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An ultrasensitive homogeneous electrochemical biosensor based on CRISPR-Cas12a

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Taking advantage of the high-efficiency indiscriminate ssDNA cleavage activity of Cas12a in combination with the diffusivity difference of methylene blue (MB)-labeled probes (short oligonucleotides/mononucleotides) toward the negatively-charged indium tin oxide (ITO) electrode, a simple, immobilization-free, highly sensitive, and homogeneous electrochemical biosensor for the detection of human papillomavirus (HPV-16) has been fabricated. At the core of the detection process, Cas12a employed ssDNA *trans*-cleavage capability to achieve short-strand nucleotide cleavage, while MB-labeled probes served as high-efficiency homogeneous electrochemical emitters to achieve differential pulse voltammetric (DPV) signal. Specifically, due to strong electrostatic repulsion, MB-labeled short oligonucleotides (reporter) cannot diffuse freely to the surface of the negatively charged ITO electrode, and only weak electrochemical signals can be detected. The presence of the target HPV-16 can activate the Cas12a complex to perform indiscriminate ssDNA cleavage of the reporter to produce MB-labeled mononucleotides. The MB-labeled mononucleotides with a smaller size have almost no negative charge, so they very easily diffuse to the surface of the ITO electrode and result in an enhanced electrochemical signal response. Different electrochemical responses (DPV peak intensity) of the CRISPR-Cas12a-assisted amplification strategy can be obtained through the diffusion rate of different MB-labeled DNA on the electrode, which is also positively correlated with the input HPV-16 concentration. Given the unique combination of the CRISPR-Cas12a system with the homogeneous electrochemical solution phase, the detection limit is determined to be 3.22 pM (wide dynamic working range from 0.01 nM to 100 nM) and the two-step detection workflow could be completed within 50 min at ambient temperature, which is superior to that of the HPV-based biosensors previously reported.

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Introduction

The latest discovery and powerful development of CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated effector proteins) systems, which were developed from the adaptive immune systems of archaea/bacteria to degrade intruding nucleic acid sequence components (plasmids or viruses) after the Cas-CRISPR RNA (crRNA) complex scans and identifies the target nucleic acid sequence, has partly revolutionized genome editing and next-generation nucleic acid biosensing.[1–4] Within the CRISPR-Cas effector family, CRISPR-Cas12a (previously called Cpf1; class 2 type V-A system) is known to have unique collateral promiscuous *trans*-cleavage nuclease activity, which can indiscriminately cleave surrounding non-target single-stranded DNA (ssDNA, near and around the target DNA) when the Cas 12a-crRNA complex is activated by a matching target DNA.[5–8] In recent reports, the collateral DNase activity of CRISPR-Cas12a has been repurposed for the development of nucleic acid and non-nucleic-acid (small organic molecules and protein molecules) biosensors due to its intrinsic high efficiency (~1250 turnovers per second), low background noise, and simplicity to fabricate.[9–11] For instance, Dai *et al.* established a universal electrochemical sensing system for portable and sensitive detection of DNA and proteins based on the CRISPR-Cas12a cleavage activity (termed E-CRISPR).[12] In this method, the target recognition process is converted into a detectable electrochemical signal by using a linear MB-labeled ssDNA reporter assembled on the surface of an electrode. Specifically, once the Cas12a-crRNA complex binds to the target, the indiscriminate ssDNA collateral cleavage activity of Cas12a is fully activated, and the MB-labeled ssDNA is degraded and separated from the electrode surface. In this case, the electrochemical response is reduced due to the separation of MB from the electrode surface and is proportional to the analyte concentration. To improve analytical sensitivity, Zhang *et al.* devised a MB-labeled hairpin DNA as a signal transduction tag assembled on the surface of an electrode instead of

