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Electrochemical deposition of dopamine–hyaluronic acid conjugates for anti-biofouling bioelectrodes†

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Bioelectrodes have been widely used to effectively mediate electrical signals with biological systems for various biomedical applications, such as biosensors and prosthetic probes. However, the electrical properties of bioelectrodes are frequently degraded in the biological milieu due to biofouling of bioelectrode surfaces. Hence, the development of simple and effective strategies for bioelectrode surface modification is important for the mitigation of biofouling. To this end, we electrochemically modify electrodes with dopamine-conjugated hyaluronic acid (DA–HA); the modified electrodes exhibit highly hydrophilic surfaces. In addition, the electrochemical impedance of the DA–HA-modified electrodes remains similar to those of bare electrodes. The DA–HA-modified electrodes showed reduced non-specific protein adsorption and minimal adhesion of fibroblasts. Our novel electrochemical passivation of electrodes using DA–HA will contribute to the further development of fouling-resistant and biocompatible bioelectrodes. The electrodeposition of DA–HA can also be potentially applied for general surface modification of other metallic and conducting materials for various applications.

Introduction

Bioelectrodes have been widely used as interfaces between biological structures and electronic devices, particularly in medical devices that record signals and/or stimulate electrical signals in biological systems. In this regard, considerable attention has been paid to implantable electronic devices including pacemakers, neurostimulators, and biosensors.[1–3] For suitable performance of such devices, the interfaces between the bioelectrodes and the biological system need to be biocompatible. For example, implanted substrates generally experience biofouling on their surfaces, triggered by the non-specific adsorption of proteins, lipids, cells, and microorganisms onto the surfaces. This biofouling eventually results in a series of foreign-body responses.[4,5] Importantly, biofouling and foreign-body reactions on bioelectrodes can typically cause the formation of insulation layers of molecules, cells, and scar tissues on the bioelectrodes, which can subsequently impair the electrical sensitivity and degrade the bioelectrode performance. To address biofouling issues of bioelectrodes, numerous studies have reported the production of antifouling electrodes using diverse materials and methods. For example, an anti-inflammatory drug (*e.g.*, dexamethasone) was loaded onto electrodes to reduce inflammatory reactions post implantation.[6,7] However, the drug was eventually consumed over time, and importantly, this approach could not effectively prevent biofouling and inflammation. Furthermore, surface modification with zwitterionic or hydrophilic polymers reportedly introduced antifouling properties in various biomaterials.[8–10] Such hydrophilic polymer layers can provide steric repulsion and a kinetic barrier that can prevent non-specific protein adsorption.[11] Yet, surface modification of bioelectrodes using antifouling polymers is a great challenge due to the lack of availability of suitable chemical functional groups on conductive materials along with difficulties in inducing chemical reactions for tethering.

Hyaluronic acid (HA) is a hydrophilic and anionic polysaccharide existing in our body. Because of its non-immunogenicity, excellent biocompatibility, and hydrophilicity, HA and its derivatives have been extensively used in biomedical applications.[12,13] In particular, its abundant negative charge and high water-interacting capacity exhibit resistance to protein and cell

