SARS-CoV-2 monitoring by automated target-driven molecular machine-based engineering

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Abstract
Biosensors based on nucleic acid-structured electrochemiluminescence are rapidly developing for medical diagnostics. Here, we build an automated DNA molecular machine on Ti₃C₂/polyethyleneimine-Ru(dcbpy)²⁺@Au composite, which alters the situation that a DNA molecular machine requires laying down motion tracks. We use this DNA molecular machine to transduce the target concentration information to enhance the electrochemiluminescence signal based on DNA hybridization calculations. Complex bioanalytical processes are centralized in a single nucleic acid probe unit, thus eliminating the tedious steps of laying down motion tracks required by the traditional molecular machine. We found a detection limit of 0.68 pM and a range of 1 pM to 1 nM for the analysis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) specific DNA target. Recoveries range between 96.4 and 104.8% for the analysis of SARS-CoV-2 in human saliva.

Keywords
SARS-CoV-2 monitoring · Ti₃C₂-based composites · Automated molecular machine · Saliva environmental testing · Modular reaction

Introduction
The coronavirus disease 2019 (COVID-19) has caused the collapse of health systems in many parts of the world. It is spreading rapidly worldwide like modern infectious diseases before it (Yan et al. 2021). Currently, countries around the globe are actively collaborating and working together to control the spread of the global outbreak. Developing and optimizing methods for rapid and accurate diagnosis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) become particularly important to test a country’s public health capacity of rapidly identifying infected individuals and taking appropriate measures to contain the virus in a pandemic (Ali and Alharbi 2020; Delafiori et al. 2021).

Recently, nucleic acid-structured electrochemiluminescence biosensing platforms have been widely applied in bioanalysis, food safety, and environmental contaminant monitoring due to the characteristics of fast response, simple operation, compact device, and high sensitivity (Fan et al. 2021b; Qi and Zhang 2020). These nucleic acid-involved electrochemiluminescence sensings rely on programmed DNA probes to participate in molecular recognition and signaling (Feng et al. 2017). Programmed DNA tetrahedron reveals enormous potential due to their stiffness and size programmability, which significantly improves the low attachment efficiency, uneven spatial distribution and reduce the aggregation of conventional DNA such as single-stranded DNA or double-stranded DNA (Fan et al. 2021a, 2020, 2022, 2021c; Li et al. 2014).

Typically, the diagnosis procedure of SARS-CoV-2 consists of the following steps. Firstly, the samples to be tested are collected by oropharyngeal or nasopharyngeal swab from the suspected COVID-19 patients. Secondly, the virus RNA is extracted through an RNA extraction step (Mattioli et al. 2020). Then, target DNA is obtained from viral RNA by enrichment steps containing reverse
transcription-polymerase chain reaction or reverse transcription recombinase polymerase amplification (Byrnes et al. 2021; Mahas et al. 2021). In detecting SARS-CoV-2, both upstream RNA and downstream DNA can be used as targets to diagnose COVID-19.

Herein, we have developed an interfacial DNA machine containing an inverted DNA tetrahedron using three capture probes as reaction probes to monitor the SARS-CoV-2 downstream DNA. When target DNA is added to the reaction system, the DNA machine produces a correlation effect on the electrochemiluminescence signal output using Ti$_3$C$_2$/polyethyleneimine-Ru(dcbpy)$_3^{2+}$@Au as electrochemiluminescence luminous material on the glassy carbon electrode based on DNA hybridization calculations, by which the concentration of the SARS-CoV-2 could be deduced.

