on the Au electrode.

Fig. 1C shows the changes in charge transfer resistance (ΔR_{ct}) measured by EIS for composites created from different volume ratios of PLL (0.1%) and 3-PBA (0.5 mM, PBS 7.4) after being exposed to 50,000 rice cells for 6 h. It can be seen from the figure that the polymerized products created from pure PLL or 3-PBA showed slightly decreased or increased ΔR_{ct} compared to bare Au electrode, respectively. The composites created from mixed solutions of PLL and 3-PBA showed increased ΔR_{ct} compared to pure 3-PBA when the ratios of PLL to 3-PBA are within the ranges of 1:11 to 2:1. The synergistic effects are obvious when the composition of 3-PBA is equal to or larger than that of PLL. When the composition of PLL is larger than 3-PBA, no significant ΔR_{ct} values were observed as compared to pure 3-PBA. The largest ΔR_{ct} occurred when PLL:3-PBA is 1:2 which is about 3.7 times of pure 3-PBA, indicating that the adhesion to rice cells is the best. Therefore, a 1:2 ratio of PLL:3-PBA was chosen as the CV polymerization condition for the rest of the experiments. QCM has become a mature analysis tool to study the adsorption phenomenon at the solid-liquid interface [21]. The mass load and viscoelastic properties of materials can be evaluated simultaneously by measuring F_r and R . It can be seen from Fig. 1D when PLL-3-PBA began to polymerize on the Au electrode, the QCM F_r decreased and R increased as a whole. Finally, F_r decreased by about 210 Hz and R increased by about 10 Ω , indicating the successful preparation of PLL-poly(3-PBA)/Au. Based on the above results and others [17,19,22,23], we propose a mechanism for CV co-polymerization of 3-PBA with PLL as shown in Fig. 1E. The mechanism for 3-PBA electro-polymerization involves the reaction of the electrogenerated phenoxy radical with a further 3-PBA molecule to yield a predominantly *para*-linked dimeric radical; subsequent reactions produce a neutral dimer, oligomers and finally a passivating insulating film as indicated by the disappearance of oxidation peak during the second cycle of CV of 3-PBA

alone (Fig. 1A). During the CV co-polymerization of 3-PBA and PLL, the electrogenerated phenoxy radical can also react with PLL in the solution [23], the O in the carboxyl group of PLL has an electron-withdrawing effect, making C partially positively charged, which favors its combination with the phenoxy radical. As the further oxidation, we speculate that amino free radical can be formed in PLL [17], which can be linked to the para- C in 3-PBA [19].

3.2. Characterizations of PLL-poly(3-PBA)/Au and adhesions to rice suspension cells

Fig. 2A shows the FTIR absorptions of PLL-poly(3-PBA)/Au and bare Au electrodes. It can be seen from the figure that there is a strong and wide absorption peak near 3329 cm^{-1} , which is associated with stretching vibration between N-H in PLL and O-H in 3-PBA, indicating that both O-H and N-H may exist, and there are absorption peaks near 2923 cm^{-1} and 2849 cm^{-1} , corresponding to the functional group $-\text{CH}_2$ in PLL. The absorption peaks near 1636 cm^{-1} and 1545 cm^{-1} indicate the existence of amide bonds occurring in PLL, the weak absorption peak near 1252 cm^{-1} is the vibrational peak of phenol O-H, which exists in 3-PBA, and the absorption peak near 1024 cm^{-1} is the stretching vibration of C-N, and this functional group exists in PLL. The above results indicate that PLL-poly(3-PBA) composite was successfully modified on the Au electrode.

As can be seen from Fig. 2B, the bare Au electrode (curve a) shows a standard redox peak, while after the composite PLL-poly(3-PBA) was modified on the electrode (curve b), the oxidation peak current was reduced by about 0.0599 mA, and the reduction peak was reduced by about 0.0738 mA. The peak currents of anode and cathode decreased, indicating that PLL-poly(3-PBA) was successfully modified on the Au electrode, which hindered the transfer of electrons to the electrode surface. After adhering to rice cells (curve c), the peak current decreased greatly, and the reversible redox peak of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ disappeared almost completely. The immobilization of cells on PLL-poly(3-PBA)/Au seriously hindered the charge transfer of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ which indicates that rice cells successfully adhered to PLL-poly(3-PBA)/Au. On the other hand, the rice cells adhering to the surface of PLL-poly(3-PBA)/Au would further affect the interfacial properties of the electrode (electron transfer velocity, resistance, etc.). Fig. 2C shows EIS curves, which further confirms the results of the CV method. The EIS of the bare Au electrode (curve a) is almost a straight line in all frequency ranges, indicating that the process is completely controlled by diffusion. The $[\text{Fe}(\text{CN})_6]^{3-/4-}$ probe molecules can easily reach the surface of the Au electrode, and the R_{ct} of the bare Au electrode is about $98.6\ \Omega$, indicating that the bare Au electrode has good conductivity. After the electrochemical modification of PLL-poly(3-PBA) (curve b), the R_{ct} increased by about $753.6\ \Omega$, indicating that PLL-poly(3-PBA) has been successfully modified on the Au electrode to form a molecular film. After adhering to rice cells (curve c), the R_{ct} was further increased to about $3380.8\ \Omega$. The CV and EIS results confirmed that PLL-poly(3-PBA) can specifically and non-specifically bind to or interact with the

