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Electrochemical sensing interfaces based on hierarchically architectured zwitterionic peptides for ultralow fouling detection of alpha fetoprotein in serum

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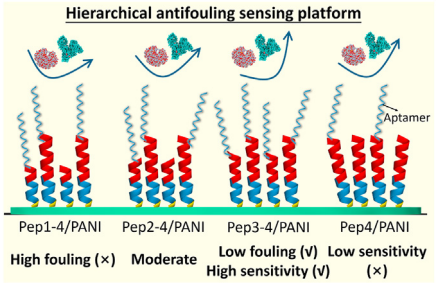
HIGHLIGHTS

- A sensitive electrochemical antifouling biosensor was prepared based on hierarchically architectured peptides.
- Peptide brushes with various hierarchical architectures showed different antifouling capabilities.
- The biosensor supported the low fouling detection of AFP in serum samples.

GRAPHICAL ABSTRACT



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ABSTRACT

Herein, an electrochemical sensing platform based on zwitterionic peptide with a hierarchical structure was constructed for ultralow fouling and highly sensitive protein quantification. Through the combination of CPPPPEKEKEKEK and CPPPPEKEKEK peptides, hierarchical antifouling peptide brushes were formed and exhibited excellent antifouling property, which can be further modified with alpha fetoprotein (AFP) aptamer to achieve highly sensitive detection of AFP. The hierarchical peptide brush-based sensor system achieved an AFP quantification range from 1.0 fg mL^{-1} to 1.0 ng mL^{-1}, with a very low limit of detection as low as 0.59 fg mL^{-1}. In addition, due to the superior antifouling property of the newly designed hierarchical peptide brushes, the electrochemical biosensor supported the quantification of AFP in solutions with a high concentration of nonspecific proteins without sacrifice in sensitivity. It is worth noting that the constructed antifouling biosensor ensured quantitative recruitment of AFP in clinical serum samples with acceptable accuracy when compared with the commonly used method in the hospital. The strategy of constructing sensing interfaces based on designed hierarchical peptide brushes provided an effective way to develop biosensors with both excellent antifouling capability and high sensitivity.

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1. Introduction

The development and application of low fouling interfaces has aroused widespread attention of researchers and has become urgent in many environmental engineering, biomedical and industrial anti-corrosion fields [1]. Clinical applications of biosensors are still facing unprecedented challenges due to the high false positive reactions caused by nonspecific protein adsorption, and the limited sensitivity from low aptamer loading [2]. Thence, many researchers have been pursuing biosensors with high signal-to-noise ratio (S/N ratio). In Recent years, we have witnessed increasing attempts on the surface modification with functional layers, such as poly(-ethylene glycol) (PEG) [3,4], zwitterionic polymers [5,6], and peptides [7,8] to achieve the antifouling property. Peptides avoid the disadvantages that PEG is easily oxidized under physiological conditions, and the complex synthesis process of zwitterionic polymers, are widely used [9].

