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Low Fouling Protein Detection in Complex Biological Media Supported by a Designed Multifunctional Peptide

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ABSTRACT: The construction of sensitive and selective biosensors capable of detecting specific targets in complex biological samples remains a challenge highly relevant to a range of sensor/diagnostic applications. Herein, we have utilized a multifunctional peptide to present an interface that supports the very specific recruitment of targets from serum. The novel peptide sequence designed contains an anchoring domain (CPPPP-), an antifouling domain (-NQNQNQNQDHWRGWVA) and a human immunoglobulin G (IgG) recognition domain (-HWRGWVA), and the whole peptide was designed to be antifouling. These were integrated into polyaniline nanowire arrays in supporting the quantification of IgG (with a limit of detection of 0.26 ng mL^{-1}) in neat serum and real clinical samples. The strategy of utilizing multi-segment peptide films to underpin highly selective target recruitment is, of course, readily extended to a broad range of targets for which an affinity sequence can be generated.

KEYWORDS: *multifunctional peptide, antifouling, nonspecific adsorption, polyaniline, immunoglobulin G, biosensor*

Since the first biosensor was created by Clark and Lyons in the 1960s, the associated market has exponentially extended into medical diagnostics, biotechnology, environmental monitoring, and food inspection.[1-3] Electrochemical biosensors are often cited as potentially supporting high levels of detection sensitivity in a format that is convenient, potentially cheap and low cost.[4,5] Because many of these ultimately operate through electrode surface confined recognition processes, real clinical application, where target recruitment must be highly selective within a concentrated complex background, can be challenging. A common approach in alleviating this is to integrate recognition into a surface that is otherwise highly non-fouling.[6] Of these, those which are hydrophilic and/or zwitterionic are most notable.[7,8] Polyethylene glycol and oligo- (PEG and OEG) motifs are common but have a tendency to oxidize in the presence of transition-metal ions and oxygen under physiological conditions (limiting their utility in long-term applications).[9-11] Zwitterionic polymers, such as those based on poly(carboxybetaine)- and poly(sulfobetaine) have excellent resistance to fouling, even in complex media.[12-14] The convenient and highly controlled synthesis of derived films, however, remains challenging.[15] In general terms, an ideal antifouling surface should also exhibit good biocompatibility, high chemical stability, convenient electrode integration and present an interface that can also be readily modified with recognition elements. Peptides are natively zwitterionic and exhibit outstanding biocompatibility.[16,17] A range of peptide-based antifouling films have been reported.[18,19] For instance, Jiang's group have reported a zwitterionic peptide composed of alternating negatively charged glutamic acid (E) and positively charged lysines (K) that exhibits a strong resistance to albumin, lysozyme and fibrinogen.[20,21] In separate work a peptide of sequence EKEKEKE-PPPPC, combining antifouling and surface anchoring functionality has been shown to be resistant to human blood serum adsorption and to prevent the adhesion of bacteria and mammalian cells.[22,23] In related work, we have shown that a specific hydrophilic peptide sequence, EESKSESKSGGGGC, exhibits similar characteristics.[18]

In vitro selected affinity peptides have been shown to be capable of supporting the detection of metal cations,[24] small molecules,[25] and antigens.[26] Recently, a number of groups have reported the use of short peptide aptamers (peptamers) to detect specific antibodies in electroanalytical configurations.[27-29] For example, the peptide sequence, DHHTQQH, has high affinity for lead salts as demonstrated by applying stringent genetic engineering protocols.[30] And a peptide aptamer (WHWQRPVSIKC) was used to fabricate a label-free EIS aptasensor for TNT detection.[28] If one can integrate both protein repelling and recognition elements into the same sequence, the generating of effective molecular films capable of selectively recruiting targets from complex mixtures, without any additional conjugation or film preparation steps becomes possible.