wall sugar molecules and negatively charged groups on the cell wall to immobilize rice cells. Therefore, we proposed the adhesion scheme of rice cells to PLL-poly(3-PBA)/Au (Fig. 2D).

Fig. 3A shows the morphology of rice suspension cells after being adhered to the surface of PLL-poly(3-PBA)/Au for 4 h. It illustrates that there is nearly 80% of the cell coverage area in the field of vision, and there are more cells overlapping together, which does not rule out the effect of natural sedimentation of the cells. After the culture media was sucked out from the Teflon well and rinsed with fresh media, the morphology of the adhered rice cells was observed again (Fig. 3B). As can be seen from the picture, the cells in the visual field are basically monolayer and the field is still covered with about 70% of the cells. The results further verified that the built PLL-poly(3-PBA)/Au has strong adhesion to rice cells.

QCM can monitor the dynamic adhesions of living cells and their interactions with the modified surfaces in real time [3]. After the composite PLL-poly(3-PBA) was modified on the Au electrode and the QCM responses became relatively stable in MS media, the adhesion process of about 50,000 rice cells followed by the treatment of 40 mM NaCl was dynamically monitored by QCM. Meanwhile, a PLL-poly(3-PBA)/Au without adhered cells was used as the control. As shown in Fig. 4A and 4B, after the addition of about 50,000 rice cells, F_r decreased rapidly, and R increased rapidly. After about 0.5 h, R tended to be stable. Within 2 h, F_r decreased by about 100 Hz and R increased by about $20\ \Omega$, indicating that PLL-poly(3-PBA)/Au has a good adhesion to rice cells. Once 40 mM NaCl was added, R dropped instantaneously and then increased overall, while F_r increased instantaneously and then decreased overall. In comparison, 40 mM NaCl was also added to the PLL-poly(3-PBA)/Au without adhered cells, and the control showed little changes in F_r and R compared to the one with the cells. Zhou et al. [24] proposed cell viscoelastic index (CVI, $\text{CVI} = \Delta R/\Delta F_r$) to semi-quantitatively characterize the viscoelasticity of cells. The lower the CVI, the smaller the elasticity of the cells' internal structure or the softer the cells. The higher the CVI, the less the energy dissipation per unit mass, the harder the cells. It was experimentally proved [25] that the stiffness of normal human umbilical vein endothelial cells increases with the increase of cell spreading area. From Fig. 4C, we can see that CVI decreased and then increased in the process of cell adhesion. It is speculated that when cells are first added, the cells have a process of sedimentation from the solution, and then the cells contact the sensor and initiate the adhesion, the cells would become harder as the cells spread more as indicated by increased CVI. It was proposed that both depolymerization and reorganization of microtubules (MTs) are important for the plant's ability to withstand salt stress as verified by the observations [26] that MTs depolymerize then repolymerize when Arabidopsis seedlings are stressed by salt. As shown in Fig. 4C, after the addition of 40 mM NaCl, the CVI of rice cells first decreased and then increased, indicating that the rice cells became softer first then harder. The cytoskeletal dynamic changes of rice cells under salt stress captured by QCM are consistent with the observed results of [26].

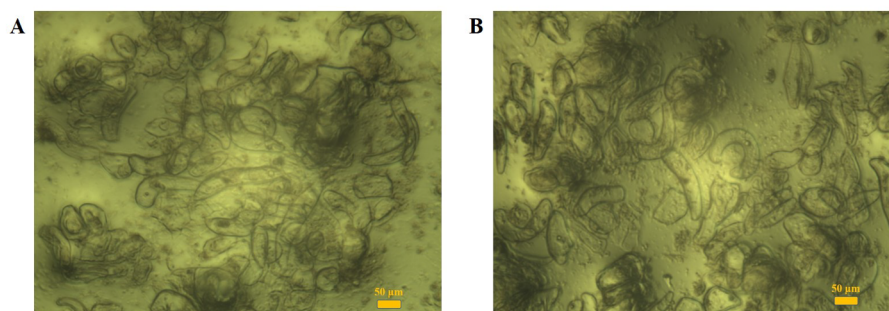


Fig. 3. Morphologies of rice cells after being adhered to the surface of PLL-poly(3-PBA)/Au for 4 h (A) and subsequently rinsed once with culture media (B) (20 ×). Scale bar: 50 μm.