Various peptides-based antifouling materials, such as hydrophilic peptoids [10], amphiphilic peptides [11,12] and zwitterionic peptides [13,14], have been developed and reported to be effective resist biofouling. Among these antifouling peptides, zwitterionic peptides contain equal and alternating negatively charged and positively charged amino acid, which is capable of binding more water molecules to form a hydration layer [15]. Compared with the zwitterionic polymers, the structure, composition, and chain length of zwitterionic peptides can be very well controlled by adjusting the peptide sequences during the peptide synthesis stage [16,17]. These advantages have been utilized to design/engineer various zwitterionic peptides-based applications, such as surface functionalization of implants in biomedicine [18], biosensors in diagnostics [19], and antifouling applications [20,21]. It has been proven that surface hydration played an important role in monitoring the antifouling property of materials. Surface hydration induced by hydrogen bonding has been considered an important factor in the antifouling performance of hydrophilic and nonionic materials [11]. Apart from hydrogen bonds, zwitterionic materials can induce strongly ionic solvation to produce additional surface hydration, which provides an extra energy barrier for other molecules to be adsorbed [22]. Therefore, these constructed new ultralow fouling interfaces must exhibit effective resistance to nonspecific protein adsorption, or have a large surface grafting density to support the formation of a hydration layer barrier between substrate and contaminants [11]. For instance, Jiang group [23] has reported that self-assembled monolayers (SAMs) anchored by cysteine (containing SH- group) exhibit high surface grafting density and ultralow fouling. Although these peptides-based platforms have been very effective in resisting biofouling from complex media, similar to other two-dimensional (2D) surfaces, they also will limit the sensor response due to the relatively low antibody/aptamer load [24]. Attributed to the limited permeability of biomolecules in the highly packed brush film, such constructed surfaces have poor target recognition capacity [25]. In view of these, we proposed a generic concept to promote the development of antifouling sensing platform, in which a hierarchical structure with regulatory functions was fabricated based on zwitterionic peptides. We designed a hierarchical peptide brushes composed of zwitterionic antifouling peptides (CPPPPEKEKEKEK, Pep4) and short-chain peptides (CPPPPEKEKEK, Pep3), and the hierarchical structure was conducive to support the long and short chains to form an orderly and dense film, which is capable of tethering more water molecules, thereby resisting biofouling. Meanwhile, the improved penetration promotes the immobilization of the probe and enhances the ability to recognize target molecules, thereby effectively improving sensitivity. The conducting polymer polyaniline (PANI) films were used to support the immobilization of hierarchical peptides brush and the subsequent modification of the aptamers, to generate not only the transducing response, but also a very selective interface. Herein, we took the merit of the clear structure of peptides and monitored the antifouling properties of a series of hierarchical structures composed of zwitterionic peptides with a well-controlled chain length. The antifouling properties of these platforms based on different peptide brushes were examined by challenging with complex biological samples. This work reported an innovative research on the effects of controlled peptide chain structure and length on sensing and antifouling capability, and provided a new methodology for biosensor and diagnostics in complex media with acceptable accuracy and satisfactory sensitivity. The construction method of the antifouling sensing platform described here can be easily applied to other antifouling materials and diagnostic applications.

2. Experimental method

2.1. Electro-polymerization of PANI films

Details of reagents and apparatus are provided in the supporting information. Conducting polymer PANI film was electrodeposited onto bare glass carbon electrode (GCE) by a galvanostatic technique at a constant current density of 0.01 mA cm^{-2} for 1 h at room temperature. The deposition solution is a 1.0 M $HClO_4$ solution with 0.1 M aniline and 2.0 mg mL^{-1} poly(sodium 4-styrenesulfonate) (PSS). The electrochemical redox peak of the electrodeposited PANI can be used as the sensing signal, and the exposed amino group at its end can be used to covalently immobilize zwitterionic peptides.

2.2. Preparation of antifouling interface

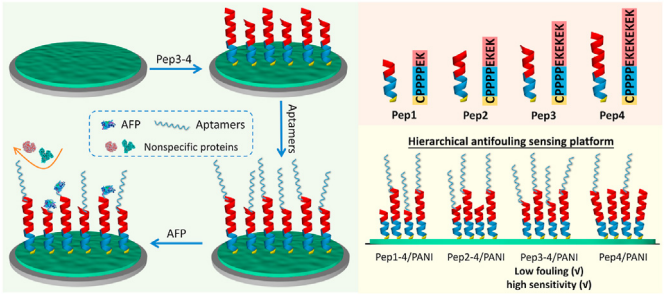
Firstly, PANI modified electrodes was immersed into 2.0 mM sulfo-SMCC (as a cross-linker) for 1 h to activate the amino group at the end of PANI. Then, the electrodes were incubated in a mixed phosphate-buffered saline (PBS) solution containing 0.2 mg mL^{-1} Pep3 and 0.2 mg mL^{-1} Pep4 for 3 h at room temperature, then obtained Pep3-4/PANI, and Pep1-4/PANI, Pep2-4/PANI and Pep4/PANI were obtained in the same way. After the above process, the terminal thiols group on the cysteine at the end of zwitterionic peptide was bonded to PANI's amino groups, thus successfully attaching the peptides onto the PANI/GCE.

