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Multivalent Capture and Detection of Cancer Cells with DNA Nanostructured Biosensors and Multibranched Hybridization Chain Reaction Amplification

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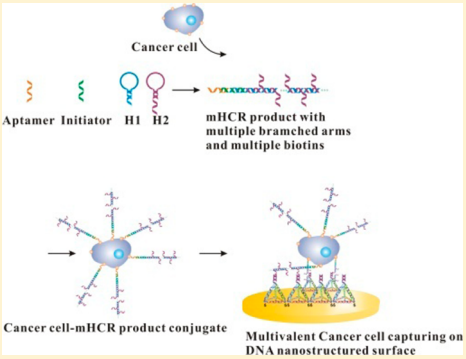
Supporting Information

ABSTRACT: Sensitive detection of cancer cells plays a critically important role in the early detection of cancer and cancer metastasis. However, because circulating tumor cells are extremely rare in peripheral blood, the detection of cancer cells with high analytical sensitivity and specificity remains challenging. Here, we have demonstrated a simple, sensitive and specific detection of cancer cells with the detection sensitivity of four cancer cells, which is lower than the cutoff value with respect to correlation with survival outcomes as well as predictive of metastatic disease in clinical diagnostics. We re-engineered the hybridization chain reaction (HCR) to multibranched HCR (mHCR) that can produce long products with multiple biotins for signal amplification and multiple branched arms for multivalent binding. The capturing gold surface is modified with DNA tetrahedral probes, which provide superior hybridization conditions for the multivalent binding. The synergetic effect of mHCR amplification and multivalent binding lead to the high sensitivity of our detection platform.



Sensitive detection of cancer cells plays a critically important role in the early detection of cancer, cancer metastasis and cancer therapies.[1−5] Even though the number of circulating tumor cells (CTC) in blood remains extremely small, the analysis of CTC can provide useful information for metastatic relapse and real time monitoring of therapies.[1−5] However, the sensitive detection of tumor cells in peripheral blood remains challenging because of their very low concentrations.[1−5] Therefore, the development of detection methods for CTCs with high analytical sensitivity and specificity is highly urgent.

There are some technologies that have been developed for the sensitive detection of cancer cells in recent years.[6−8] Typically, they are based on the detection of the molecular biomarkers of cells such as microRNAs or telomerase.[6−8] However, these methods require the lysis of the cancer cells and the following polymerase chain reaction (PCR) process, which are complicated, expensive and not suitable for point of care (POC) detection.

By avoiding the lysis step and the PCR process, direct detection of whole cancer cells provides an effective and simple approach for cancer cell detection.[1−4,9−20] However, the realistic demands require that these approaches are highly sensitive and specific, which should have dual functions of capturing and detection. A high capturing capability minimizes the loss of rare cancer cells and a strong detection capability ensures the efficient detection of cancer cells at ultralow concentrations. In most existing methods, the antibody or aptamer against EpCAM (epithelial cell adhesion molecule) is widely used to capture the cancer cells.[1−4,21] One EpCAM can bind only a single antibody or aptamer for capturing and detection, which may limit the capturing capability and analytical sensitivity.[21] Multivalent binding may improve the capturing efficiency.

Here, we re-engineered the hybridization chain reaction (HCR[22−25]) to multibranched HCR (mHCR) that can produce modified long products with multiple biotin and multiple branched arms, through which multiple avidin-HRP (avidin-horseradish peroxidase conjugation) can be attached for efficient signal amplification and multiple branched arms can be used for multivalent binding on the DNA nanostructured gold electrode surface (Figure 1). The aptamer of EpCAM that partially hybridized to an initiator of HCR was employed to

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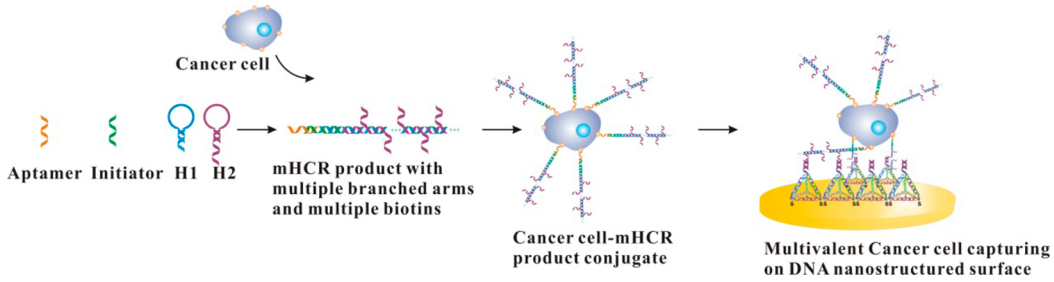