Conducting polymers have been widely used as substrates for the construction of electrochemical biosensors. Among various conducting polymers, polyaniline (PANI) materials have attracted much attention as they possess

many promising characteristics including high electronic conductivity, reversibility between redox states through doping/dedoping processes, and good biocompatibility.[31] Moreover, amino groups (-NH_2) of PANI can be readily functionalized with biomolecules, which make it very attractive for biosensor applications.[32,33] In particular, PANI nanowires have been utilized for the construction of sensitive electrochemical biosensors as they possess very high surface area.[34,35] Therefore, PANI nanowires were used in this work as the substrate for the immobilization of peptides, and at the same time their redox currents were used as the sensing signals.

Herein, we have designed and synthesized a novel a peptide (CPPPPNQNQNQNQDHWRGWVA) with three functional domains enabling the single step fabrication of a surface tethered, highly selective target recruiting monolayer film. The terminal sequence HWRGWVA has been identified as a candidate ligand to selectively bind the Fc region of human immunoglobulin G (IgG) by Carbonell,[36-38] where it has been shown to be capable of supporting the purification of IgG from mammalian cell culture medium (cMEM) and commercial Chinese hamster ovary (CHO) cell culture supernatant.[39,40] The multifunctional peptides were covalently coupled to PANI nanowires in generating electrochemical sensors for which the wire redox activity was used to transduce target recognition. The constructed films are demonstrably capable of supporting IgG quantification in blood serum.

EXPERIMENTAL METHODS

Reagents. Multifunctional peptides and blocking peptides (purity > 98%) were both synthesized and purified from Bankpeptide biological technology Co. Ltd. (Hefei, China). Human immunoglobulin G (IgG) lyophilized powder (> 97%) was obtained from Equitech-Bio (Kerrville, TX). Fetal bovine serum (FBS), alpha-fetoprotein (AFP), myoglobin (Mb), carcinoembryonic antigen (CEA), thrombin (TB), human serum albumin (HSA), hemoglobin (Hb), bovine serum albumin (BSA), lysozyme (Lys) and Immunoglobulin E (IgE) were purchased from Beijing Bo Yang Hongda Technology Co. Ltd. (Beijing, China). 4-(N-maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt (sulfo-SMCC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aniline was obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Clinical human serum samples were supplied by the Eighth People's Hospital of Qingdao (Qingdao, China). Other chemicals were of analytical grade and ordered from Aladdin Reagent Co., Ltd. (Shanghai, China).

Synthesis of PANI Nanowire Arrays. PANI nanowire arrays were electropolymerized through the galvanostatic technique onto the glassy carbon electrode (GCE, diameter 3.0 mm), which was polished with 0.3 μm and 0.05 μm alumina powder in sequence prior to an ultrasonic washing in absolute ethanol and water for 2 min, respectively. The electropolymerization was performed at a constant current density of 0.01 mA cm^{-2} at room temperature for 1 hour, and the typical electrolyte was 1.0 M HClO4 solution containing 0.1 M aniline.[41]

Immobilization of the Peptides. Firstly, PANI nanowire arrays modified electrode was immersed into 2 mM solution of sulfo-SMCC as cross-linker for 1 h at room temperature. Secondly, the modified electrode was incubated in phosphate buffered saline (PBS, 0.2 M, pH 7.4) containing 2.0 mg mL^{-1} multifunctional peptides and 2.0 mg mL^{-1} blocking peptides for 3 h. The peptides were immobilized on the PANI nanowire arrays modified electrode via covalent bonds formed between the cys-terminal thiols and amino groups (from PANI). Finally, the fabricated antifouling electrochemical biosensor (peptides/PANI/GCE) was equilibrated in PBS (0.2 M, pH 7.4) for 24 h at room temperature before use.

