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Ultrasensitive amperometric aptasensor for the epithelial cell adhesion molecule by using target-driven toehold-mediated DNA recycling amplification

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Abstract

An amperometric aptasensor is reported for the electrochemical determination of the epithelial cell adhesion molecule (EpCAM). It is based on a combination of EpCAM-driven toehold-mediated DNA recycling amplification, the specific recognition of EpCAM aptamer, and its binding to EpCAM. Hairpin probe 1 (Hp1) with a toehold region was modified with a 5′-thiol group (5'-SH) and self-assembled onto the surface of a gold electrode. Upon addition of EpCAM, the probe A (a 15-mer) is liberated from the aptamer/probe A complex and then hybridizes with the toehold domain of Hp1. This results in the exposure of another toehold for further hybridizing with hairpin probe 2 (Hp2) to displace probe A in the presence of Hp2 that was labeled with the electrochemical probe Methylene Blue (MB). Subsequently, liberated probe A is hybridized again with another Hp1 to start the next round of DNA recycling amplification by reusing probe A. This leads to the formation of plenty of MB-labeled DNA strands on the electrode surface and generates an amplified current. This 1:N probe-response amplification results in ultrasensitive and specific detection of EpCAM, with a 20 pg·mL^{-1} detection limit. The electrode is highly stable and regenerable. It was successfully applied to the determination of EpCAM in spiked human serum, urine and saliva, and thus provides a promising tool for early clinical diagnosis.

Introduction

Cancer-associated morbidity and mortality are primarily caused by cancer metastasis which involves the dissemination of cancer cells from the primary tumor to sur-

rounding tissues and bloodstream via blood or lymph nodes [1]. Clinical diagnosis of cancer primarily depends on imaging techniques or biopsy of diseased cells or tissues [2]. However, these methods are limited to the low sensitivity, capacity of discriminating benign and malignant lesions and diagnosis of cancer at early stage [2]. Biomarkers, including cells [3], proteins [4], nucleic acids [5] and so on, are molecules that differential expression or activity would be observed in pathologic conditions. It can be used as an indicator for evaluation of either normal or pathologic processes [6]. The epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein involved in cellular signaling, proliferation, differentiation and migration. It is primarily expressed at low levels in a majority of normal epithelial tissues [7]. However, overexpression of EpCAM was found in several human carcinomas [8], malignant effusions [9], and cancer stem cells [10]. Therefore, it is considered as a cancer biomarker for cancer diagnosis, therapy and prognosis. The

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sensitive and specific detection of EpCAM may provide a potential reference for understanding mechanisms of tumorigenesis, tumor development and metastasis and assisting clinicians to make effective therapy strategies. Methods such as enzyme-linked immunosorbent assay (ELISA) [11], flow cytometry [9, 12], quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) [13] and immuno-histochemistry (IHC) [14] were used for quantification of EpCAM or EpCAM-based circulating tumor cells (CTCs). Besides, several novel biosensor strategies have emerged for detection of EpCAM, including fluorescence resonance energy transfer-based bioassay [4, 15], microfluidic immunosensor [11, 16, 17], fluorescent biosensor [18]. Among these biosensors, electrochemical techniques were frequently adopted for EpCAM detection because of its merits of low cost, sensitivity, wide dynamic detection range and compatibility with many miniature devices including microfluidic immunosensor devices [11]. However, these microfluidic immunosensors relying on anti-EpCAM antibody were limited due to its instability and low sensitivity caused by its large size interfering with target binding ability and immune response [12]. Aptamers are single-stranded DNAs or RNAs which possesses several merits of specific recognizing and binding its targets (metal ions, small molecule, proteins and even cells), high stability and easy functionalization. It has been considered as potential alternatives to antibodies for bioanalysis [19]. Similarly, EpCAM aptamer can specifically recognize and bind EpCAM with high affinity to form aptamer-EpCAM complex, which was successfully identified by Song Yanling and its co-workers [12], providing a possibility for establishment of aptamer-based biosensors.