**Experimental**

**Principle of the proposed electrochemiluminescence biosensor**

The electrochemiluminescence emitting material (Ti$_3$C$_2$/polyethyleneimine-Ru(dcbpy)$_3^{2+}$@Au) is coated onto the glassy carbon electrode surface, and the electrochemiluminescence intensity is used as the sensor’s signal output (Fig. 1). Then, we modify the automated molecular machine on the surface of Ti$_3$C$_2$/polyethyleneimine-Ru(dcbpy)$_3^{2+}$@Au. The inverted tetrahedron has only one vertex modified by the thiol group, while possessing three capture probes where two of the strands (H and P) are partially complementarily paired with H’ and P’, respectively. The one remaining capture probe (S’-S) forms a hairpin structure by itself and its S’ end carries the quenching motif ferrocene of the electrochemiluminescence signal. When the target DNA binds to the exposed toehold sequence of the anticodon H’, H is released. Then, H binds to the toehold sequence of the anticodon P’, which allows P to be released. Immediately afterward, P binds to the anticodon S’, producing a site that is recognized and cleaved by the nuclear endonuclease (Nt. BbvCI), resulting in the release of quenched motif ferrocene and reuse of the P. In this process, the repeatedly generated P enables the cyclic cleavage of the S’-S and the cyclic release of the ferrocene, resulting in the generation of a transduction signal that allows the assessment and quantitative analysis of target concentration.

**Fig. 1** Synthesis of the Ti$_3$C$_2$/polyethyleneimine-Ru(dcbpy)$_3^{2+}$@Au composite (Ti$_3$C$_2$/PEI-Ru@Au) and the electrochemiluminescence (ECL) process of automated molecular machine in the case of target (T) input on glassy carbon electrode (GCE). The box in the middle of the scheme is a concise frame diagram of the actual electrode reaction presented at the bottom of the scheme. The triangles represent the plane of the tetrahedron connecting the H’-H, P’-P, and ferrocene-S’-S’ (Fc-S’-S’) and the dashed arrows represent the direction in which the nucleic acid chain is to attack.
Characterization of Ti$_3$C$_2$/polyethyleneimine-Ru(dcbpy)$_3^{2+}$@Au

We have characterized the synthesis of Ti$_3$C$_2$/polyethyleneimine-Ru(dcbpy)$_3^{2+}$@Au. Ti$_3$C$_2$ transmission electron microscope image (Fig. 2A) shows monolayers or multilayers of nanosheets. In contrast, the Ti$_3$C$_2$/polyethyleneimine-Ru(dcbpy)$_3^{2+}$@Au transmission electron microscope image shows that the gold nanoparticles are spread on the surface of Ti$_3$C$_2$ (Fig. 2B). The magnified Ti$_3$C$_2$/polyethyleneimine-Ru(dcbpy)$_3^{2+}$@Au transmission electron microscope image (Fig. 2C) and its inset show that the gold nanoparticles are uniformly distributed with a particle size of approximately 4.3 nm. To further validate the synthesis of Ti$_3$C$_2$/polyethyleneimine-Ru(dcbpy)$_3^{2+}$@Au, we characterized the elemental distribution (Figs. S1A-H). The elemental distribution also shows that Au (Fig. S1H) and Ru elements (Fig. S1E) are distributed on the surface of Ti (Fig. S1C) and C (Fig. S1D), further indicating that Au particles and Ru(dcbpy)$_3^{2+}$ are uniformly distributed on the Ti$_3$C$_2$ nanosheets.