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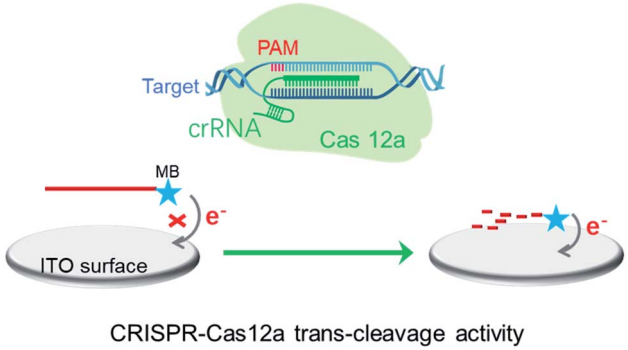
traditional linear DNA.[13] The improvement is conducive to shortening the distance between the MB and the electrode surface, which can increase the electron transfer rate constant and lead to a higher electrochemical response. Despite these spectacular advances, the application of functionalized probe DNA onto the electrode surface still requires very strict control of its batch-to-batch assembly efficiency in order to achieve reproducible signal readout. Besides, the interaction between Cas12a and the corresponding probe DNA occurs at the electrode surface/solution, and the steric hindrance of the probe DNA on the electrode surface may affect the reaction efficiency. In this regard, the follow-up work is to explore CRISPR-Cas12a-based electrochemical strategies to achieve simplicity and repeatability, while preserving the basic benefits of sensitivity and broad applicability.

Homogeneous electrochemical biosensors produce electrochemical signals through the diffusion of electroactive materials to the electrode surface without the immobilization of biorecognizable molecules (antibodies or aptamers), and have attracted particular attention due to unique superiorities (*e.g.*, rapid response, sensitive recognition, no time-consuming modification processes and relatively low cost).[14–16] More importantly, compared to heterogeneous electrochemical methods, the analyte recognition and signal amplification/transduction are performed in the homogeneous solution phase, which simplifies the experimental procedure and effectively improves the reliability and repeatability of the biosensors. So far, diverse schemes involving homogeneous electrochemical systems have been reported in the field of bioanalysis (*e.g.*, nucleic acids, disease markers, enzyme activities, drugs, *etc.*), by using the ITO electrode surface and DNA negative charge repulsion.[17–20] Therefore, the use of a homogeneous electrochemical system is an ideal choice for improving the CRISPR-Cas12a electrochemical biosensor.

In this work, by utilizing the diffusivity difference of mononucleotides and ssDNA toward the ITO electrode, a versatile and immobilization-free homogeneous electroanalytical strategy for sensitive sensing of human papillomavirus (HPV-16) was elaborately constructed. The homogeneous electrochemical system is carried out on the basis of a simple CRISPR-Cas12a system, whereas the electrochemical signal is derived from the MB-labeled short oligonucleotides (reporter). When the target HPV-16 DNA is present (shown in Scheme 1), with the assistance of the Cas12a-crRNA system, the resulting Cas12a-crRNA-HPV-16 is activated and shows *trans*-cleavage ability on surrounding ssDNA, leading to the indiscriminate cleavage of the reporter molecule into mononucleotides. The different adsorption of DNA fragments with different lengths on the surface of ITO leads to changes in the electrochemical signal, which is positively correlated with the concentration of HPV-16. By taking full advantage of the unique *trans*-cleavage features of the CRISPR-Cas12a system, as well as the simple and fast homogeneous electrochemical detection workflow, the proposed strategy provides a biosensing platform for HPV-16 assay with favorable stability, great anti-interference ability, and satisfactory accuracy.



Scheme 1 Schematic illustration of the homogeneous electrochemical biosensor toward the human papillomavirus (HPV-16) analyte on the negatively-charged indium tin oxide (ITO) electrode based on an HPV-induced Cas 12a-assisted signal amplification strategy, using the electrochemical response of methylene blue (MB, electrochemical indicator) for quantification.