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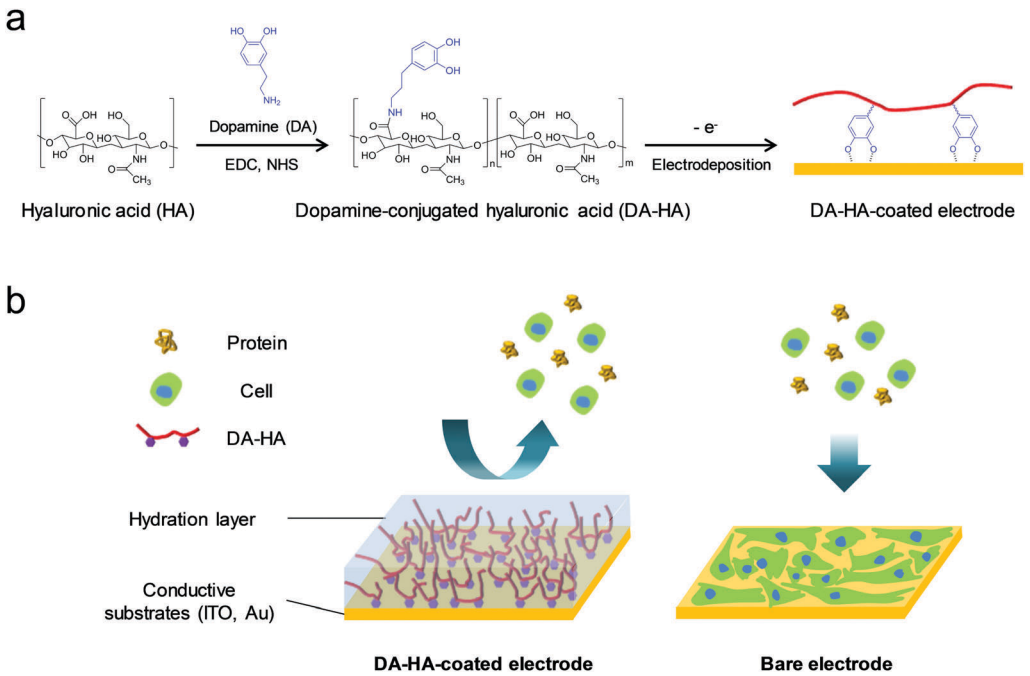

Scheme 1 (a) Chemistry of dopamine–hyaluronic acid conjugate (DA–HA) synthesis and electrodeposition. (b) Scheme for fabricating electrochemical DA–HA-coated ITO surfaces with antifouling properties.

adsorption.[14,15] Accordingly, the fouling-resistant properties of HA have been the focus of several previous reports. For instance, Zhimin *et al.* chemically grafted HA onto gold surfaces, which yielded greatly improved protein-repelling properties of the HA-coated surface when compared with the uncoated substrates.[16–18] Chemical modification methods generally allow for strong and selective coatings; however, the use of such chemical coatings is often not feasible and effective because most electrode surfaces do not present appropriate chemical functional groups for conjugation. Furthermore, selective and fine control over the location and thickness of the coating is difficult to achieve.

On the other hand, electrochemical methods can offer versatile and simple modification of electrode surfaces with advantages such as the controllable deposition of coatings and spatially selective modification of electrodes. In this regard, Lee *et al.* demonstrated the electrochemical coating of pyrrole–HA conjugates (PyHA) on electrodes to prevent cell attachment to conductive materials.[19] They also found that electrochemical PyHA-coated neural prosthetic probes could attenuate glial scarring in the cortex compared with unmodified probes five weeks post implantation.[20] However, the pyrrole moiety, utilized as an electroactive molecule, is not very adhesive, and its biological activity remains unclear.

In this study, we synthesized dopamine–HA conjugates (DA–HA) and deposited them electrochemically on electrodes to fabricate antifouling bioelectrodes (Scheme 1). Dopamine (DA) is a widely used bio-inspired adhesive material that can be chemically or electrochemically oxidized and polymerized to polydopamine (pDA). Interestingly, pDA and DA-modified substances can firmly adhere to various substrates, including metals, even in aqueous solution.[21–24] Hyang *et al.* chemically polymerized DA in the presence of HA at alkaline pH to trap HA within pDA layers. The pDA/HA films exhibited reduced protein adsorption properties.[25] This chemical modification, however, is inappropriate for bioelectrode application because this approach cannot enable fine control over deposition sites and the extent of deposition on electrodes. Moreover, the presence of the electrically insulating pDA layer can degrade the electrical properties when employed for bioelectrode modification.