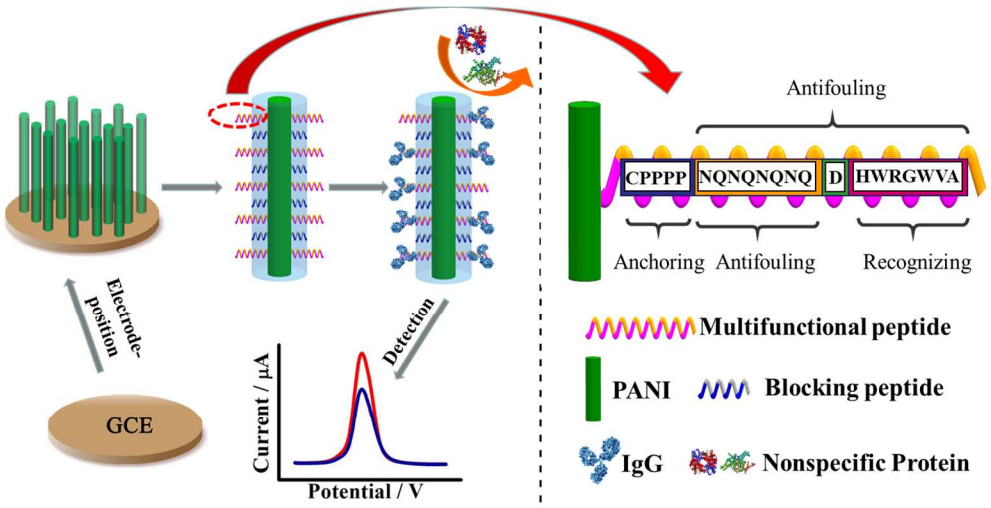
Characterization of the Antifouling Ability. To assess the antifouling performance of the modified electrode, relatively complex media were tested. Differential pulse voltammetry (DPV) technique was utilized to monitor the nonspecific adsorption on the peptide-based biosensor interface. The DPV responses of modified electrodes before and after soaking in FBS (immersed with FBS solution for 30 min and then washing with water) were recorded in PBS (0.2 M, pH 7.4). FBS samples were diluted with PBS (10 mM, pH 7.4) to different dilution ratio (V/V) solutions.

Secondary Structure and Charge Neutrality of the Designed Peptides. Circular dichroism (CD) spectra were recorded over a wavelength range of 190-270 nm with a step resolution of 0.5 nm on 0.3 mg mL^{-1} multifunctional peptides in PBS solution (pH 7.4) at 25 °C using a Jasco J-810 CD spectropolarimeter (Jasco Inc., Japan). Buffer spectra were recorded and substracted from the sample spectra. Each experiment was repeated three times.

Zeta potential measurement of multifunctional peptide was performed with a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK). The zeta potential, when the peptides were dissolved in PBS (0.2 M, pH 7.4) solution, was investigated.

Surface Characterization. Scanning electron microscope (SEM) images of PANI nanowires were recorded using JEOL JSM-7500F SEM instrument (Hitachi High-Technology Co., Ltd., Japan) with an accelerating voltage of 5.0 kV. Hydrophilicity characterizations of different modified surfaces were performed on a static water contact angle (WCA) measurement (JC2000D1 Shanghai Zhongchen Instrument Co., China). Water drops with a volume of 10 μL was used, and the water drops were equilibrated in contact with surfaces for 10 s prior to data collection.

Sensing with the Biosensor. For the detection of target molecular, IgG solutions with different concentrations in PBS (0.2 M, pH 7.4) were prepared. The peptide modified surfaces were incubated in different concentrations of IgG for 90 min at room temperature, followed by rinsing with PBS and water. The signal suppression as the ratio before and after hybridization was measured by differential pulse voltammetry (DPV) in the range of -0.4 to 0.6 V.



Scheme 1. Schematic illustration of the preparation of a PANI supported peptides-based amperometric biosensor.

Fluorescence Microscopy. The fluorescence was monitored using TCS SP5 confocal laser microscope (Leica, Germany). Bare electrode without peptide was used as controls. All interfaces were incubated in 0.1 mg mL^{-1} fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA) solution for 2h at room temperature and gently rinsed three times with PBS and Milli-Q water thoroughly afterwards. Finally, the images of three interfaces were taken under a confocal fluorescence microscope.