Figure 1. Multibranched HCR (mHCR) reaction was employed to synthesize long products with multiple biotin labels and multiple branched arms. After the conjugation of cancer cells and mHCR products, the cancer cells can be attached on the DNA nanostructured surface through multivalent binding. A large number of HRPs were attached on the mHCR products to amplify the electrochemical signal through enzyme catalytic reaction, which increased the detection sensitivity.

Table 1. DNA Sequences Used in Our Experiments

	DNA sequence (5′-3′)
A	ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTATTTTTTTTTTGTATCCAGTGGCTCA
B	HS-TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAGGGTCCAATAC
C	HS-TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATGGCGGCTCTTC
D	HS-TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCTCGCAT
E	HS-TTTTTTTTTTGTATCCAGTGGCTCA
L-aptamer	CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTGTTTTTTTTTTTGAGCCACTGGATAC
2-biotin-aptamer	biotin-CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTG-biotin
L-2-biotin-aptamer	biotin-CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTGTTTTTTTTTTTGAGCCACTGGATAC-biotin
aptamer	CACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTGTTTGCAAAGCTTACGGCATACGT
I	CTAGAGCACAATCACAGGAGCCAGTTTACGTATGCCGTAAGCTTTGC
H1	biotin-TTTTTTTTTTCTGGCTCCTGTGATTGTGCTCTAGTTTACATCGCTAGAGCACAATCACAGG
H2	biontin-TTTTCTAGAGCACAATCACAGGAGCCAGTTACCTGTGATTGTGCTCTAGCGATGTTTTTTTTGAGCCACTGGATAC

trigger the mHCR reaction. The long products were employed for the efficient signal amplification and the multivalent binding. As a result, we can sensitively detect as few as four MCF-7 cells with excellent specificity (Figure 1). In our design, mHCR is used for both signal amplification and multivalent binding in the capturing of rare cancer cells, which has not been investigated previously[22−25] and was proved to improve the detection sensitivity by our results.

EXPERIMENTAL SECTION

Materials. MCF-7 cells were obtained from Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences. All DNA sequences shown in Table 1 were synthesized and modified by Sangon Biotech. 6-Mercapto-1-hexanol (MCH), Hoechst, casein and horseradish peroxidase coupled with avidin (Avidin-HRP) were purchased from Sigma-Aldrich. 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was purchased from Neogen. The washing buffer was phosphate buffered saline (PBS) solution (10 mM phosphate buffer, 0.14 M NaCl, 2.7 mM KCl, pH 7.4); the hybridization chain reaction buffer (TM buffer) was Tris buffer (20 mM, pH8.0) containing 50 mM $MgCl_2$; dilution solution for Avidin-HRP (10 mM phosphate sodium buffer solution at pH 7.4, 0.1 M NaCl, 1% casein); substrate solution for Avidin-HRP catalyzed reactions in a 96-well plate (TMB, enhanced K-blue activity substrate, H_2O_2 included, pH 5.0). The chemicals mentioned were all used as received, and Milli-Q water (18 MΩ·cm^{-1} resistivity) was used throughout all experiments.

Instruments. A CHI 1130b electrochemical workstation was employed for cyclic voltammetry detection, which was performed from 0 to 0.7 V at a scan rate of 100 mV/s. And it was also used for amperometric detection, which was obtained at 100 mV within 100 s. A conventional three-electrode system was used (gold disk electrode was used as the working electrode; Ag/AgCl electrode was used as the reference electrode; platinum wire was used as the counter electrode). Oligonucleotides were quantified with a UV−vis absorption spectrophotometer (Hitachi U-3010, Japan). MCF-7 cells isolated on electrodes were directly monitored by imaging a gold surface under a microscope (Axioskop2, Carl Zeiss Shanghai Co., Ltd.). For the colorimetric assay, 50 mL of solution with different numbers of HRP-aptamer-cells was mixed with 50 mL of TMB solution (H_2O_2 included, pH 5.0). The absorbance at 652 nm of this mixture was monitored via a Tecan microplate reader over 30 min.