2.3. Immobilization of AFP aptamers

The same volume of 1.0 μM AFP aptamers modified with carboxyl groups was mixed with 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.1 M N-Hydroxysuccinimide (NHS) mixed solution and stabilized for 30 min, and then, the peptides modified surfaces were soaked into the above mixed solution for 4 h at 37 °C. Finally, the successfully constructed sensing platform (Apt/Peptide/PANI/GCE) was stabilized in PBS solution for 12 h at room temperature to neutralize the impurity ions before detection.

2.4. Sensing of AFP

The antifouling modified electrodes (Apt/Peptide/PANI/GCE) were incubated in various concentrations of AFP solutions for 60 min, and then rinsed with PBS and water. Signal suppression was measured by differential pulse voltammetry (DPV) response before and after incubation in the range of −0.4 to 0.5 V.



Scheme 1. Schematic diagram of the fabrication of antifouling electrochemical AFP biosensor based on hierarchical zwitterionic peptides.

3. Results and discussion

3.1. Design and fabrication of the antifouling sensing interface

The antifouling platform was prepared as shown in Scheme 1. PANI film was electropolymerized onto the bare GCE by the galvanostatic method, and was used as a conductive carrier in this sensing platform and supported a transducing electrochemical signal, without the need for additional redox probes. The antifouling layer composed of peptides film with interlaced long and short chains (Pep1-4, Pep2-4, Pep3-4), or a single-layer with long-chain Pep4 was expected to alleviate the nonspecific protein adsorption and support more aptamer loading to improve the biosensor sensitivity for AFP quantification.

Scanning electron microscope (SEM) was used to characterize the micromorphology of the PANI film. PANI film exhibited a microstructure of particle accumulation (50–100 nm particles), and was uniformly covered on the electrode surface, as shown in Fig. 1A. With the aid of the sulfo-SMCC crosslinking agent, the antifouling peptides were covalently bound to the PANI film, by activating the amino group of PANI and then reacting with the Cys-terminal thiols. The successful covalent bonding of zwitterionic peptide and PANI was conducted by fourier transform infrared spectroscopy (FTIR). As shown in Fig. 1B, the band at 1476 and 1558 cm^{-1} represented clear quinoid ring and benzenoid stretching bands, respectively. Besides, the bands at 1301 and 1122 cm^{-1} were aligned with C–N stretching of the secondary aromatic amine, verifying successful electro-polymerization of PANI film. A new band appeared at 1705 cm^{-1} ascribed to the generated amides, after antifouling peptide was covalently bound onto PANI.

3.2. Investigation of zwitterionic peptides

It has been proved that, in order to achieve effective antifouling properties, the designed molecular films should be orderly and dense, highly hydrophilic, and electrically neutral. The antifouling layer was composed of reported zwitterionic antifouling peptides (CPPPPEKEKEKEK (Pep4)) and short-chain peptides of different lengths (e.g. Pep1, Pep2, Pep3). The secondary structures of these zwitterionic peptides were evaluated by circular dichroism (CD), in which a strong negative band was present at ~200 nm and a weak positive band at ~220 nm (Fig. 1C), and it was clear that the peptide sequence with a relatively higher proportion of proline showed stronger peaks, indicating that characteristic polyproline (P) helix conformation, one that supports a dense and ordered film [23]. The isoelectric points (pI) of the four peptides were all around 6.56, and the experimentally tested zeta potentials were all close to 0.0 mV, as displayed in Fig. 1D. Interface wettability of different modified electrodes was tested by static water angle (WCA). These interfaces based on zwitterionic peptides all presented much smaller contact angle (Pep1-4/PANI: 26.68°, Pep2-4/PANI: 24.45°, Pep3-4/PANI: 23.4°, and Pep4/PANI: 24.54°), as shown in Fig. 1E and Table S1, compared to those from the PANI/GCE (58.90°) and the bare GCE (66.73°), proving the hydrophilicity of the zwitterionic peptides. And the Pep3-4/PANI modified electrode exhibited excellent hydrophilicity, which may be attributed to the more hydrophilic EK of Pep3-4. And the hierarchical structure support good penetration and enable peptides with different chain lengths to support each other to form an orderly and dense antifouling layer, that improves the interface hydrophilicity.