Electrochemical Measurements. All electrochemical experiments were carried out with a CHI660E Electrochemical Workstation (Shanghai CH Instrument Co., China) at room temperature with a conventional three-electrode system: a GCE acted as the working electrode, a saturated calomel electrode (SCE) acted as the reference electrode, and a platinum wire acted as the auxiliary electrode. Cyclic voltammetry (CV) measurements were conducted with a potential from -0.4 to 0.6 V and a scan rate of 100 mV s^{-1}. Biosensor response data were recorded using DPV, with the amplitude of 50 mV, potential increment of 4.0 mV, and pulse period of 0.5 s by scanning the potential in the range from -0.4 V to 0.6 V. The signal suppression (%) was expressed as the ratio between the peak current of DPV detected in the absence and presence of target molecule (signal suppression (%) = $[(i_{target} - i_{blank})/i_{blank}] \times 100$).

RESULTS AND DISCUSSION

Investigation of the Newly Designed Peptides. In order to enable effective antifouling character, it has been established that molecular films should be highly hydrophilic and of low net charge. Here the antifouling and recognition domains were connected through a negatively charged aspartate. The isoelectric point (pI) of the peptide is 7.1. As shown in **Figure S1**, the resolved zeta potential of the peptide solution was pleasingly close to 0.0 mV.

The peptide secondary structure of was characterized using circular dichroism (CD), where (**Figure 1A**) the extended polyproline-generated helix conformation (strong negative band around 200 nm and a weaker positive band near 220 nm), ascribed to the rigidity imparted to the backbone by the proline linker, are resolved.[42,43]

Construction of the Antifouling Electrochemical Biosensor. Derived surface fabrication is schematically illustrated in **Scheme 1**. A short peptide sequence (CPPPPNQNQNQNQ) is used as blocking reagent to block the unmodified sites and resist nonspecific adsorption. Multifunctional peptides and the blocking peptides were covalently coupled on the PANI nanowire arrays through the reaction between the Cys-terminal thiols and amino groups (from PANI) with the assistance of a sulfo-SMCC heterobifunctional crosslinker. PANI nanowire arrays were electropolymerized through the galvanostatic technique onto the glassy carbon electrode (GCE) and used herein as a high surface area conductive support to generate a transducing electrochemical signal without the need to add or integrate additional redox probes.[44]

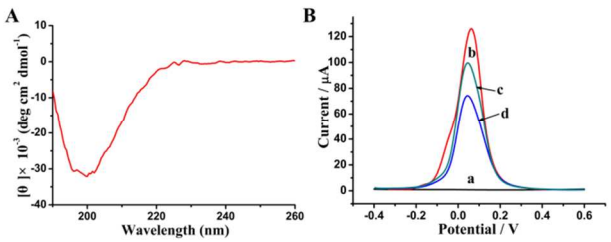


Figure 1. (A) CD spectra of multifunctional peptide in PBS at a concentration of 0.3 mg mL^{-1}. (B) DPV curves recorded in PBS (0.2M, pH 7.4). (a: the bare GCE; b: PANI/GCE; c: peptides/PANI/GCE; d: IgG/peptides/PANI/GCE).