MCF-7 cells were cultured by following instructions supplied by the supplier. The aptamer linked hybridization chain reaction (HCR-apt) was carried out similar to the reported method created by Pierce's group.[24] Briefly, aptamer and initiator strands were mixed in TM buffer with a final concentration of 5 μM. Stock solutions of H1 and H2 were diluted in TM buffer to a final concentration of 20 μM. Then, they were heated to 95 °C for 10 min and cooled to 4 °C within 30 s by using a PTC-200 thermal cycler (MJ Research Inc., Waltham, MA). After that, 100 μL of aptamer and initiator strands solution was added to 1 mL of H1 and H2 (1:1) with approximate final concentrations of 0.5, 0.5, 10 and 10 μM for aptamer, initiator, H1 and H2, respectively. The prepared hybridization mixture was reacted overnight at 37 °C. Finally, HCR-apt/cells were obtained by adding 1 mL of HCR-aptamer products to about 1 million MCF-7 cells, whose incubating condition was under smoothly shaking at 4 °C. The washing procedure was repeated four times to thoroughly removing the excess HCR-aptamer. The apt/cells were

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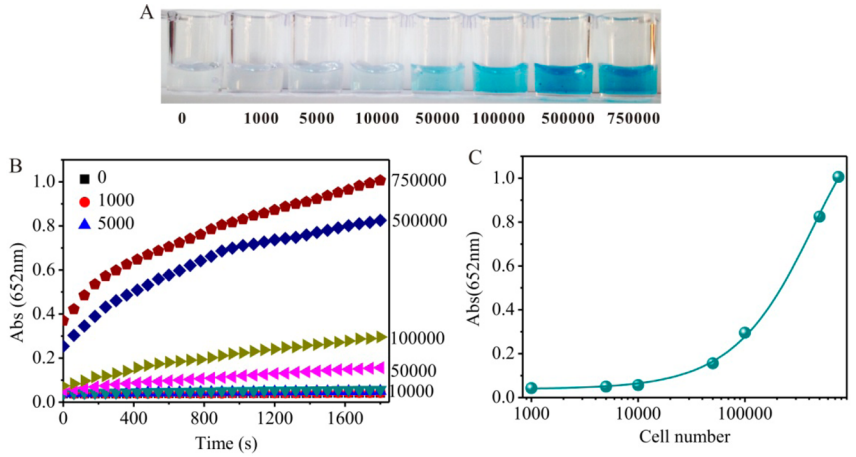

Figure 2. (A) MCF-7 cells that bound with aptamer and HRP initiated the enzymatic reaction in the solution containing enzyme substrates. The color change is related to the cell number. (B) Kinetics of the enzymatic reaction of various cell numbers. (C) UV−vis adsorption at 652 nm increased along with the increase of cell number.

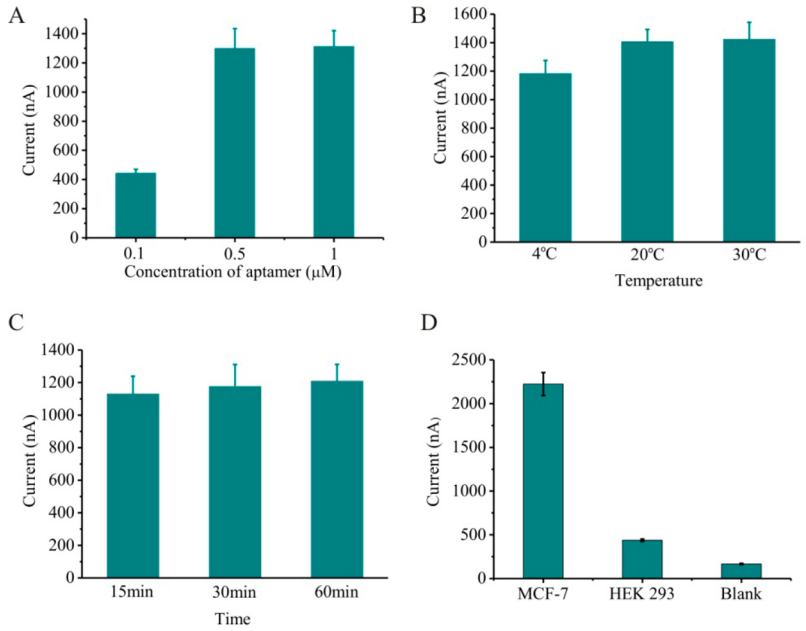

Figure 3. (A) Optimized concentration of aptamer was selected. 5000 MCF-7 cells were used in the optimization experiments. The final electrocatalytic current originated from the enzyme catalytic reaction was collected. (B and C) Incubation temperature and incubation time of apt/cells and tetrahedral probes modified electrode were investigated, respectively. (D) Electrocatalytic current from 10 000 HEK293 cells was significantly lower than that from 10 000 MCF-7 cells, which indicated the high specificity of our detection.

prepared the same as HCR-apt/cells, except the HCR-aptamer was replaced by 2-biotin-aptamer (or L-2-biotin-aptamer), which was modified with biotin at the 5′ and 3′ ends.