To obtain higher biosensing performance of biosensors, aptamer-based amplification strategies were adopted for enhancing signal readout, such as hybridization chain reactions(HCR) [20], catalytic hairpin assembly(CHA) [21], strand displacement amplification (SDA) [22], rolling circle amplification (RCA) [23], enzyme-assisted signal amplification [24]. Most of these amplification strategies are triggered by toehold domain. Toehold is a short stretching single-stranded domain typically composed of 5–8 nucleotides in a partially hybridized duplex, which is used as a trigger point for initiating hybridization by base-pairing [25]. The invading strand firstly hybridizes with a toehold at one end of partially hybridized duplex and then displaces target stand. This leads to a toehold-mediated strand displacement reaction (TSDR), which can increase the kinetic rate by 10^6 fold [26]. In the aforementioned amplification strategies, toehold-mediated SDA has been widely applied for signal amplification because of its intriguing merits of simplicity, low cost, enzyme-free operation

and highly specific self-powered dynamic self-assembly mechanism [27]. This toehold-mediated SDA includes toehold-mediated DNA recycling amplification [28] and toehold-mediated target recycling amplification [29]. However, no biosensor using aptamer-based amplification strategies has been reported for detecting EpCAM.

An ultrasensitive amperometric aptasensor is described here for electrochemical detection of EpCAM by combining EpCAM-driven toehold-mediated DNA recycling amplification and the specific recognizing and binding of EpCAM aptamer to EpCAM. Upon addition of EpCAM, probe A is firstly liberated from probe A/aptamer complex and hybridizes with toehold domain of Hp1 to open the hairpin structure of Hp1 and expose another toehold domain of Hp1. Subsequently, toehold-mediated SDA is initiated by recycling probe A when the exposed toehold domain of Hp1 further hybridizes with electro-active MB-labeled Hp2 to liberate probe A. Thus, plenty of electro-active MB-labeled Hp2 is brought in proximity to the gold electrode surface for highly efficient electron transfer and then generates the amplified current response. Thus, this electrochemical biosensor has a great potential for ultrasensitive detection of EpCAM.

Experimental section

Materials

All synthetic DNA sequences and aptamer purified by HPLC, were purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China; www.sangon.com) and listed in Table S1. 6-Mercapto-1-hexanol (MCH), Tris(hydroxymethyl) aminomethane (Tris) and tris (2-carboxyethyl) phosphine hydrochloride(TCEP) were all provided by Sigma-Aldrich (St. Louis, MO, USA; www.sigmaaldrich.com). The EpCAM, bovine serum albumin(BSA), CD86 and prostate specific antigen(PSA) for verifying the specificity of the sensor were purchased from Cusabio Biotech Co. (USA; www.cusabio.com). Other reagents were all of analytical grade and used without further purification. The biological samples such as health human serum, saliva and urine were obtained from Dongfeng General Hospital. Ultrapure water used in all runs was prepared by a Millipore water purification system (18.2 MΩ•cm resistivity, Milli-Q Direct 8).

Preparation of the electrochemical biosensor

The gold working electrodes (2 mm in diameter, CH Instruments, Inc., Austin, TX) were first immersed in piranha solution at 90 °C for 5 min for chemical pretreatment

before assembling of probes on its surface, and then adequately washed with ultrapure water [30, 31]. Subsequently, the mirror-like surface of gold electrode was obtained by polishing with 0.05 μm γ-alumina powder (CH Instruments, Inc.) and then sonicated with water and ethanol for 5 min, respectively, to remove residual alumina powder. After electrochemically cleaned with 0.5 M H_2SO_4, the pretreated gold electrodes were obtained by washing with ultrapure water and finally drying with a stream of nitrogen [25]. For the immobilization of probe onto the gold electrode surface, the 10 μM Hp1 was dissolved in 20 mM Tris–HCl buffer (containing 100 mM NaCl, 1.0 mM TCEP, pH 7.4) and incubated in the dark for 1 h to decrease the disulfide bonds of the Hp1. The prepared Hp1 Tris–HCl buffer (5 μM) was diluted to a series of the required concentration before assembling. Subsequently, 10 μL of Hp1 solution was dropped on the surface of the pretreated electrode for Hp1 self-assembly overnight at room temperature. After assembly of Hp1, the Hp1/gold electrodes were obtained by thoroughly rinsing with wash buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.4) and gently drying with nitrogen. Finally, The obtained Hp1/gold electrode was immersed into 1 mM freshly prepared MCH solution for 1 h to fill the unoccupied region and thus to inhibit the nonspecific DNA adsorption. The modified gold electrodes were finally named MCH/Hp1/AuE. Of note, the modified electrodes were rinsed with buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.4) and dried with nitrogen before detecting EpCAM.