Characterization of the Developed Biosensing interfaces. PANI nanowire arrays were electropolymerized onto GCE through the galvanostatic technique. The micromorphology of the PANI nanowire arrays were evaluated by SEM. As shown in **Figure S2**, the PANI nanowire arrays (50-60 nm height) formed a relatively uniform and dense, vertically orientated, layer on the underlying GCE presenting a very high surface area to solution. Hydrophilicity and wetting characteristics were mapped through static water contact angle assessment. As shown in **Figure S3** and **Table S1**, the electropolymerised arrays (PANI/GCE), are associated with a water contact angle value decrease from 73.8±0.3° to 57.1±0.4°, in accordance with hydrophilicity.[45] Subsequent peptide coupling (peptide/PANI/GCE) is associated with a further significant increase in wetting (WCA 27.2±0.5°), in a manner consistent with reports at other peptides films.[23,46]

DPV was used to follow film formation in the presence of a solution phase redox probe response (**Figure 1B**). It is initially worth noting that the PANI/GCE (curve b) surface displays a markedly increased DPV peak current compared with the bare GCE (curve a), with a peak potential at 0.05 V corresponding to the transition of leucoemeraldine/emeraldine. This is in line with the conductive high area support provided. The integration of peptides is predictably associated with a decrease in charge transfer capability from solution (curve c). Subsequent capture of target IgG results in a stepwise calibratable further decrease in current due to the progressive steric restrictions IgG binding imparts on electrolyte access to the film redox centres.

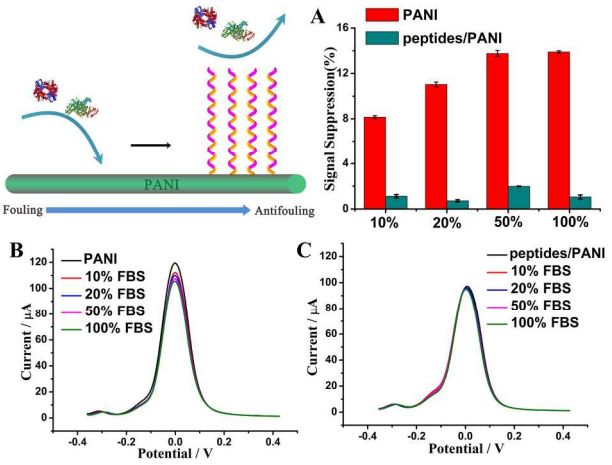


Figure 2. The antifouling property of the PANI (red) and peptides/PANI (cyan) for FBS samples of different concentrations (V/V) (A). DPV responses of PANI (B) and peptides/PANI (C) before and after incubation in different concentrations of FBS samples. Error bars represent the standard deviations of three repeated determinations.

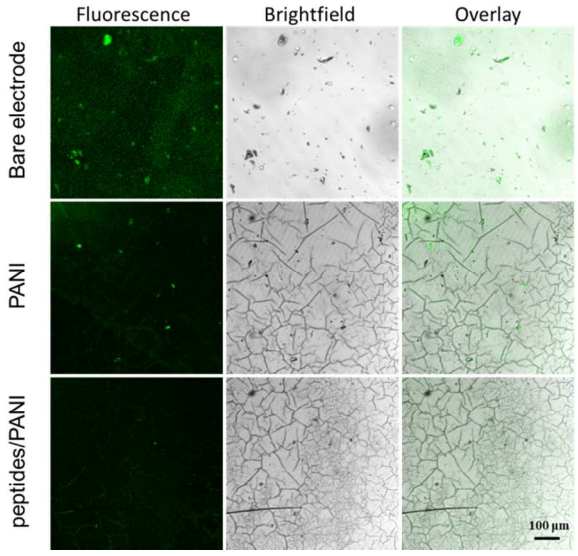


Figure 3. Representative fluorescence microscopy images of bare GCE, PANI and peptides/PANI modified electrodes after incubation in 0.1 mg mL^{-1} FITC-BSA solution for 2h.

