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A simple fluorescence aptasensor for gastric cancer exosome detection based on branched rolling circle amplification

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Exosomes are membrane nanovesicles carrying molecular information that may reflect the biological and genetic characteristics of their parent cells. Numerous studies have demonstrated the potential of exosomes as noninvasive cancer biomarkers. Hence, specific detection of cancer cell-derived exosomes is of significant importance. Here, we developed a fluorescence assay for the determination of gastric cancer exosomes based on branched rolling circle amplification (BRCA) and an aptamer to target specific exosomes. The designed padlock probe was cyclized after incubation with an aptamer binding with the target exosome. BRCA was triggered by adding a second primer and the resulting long tandem double-stranded DNA product was detected using SYBR Green I as the fluorescent dye. This method demonstrated a high specificity for target exosomes with a detection limit of 4.27×10^4 exosomes per mL. Moreover, plasma from gastric cancer patients was tested to verify the clinical applicability of this assay. Our results demonstrated that this aptamer-based biosensor may show potential for the early diagnosis of gastric cancer.

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Introduction

Globally, gastric cancer is one of the most common cancers. It is also the 3rd most common cause of cancer-related mortality.[1] Therefore, early gastric cancer detection methods are urgently needed in order to develop more effective treatment strategies and reduce mortality rates. Exosomes are membrane enclosed nanovesicles that are secreted into extracellular spaces to enter the circulation *via* almost all cell types.[2] Exosomes contain functional biomolecules including nucleic acids, proteins, and lipids. These extracellular vesicles play a significant role in reciprocal communication between tumor cells and the cells surrounding tumor cells and are closely associated with angiogenesis, tumor progression, and metastasis.[3,4] A lot of evidence has been found revealing the impact of exosomes on the modulation of gastric cancer initiation, progression and metastasis.[5] Although the bioactive molecules contained in exosomes are specific to their parent cells, the concentrations of these molecules are also much higher than those of biological fluids. Therefore, exosomes are considered to be potential cancer biomarkers for clinical diagnostics and assessment of treatment.[6,7]

Considering the importance of exosomes in the liquid biopsy of cancer, many studies have explored new detection techniques over the past few decades. Besides traditional methods such as nanoparticle tracking analyses, a series of novel biosensors using aptamers as sensing components have been developed for the determination of exosomes. These aptasensors are based on different detection principles, such as electrochemistry, fluorescence, surface plasmon resonance, Raman scattering, nuclear magnetic resonance and mechanics.[8–11] By exploiting new strategies, the determination of exosomes can be achieved without the purification of samples, or without labeling, or with high sensitivity, or with streamlined and simplified detection procedures.[12,13] However, many of these strategies are unable to distinguish cancer cell-derived exosomes from normal exosomes, which limits their application in clinical cancer diagnosis. Recently,

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several proteins have been identified as reliable candidates that may be useful for detecting and isolating cancer-related exosomes, such as glypican-1 for pancreatic cancer,[14] claudin-4 for ovarian cancer,[15] HER2 for breast cancer[16] and annexinV/EpCAM/ASGPR1 for liver malignancies.[17] Studies based on these protein markers may offer opportunities for early diagnosis as well as designing potential curative surgical options for cancers.

Rolling circle amplification (RCA) is a novel nucleic acid amplification technique carried out at room temperature and able to generate high molecular weight products. Due to its simplicity, robustness and high sensitivity, RCA is considered to be an efficient tool in biochemical analysis.[20] In this article, we developed a fast and simple method for the quantitative determination of gastric cancer exosomes. Mucin 1 (MUC1) is a cell surface glycoprotein that is overexpressed in gastric cancers. In our previous article, we have demonstrated that the MUC1 specific aptamer could identify gastric cancer exosomes.[20] However, the assay we described earlier had several shortcomings such as complicated and error-prone steps, and large testing agent and time consumption. A constant humid environment was also needed during the modification of the detection platform. To overcome these issues, we designed the following experiment (Fig. 1). First, the aptamer was incubated with different concentrations of exosomes, following which the unbound aptamer was removed using a 22 nm pore size filter. The aptamer binding with exosomes was separated through high temperature treatment. Then, a designed padlock probe which was partially complementary to the aptamer was added. BRCA was triggered by adding the second primer and the resulting long tandem double-stranded DNA product was detected using SYBR Green I (SG) as fluorescent dye. This method demonstrated a high specificity for target exosomes with a linear response ranging from 10^5 to 10^9 exosomes per mL.

Materials and methods

DNA sequences were purchased from Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). The sequences are as follows:

MUC1 specific aptamer: 5′-TACTGCATGCACACCACTTCAACTA-3′

Padlock probe: 5′-phosphate – TGTGCATGCAGTATTTGCAT TTCAGTTTACGGTTTAGCATT – TCGCAATTTTTAGTTGAAGTGG-3′

Second primer: 5′-TAGTTGTGCATGCAGTATTTGC-3′

ssDNA: 5′-N (25)-3′

The underlined sequences and the dotted sequences in the aptamer were complementary to those in the padlock probe. The ssDNA sequence was used in control experiments.