3.3. Assessment of antifouling performance

The concentration of zwitterionic peptide was optimized to obtain the optimized antifouling conditions (Fig. S2). DPV was used to evaluate the degree of protein adsorption in complex medium fetal bovine serum (FBS). The bare GCE and the different antifouling materials modified PANI film, e.g. commonly used blocking reagent bovine serum albumin (BSA), hierarchical antifouling peptides (Pep1-4, Pep2-4, Pep3-4), and Pep4, were incubated in different diluted concentration FBS samples (1–100%) for 0.5 h, respectively.

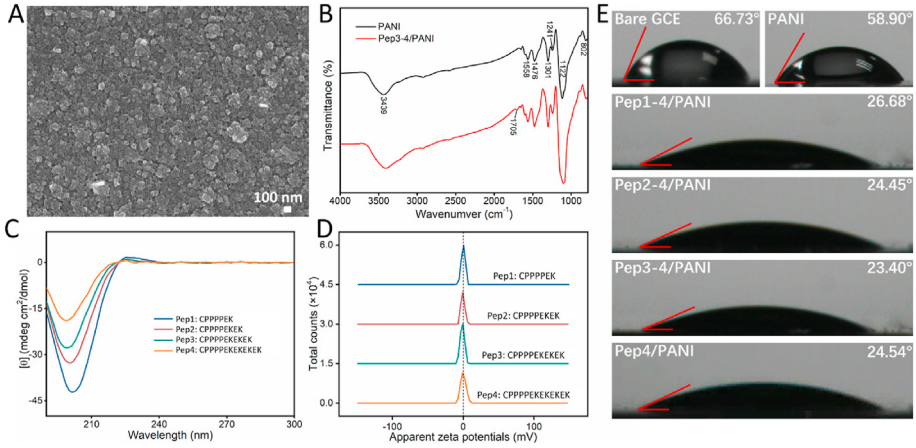


Fig. 1. (A) SEM image of the PANI/GCE. (B) FTIR of PANI (black curve) and Pep3-4/PANI (red curve) modified GCE. (C) CD spectra of 0.3 mg mL^{-1} zwitterionic peptides. (D) Zeta potential of zwitterionic peptides with different chain lengths. (E) WCA images of bare GCE, PANI film, and Pep1-4, Pep2-4, Pep3-4 and Pep4 modified PANI/GCE. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