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In our study, we electrochemically oxidized and coated DA–HA on electrode surfaces (Scheme 1). The DA moieties in DA–HA can be electrochemically oxidized to afford strong bonding with conductive materials. Furthermore, HA was expected to display non-cell adhesive properties and resistance to non-specific protein adsorption. The deposition of DA–HA films on electrode surfaces was monitored with a quartz crystal microbalance (QCM) during electrochemical deposition. The DA–HA-coated surfaces were further characterized *via* fluorescence images using a fluorescein-conjugated HA-binding peptide (HABP–F), water contact angle measurement, atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), and electrochemical impedance spectroscopy (EIS). Subsequently, we tested the electrode anti-biofouling properties using a serum protein adsorption test and *in vitro* cell adhesion to evaluate the suitability of the modified electrodes for biocompatible bioelectrode applications.

Experimental

Materials

Dopamine hydrochloride (DA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), *N*-hydroxy succinimide (NHS), and fluorescein-isothiocyanate-labeled bovine serum albumin (BSA-FITC) were purchased from Sigma Aldrich (St. Louis, MO, USA). Hyaluronic acid (HA, 200 kDa) was purchased from Lifecore Co. (Chaska, MN, USA), and an FITC-labeled hyaluronic acid binding peptide (FITC-HABP) was purchased from ChinaPeptides (China).

Synthesis of DA–HA conjugates

HA (1 g) was completely dissolved in 100 mL of double-deionized (DDI) water overnight. EDC (485.3 mg) and NHS (291.4 mg) were slowly added to the HA solution. After 30 min of stirring, 0.5 g of DA was added and the pH was adjusted to 5.5 using 1 M HCl. The solution was maintained in the pH range of 4–6 during the reaction. After 9 h of reaction, the solution was dialyzed (Spectrum, Houston, TX, MWCO = 10 000) at room temperature in acidified DDI water (pH $<$ 5) for 3 days. The solution was freeze-dried and stored at -20 °C. DA–HA was characterized using ^{1}H NMR in D_2O (400 MHz, JNM-ECX-400P, JEOL, Japan) and ultraviolet-visible spectroscopy (UV-vis, Biodrop Duo, Biodrop, UK). The content of the DA group in the product was determined by absorbance at 280 nm with a DA standard.[26] Our results showed a DA substitution degree of 5.1 $\pm$ 0.4%.

Electrochemical coating of DA–HA on electrodes

DA–HA was electrochemically coated on indium tin oxide (ITO) glass slides or gold-coated glass slides. Gold-coated glass slides were prepared by sputter coating of gold (80 nm in thickness) onto Ti-coated glass slides using an e-beam evaporator (MEP 5000, SNTEK). The Au-coated slides were cleaned by soaking in acetone for 10 min, followed by washing with methanol for 10 min in a bath sonicator (Branson 2510). DA–HA was electrochemically deposited on the electrodes by application of a constant potential of 1.5 V (*vs.* standard calomel electrode (SCE)) for 5 min in an aqueous solution containing 5 mg mL^{-1} of DA–HA in phosphate-buffered saline (PBS, pH 5). A computer-assisted potentiostat (VersaSTAT3 electrochemical working station, Princeton Applied Research) was utilized in a three-electrode configuration with Pt wire as a counter-electrode and a SCE as a reference electrode. The ITO or gold-coated slide served as the working electrode. A cycling potential in the range of -0.5 V to 1.5 V, *vs.* SCE, was applied at a scan rate of 0.02 V s^{-1}. Ten cycles were applied for the coating on each substrate. For comparison, the electrodes were also treated with electrochemical applications in either unmodified HA solution or DA solution under the same conditions as those of the electrochemical DA–HA coating process. After coating, the samples were washed with DDI water and then dried at room temperature.

Fluorescence and image analysis

For the immunostaining of HA, the substrates were incubated in blocking solution (1% BSA in PBS) at room temperature for 30 min, followed by incubation in HABP–F (5 μg mL^{-1} in PBS) at room temperature for 45 min. After the substrates were washed three times with PBS, the fluorescence images of the substrates were acquired with a ChemiDoc MP imaging system (Bio-Rad) with a 0.5 s exposure time for all samples.

Water contact angle measurement

Water contact angles were measured with a goniometer (Phoenix 300, SEO Co.) assembled with a camera to analyze the wettability of the electrodes. A 2 μL drop of pure DDI water was placed on the surface of the substrate at room temperature. Experiments were performed in triplicate (n = 3) for each condition.