EpCAM-driven DNA recycling amplification detection of EpCAM

Prior to EpCAM detection, the probe A was hybridized with EpCAM aptamer to form aptamer/probe A duplex. Then, Tris-HCl buffer A containing the complex of EpCAM aptamer/probe A (20 μL), Hp2 (20 μL) and various concentrations of EpCAM (10 μL) were incubated at 37 °C for 20 min. Subsequently, 10 μL of Tris-HCl buffer A was dropped on the MCH/Hp1/AuE in a hybridization oven (UVP, HB-1000) with constant temperature of 37 °C and humidity for 40 min. Subsequently, the electrodes were washed with buffer and transferred into electrochemical cells for measurements.

Electrochemical measurements

Electrochemical measurements were conducted by a CHI660D workstation (CH Instruments Inc., Shanghai, China) with a conventional three-electrode system, including a platinum wire auxiliary electrode, an Ag/AgCl reference electrode, and a modified working gold

electrode. Square wave voltammetry (SWV) measurements were conducted in 20 mM Tris-HCl buffer (0.1 M NaCl, pH 7.4) by scanning the potential from −0.4 to 0 V with an amplitude of 25 mV, step potential of 4 mV and frequency of 25 Hz. The electrochemical impedance spectra (EIS) and cyclic voltammetry experiments (CV) were obtained in 0.01 M PBS containing 0.1 M KCl and 5 mM [Fe(CN) 6] $^{3-/4-}$ in the frequency range from 0.1 Hz to 10 kHz, with the potential window from −0.2 to 0.6 V under a scan rate of 0.1 $V \cdot s^{-1}$ and amplitude of 5 mV, respectively.

Results and discussion

Sensor design and mechanism of signal amplification

An electrochemical biosensor for ultrasensitive detection of EpCAM was designed. The architecture of electrochemical EpCAM sensor and mechanism of its signal amplification is illustrated in Scheme 1. The Hp1 with a toehold region was labeled with 5'-SH and self-assembled on the gold electrode surface. Subsequently, the unoccupied region of the electrode was passivated with MCH to inhibit the nonspecific DNA adsorption. In this case, the rigid hairpin structures of Hp1 may maintain DNA strands in a relative upright orientation to enhance DNA recognition and hybridization efficiency [32]. Prior to EpCAM detection, the probe A was hybridized with EpCAM aptamer to form aptamer/probe A duplex. In the absence of EpCAM, the occurrence of strand displacement reaction is restrained and thus only a negligible peak current signal is generated. Upon addition of EpCAM, probe A is liberated from the aptamer/probe A duplex
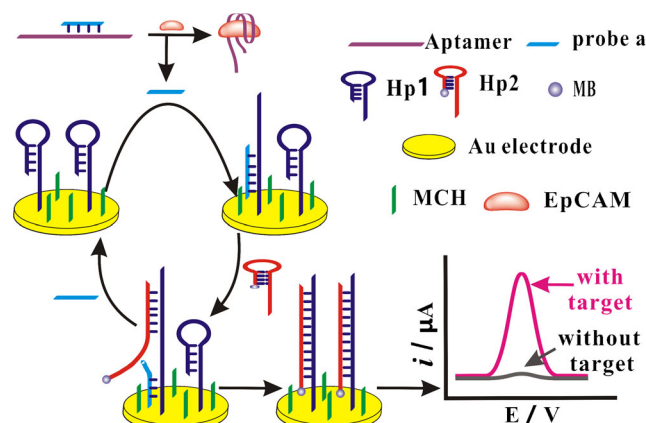
Scheme 1 Schematic illustration of sensor design and mechanism of its signal amplification on the basis of EpCAM-driven toehold-mediated DNA recycling amplification. Hp1: Hairpin probe 1; Hp2: Hairpin probe 2; MB: Methylene blue; MCH: 6-Mercapto-1-hexanol; EpCAM: Epithelial cell adhesion molecule
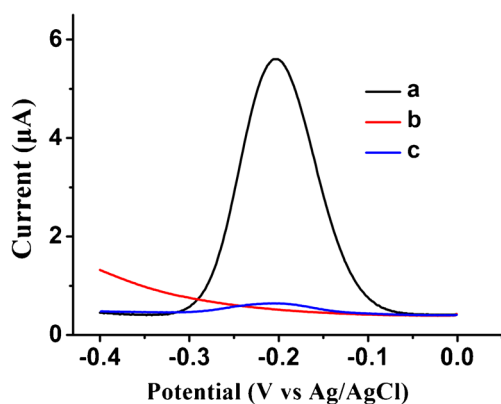
Fig. 1 Typical SWV current responses of (**a**) MCH/Hp1/gold electrode incubated with complex of EpCAM aptamer/probe A and Hp2 in the presence of EpCAM, (**b**) MCH/probe/AuE, (**c**)MCH/Hp1/AuE incubated with complex of EpCAM aptamer/probe A and Hp2 in the absence of EpCAM