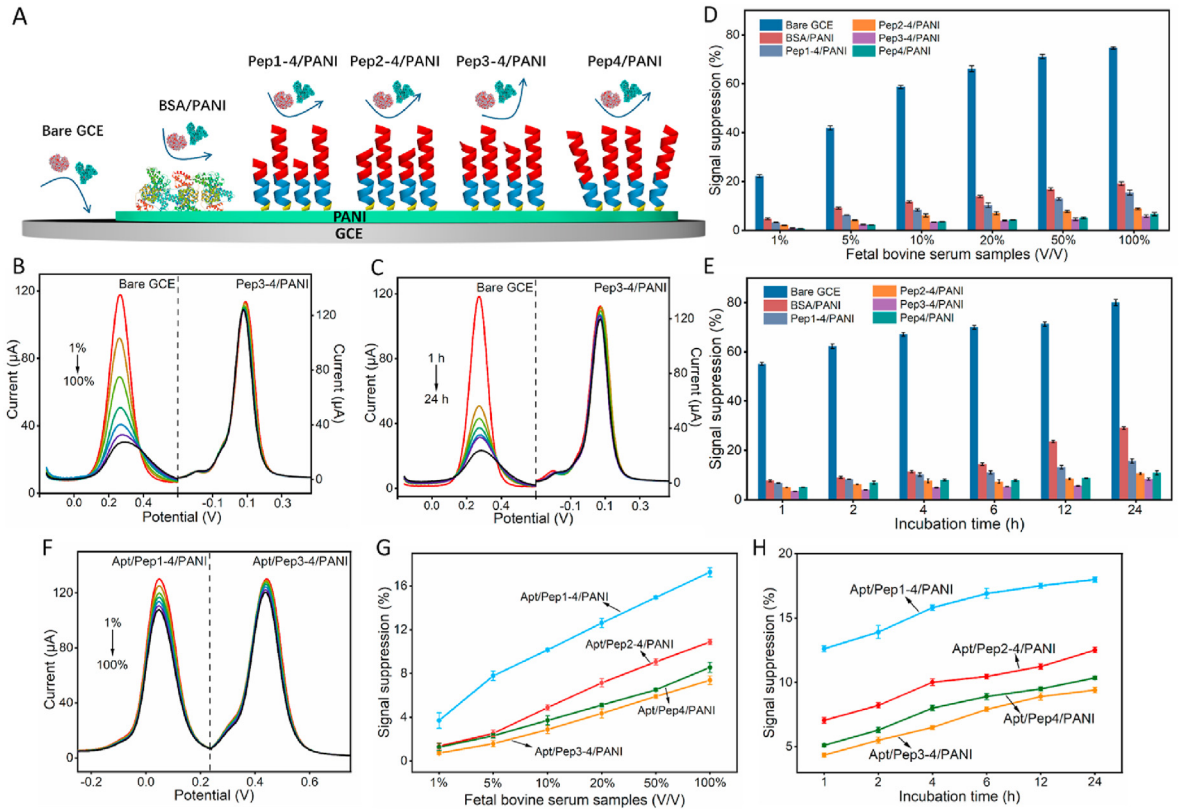


Fig. 2. (A) Schematic of the antifouling performance of different modified electrodes (e.g. bare GCE, BSA/PANI, Pep1-4/PANI, Pep2-4/PANI, Pep3-4/PANI, Pep4/PANI). (B) DPV curves of bare GCE (left) and Pep3-4/PANI/GCE (right) after immersing in FBS samples of different dilution concentrations. (C) DPV responses of bare GCE (left) and Pep3-4/PANI/GCE (right) against 1–24 h incubation in 20% FBS samples. (D) Antifouling performances of these interfaces after incubation in FBS samples for 30 min(E) Long-term antifouling performances after incubation in 20% FBS for 1–24 h. (F) DPV of Apt/Pep1-4/PANI (left) and Apt/Pep3-4/PANI (right) after immersing in FBS samples. (G) Short-term in FBS samples for 30 min and (H) long-term antifouling performances in 20% FBS for 1–24 h of these interfaces modified with aptamer.

As displayed in Fig. 2B, bare GCE obviously suffered from biofouling, the current response showed sharp decreases (about 74.68% after 100% FBS incubation). While for the Pep3-4/PANI, the current reduction was only 5.86% due to the efficient prevention of proteins from blocking the electroactive surface. The DPV responses of BSA, Pep1-4, Pep2-4, and Pep4 modified PANI films after incubation with FBS were shown in Fig. S3, respectively, the antifouling performance was displayed in Fig. 2D. The antifouling performance of the five surfaces are in the order: Pep3-4/PANI ≈ Pep4/PANI > Pep2-4/PANI > Pep1-4/PANI > BSA/PANI. Moreover, long-term antifouling performance of these interfaces in 20% FBS was displayed in Fig. 2C, E and S3. The signal response of bare GCE decreased by 80.10% after 24 h incubation, whereas the oxidative current of Pep3-4/PANI was well maintained (decreases by 8.41% after 24 h in 20% FBS), highlighting the advantage of the hierarchical peptides structure in resisting the long-term biofouling. The Pep3-4/PANI interface showed better antifouling ability than Pep1-4 and Pep2-4 modified PANI because of more hydrophilic amino groups and carboxyl groups of Pep3-4. Notably, the hierarchical structure is conducive to the mutual support between the long and short peptides to form an ordered and dense film, which is capable of tethering water molecules to resist protein adsorption, however, the Pep4-based antifouling layer is prone to collapse or curl due to the same peptide chain length, thereby reducing the interface density and the hydration density.