X-ray photoelectron spectroscopy (XPS)

XPS measurements were performed by using a VG Multilab 2000 spectrometer (Thermo VG Scientific) under ultra-high vacuum. The survey scan data were collected with a pass energy of 50 eV.

Electrochemical impedance spectroscopy (EIS)

For EIS analysis, an electrode was covered with a tape having a circular hole (6 mm in diameter) to ensure a consistent electrode area (0.2829 cm^2). A three-electrode setup was employed using the VersaSTAT3 electrochemical working station, as described earlier. Experiments were performed in PBS (pH = 7.4) with the application of an alternative sinusoidal potential of 10 mV and a DC potential of 0 V (*vs.* open circuit voltage) in the range of 1–10^5 Hz.

Pencil hardness test

Pencil hardness tests were performed with an Elcometer 501 Pencil Hardness Tester following previous studies.[27,28] Sample surfaces were scratched with various pencils of different

hardness grades (9B–9H) with the same load. After the scratch test, the substrate was examined for scratching.

Protein adsorption test

BSA-FITC was used to investigate non-specific protein adsorption onto the electrodes. Samples were incubated in BSA–FITC solution (0.1 mg mL^{-1} in PBS (pH 7.4)) at 37 °C for 1 h, followed by three careful washings with PBS. Fluorescence images were acquired with the ChemiDoc MP imaging system (Bio-Rad) with a 0.5 s exposure time. The image intensity was analyzed with ImageJ software (NIH). For further investigation of protein adsorption onto electrode samples and its effects on electrochemical impedance, the impedances of the electrodes were measured in frequency ranges of 1–10^5 Hz with an amplitude of 5 mV. Bare and modified electrodes were incubated in 10% fetal bovine serum (FBS) in PBS (pH 7.4) for 1 h at 37 °C. The impedance spectra were obtained before and after protein adsorption experiments. Nyquist plots of the impedance data were fitted with an equivalent circuit with the ZsimpWin 3.21 (Princeton Applied Research) software package. The charge transfer resistance (R_{ct}) was calculated from fitting the impedance data.[29]

Cell culture

Mouse NIH 3T3 fibroblasts were maintained in a tissue culture plate with 5% CO_2 at 37 °C in a culture medium consisting of Dulbecco's modified Eagle's medium (DMEM, Gibco), 10% heat-inactivated fatal bovine serum (FBS), and 1% antibiotic/antimycotic solution. The substrates were exposed to UV for sterilization for 30 min. The cells were seeded at a density of 3×10^4 cells per cm and incubated in a humidified incubator with 5% CO_2 at 37 °C. The cells were cultured in the culture medium for 3 days.

Results and discussion

Synthesis and electrodeposition of DA–HA on electrodes

HA was first chemically modified by conjugation with DA to prepare an HA derivative (DA–HA) that can be electrochemically and strongly deposited on electrode surfaces (Scheme 1). DA-functionalized molecules bind firmly with various substrates, including metals and metal oxides.[30] Our ^{1}H NMR analysis exhibited peaks at δ 6.95–6.60 and δ 1.80–2.0, which correspond to the hydrogen atoms in the aromatic rings of catechol moieties and acetyl groups of HA, respectively (Fig. S1, ESI†). Corresponding to the adsorption at 280 nm, the degree of DA incorporation in the synthesized DA–HA was calculated to be 5.1 ± 0.4% (Fig. S2, ESI†). The pH of the PBS solution containing DA–HA was adjusted to 5 to prevent self-polymerization of DA to pDA during electrodeposition because DA can be spontaneously oxidized at alkaline pH.[30] This mildly acidic DA–HA solution used in our study did not cause spontaneous oxidation or polymerization, which was confirmed by there being no changes in the peak at 280 nm in the UV-vis spectrum (Fig. S3, ESI†). Cyclic voltammetry (CV) was employed to study the redox properties of HA and DA–HA in the voltage range from −0.5 V to 1.5 V *vs.* SCE (Fig. 1a and b). Unmodified HA did not show any substantial oxidation and reduction peaks. On the other hand, the cyclic voltammograms of DA and DA–HA exhibited distinct oxidation peaks at 1–1.2 V and reduction peaks at around −0.1 V, indicating the electrochemical formation of dopaquinone (DQ) moieties from DA–HA. The intermediates of DA and DQ are known to form strong bidentate binuclear complexes with substrates.[31,32] We further investigated the electrochemical deposition of DA–HA in the galvanostatic mode by applying a constant potential of 1.5 V (*vs.* SCE) for 5 min. The current decreased over time during electrodeposition, possibly due to the gradual formation of DA–HA layers and the local depletion of DA–HA in the solution (Fig. 1c). In addition, the mass gradually increased; thus, our results indicated successful electrochemical deposition of DA–HA onto electrodes.