because of the specific binding of EpCAM to its aptamer and then hybridizes with toehold domain of Hp1 to open the hairpin structure of Hp1. This results in the exposure of another toehold domain of Hp1 for further hybridizing with Hp2 to form the Hp1-Hp2 duplex which brings electro-active reporter MB in proximity to the gold electrode surface [25]. In addition, the dissociated probe A can hybridize with another Hp1 to start next round of DNA recycling amplification reaction. Finally, plenty of MB labeled DNA strands are formed on the electrode surface and generates the amplified current response.

Electrochemical characterization

Electrochemical impedance spectroscopy (EIS) is usually used to characterize the modification process of electrochemical biosensors. A typical Nyquist plot of EIS

contains a semicircle region related to electron transfer resistance at higher frequencies and a linear region at lower frequencies. The enhancement of semicircle diameter implies the enhancement of electron transfer resistance (R_{et}) on the surface of electrode [30]. To characterize the modification process of the gold electrode at different stages, the Nyquist plots of EIS measurements were obtained. In addition, cyclic voltammetry experiment (CV) was also conducted to further characterize the modification process. The results of EIS and CV are discussed in Electronic Supporting Materials.

The feasibility of the sensor

After successful modification of the gold electrode, the feasibility for ultrasensitive detection of EpCAM was verified via square wave voltammetry (SWV). As depicted in Fig. 1, no obvious current response is observed in the absence of EpCAM/probe duplex and Hp2 due to the lack of electro-active reporter MB on the modified electrode (curve b). When the electrode was incubated with EpCAM aptamer/probe and Hp2, the negligible current changes is exhibited due to inhibition the occurrence of the TSDR triggered by probe A in the absence of EpCAM (curve c). Interestingly, a significant enhancement of current response is exhibited when the modified electrode was incubated with Tris-HCl buffer containing EpCAM aptamer/probe A, Hp2 and EpCAM(curve a). This significant enhancement of current response proves the occurrence of DNA recycling amplification triggered by probe A and the formation of more Hp1-Hp2 duplexes, which brought more electro-active reporter MB proximity to the gold electrode surface for highly efficient electron transfer. Therefore, the aforementioned results further