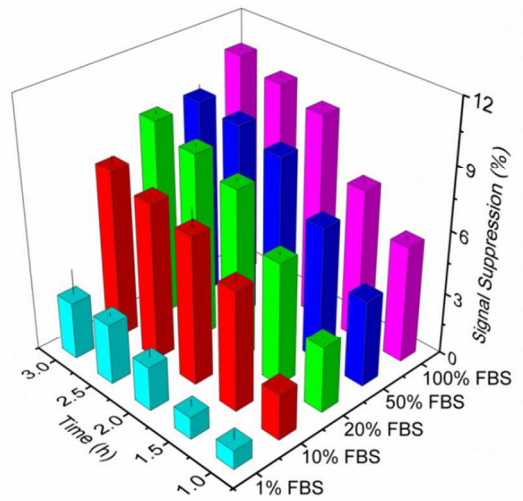


Figure 4. Antifouling analysis of the peptide/PANI modified electrodes in FBS samples for a considerably long time of 1-3 h.

Generic Antifouling Characteristics. In order to evaluate the antifouling ability of the as-prepared surfaces, DPV responses of PANI, and peptide film modified PANI interfaces were compared before and after incubation in FBS (30 minutes), where (**Figure 2 and S4**), the DPV resolved signal decrease directly reports on nonspecific association (these films are receptor free at this stage). Significantly, even after incubation in 100% FBS, the current response of the peptide/PANI modified electrode

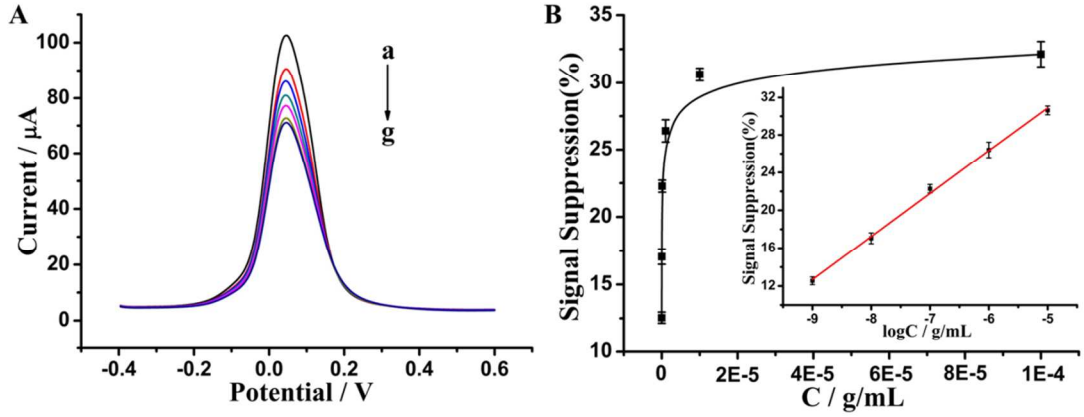


Figure 5. (A) DPV resolved responses of the sensor interfaces to IgG. Curve a refers to the blank solution and curves b-g to IgG at concentrations from 1.0 ng mL^{-1} to 10 μg mL^{-1}. (B) Peak current change of the interface after incubation with controlled concentrations of IgG in PBS (0.2M, pH 7.4). Inset shows the corresponding calibration curve (R^2 = 0.9975).