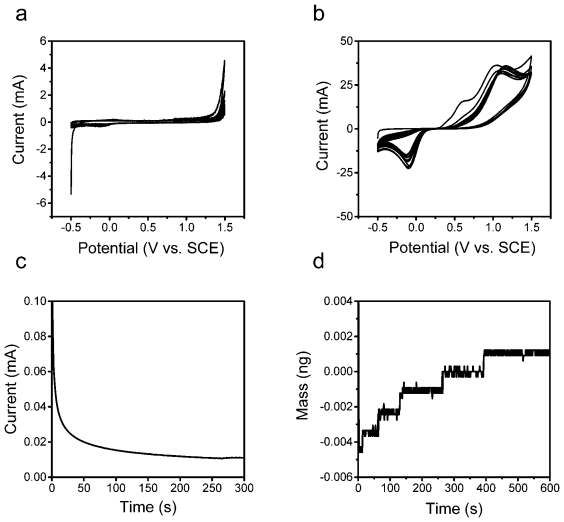

Fig. 1 Electrodeposition of dopamine–hyaluronic acid conjugates (DA–HA). Cyclic voltammograms of (a) HA and (b) DA–HA. Cyclic voltammetery was performed with 5 mg mL^{-1} solutions (PBS, pH 5) at a scan rate of 0.02 V s^{-1}. (c) Chronoamperometry (1.5 V) in DA–HA solutions. (d) Electrochemical quartz crystal microbalance (QCM) profiles during chronoamperometric deposition (1.5 V) of DA–HA.

Surface characterization of DA–HA-coated electrodes

The modified electrodes were characterized by multiple methods. First, immunostaining of HA was performed with hyaluronic acid binding peptide–fluorescein conjugates (HABP–F) to confirm the presence of HA moieties on the modified electrodes (Fig. 2a). Image analysis of the HABP–F-stained electrode indicated that the DA–HA-coated region showed an approximately 4.2-fold higher intensity than the uncoated bare region, thus suggesting the successful coating of the DA–HA film and introduction of HA moieties onto the electrode surfaces. In addition, we scratched the ITO electrode to obtain electrically non-connected areas on ITO and their possible deposition of DA–HA *via* physisorption. This modified area showed faint fluorescence, which was different from the bright fluorescence