Experimental section

Chemicals and reagents

Engen® Lba Cas12a (catalog no. M0653) and the corresponding 10× NEBuffer 2.1 were purchased from New England Biolabs Inc. (Ipswich, MA). All oligonucleotides used in this work were synthesized and purified by high-performance liquid chromatography (HPLC) by Shanghai Sangon Biotechnology Co., Ltd., and the sequences (5′-3′) were as follows:

crRNA-16:
UAAUUUCUACUAAGUGUAGAUUGAAGUAGAUAUGGCAGCAC

crRNA-18:
UAAUUUCUACUAAGUGUAGAUACAAUAUGUGCUUCUACACA

HPV-16_TS: TACAAATATGTCATTATGTGCTGCCATAT
CTACTTCAGAAACTACATATAAAAATACT

HPV-16_NTS: AGTATTTTTATATGTAGTTTCT
GAAGTAGATATGGCAGCACATAATGACATATTTGTA

HPV-18_TS: TGCCCAGGTACAGGA
GACTGTGTAGAAGCACATATTGTTAAATTGGTACTGCGAGTGG

HPV-18_NTS: CCACTCGCAGTAC
CAATTTAACAATATGTGCTTCTACACAGTCTCCTGTA
CCTGGGCA

Random DNA:
ATCGGCTATTAGCGATTAGCGTAGCTCAGTGCATGCCAT

Reporter: TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-MB

RNase-free water was employed as the storage solvent for crRNA throughout the experiment. TE buffer (pH = 7.8–8.2, 10 mM Tris–HCl, 1 mM EDTA and 10 μg mL^{-1} BSA) was used as the solution medium of oligonucleotides.

Electrode pretreatment and electrochemical measurement

Prior to homogeneous electrochemical measurements, the ITO electrode was successively sonicated and washed in Alconox solution (20 g L^{-1}, 15 min), propan-2-ol (15 min), and ultrapure water (15 min). Then, the treated ITO electrode was further immersed in NaOH solution (1 mM, 5 h) and sonicated in deionized water for 15 min to obtain a clean negative electrode

surface. Differential pulse voltammetric (DPV) experiments were performed using an AutoLab electrochemical workstation (μAUTtIII.FRA2.v, Eco Chemie) with a conventional three-electrode cell (*i.e.*, an ITO electrode with an active surface area of 15 mm^2 as a working electrode, a Pt-wire auxiliary electrode, and an Ag/AgCl reference electrode). Among them, the potential window range, pulse height, pulse width, and pulse period of DPV signals were set to values as follows: from −0.35 V to −0.05 V, 0.05 V, 0.05 s, and 0.5 s, respectively. Each DPV measurement result was measured three times and averaged for quantitative analysis at room temperature, as indicated with error bars.

CRISPR-Cas12a-assisted homogeneous electrochemical HPV-16 detection

Briefly, non-activated Cas12a-crRNA was pre-assembled by incubating Cas12a (final concentration of 200 nM, 20 μL, 4 μL of 10× NEBuffer 2.1) with crRNA (final concentration of 150 nM, 16 μL, in RNase free buffer) at 37 °C for 30 min. Subsequently, 1× NEB 2.1 reaction buffer (40 μL) containing different concentrations of the target (final concentrations of 0 nM, 0.01 nM, 0.1 nM, 1.0 nM, 10 nM, 50 nM and 100 nM, 4 μL), Cas12a-crRNA (final concentration of 100 nM, 4 μL), reporter (final concentration of 1.0 μM, 10 μL) and nuclease-free water were incubated at 37 °C in a constant temperature incubator for 50 min. Finally, the electrochemical detection of the system from the mixture was recorded.

Native polyacrylamide gel electrophoresis

The cleavage activity of CRISPR-Cas12a and the corresponding products were confirmed by 20% native polyacrylamide gel electrophoresis (PAGE) in 0.5× TBE buffer at a constant voltage of 95 V for 35 min. SYBR Green I (final concentration of 100×, 50 μL) and loading buffer (10 mM Tris–HCl (pH 7.6), 10 mM EDTA, 0.01% bromophenol blue, 0.01% xylene cyanol FF and 15% glycerol, 50 μL) were mixed to obtain a fluorescent dye. Different DNA samples (5 μM, 5 μL) were mixed with the fluorescent dye (5 μL) for 3 min in the dark, and then the mixtures (6 μL) were loaded into the lanes. Finally, a Bio-Rad gel imaging system was used to scan and record the bands.