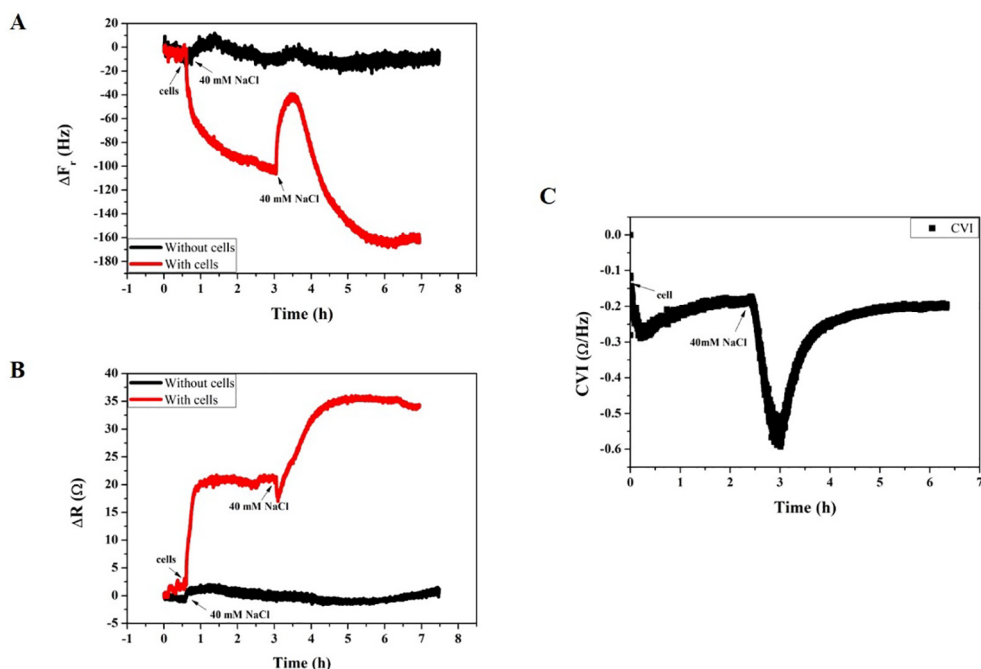


Fig. 4. Dynamic QCM responses during the adhesion of rice suspension cells to PLL-poly(3-PBA)/Au followed by the treatment of 40 mM NaCl. (A) Resonant frequency change; (B) motional resistance change; (C) CVI change. QCM responses of PLL-poly(3-PBA)/Au without adhered cells as a control are shown in black curves of Fig. 4A and B.

4. Conclusions

Using cationic reagent PLL and 3-PBA which can interact with saccharides as raw materials, the PLL-poly(3-PBA)/Au having synergistic adhesion effects to plant cells was prepared by the electrochemical method. By adjusting the volume ratio of PLL (0.1% w/v) to 3-PBA (0.5 mM), it was found that optimized adhesion results to rice cells were obtained when the volume ratio of PLL to 3-PBA was 1: 2. The changes of F_r and R during the electrochemical polymerization confirmed the successful deposition of the new polymerized composite. The results were further supported by FTIR. A mechanism for the CV copolymerization of 3-PBA with PLL was proposed. The prepared composites and their adhesions to rice cells were characterized and confirmed by CV and EIS. QCM was used to monitor the adhesion process in real time and demonstrated that PLL-poly(3-PBA)/Au is effective to adhere rice cells. Moreover, the adhered rice cells became softer then harder under salt stress of 40 mM NaCl, which is consistent with the observed changes in MTs structure in literature. The purpose of this study is to build a conventional sensing interface that can adhere plant cells which lack the autonomous adhesion function of animal cells. We expect that interfacial sensor techniques including QCM and EIS can then be used to study plant structure and function at the cellular level in real time. One important application is to monitor dynamic changes of living plant cells subjected to different kinds of abiotic stresses including heavy metals, saline-alkali, temperature, drought, etc., a field rarely being explored owing to the lack of suitable techniques.

CRedit authorship contribution statement

Mei Zeng: Methodology, Investigation, Writing - original draft. **Tiean Zhou:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition. **Zhaohong Su:** Conceptualization, Methodology. **Weisong Pan:** Conceptualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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