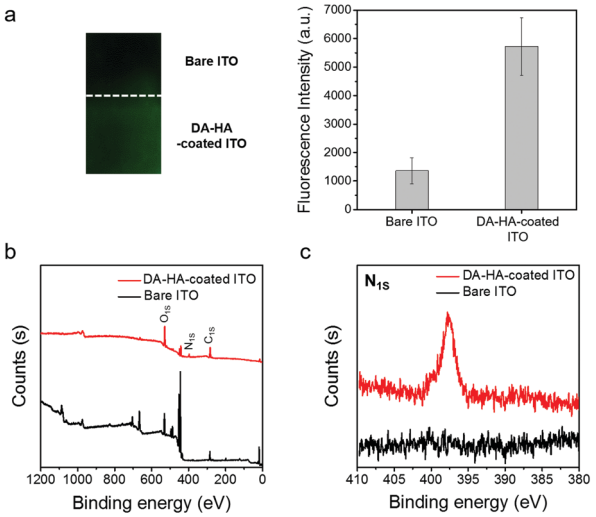

Fig. 2 Characterization of dopamine–hyaluronic acid conjugate (DA–HA)-modified electrodes. (a) Fluorescence image of the DA–HA-coated electrode by immunostaining with HABP–F. (b) X-ray photoelectron spectroscopy (XPS) survey spectra of bare ITO and DA–HA-coated ITO. (c) N_{1s} XPS spectra of ITO and DA–HA-coated ITO. Electrodeposition was carried out with 5 mg mL^{-1} of DA–HA in PBS (pH 5) with the use of chronoamperometry at 1.5 V for 300 s.

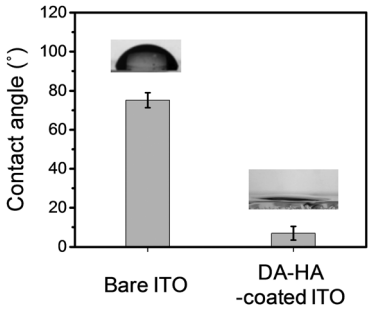

Fig. 3 Water contact angles on bare indium tin oxide (ITO) and dopamine–hyaluronic acid conjugate (DA–HA)-coated ITO.

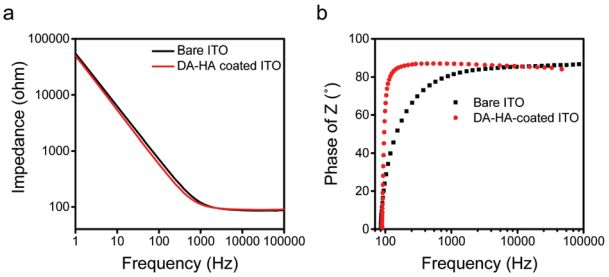

Fig. 4 (a) Bode and (b) phase-angle plot of bare indium tin oxide (ITO) and dopamine–hyaluronic acid conjugate (DA–HA)-coated ITO.

from the area electrochemically coated with DA–HA (Fig. S4, ESI†). Therefore, the results suggest that the electrochemical method can be useful for applying spatially selective coatings onto conductive substrates.

The surface elemental composition of the samples was characterized by XPS. For the DA–HA-modified electrode, a new nitrogen (N_{1s}) peak was observed at 397.7 eV, while no significant nitrogen peak was observed from the bare ITO (Fig. 2b and c). XPS analysis also confirmed the presence of a DA–HA film on the ITO electrode, which is in good agreement with the results of immunostaining with HABP–F.

The wettability of the DA–HA-coated electrode surface was examined by measuring the static water contact angles of the bare ITO and DA–HA surfaces. As shown in Fig. 3, the bare surface was hydrophobic with a water contact angle (WCA) of $75.2 \pm 4^{\circ}$. On the other hand, electrode deposition of DA–HA significantly decreased the WCA to $7 \pm 3^{\circ}$. The extremely hydrophilic surfaces of DA–HA-modified electrodes can be attributed to the high hydrophilicity of the tethered HA. Since hydrophilic surfaces can prevent non-specific protein adsorption,[33,34] our DA–HA-modified electrode was expected to exhibit improved antifouling properties that may reduce foreign-body responses. In addition, toluidine blue O staining indicates the presence of carboxylic groups on the DA–HA-modified electrodes (Fig. S5, ESI†). The morphology and thickness of the coatings were characterized using atomic force microscopy (AFM). The coated surface was found to be slightly smoother than the unmodified ITO surfaces. The thickness of the coating was approximately 32 nm (Fig. S6, ESI†).