Results and discussion

Feasibility of the CRISPR-Cas12a-based homogeneous electrochemical biosensor for HPV-16 detection

In our design, the change in the DPV value of the MB-labeled reporter is derived from the cleavage activity caused by CRISPR-Cas12a. Thus, one inevitable question arises as to whether the CRISPR-Cas12a system could be readily implemented with the high cleavage activity. To clarify this point, native-polyacrylamide gel electrophoresis (PAGE, 20%, Fig. 1A) was first used to verify the change in CRISPR-Cas12a-based recognition and *trans*-cleavage ability activity after the analyte was identified. To make the result clearer, a 32 nt linear reporter was employed as the non-specific substrate. The designed reporter only shows one clearly defined band on lane "a". The Cas12a-crRNA duplex when added to the above system does not cut the reporter well (lane "b"). The above results mean that in the absence of target HPV-16, the co-incubated Cas12a-crRNA duplex and 32 nt reporter substrate were inactive. Impressively, when 100 nM HPV-16 was co-incubated with the Cas12a-crRNA duplex and reporter system (lane "c"), the position band corresponding to the reporter substrate was completely degraded. The PAGE results strongly proved that the assembled Cas12a-crRNA-HPV-16 triplex can successfully activate the ability to cut the reporter after the analyte is identified.

Logically, another puzzling question arises as whether the *trans*-cleavage ability of Cas12a could be used for the homogeneous electrochemical detection of target HPV-16. To further realize our design, DPV measurements of the platform were implemented under different conditions (Fig. 1B). As seen from curve 'd', a very low DPV peak current was obtained in the reporter. Such a low current was ascribed to the electrostatic repulsion between the long-stranded DNA and the electrode

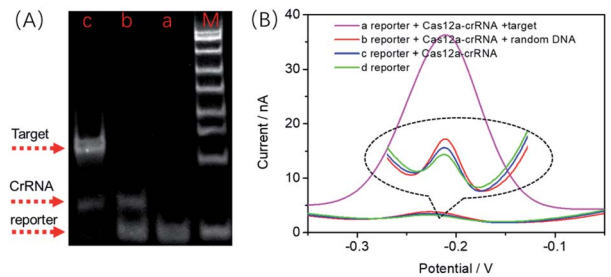


Fig. 1 (A) PAGE (20%) results for different samples (lane M: DNA marker; lane 'a': reporter as the substrate; lane 'b': crRNA + Cas 12a + reporter; lane 'c': crRNA + Cas 12a + target + reporter); (B) DPV signal of the proposed CRISPR-Cas12a biosensor in the presence of (a) 100 nM HPV-16 + crRNA + Cas 12a + reporter, (b) crRNA + Cas 12a + random DNA + reporter, (c) crRNA + Cas 12a + reporter, and (d) reporter.

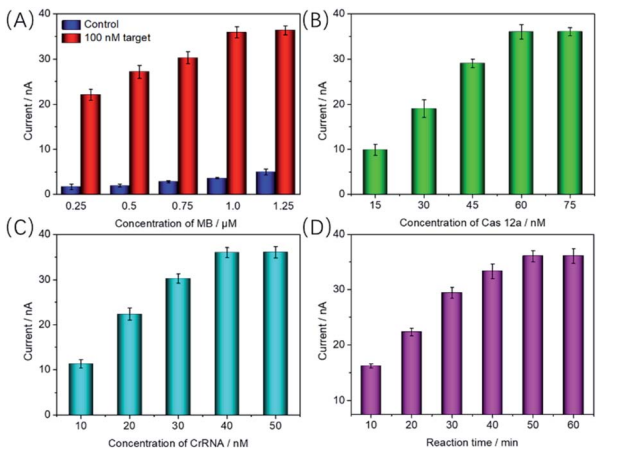


Fig. 2 Influence of (A) reporter concentration, (B) Cas12a concentration, (C) crRNA concentration, and (D) cleavage time of Cas12a on the reporter on the DPV peak current of the homogeneous electrochemical platform by using 100 nM HPV-16 as an example. The error bars represent the standard deviation (SD, $n = 3$).

