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A novel ultrasensitive competition strategy for electrochemical and colorimetric cytosensing of acute leukemia cells

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ABSTRACT

Selective and reliable method is of vital importance for acute leukemia detection. In this work, a robust competitive electrochemical and colorimetric cytosensing platform for detection of acute leukemia cells was developed with high sensitivity, selectivity, acceptable rapidity and excellent extensibility. The joint effects of the high catalytic activity of horseradish peroxidase (HRP)-mimiking DNAzyme toward H_2O_2 reduction and the excellent competing reaction contributed to the greatly enhanced sensitivity. Such a dual mode sensing strategy allows for ultrasensitive acute leukemia cytosensing with a detection limit as low as ~4 cells and a wide linear response range from 10^2 to 10^7 cells per mL. This electrochemical and colorimetric cytosensing approach holds great promise as a new point-of-care diagnostic tool for early detection of human acute leukemia and may be readily expanded to multiplex cytosensing of other cancer cells.

1. Introduction

Leukemia is a type of fatal cancer that affects the bone marrow, the blood cells, and other parts of the lymphatic system [1]. Early detection of acute leukemia is of vital importance because acute leukemia has a rapid clinical course and progresses over several weeks to months, ultimately culminating in bone marrow failure [2]. The American Cancer Society has estimated about 20,000 new cases with expected 11,800 deaths from acute leukemia, respectively, in the United States in 2016 [3]. A variety of analytical tools have been developed for acute leukemia diagnosis, such as immunohistochemistry [4], cytochemistry test [5], microarrays [6], polymerase chain reaction (PCR) [7], the complete blood count and peripheral blood smear [8]. However, these methods may be relatively costly, time-consuming, labor-intensive, or requiring sophisticated instrumentation, limiting their use as point-of-care diagnostic tools. The amplification of malignant cell mutations by PCR may even lead to false-negative results in some cases [7]. Consequently, there is an urgent need to develop an innovative and appropriate detection approach to overcome the limitations associated with the conventional methods mentioned above.

In most practical detections or diagnoses, a fast and cheap qualitative test is often preferable before a more elaborate and quantitative test [9]. Transforming the detection events into color changes, colorimetric biosensing is fast responding, low-cost and readable to the naked eye. These advantages gain colorimetric sensors great popularity in qualitative self-help tests. However, the sensitivity and linearity of colorimetric sensors are still not competitive to electrochemical cytosors, which though, are often delicate, require the connection to signal analysis instruments [10]. On the other hand, the competition assay has the potential to identify ligands with high affinity for the target, reduce the problem of false-positive results, and enhance the detection selectivity [11–14]. For instance, Wang and co-workers developed a three-layer, competition-based, giant magneto-resistive assay to detect urinary endoglin with high specificity, and distinguish between different grades of prostate cancer [15]. As noted above, in this work, a novel ultrasensitive competition strategy was developed integrating the two colorimetric and electrochemical orthogonal sensing methods for low-abundance acute leukemia cell detection. The high detection sensitivity, integrated with the multiplex sensing capabilities, are achieved essentially by hierarchically assembling an aptamer-functionalized DNA nanoprobe on gold electrode surface, one end of which is specially designed as a horseradish peroxidase (HRP)-mimicking DNAzyme. Upon stacking with hemin, the DNAzyme could catalyze the oxidation of blue-colored 3,3′,5,5′-tetramethyl benzidine (TMB), by H_2O_2 to a yellow-colored product 3,3′5,5′-Tetramethyl-4,4′-diphenoquinone (TMB^+) as a

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readout [16–18]. This multiplex electrochemical and colorimetric cytosensing approach is of great clinical value for the high throughput early detection of human acute leukemia.

2. Experimental

2.1. Reagents and chemicals

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), hemin and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. The 3,3′,5,5′-tetramethyl benzidine hydrochloride dehydrate (TMB·2HCl) was purchased from Beyotime Institute of Biotechnology (Haimen, China). Nt. BbvCI and nicking endonuclease buffer (NEB) containing 33 mM Tris-Ac, 10 mM $MgCl_2$, 66 mM KAc, 1% Tween 20, 1 mM DTT (pH 7.9) was purchased from New England Biolabs (Beijing). Phosphate buffer saline (PBS, pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 8.7 mM Na_2HPO_4, and 1.4 mM KH_2PO_4 was used as incubation buffer. Citrate buffer (pH 5.0) containing 26.6 mM citric acid, 51.4 mM Na_2HPO_4 and 20 mM KCl was used as electrolyte. All other reagents were of analytical grade. All aqueous solutions were prepared using ultrapure water (Milli-Q, Millipore). All DNA sequences were obtained from Sangon Biotechnology Co. Ltd. (Shanghai, China). The sequence of the aptamer-functional DNA was 5′-HS-TTT TTT-ATC TAA CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG TTA GAC CTC AGC GGG TAG GGC GGG TTG GG-3′. The sequence of cDNA was 5′-GCT GAG GTC TA-3′.

2.2. Cell lines and cell culture

CCRF-CEM, K562 and Hela cells were obtained from Nanjing KeyGen Biotech Co., Ltd. and cultured in a fask in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Sigma), penicillin (100 mg mL^{-1}) and streptomycin (100 mg mL^{-1}) in an incubator (5% CO_2, 37 °C). At the logarithmic growth phase, the cells were collected and separated from the medium by centrifugation at 1000 rpm for 2 min and then resuspended in the binding buffer (4.5 g L^{-1} glucose, 5 mM $MgCl_2$, 0.1 mg mL^{-1} tRNA and 1 mg mL^{-1} BSA, all dissolved in Dulbecco's phosphate-buffer saline with $CaCl_2$ and $MgCl_2$) to obtain a homogeneous cell suspension. The binding buffer was used to ensure the effective binding affinity between cells and aptamers.