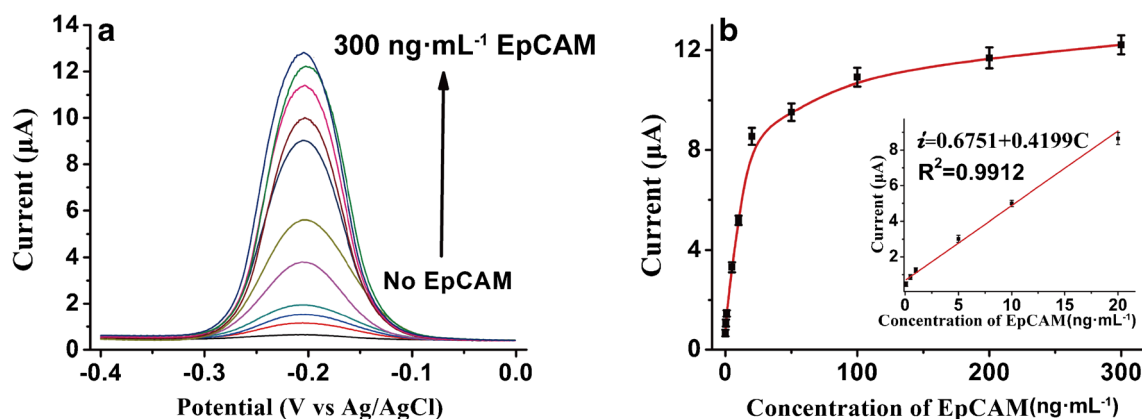

Fig. 2 **a** Typical SWV current response of the modified electrode to different concentrations of EpCAM in the presence of EpCAM aptamer/probe A and Hp2 (from bottom to top: 0 to 300 ng·mL^{-1}); **b**The relationship curve of SWV current responses vs. the concentrations of EpCAM from 0 to 300 ng·mL^{-1}. Inset of (**b**): linear relationship

between SWV current responses and the concentrations of EpCAM in the range from 0.1 ng·mL^{-1} to 20 ng·mL^{-1}. The measurements were conducted by scanning the potential from −0.4 to 0 V with an amplitude of 25 mV, step potential of 4 mV and frequency of 25 Hz

Table 1 The comparison of designed method with other reported biosensors

method	signal mechanism[a]	Detection limit	Linear range	Ref.
Fluorescence	Cellulosepaper-based substrate; QDs-Apt, Cy3 labeled cDNA	10 ng·mL^{-1} in buffer and 24 ng·mL^{-1} in 10% serum	40–4000 ng·mL^{-1}	[15]
Turn-on fluorescence biosensor	GQDs-PEG-aptamer/MoS2	18 ng·mL^{-1}	120–2160 ng·mL^{-1}	[4]
Microfluidic immunosensor	HRP-conjugated anti-EpCAM-antibody; AgNPs-PVA-3-APTES	0.8 fg·mL^{-1}	2–2000 pg·mL^{-1}	[11]
Microfluidic immunosensor,	HRP-conjugated anti-EpCAM-antibody;AgNPs-Cts	2.7 pg·mL^{-1}	–	[16]
Microfluidic immunosensor,	ZnONPs-PVAcoupled to LIF	1.2 pg·mL^{-1}	2.5–2000 pg·mL^{-1}	[17]
Fluorescence	ACP probes; fluorescence switching	1.0 ng·mL^{-1}	–	[18]
Electrochemical biosensor	EpCAM-driven toehold-mediated DNA recycling amplification	20 pg·mL^{-1}	0.1–20 ng·mL^{-1}	This work

[a] Abbreviation: GQDs, graphene quantum dots; MoS2, molybdenum disulfide; 4-TBC, 4-tertbutylcatechol; AgNPs, silver nanoparticles; PVA,polyvinyl alcohol; APTES, 3-aminopropyl triethoxysilane; Cts, chitosan; ZnONPs, zinc oxidenanoparticles; LIF, laser-induced fluorescence; Apt, aptamer; ACP, aptamer-conjugated polydiacetylene imaging probe

reveal the feasibility of the sensor for ultrasensitive detection of EpCAM.