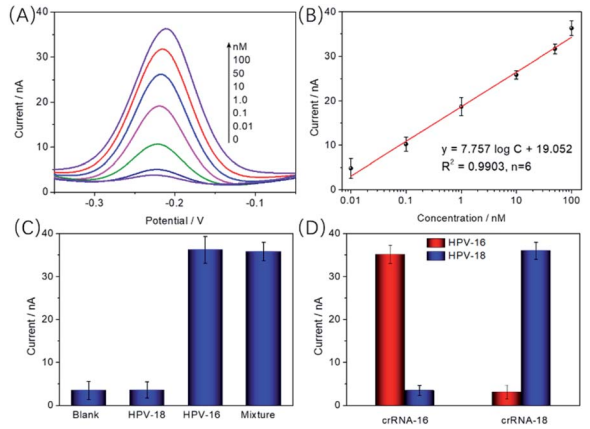


Fig. 3 (A) DPV of the CRISPR-Cas12a-based homogeneous electroanalytical biosensor after reaction with target HPV-16 standards including 0, 0.01, 0.1, 1.0, 10, 50 and 100 nM; and (B) calibration plots corresponding to DPV peak current intensity as a function of HPV-16 level (nM); the specificity (C) and universal ability (D) of the electroanalytical biosensor including 100 nM HPV-16, 100 nM HPV-18 and the mixture (100 nM HPV-16 + 100 nM HPV-18). All error bars represent the standard deviation (SD, $n = 3$).

surface. Also, adding the Cas12a-crRNA duplex to the reporter will not cause significant changes in DPV peak intensity (curve 'c'). Significantly, the DPV peak intensity largely increased in the presence of target HPV-16 (100 nM) (curve 'a'). In addition, random sequence DNA cannot cause obvious changes in electrical signals (curve 'b'). Namely, only the mixture containing target HPV-16, crRNA and Cas12a could form the Cas12a-crRNA-HPV-16 triplex and activate the *trans*-cleavage ability of Cas12a, which leads to the cleavage of the MB-labeled reporter and correspondingly increases the electrochemical signal. Both the results of PAGE and DPV measurement corroborated that the *trans*-cleavage ability of Cas12a can only be activated when the target is present, indicating that the homogeneous electrochemical platform could be used for the detection of target HPV-16.

Optimization of experimental conditions

In this system, the homogeneous electrochemical biosensor mainly consisted of the reporter and CRISPR-Cas12a-based reaction. To achieve the best analytical performance, some experimental conditions were carefully optimized through variable control methods by using 100 nM HPV-16 as an example. Generally, an insufficient amount of the reporter may result in a low yield of MB-labeled nucleotides after digestion, whereas excessive amounts of the reporter may cause a high background signal. Therefore, the control group and analyte are set to optimize the influence of reporter concentration on the detection signal. As shown in Fig. 2A, the results showed that the detected DPV signal-to-noise ratio increased gradually with the reporter concentration in the range from 0.25 to 1.0 μM and then reached saturation at 1.0 μM. But if the concentration of the reporter is greater than 1.0 μM, the detection background signal will be significantly stronger than the detection signal. Therefore, 1.0 μM reporter was chosen as the optimized substrate concentration.