We have performed X-ray photoelectron spectroscopy analysis of Ti$_3$C$_2$/polyethyleneimine-Ru(dcbpy)$_3^{2+}$@Au nanocomposite. The X-ray photoelectron spectroscopy survey spectrum of the complex (Fig. 2D) exhibits Ti 2p and C 1s peaks, which are the elemental peaks of Ti$_3$C$_2$. The formation process of Ti$_3$C$_2$ produces F and Cl elements; therefore, F 1s and Cl 2p peaks are observed. The complex also has Ru 3p, Ru 3d, and Au 4f characteristic peaks due to the introduction of Ru(dcbpy)$_3^{2+}$ and the reduction of Au$^{3+}$ to gold nanoparticles. We also elaborate the bonding distribution of Au nanoparticles in Fig. 2C. D X-ray photoelectron spectroscopy survey and high-resolution C 1s (E), O 1s (F), N 1s (G), Au 4f (H). I The ultraviolet-visible absorption spectra of Ti$_3$C$_2$, Au nanoparticles (Au NPs), polyethyleneimine (PEI), polyethyleneimine and Ru(dcbpy)$_3^{2+}$ composite (Ru + PEI) and Ti$_3$C$_2$/PEI-Ru@Au.
interaction between the complexes by fitting C 1 s, O 1 s, N 1 s and Au 4f. Four fitted peaks have appeared in the C 1 s peak of Ti3C2/polyethyleneimine-Ru(dcbpy)32+@Au (Fig. 2E), which mainly correspond to the characteristic bonds of O-C=O, C-O/C-N, C–C and C-Ti. Similarly, the X-ray photoelectron spectroscopy spectra at O 1 s (Fig. 2F) show the typical bonds of C-Ti-(OH), C-Ti-Ox and TiO2. The X-ray photoelectron spectroscopy spectra at N 1 s (Fig. 2G) also show the characteristic bonds of R-NH2, R-NH-R, R = N-R and N-Ti. The double peaks of Au (Fig. 2H) at Au 4f7/2 (82.83 eV) and Au 4f7/2 (86.51 eV) also verify the presence of gold particles. All these X-ray photoelectron spectroscopy analysis data indicate the formation of the Ti3C2/polyethyleneimine-Ru(dcbpy)32+@Au complexes. We also confirm the synthesis of the complex by ultraviolet–visible spectral analysis (Fig. 2I). It shows that the Ti3C2/polyethyleneimine-Ru(dcbpy)32+@Au complex has both Ru(dcbpy)32+-polyethyleneimine (characteristic peaks at 300 nm and 475 nm) and Au nanoparticles (characteristic absorption peak at 525 nm) peaks, also verifying the synthesis of Ti3C2/polyethyleneimine-Ru(dcbpy)32+@Au complex.

Characterization of the inverted DNA tetrahedral scaffold

We also characterize the inverted DNA tetrahedra by atomic force microscope (Fig. 3A). The DNA tetrahedra formed by four strands (H-T1, P-T2, S’-S-T3, T4) appear granular and uniform in size. Meanwhile, we characterize the heights of the selected tetrahedral particles relative to the silicon wafer base (Fig. 3B–F). We define the silicon wafer substrate as the control, and the height of the tetrahedra relative to the substrate as the actual height of the DNA tetrahedra. The statistical height ranges between 8.43 nm and 9.82 nm, indicating the successful synthesis of inverted tetrahedra with relatively homogeneous dimensions.

Feasibility of DNA reactions exploited by the automated molecular machine

We next verify the possibility of cascade reactions on the automated molecular machine (Fig. S2A). Lane 4 shows that when T and H’-H-T1 are present together, which can be effective against P’-P-T2, eventually producing three major new bands: T-H’, T1-H-P’ and P-T2. However, lane 2 indicates that H’-H-T1 and P’-P-T2 do not react when T is absent. Likewise, lane 3 T does not act on P’-P-T2 when the H’-H-T1 intermediate is absent.

Lanes 1–3 are raw material strips. Upon adding H-T1 with P’-P-T2 to S’-S-T3 with nicking endonuclease (lane 4), we observe the disappearance of S’-S-T3 and the appearance of three new bands: P-T2, T1-H-P’ and cleaved S’-S-T3 (Fig. S2B). It indicates the successful linkage of the nicking reaction to the previous one. Owing to the essential H-T1 linkage, the modular cascade reaction on the DNA molecular
machine proceeds smoothly. At the same time, the newly generated P-T2 is available for the continuous action of S'-S-T3 until its depletion. The evidences provided by the two images suggest that the reaction occurs as expected on the automated molecular machine.