The electrical impedances of the DA–HA-coated electrodes were measured to evaluate their electrical properties as an important factor in their application as bioelectrodes. Surprisingly, the impedances of the DA–HA-modified electrodes showed no significant differences from that of the bare ITO electrode (Fig. 4). In general, the presence of polymer coatings on a conducting substrate often leads to drastic increases in impedance and a consequent loss of electrical performance. We speculate that this maintenance of the original electrical properties of the electrodes resulted from the deposition of a very thin layer of highly charged HA.[19]

A strong adhesion of coatings on electrode surfaces is important to ensure long-term and reproducible applications of bioelectrodes. The surface mechanical properties of the coating were measured and compared using pencil hardness tests (Fig. 5 and Fig. S7, ESI†). The DA–HA film surface showed scratches with a harder "H" pencil, whereas the PyHA film showed scratches with a softer "2B" pencil (Fig. 5). It should be noted that we tested the PyHA-coated electrode as a control, which was previously demonstrated to electrochemically form non-cell adhesive HA coatings.[19] DA–HA was strongly coated on

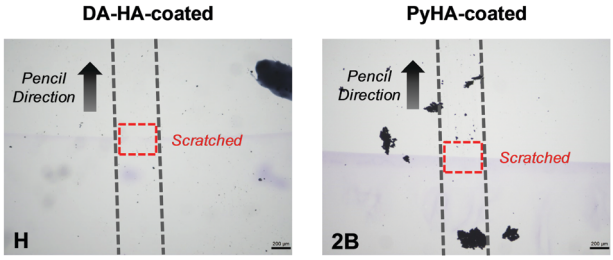

Fig. 5 Optical images of pencil-scratched dopamine–hyaluronic acid conjugate (DA–HA)- and pyrrole–HA conjugate (PyHA)-coated electrodes.

ITO likely due to the contribution of the adhesive DA moieties tethered on the HA backbone. This strong adhesion of DA–HA-modified films can overcome the issue of structural and functional failure during and after substrate implantation in a living body.

Non-specific protein adsorption

Non-specific protein adsorption onto the surfaces of biomaterials is an important initial step of biofouling because protein adsorption triggers cell attachment and eventual foreign-body responses.[5] Bare and DA–HA-coated ITO substrates were incubated with fluorescein-conjugated BSA solution, and the adsorption between the two substrates was compared. Bright fluorescence intensity was observed from bare ITO, whereas a slightly weak signal intensity was observed from the DA–HA-coated electrode (Fig. 6a); the fluorescence intensity of the DA–HA-coated electrode was 61% of that of the bare electrode. This reduced non-specific protein adsorption can be accounted for by the presence of HA. An HA moiety with highly hydrated and dynamic chains appears to play a key role in preventing proteins from accessing and binding with electrode surfaces.

Next, we performed further electrochemical analyses to study the non-specific protein adsorption and its effects on electrical properties of the electrodes using an EIS method.[29,35] The DA–HA-modified and bare electrode surfaces were exposed to 10% FBS for 1 h at 37 °C for protein adsorption. Subsequently, the electrical properties were analyzed in the presence of 5 mM $[Fe(CN)_6]^{3-/4-}$. Protein adsorption usually alters the impedance by hindering the current flow from redox probes in solution across an electrode/electrolyte interface. Consequently, the charge transfer resistance (R_{ct}) of the electrode increases. Less protein adsorption and thus smaller increases in R_{ct} were observed for the DA–HA-coated electrodes than the bare electrodes (Fig. 6c). The R_{ct} values of the bare and DA–HA-modified electrodes were 8440 and 4187 Ω, respectively, (Fig. 6d). The observed relatively smaller increases in resistance of the DA–HA-modified electrode are likely due to the contribution of HA in preventing protein adsorption. However, some amounts of protein were adsorbed to a certain extent onto the DA–HA-modified electrodes; we speculate that the DA–HA did not fully cover the entire electrode surface and small molecules could partly diffuse through the coating to access the electrode surface for binding.