2.3. Cytosensor assembly

The Au electrode (3 mm diameter) was first pretreated with freshly made piranha solution (98% H_2SO_4: 30% H_2O_2 = 7: 3, v/v) for 5 min twice and then rinsed with water (CAUTION: piranha solution should be handled with great care). After successive sonication in 1:1 nitric acid/water, acetone, and doubly distilled water, the electrode was rinsed with doubly distilled water and allowed to dry at room temperature. After that, the electrode was scanned in 0.5 M H_2SO_4 between −0.2 and 1.5 V at 100 mV s^{-1} until a reproducible cyclic voltammogram (CV) was obtained.

Before modification, the aptamer-functionalized DNA was activated with 1.5 μL of 10 mM TCEP in pH 5.2 acetate buffer for 1 h to prevent terminal cysteine from forming disulphide bonds. Afterwards, 10 μL of 5 mM DNA nanoprobe was spread on the pre-cleaned Au electrode surface for 12 h at 4 °C in 100% humidity. Then, 10 μL cDNA suspension was introduced for sufficient hybridization, which was carried out for 50 min at 37 °C. Following a careful rinse with incubation buffer, the cDNA hybridization electrode was immersed in BSA (1%) solution for 1 h to block the nonspecific binding sites. After that, the electrode soaked in 100 μL of cell suspension at a certain concentration and incubated at 37 °C for 1 h to capture the cells via the specific binding of sgc8c aptamers. Then the electrode was taken out and rinsed with incubation buffer to remove the noncaptured cells. The nicking endonuclease was performed by incubating the resulting mixture with 10 unit of Nt. BbvC I at 37 °C for another 2 h in NEB buffer. The obtained electrode was used for subsequent assays.

2.4. Electrochemical detection of captured cells

After endonuclease nicking, the electrode were incubated in a HEPES buffer solution 5 mM, pH = 7.2, containing 20 mM KNO_3, 200 mM $NaNO_3$, and 2×10^{-6} M hemin, for 60 min at 37 °C, to stabilize the hemin/G-quadruplex complex. Before electrochemical measurements, the cyto-sensor was washed thoroughly with the incubation buffer to remove nonspecifically bound hemin to minimize the background response. For cytosensing, the electrode was placed in 1 mL citrate buffer (pH 5.0, containing 50 μL TMB, 10 μL H_2O_2) at 37 °C for 30 min. After terminating the reaction with 400 μL sulfuric acid (2 M), differential pulse voltammetry (DPV) measurements were performed from 700 mV to 300 mV (vs. SCE) on a CHI 660C workstation (CH Instruments, USA) with a conventional three-electrode system composed of a platinum wire as the auxiliary, a saturated calomel electrode as the reference, and the modified gold electrode as the working electrode with a pulse amplitude of 50 mV and a pulse width of 50 ms.

2.5. Colorimetric detection of captured cells

After endonuclease nicking, 5 μL reaction liquid was taken from different electrodes and incubated in 390 μL citrate buffer solution (pH 5.0, containing 5 μL, 5 μM hemin) at 37 °C for 60 min to stabilize the hemin/free G-quadruplex complex. The colorimetric assay was performed by adding 5 μL TMB (0.5 wt%), 5 μL H_2O_2 (3%) into the hemin/free G-quadruplex system and incubated at 37 °C in the dark for 30 min. After terminating the reaction with 400 μL sulfuric acid (2 M), the absorption of reaction product was recorded on a UV-3600 spectrophotometer (Shimadzu, Kyoto, Japan) as readout. All reactions were processing in a 0.2 mL PCR tube and the total volume of the reaction is 80 μL.

3. Results and discussion

3.1. Detection principle of the dual colorimetric and electrochemical cytosensing platform

The detailed principle of the proposed competitive cytosensing system is illustrated schematically in Fig. 1. We designed an aptamer-functionalized DNA nanoprobe with three functional regions (A, D and G). Region A (blue) is a sgc8c aptamer binding specifically to CCRF-CEM cells with high affinity. Region D, embedded a (5′-CCTCAGC-3′) sequence, is a trigger region to initiate the endonuclease nicking reaction. Nicking enzyme is a special family of restriction endonuclease, which recognizes a specific sequence along double-strand DNA and hydrolyzes only one specific strand instead of both stands, leaving a nicking in the DNA strand [19–22]. In the present study, nicking enzyme, Nt. BbvC I, which recognizes (5′-CCTCAGC-3′/3′-GGAGTCG-5′) and specifically cleaves the upper strand, is introduced to “free” the signal DNA (region G). Region G (red) is a G-quadruplex which can form the HRP-mimicking DNAzyme in the presence of hemin.

To fabricate the cytosensor, the aptamer-functionalized DNA nanoprobe was first assembled on the surface of gold electrode by strong Au-S linkage. Then the hybridization with cDNA (gray, 5′-CCTCAGC-3′) would result in the formation of Nt. BbvC I recognition site. Consequently, aptamer-functionalized DNA/cDNA duplex is cleaved by Nt. BbvC I, freeing region G off the electrode, which forms G-quadruplex HRP-mimicking DNAzyme upon stacking with hemin, enzymatically catalyzing a colorimetric reaction.

On the other hand, for an aptamer, the consensus secondary structure motif upon a target molecule is a tertiary folding conformation, whereas, in the presence of a cDNA sequence, only a DNA duplex structure is formed. We assume that the aptamer is in a dynamic

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