Results and discussion

Performance, specificity and stability

The automated molecular machine exhibits highly sensitive electrochemiluminescence variations for target concentrations (Fig. 4A, B). The increased electrochemiluminescence intensity is enhanced with the logarithmic value of the target concentration ranging from 1 pM to 1 nM. The linear equation of the calibration plot is $y = 431.5 + 1255.9\lg C_{\text{target}}$, where $y$ represents the increased electrochemiluminescence intensity. The calculated limit of detection is 0.68 pM according to $3\sigma$ method. We compare our strategy in terms of detection methods, sensitivity and detection range with other viral DNA detection methods (Table S1). It notes that our detecting method performs well in these three aspects. Besides, this method as a fundamental strategy in combination with other signal amplification techniques such as hybridization chain reaction, loop-mediated isothermal amplification (LAMP), the catalytic hairpin assembly will achieve higher sensitivity. We further evaluate the specificity of DNA automated molecular machine for the target monitoring. The sensing performances of three non-specific strands, including Bat SARS-related CoV isolate bat-SL-CoVZC45 (M1), BM48-31/BGR/2008 (M2) and

![Figure 4](image-url)

*Fig. 4* Assay performance (A, B), specificity (C) and repeatability (D) are investigated to validate the superior characteristics of our sensors. A Electrochemiluminescence-Time (ECL-Time) and Electrochemiluminescence (ECL)-Potential (ECL-Potential, the inset of Fig. 4A) curves of the strategy when monitoring target with different concentrations (1 pM, 10 pM, 20 pM, 50 pM, 0.1 nM, 0.2 nM, 0.5 nM and 1 nM). B The variation of increased electrochemiluminescence intensity ($\Delta ECL$) with target concentration and the logarithm of target concentration (the inset of Fig. 4B) in the detection region from 1 pM to 1 nM. C Specificity of strategy at 0.1 nM target and 1 nM non-targets. D Sensor stability over 15 cycles of potential sweeps in the presence of 50 pM (blue curve) and 0.5 nM target (red curve). The relative standard deviations (RSD) were obtained by the peak values of the 15 cycles at the corresponding concentrations.
SARS-CoV, are investigated (Fig. 4C). We see that the electrochemiluminescence intensity of the target is higher than the DNA intensity of the various mismatched strands, which also illustrates that our automated molecular machine has superb accuracy. Besides, our automated molecular machine possesses excellent reproducibility (Fig. 4D) by performing continuous potential scans for 15 cycles, with relative standard deviations of only 2.54% and 2.13% for 50 pM and 0.5 nM, respectively.

**Analysis of saliva dilutions**

Since viral DNA is usually obtained from viral RNA by techniques such as reverse transcription-polymerase chain reaction or reverse transcription recombinase polymerase amplification during actual virus detection, some non-specific substances interfere with the diagnosis of COVID-19. Therefore, we discuss the feasibility of the assay under complex environments. The pharyngeal swabs method usually collects viral or bacterial samples in saliva. To verify the ability of the DNA automated molecular machine to monitor targets in the saliva environment, we add targets with different concentrations to 20-fold or 50-fold human saliva dilutions and verify their feasibility (Table S2). After repeated experiments (n = 3), the obtained recoveries are between 96.4 and 104.8%, which further validates the reliability of our DNA automated molecular machine platform for target monitoring. During the experiments, targets in the range of 5 pM to 5 nM are successfully monitored, demonstrating the universality of our DNA automated molecular machine in the complex saliva environments.

**Conclusion**

Herein, we have developed an inverted tetrahedron-based DNA molecular machine for SARS-CoV-2 target monitoring, which demonstrates excellent interference resistance and performs well in a saliva dilution environment. Only one thiol group is required for inverted tetrahedra, significantly reducing the experimental fee due to nucleic acid modifications. Moreover, the sophisticated biochemical reactions are amplified on a molecular machine with high programmability and controllability, which is expected to be promoted to medical diagnostics and environmental monitoring. This assay strategy has a promising application in determining other viral and protein biomarkers by designing the three probes that extend out. The molecular device enables the detection of contaminants in the environment and clinical specimen dilutions, which paves a novel way to advance public health development and assess their hazards in the environment.
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