Actually, the *trans*-cleavage efficiency of the reporter depends on the CRISPR-Cas12a system, which further affects the number of MB-labeled nucleotides produced. In this regard, the concentration of Cas12a protein and crRNA is another interrelated factor and key to restricting the whole biosensing system. As shown in Fig. 2B and C, the peak DPV intensity increases continuously with the concentration of Cas12a and crRNA, and reaches a plateau at 60 nM and 40 nM, respectively. Moreover, the introduction of high concentrations Cas12a and crRNA did not significantly increase the peak DPV intensity. To save cost, 60 nM Cas12a and 40 nM crRNA are utilized as optimized parameters for the strand cleavage. By the same token, the *trans*-cleavage time of the Cas12a complex on the reporter was highly correlated with the performance of the system, which was strictly studied from 10 min to 60 min. Similarly, as displayed in Fig. 2D, the DPV intensity continued to increase with the *trans*-cleavage reaction time of Cas12a, and the appropriate reaction time was observed to be within 50 min to acquire an adequate signal.

Responses of the CRISPR-Cas12a-based homogeneous electrochemical biosensor toward target HPV-16

To evaluate the analytical performance and the dynamic range of the developed CRISPR-Cas12a-based homogeneous electrochemical biosensor, different levels of target HPV-16 (0 to 100 nM) were systematically detected under the optimal experimental conditions. The DPV peak intensity increased dynamically with the increasing target HPV-16 concentration which indicated that the Cas12a catalyzes the cleavage of the MB-labeled reporter and leads to the release of mononucleotides

Table 1 Comparison of CRISPR-Cas12a and other assays for HPV-16 determination

Transduced signal	Linear range	Detection limit	Ref.
CRISPR-Cas12a electrochemical biosensor with a linear reporter	0.1–100 nM	50 pM	12
CRISPR-Cas12a electrochemical biosensor with a hairpin reporter	0.05–100 nM	10 pM	13
CRISPR-Cas12a MXene-PEDOT:PSS piezoresistive biosensor	0.02–50 nM	15.22 pM	21
Zn-doped MoS_2 QDs/reductive Cu(I) particle electrochemiluminescence	0.1–200 nM	30 pM	22
Gold nanoparticle-modified nanomaghemite/quantum dot fluorometric sensor	0.05–200 μM	100 pM	23
Reduced graphene oxide-based field effect transistors	7.5–1000 nM	1.75 nM	24
CRISPR-Cas12a homogeneous electrochemical biosensor	0.01–100 nM	3.22 pM	This work

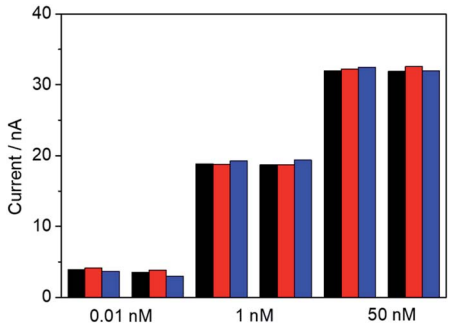


Fig. 4 Histogram of the peak current data to investigate the reproducibility (the same batch and different batches correspond to different concentrations).

(Fig. 3A). DPV peak current–HPV-16 concentration curves showing good linear dependence were acquired and the linear regression equation was I (μA) = 7.757 × log $C_{[HPV\text{-}16]}$ + 19.052 (nM) (correlation coefficient R^2 = 0.9903, n = 6). The limit of detection (LOD) of the CRISPR-Cas12a-based homogeneous electrochemical strategy was calculated to be 3.22 pM at a signal-to-noise ratio of 3, which is lower than that of recent amplification-free CRISPR-powered electrochemical methods and other existing HPV-16 detection strategies (Table 1). Such an exceptional performance of the homogeneous electroanalytical platform was ascribed to the high *trans*-cleavage efficiency of the CRISPR-Cas12a-assisted amplification strategy.