Optimization of the assay

The following parameters were optimized: (a) concentration of Hp1; (b) concentration of EpCAM aptamer; (c) reaction time; (d) pH; (e) frequency of SWV. Respective data and Figures are given in the Electronic Supporting Materials. The following experimental conditions were found to give best results: (a) Optimal concentration of Hp1: 1.0 μM; (b) Optimal concentration of EpCAM aptamer: 40 nM; (c) Optimal reaction time: 40 min; (d) pH: 7.4; (e) Frequency of SWV: 25 Hz.

Sensitivity and specificity

Under the optimal experimental conditions, the sensitivity of the sensor for detection of EpCAM was investigated in the presence of EpCAM aptamer/probe A and Hp2. Fig. 2a shows SWV current response to EpCAM gradually increases with increasing EpCAM concentration ranging from 0 to 300 ng·mL^{-1}, indicating a highly concentration-dependent current response for detection of EpCAM. The relationship curve of SWV current responses vs. the concentrations of EpCAM was also plotted (Fig. 2b), which exhibits good linear relationship between current response and the concentrations of EpCAM in the range from 0.1 ng·mL^{-1} to 20 ng·mL^{-1} (inset in Fig. 2b). The regression equation was expressed as $i = 0.6751 + 0.4199C$ ($R^2 = 0.992$), where i is the SWV current response and C is the EpCAM concentration. The detection limit was calculated

to be 20 pg·mL^{-1} according to 3σ rule. In comparison, such a low detection limit was comparable or even superior to reported literatures listed in Table 1.

To verify the specific recognition and affinity of EpCAM aptamer to EpCAM, the specificity of the sensor for EpCAM detection was also investigated using other proteins as control, such as BSA (100 ng·mL^{-1}), CD86 (100 ng·mL^{-1}), PSA (100 ng·mL^{-1}). As shown in Fig. 3, the response of the modified electrode to control proteins (100 ng·mL^{-1} of each control protein) exhibits negligible SWV current signal which can almost be the same as that of the blank control ($n = 3$). However, the significant increase of SWV current signal is observed in the presence of EpCAM (10 ng·mL^{-1}). These results suggest the satisfactory specificity for EpCAM detection.
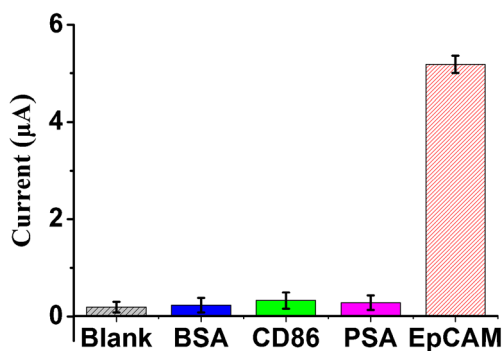

Fig. 3 The specific SWV current response of the sensor for different proteins including EpCAM (10 ng·mL^{-1}), BSA (100 ng·mL^{-1}), CD86 (100 ng·mL^{-1}) and PSA (100 ng·mL^{-1}). Error bars: SD, $n = 3$

Fig. 4 **a** Investigation of the stability of the sensor by recording the SWV current response to EpCAM every 24 h for 5 days; **b** Regenerability of the sensor for repeated detection of EpCAM over five cycle uses. Error bars: SD, n = 3
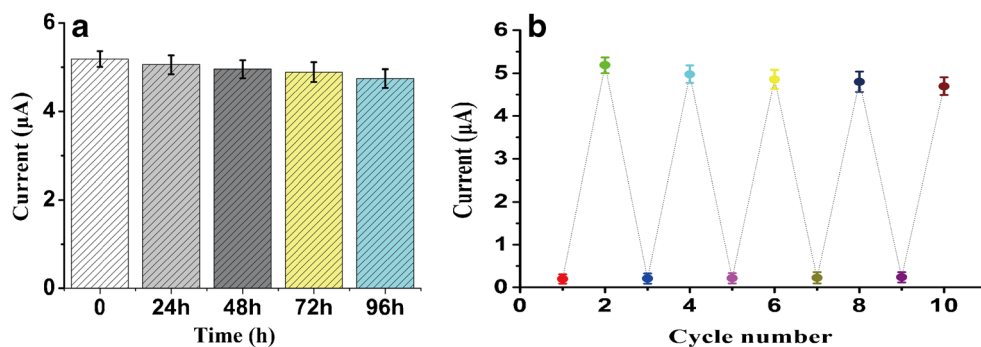
Stability and regenerability of the sensor