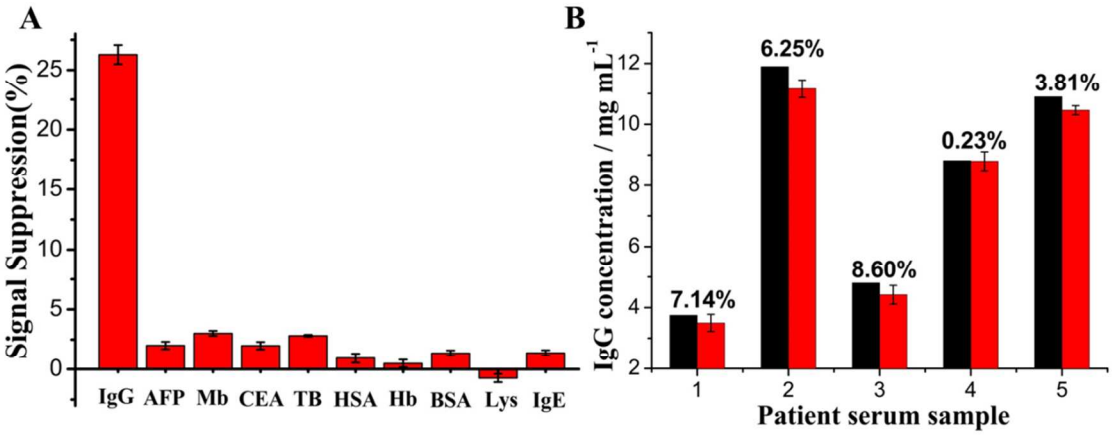


Figure 6. (A) Responses of the IgG biosensor to 1.0 μg mL^{-1} IgG, and 1.0 mg mL^{-1} of AFP, Mb, CEA, TB, HSA, Hb, BSA, Lys, and IgE, respectively. (B) Assay results of clinical serum samples using the developed and reference (left column) methods. The % differences are shown.

is modulated by < 2.0%, a performance equivalent to that of the very best antifouling surfaces.[45,47] This was further evaluated using fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA) (**Figure 3**) where almost no detectable FITC-BSA is evident at the peptide interfaces. An evaluation of fouling prolonged periods relevant to detailed calibration and assay experiments is shown in **Figure 4**.

Electrochemical Detection of IgG. Under the optimized sensing conditions (see the Supporting Information), an evaluation of the peptides/PANI/GCE selective response to IgG is shown in **Figure 5A**, with the transducing PANI oxidation peak responding across 1.0 ng mL^{-1} to 10 μg mL^{-1} 4 order dynamic ranges (**Figure 5B**). The detection limit (0.26 ng mL^{-1} where S/N = 3), is lower than that of many previous reports (**Table S2**).[48-55] This result indicates that the sensing interface possesses high bio-affinity toward the target IgG and it can detect IgG at very low level, which may be ascribed to the peptide sequence itself and the biocompatible microenvironment provided by the whole peptide based antifouling interface.

Specificity and Stability of the Biosensor. The ability of this peptide-based interface to selectively respond to IgG was evaluated by monitoring its exposure to a range of proteins of different molecular weight and pI/charge (**Figure 6A**). Even at 1.0 mg mL^{-1} levels interfacial amperometric response is modulated by < 3.0%. An analysis of sensor stability was carried out by continuous CV measurement in PBS (0.2 M, pH 7.4),[45] with the potential range from -0.4 to 0.4 V. As shown in **Figure S6**, the transducing signal is largely unchanged after 50 cycles. A longer term analysis of peak current across 10 days, shows a retention of >90% baseline signal (**Figure S7**).

Clinical Serum Sample Analysis. The ability of these electroactive peptide interfaces to support the reliable quanti-

fication of IgG from real patient serum samples was tested and cross referenced to analyses to an established hospital nephelometry test. As shown in **Figure 6B**, a very good correlation was obtained across 5 samples analysed, indicating an acceptable accuracy of the present method for IgG assay in practical serum samples.

CONCLUSIONS

Based on the designed multifunctional peptide, an electrochemical biosensor has been designed to enable the sensitive detection of IgG at high surface area polymer modified carbon electrodes. Newly designed multifunctional peptides possessed excellent antifouling property in complex serum samples. More importantly, the recognizing peptide segment was integrated into the whole antifouling peptide, offering the designed single peptide sequence both antifouling and biosensing functions. The designed biosensor showed a detection range over 4 orders of magnitude, with satisfying selectivity, high stability and a very low LOD, and it can be applied for the assay of real serum samples with acceptable accuracy, showing promising potential to be applied in clinical diagnosis and bioanalysis. In addition, this designed sensing platform with multifunctional peptides may be extended to generic mechanism for sensitive detection of other target biomolecules without encountering biofouling.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Zeta potentials, characterization of the developed biosensing interfaces, antifouling ability of different surfaces, optimization of sensing conditions, stability of the biosensor, and comparison with other reports (PDF)

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