More importantly, after immobilization of the AFP aptamer, the signal suppression of Apt/Pep3-4/PANI was still very low, as shown in Fig. 3F and G, the signal only droped by 9.4% after being soaked in 100% FBS for 30 min. And the antifouling performance of long-term immersion in 20% FBS was also superior to other peptide modified electrodes. These results indicated that the PANI film functionalized with the hierarchical-structured antifouling peptide performs excellent antifouling capability even after the further immobilization of biorecognition element onto the modified interface.

3.4. Sensing of target AFP

DPV was performed to characterize the preparation process without a solution phase redox probe (Fig. 3A). Particularly, the electropolymerized PANI film (143.31 μA, curve red) showed a significant current response at 0.087 V compared to bare GCE (around 0.0 μA, curve green), which is attributed to the transition of PANI leucoemeraldine/emeraldine. The peak current of the PANI/GCE was linearly related to the scan rate, which proved that PANI redox processes is a surface-controlled processes with fast electron transfer, as shown in Fig. 3B. Then, the immobilization of Pep3-4 and AFP aptamer reduced the current response as expected (129.45 μA, curve blue; 105.64 μA curve orange) because they will significantly hinder electron transfer as non-conductive biomolecules, respectively. The subsequent capture of target AFP leads to a further reduce in the current signal (86.40 μA), because the specific recognition between the target and aptamer results large steric hindrance to hamper electron transfer and ion diffusion. The different current responses in each above step illustrated the successful fabrication of the sensing system, and the current changes related to the specific binding of the target proved the feasibility of the biosensor.