The stability and regenerability of the sensor are two critical indicators for analysis in real biological samples. To investigate the stability of the biosensor, the SWV current responses to EpCAM in the first continuous five days were measured every 24 h for 5 days after storage at 4 °C. As shown in Fig. 4a, compared with its original current response, only a negligible current response changes is observed, indicating the satisfactory stability of the designed biosensor. Moreover, we further investigated the regenerability of the sensor [33]. Prior to rinse with Tris-HCl buffer (10 min), the modified electrode was firstly dipped in 5 M urea solution at temperature of 37 °C for 5 min and then rinsed with 5 M urea solution for 2 min. The results show that the sensor can repeatedly detect EpCAM over 5 times (Fig. 4b). This indicates the good regenerability of assay.

Application to biological samples

To further verify the applicability and selectivity of the sensor system in biological samples, the biological samples were prepared by spiking EpCAM (10 ng) into

200 μL of 50% blank biological samples (human serum, urine and saliva, respectively). Then SWV measurements were separately performed. As shown in Fig. 5, compared with blank biological samples, the significant SWV current enhancement is observed in various biological samples in the presence of 10 ng·mL^{-1} EpCAM, respectively. In addition, the recoveries for spiking EpCAM into various biological samples were calculated and the negligible current changes (RSD are less than 9.6%) were exhibited in various biological samples with EpCAM concentration of 10 ng·mL^{-1} (Table 2). The above results suggest the better applicability and selectivity of the sensor system, which is due to the specific recognition and affinity of EpCAM aptamer to EpCAM and the high efficiency toehold-mediated SDA.

Conclusion

In summary, we have designed an amperometric aptasensor for ultrasensitive detection of EpCAM. The design strategy is based on the specific recognition and affinity of EpCAM aptamer to EpCAM and high specific toehold-mediated DNA recycling amplification. The designed sensor exhibits good stability, specificity and regenerability. The sensitivity was also obtained, with the detection limit low to 20 pg·mL^{-1}. Moreover, the designed method was successfully applied for detection of EpCAM in various spiked biological samples, implying the
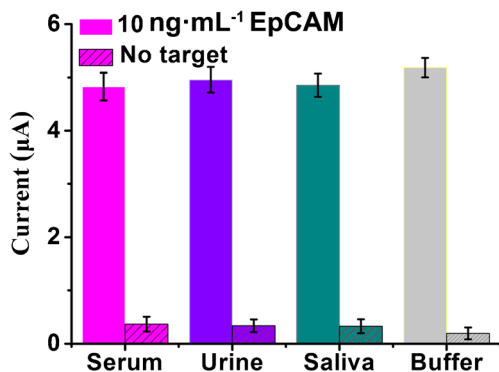

Fig. 5 The SWV current responses of the sensor for detection of EpCAM in buffer and 50% blank biological samples (human serum, urine and saliva, respectively). Error bars: SD, n = 3

Table 2   Recoveries of EpCAM spiked to different matrices (n = 3)

Matrice	EpCAM added/ng	EpCAM found/ng	Recovery (100%)	RSD (%)
Buffer	10.0	9.96[a]	99.6	6.7
Serum	10.0	9.32[a]	93.2	9.6
Saliva	10.0	9.37[a]	93.7	9.2
Urine	10.0	9.56[a]	95.6	8.5

[a] mean values of three measurements

possibility of application of this method in early clinical diagnosis. It is worthy of note that, prior to EpCAM detection, we diluted the human biological samples with the buffer to achieve the desired results. We believe that this design strategy will be a prominent potential for monitoring other biomarkers directly by altering corresponding aptamers.

Compliance with ethical standards The author(s) declare that they have no competing interests.

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