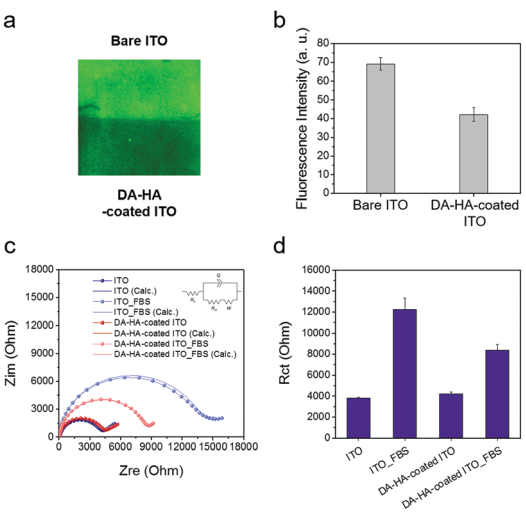

Fig. 6 Protein adsorption test of indium tin oxide (ITO) and dopamine–hyaluronic acid conjugate (DA–HA)-coated ITO with BSA-FITC. (a) Fluorescence images and (b) fluorescence intensity of bare ITO and DA–HA-coated ITO. (c) The Nyquist plot of protein adsorption onto DA–HA-modified ITO and bare ITO. (d) Plot of the R_{ct} value during protein adsorption onto modified and bare ITO.

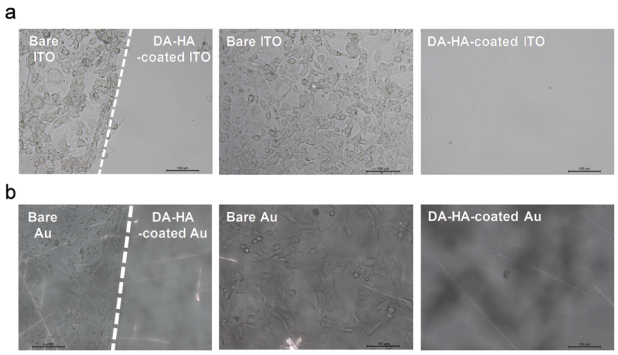

Fig. 7 *In vitro* cell culture on bare ITO and DA–HA-coated ITO surfaces. (a) NIH 3T3 cells were cultured for 3 days on ITO and DA–HA-coated ITO. (b) NIH 3T3 cells were also cultured for 3 days on gold and DA–HA-coated gold.

In vitro cell culture on DA–HA-coated substrates

The cell adhesion of DA–HA-coated substrates was explored by means of an *in vitro* culture of fibroblasts. The post implantation of biomaterials generally leads to attachment of various cells such as macrophages and fibroblasts, which is followed by scar tissue deposition in the vicinity of the implanted materials.[5] As shown in Fig. 7, no fibroblasts are attached to the DA–HA-coated electrodes, whereas most cells are attached to the bare electrodes. These results suggest that the DA–HA coating effectively resists cell attachment. Therefore, DA–HA-modified electrodes can improve biocompatibility and bioelectrode performance. Unlike protein adsorption, cell attachment could be completely prevented by the electrochemical deposition of DA–HA. The DA–HA coating does not allow access of large objects, such as cells. Moreover, the large hydrophilicity and the abundant polyanioinic character of HA prevent cell binding. Hence, our DA–HA-modified bioelectrodes can be useful in preventing cell binding and potential scarring. In addition, we studied the *in vitro* cytotoxicity of the DA–HA modified electrodes by a direct contact method.[36] The fibroblasts cultured in contact with the modified electrodes did not show any substantial changes in their viability (Fig. S8, ESI†), indicating their good cytocompatibility.

Conclusions

In this study, we introduced a bio-inspired adhesive DA moiety to biocompatible HA. This DA–HA conjugate could be

electrochemically coated onto conducting substrates (*e.g.*, ITO, Au) based on the redox activity of the pendant DA moiety. The DA–HA-coated electrodes exhibited fouling-resistant properties. Multiple characterizations of the DA–HA-modified substrates revealed the successful deposition of HA and a resulting highly hydrophilic surface. Importantly, the electrical impedances remained unchanged even after surface modification. Furthermore, the non-specific adsorption of BSA and cell attachment could be significantly reduced by DA–HA deposition. We believe that this new and simple approach for bioelectrode surface modification can be utilized in diverse applications including implantable bioelectrodes, biosensors, and prosthetics. Future studies will focus on the application of electrochemical surface modifications of bioelectrodes with DA–HA and their *in vivo* performance tests.

Acknowledgements

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