After optimizing the sensing conditions (see Supporting

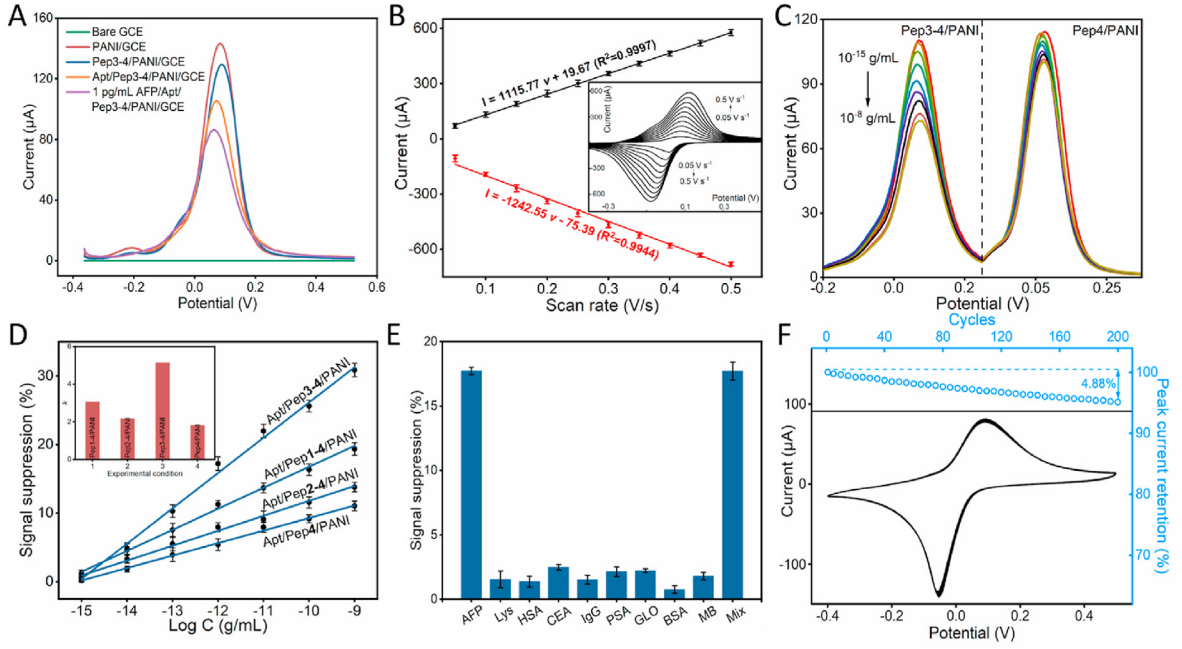


Fig. 3. (A) The DPV curves of different prepared electrodes in PBS solution. (B) Linear relationship of PANI/GCE between the cathodic/anodic peak currents and scan rate. Inset shows the cyclic voltammetry (CV) of PANI/GCE at different scan rate. (C) DPV responses of the Pep3-4/PANI (left) and Pep4/PANI/GCE (right) to target AFP. (D) Peak current signal suppressions of the biosensors after incubation with various concentrations of AFP. Inset shows the kinetic rate constant (k). (E) Signal suppression of this AFP sensing platform to 1.0 pg mL^{-1} target AFP, and 1.0 mg mL^{-1} of Lys, HSA, CEA, IgG, PSA, GLO, BSA, MB, and their mixture (Mix), respectively. (F) Stability of the prepared electrochemical biosensor by continuous CV scanning in PBS solution. Inset shows the peak current retention corresponding to different CV cycles.

Information), a quantitative assessment of various aptasensors based on the hierarchical peptide films to AFP were shown in Fig. 3C and S4. These corresponding regressions were shown in Fig. 3D and Table S2. Notably, the kinetic rate constant k (derived as the slope of the $\triangle i/i_0 - \text{Log } C$ plot, $\triangle i/i_0 = k \times \text{Log } C + A$) of these surfaces are in the order: Pep3-4/PANI > Pep1-4/PANI > Pep2-4/PANI > Pep4/PANI, indicating a better sensitivity of Pep3-4/PANI interface, owing to the hierarchical interlaced structure is conducive to target identification. The regression equation of the Pep3-4/PANI was $\triangle i/i_0 = 3.06 \times \text{Log C} + 47.4$ ($R^2 = 0.996$). The limit of detection (LOD) of this AFP assaying (0.59 fg mL^{-1}) was lower than that of many reported biosensors or sensing platforms (Table S3) [13,26–32]. The low LOD and high sensitivity may be attributed to large electroactive surface area of the conducting polymer PANI (supporting sensitive electrochemical response), hierarchical structure supporting high target recognition, and the good biocompatibility provided by zwitterionic peptides (generating a biocompatible environment for aptamer and target).

Then, the selectivity of this hierarchical peptide-based AFP biosensor was monitored by detecting a series of proteins with different molecular weight or pI/charge. At an interference concentration of 1.0 mg mL^{-1} level, the current responses were far lower than the specific response to target AFP, as shown in Fig. 3E, indicating outstanding selectivity. Fig. 3F showed the stability of the prepared biosensor after 200 cycles of continuous CV scanning (range from −0.4 to 0.5 V), displaying only a slight reduce of the peak current (4.88%). A longer-term monitoring of the basal current signal within 20 days, found that >95% of the baseline signal remained after 20 days of storage in PBS solution (Fig. S5A). As shown in Fig. S5B, the biosensor also exhibited good reproducibility in response to 1.0 pg mL^{-1} AFP through five independently prepared biosensors with the relative standard deviation (RSD) of 2.0%.