The procedure for the immobilization of single stranded probes (ssP) and tetrahedral probes (TSP) on gold electrode surfaces was the same as reported papers published by our group,[26] whereas the ssP modified electrodes were further treated with 2 mM MCH for 30 min to remove nonspecific DNA adsorption. Each TSP or ssP electrode was incubated with 10 μL of pretreated apt/cells or HCR-apt/cells (cell number, 0−100 000) for 30 min. Uncaptured cells were washed away with PBS, then 3 μL of avidin-HRP (10 μg/mL) was dropped onto the electrode surface with another 15 min. Rinsed thoroughly and steeped with PBS, sensors were then ready for electrochemical measurements in 3 mL of TMB solution. To verify the MCF-7 cells captured on the electrode surface, stained cells with Hoechst that were captured on the gold electrode were imaged under an ultraviolet microscope.

RESULTS AND DISCUSSION

The aptamer we employed was selected for EpCAM (which is part of the membrane transported from the cytoplasm through very complicated vesicular transport mechanism).[27] To test the binding of this aptamer (with biotin label) to cancer cells in homogeneous solution, we employed MCF-7 cells (a breast cancer cell line. Because metastases is the major cause of death in breast cancer patients, we employed MCF-7 as a model to demonstrate our detection platform), which has high EpCAM expression levels on their cell membrane, to bind the aptamer with the biotin label. Then, we attached avdin-HRP (HRP catalyzes the conversion of chromogenic substrates into colored products) on the biotin labeled aptamer. We detected the

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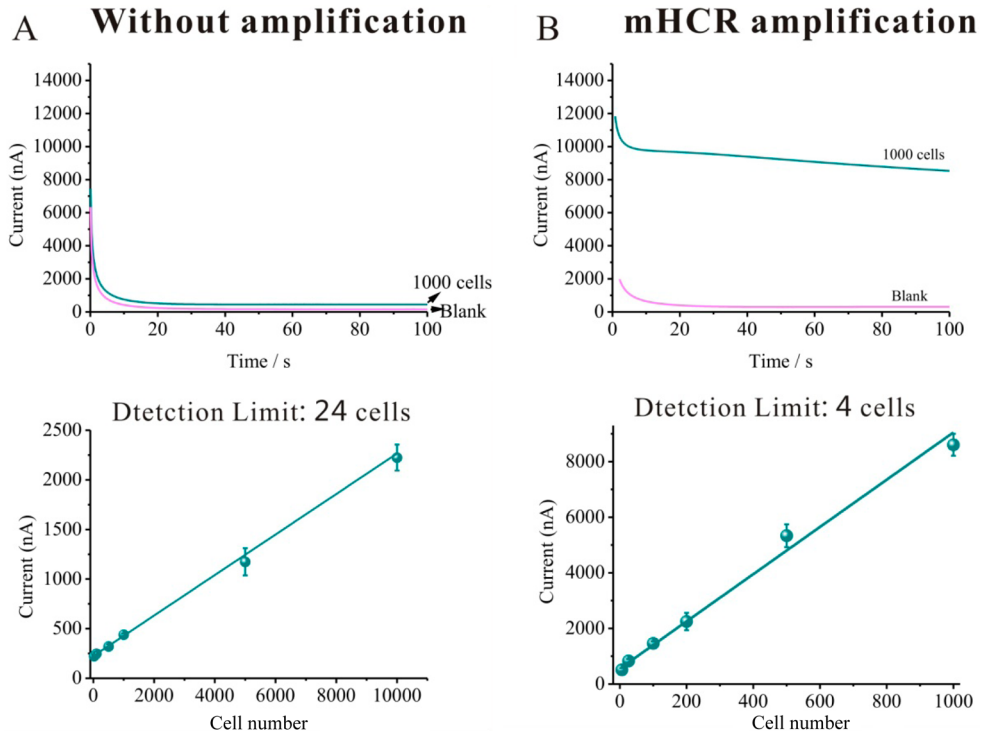