Method specificity and reproducibility of the CRISPR-Cas12a-based homogeneous electrochemical biosensor

The specificity of the CRISPR-Cas12a-based homogeneous electroanalytical system was evaluated by assaying another high-risk HPV genotype (HPV-18) as an interference agent under the same experimental conditions. Compared to significant DPV peak current enhancement with the addition of HPV-16 and the mixture (100 nM HPV-16 + 100 nM HPV-18), only a very weak DPV peak current was observed upon introduction of HPV-18 and the blank sample (PBS) (Fig. 3C), revealing the brilliant recognition ability of the Cas12a system. Additionally, to further study the versatility and specificity of the construction system, we used two crRNAs to detect two HPVs. As illustrated in Fig. 3D, HPV-16 and HPV-18 with their corresponding crRNA can induce specific DPV peak current without significant cross-reactivity. Meanwhile, this CRISPR-Cas12a electroanalytical methodology can be easily extended for the detection of any other nucleic-acid targets and/or non-nucleic-acid targets by easily changing the guide region sequences of the corresponding crRNA.

The reproducibility and batch-to-batch precision of the CRISPR-Cas12a-based homogeneous electroanalytical biosensor were monitored by analyzing low/middle/high HPV-16 concentrations including 0.01 nM, 1.0 nM, and 50 nM, respectively (Fig. 4). The relative standard deviations (RSDs, three independent tests) of using the within-batch (intra-assay) were 5.37%, 6.19%, and 5.88%, whereas those of using the various-batch (inter-assay) were 10.11%, 9.73%, and 9.42% toward the aforementioned concentrations, respectively, undoubtedly demonstrating good reproducibility and ideal accuracy.

Table 2 Detection of HPV-16 in spiked human serum samples using the homogeneous electroanalytical system

Sample	Added (nM)	Measured (mean ± SD, nM, n = 3) (with/without Cas 12a)	Recovery (%, n = 3)
HPV-16	0.1	0.094 ± 0.01/0.003 ± 0.004	94.0
	1.0	1.06 ± 0.04/0.005 ± 0.002	106.0
	2.0	2.16 ± 0.08/0.009 ± 0.003	108.0
	5.0	4.72 ± 0.07/0.008 ± 0.005	94.4
	10	9.97 ± 0.09/0.006 ± 0.01	99.7
	50	50.8 ± 0.11/0.010 ± 0.03	101.6

Detection of HPV-16 DNA in human serum samples

Inspired by the above-mentioned results, the detection reliability and possible practical application of the CRISPR-Cas12a-based homogeneous electroanalytical biosensor in clinical sample matrices were investigated. A spike-and-recovery test was conducted in bodily liquid (serum samples) by spiking known different concentrations of target HPV-16. The concentration of the target in the obtained serum samples was determined by using the developed homogeneous electroanalytical system. The high detection average recoveries (ranging from 94.0 to 108.0%, Table 2) also indicated good accuracy for the determination of target HPV-16 in clinical serum samples.

Conclusions

This contribution describes a facile and homogeneous electroanalytical bioanalysis for a sensitive and cost-effective determination of HPV-16 by using the CRISPR-Cas12a system for signal transduction and signal amplification. Compared with other CRISPR-Cas12a-based electrochemical methods for HPV-16 detection, this sensing system possesses the merits of simplicity and good repeatability, because just one probe (reporter) and the CRISPR-Cas12a system are introduced. Additionally, the detection process is carried out in the homogeneous solution phase under isothermal conditions with simplified operation, making this approach a promising candidate for developing simple, reliable, and highly sensitive molecular diagnostic tools. Under these circumstances, this method exhibits high sensitivity (a LOD of 3.22 pM could be obtained) and good selectivity (having discrimination ability for another high-risk HPV genotype, HPV-18) toward HPV-16 detection, which can be attributed to the powerful CRISPR-Cas12a-based signal transduction and signal amplification, high sensitivity of the homogeneous electroanalytical bioanalysis technology, and the brilliant recognition ability of the Cas12a system. Impressively, a universal biosensor can be constructed for the detection of other analytes by modifying the sequence of crRNA.

Ethical statement

All experiments were performed in accordance with the Guidelines of Fuzhou University (China) and approved by the ethics committee at Fuzhou University (China). Informed consent was obtained from the human participants of this study.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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