The regeneration of the sensing platform was tested by immersing the modified electrode into glycine–HCl (0.1 M, pH 2.2) for 10 min, and then performing the next detection. As displayed in Fig. 4A, the current values of the biosensor showed only slight decreases after 5 regeneration runs, revealing the facile regeneration of the peptide-based biosensor. To explore its applicability in actual biological samples, the antifouling biosensor response to various concentrations of AFP in the presence of 1.0 mg mL^{-1} HSA

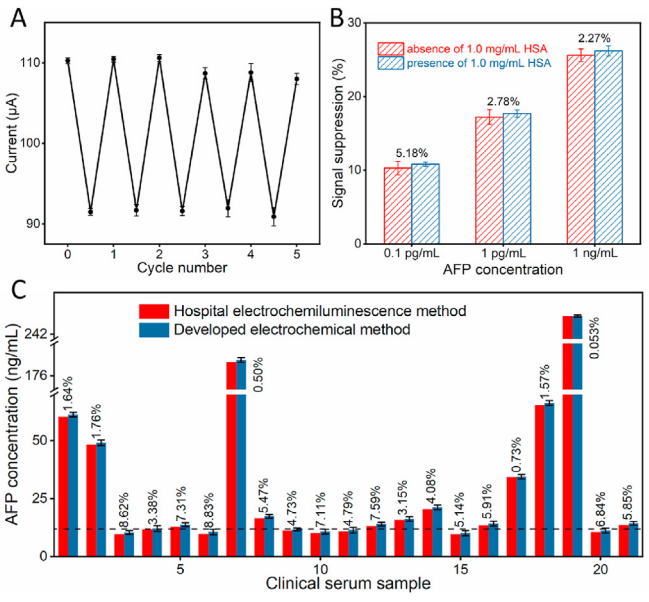


Fig. 4. (A) The regeneration of the biosensor (responses to 1.0 pg mL^{-1} AFP). (B) Signal suppressions of this biosensor after incubation with AFP in the absence (red) and presence (blue) of 1.0 mg mL^{-1} HSA in PBS. (C) Results of testing clinical serum samples using the developed electrochemical assaying (red column) and reference hospital electrochemiluminescence assaying (blue column). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

was studied. Clearly, even in a solution with a high concentration of nonspecific proteins, the sensing performance of the sensing platform was almost identical to that in pure buffer (similar signal suppression in Fig. 4B). These results validated that the biosensor is capable of recruiting targets very specifically. Notably, the constructed biosensor demonstrated the possibility of its practical application by testing clinical samples, and compared it with the results obtained by the electrochemiluminescence method established by the hospital. As shown in Fig. 4C, across the analysis of 21 real clinical samples, a very good correlation was obtained, indicating acceptable accuracy of the developed method for AFP quantification in real samples.

4. Conclusion

In conclusion, a zwitterionic peptides-based sensing platform with hierarchical architecture for ultralow fouling and high target binding was fabricated for AFP quantification. It was found that the sensitivity of the biosensor was greatly enhanced through the controlled formation of antifouling peptide brushes with suitable hierarchical structure to achieve the high target binding ability. More importantly, in the presence of high concentrations of nonspecific proteins, the electrochemical biosensor was able to maintain excellent antifouling property and high sensitivity. And it also ensured reliable accuracy for AFP detection in human serum when compared with the hospital reference method. This work demonstrated an effective surface modification strategy that aims to reconcile two conflicting properties to maintain sensing interfaces with ultralow fouling and high sensitivity.

CRediT authorship contribution statement

Nianzu Liu: Conceptualization, Methodology, Investigation, Writing - original draft, Visualization, Data curation. **Xiaojian Fan:** Conceptualization, Resources, Writing - review & editing. **Haiqing Hou:** Resources, Writing - review & editing. **Fengxian Gao:** Methodology, Supervision, Writing - review & editing. **Xiliang Luo:** Conceptualization, Methodology, Investigation, Writing - review & editing, Visualization, Supervision, 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2020.12.031.

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