Figure 4. (A) Electrocatalytic current was collected by i−t method (top). Shown are i−t curves for 1000 MCF-7 cells and 0 MCF-7 cells, respectively. The electrocatalytic current increased along with the increase of cell number (bottom). The detection limit was 24 cancer cells. (B) mHCR was employed to amplify the signal and realize multivalent binding. The catalytic signal was significantly higher than that without mHCR amplification (top). Catalytic current is proportional to the cell numbers. As few as four cancer cells can be detected (bottom).

catalytic reaction originated from the HRP enzyme that attached on the surface of the MCF-7 cells (Figure 2). We observed an obvious color change when we employed 750 000 cells, which indicated the efficient enzymatic reaction in the presence of H_2O_2 and cosubstrates (TMB). We concluded that the aptamers were successfully bound to the MCF-7. We also observed that the color changed from blue to colorless with the decrease of the cell number (Figure 2A,B). The detection limit of the enzyme that induced colorimetric detection was ∼5000 cells (Figure 2C).

Next, we attempted to detect cancer cells through an electrochemical method. Electrochemical methods are widely used in biosensors with high sensitivity, rapid speed and low cost.[28−30] Especially, electrocatalysis is well suited for the detection of enzymatic reactions. First, we combined a DNA fragment with the aptamer of EpCAM (L-aptamer), which the capturing probes that immobilized on the gold electrode surface can bind to. Then, after the incubation of cancer cells with biotin labeled L-aptamers and avdin-HRP, washing steps were employed to minimize the nonspecific adsorption of avdin-HRP on the cell surface. After that, a DNA nanostructure (here, we employed a DNA tetrahedron with a pendant capturing probe) modified macroscopic gold electrode (2 mm in diameter) was employed to capture the cancer cells and transduce the electrocatalytic current of the enzymatic reaction through a potentiostat. We first investigated the influence of the concentration of aptamer, the reaction temperature and reaction time on the final electrochemical signal (Figure 3). We found that 0.5 μM aptamer was sufficient to produce the electrochemical signal (Figure 3A). The reactions were taking place well either at 20 or 30 °C (Figure 3B). We only need 15 min to obtain a nearly saturated signal, which is fast (Figure 3C). We detected the HEK293 cells (10 000 cells, human embryonic kidney cell line with low EpCAM expression) by using our detection platform and obtained a much lower signal than that with MCF-7 cells (10 000 cells), which indicated the excellent specificity of our detection (Figure 3D).

Under our optimized conditions, we observed an electrocatalytic current of ∼450 nA with 1000 cancer cells, whereas a control experiment without cancer cells was carried out and only ∼150 nA of current was obtained (Figure 4A). When we decreased the number of cancer cells, we obtained a decreased electrocatalytic current. Interestingly, when the cell number was as low as 24, we obtained a distinguishable signal (∼222 nA) to the control experiment (∼150 nA), which is more sensitive than the colorimetric method (Figure 4A,B).

We should point out that the DNA tetrahedron modified gold surface provided superior surface hybridization capability for capturing.[26,31−33] As a contrast, we employed single stranded DNA as capturing probes and immobilized them directly on the gold electrode via Au−S bonds. With 100 000 MCF-7 cells, we obtained an electrocatalytic signal of ∼650 nA, which was dramatically lower than that obtained on the DNA-nanostructured surface. The detection sensitivity was 500 cells (Figure S1, Supporting Information). As indicated before, the surface status of the capturing probes heavily affected the capturing performance such as hybridization kinetics and efficiency.[6,26,29,31,33−35] The surface hybridization is usually limited by steric effect, undesirable entanglement between probes, lateral spacing and the nonspecific interaction between probes and surface.[6,26,29,31,33−38] Here, DNA tetrahedral probes provided an appropriate environment for hybridization, and thus for cancer cell capturing.

A critical requirement in clinical diagnostics and therapy monitoring is that five cancer cells are usually considered as the cutoff value with respect to correlation with survival outcomes

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as well as predictive of metastatic disease.[39−42] The sensitivity of 24 cancer cells is not sensitive enough to meet this requirement. To further improve the sensitivity, we employed mHCR reaction to produce long products (over 1500 base pairs, Figure S2, Supporting Information) with multiple biotins to amplify the signal and multiple branched arms to realize the multivalent binding on the surface. The multivalent binding of mHCR products and tetrahedral probes in homogeneous solution was proved by gel results and AFM images (Figures S3 and S4, Supporting Information). However, the multivalent binding on the surface may have a slight difference than that in solution. The overall electrochemical signal was greatly improved to ∼8600 nA with 1000 cancer cells (Figure 4B), whereas, without HCR amplification, the electrochemical signal from 1000 cancer cells was only ∼450 nA. The detection limit was improved down to four cancer cells as well (Figure 4B). Interestingly, with increasing the number of cancer cells, we observed increased density of cancer cells on the electrode surface after staining of cancer cells (Figure 5A−E). With four times of extensively washing, we did not observe an obvious loss of cancer cells (Figure 5A′−E′), which indicated the strong binding of cell-mHCR conjugates on the capturing surface. The improved sensitivity by using mHCR may originate from the synergetic effect of the multivalent binding and the amplification of multiple HRP attached on the mHCR products. However, we would state that it is difficult to quantify the contribution from each effect. The significant signal increase demonstrated in Figure 4 proved that the amplification of multiple enzymes contributed to the improvement of sensitivity. On the other side, the number of cells captured on the gold surface was almost unchanged before and after washing steps, indicating that the multivalent binding provided a solid method to capture cancer cells (Figure 5). Our method could be used for direct cancer cell detection in complicated samples such as serum and blood, because the MCF-7 cells we employed originated from cell culture medium, which could be considered as a complicated matrix.

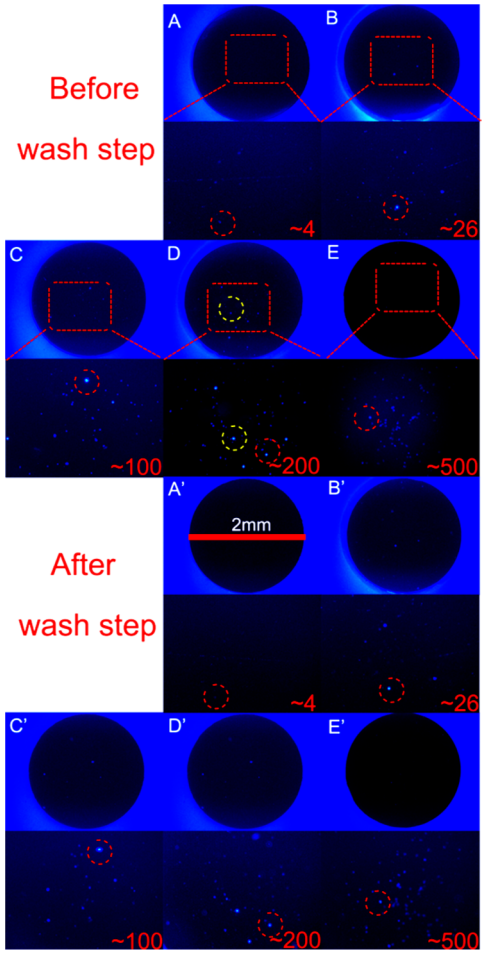

Figure 5. Cancer cells captured on the surface of gold electrode were stained with Hoechst and imaged (A−E, the diameter of the gold electrode is 2 mm and the size of the enlarged images is ∼687 × 513 μm). Panels A′−E′ represent the same electrode after extensive washing steps, respectively. The solution of cells (which is used to be captured) contained 4, 26, 100, 200 and 500 cells, respectively. A red circle is used to indicate the same cell on the corresponding electrode before and after the washing step.

CONCLUSION

Here, we have demonstrated a simple, sensitive electrochemical detection platform for cancer cells. With rational redesign, a multibranched HCR (mHCR) reaction was employed to produce a long nicked DNA helix with multiple biotins for multiple enzymes binding and multiple branched arms for multivalent binding on the surface. Some other methods based on HCR and DNA assembly provided efficient signal amplification;[43−46] however, our present method employed a dual-functional mHCR to realize signal amplification and multivalent binding, which could be used for many other detection platforms. The DNA nanostructured gold surface provided favorable hybridization for the multivalent binding. We can sensitively detect as few as four cancer cells.

ASSOCIATED CONTENT

Supporting Information

Cancer cell detection by using the single stranded DNA as capturing probes, i−t curves that were obtained from different cancer cells, gel results, and AFM images of tetrahedral probe and HCR products before and after hybridization with tetrahedral probes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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