Salmon sperm DNA, aldehyde/sulfate latex beads, T4 DNA ligase, 10× T4 DNA ligase reaction buffer, exonuclease I, exonuclease III, phi29 DNA polymerase and 10× phi29 DNA polymerase reaction buffer were purchased from Thermo Fisher Scientific (MA, USA). SYBR Green I (10 000×), BSA, diethylprocarbonated (DEPC) treated water and dNTPs were purchased from Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). Gold nanoparticles were

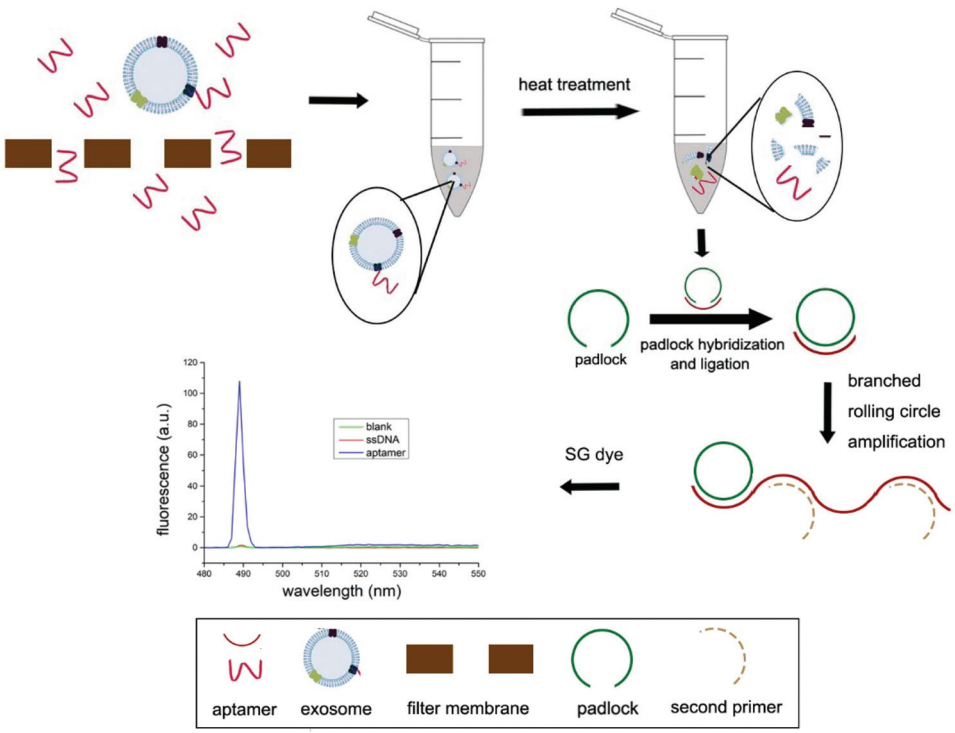

Fig. 1 Schematic illustration of BRCA for gastric cancer exosome detection.

synthesized in our lab. Dulbecco's modified Eagle's medium (DMEM, high glucose), Roswell Park Memorial Institute 1640 (RPMI 1640, high glucose), and fetal bovine serum (FBS) were purchased from Hyclone (UT, USA). Exosome-depleted FBS Media Supplement was purchased from System Biosciences, SBI (CA, USA). All cell lines were purchased from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China).

Exosome isolation and characterization

Exosomes were prepared with a method we described in our earlier article. Briefly, the human gastric cancer cell line, SGC7901, was cultured in RPMI 1640 medium supplemented with 10% exosome-depleted FBS and incubated at 37 °C with 5% CO_2. The human gastric epithelial mucosa cell line, GES-1, was cultured in high glucose DMEM. The cell supernatant was collected and filtered using a 600 nm and 22 nm pore size filter.[18,19,21] Exosome concentration was evaluated using a Malvern Nanosight NS300 instrument.

Branched rolling circle amplification

To prepare a circular template, 1 μL of the padlock probe (100 μM) was hybridized with 1 μL aptamer (100 μM) at 55 °C for 5 min and annealed at 37 °C for 30 min, following which it was slowly cooled to room temperature. The hybridization product was added to a ligation mixture containing 2 μL 10× T4 DNA ligase reaction buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8 at 25 °C), 3 μL T4 DNA ligase (30 U μL^{-1}) and 13 μL DEPC treated water. The ligation process was performed at 37 °C for 90 min. Instead of an aptamer, DEPC-treated water and a ssDNA sequence were used in control experiments.

The BRCA reaction was carried out in a mixture containing 1 μL circular template, 1 μL 10× phi29 DNA polymerase reaction buffer (330 mM Tris-acetate (pH 7.5), 100 mM Mg-acetate, 660 mM K-acetate, 1% (v/v) Tween 20, 10 mM DTT), 1 μL dNTPs (10 mM for each of dATP, dGTP, dCTP and dTTP), 1 μL second primer (2 μM), 1 μL phi29 DNA polymerase (10 U μL^{-1}) and 5 μL DEPC treated water. The mixture was incubated at 37 °C for 2 h and then heated at 65 °C for 15 min to stop the reaction. The BRCA product was verified *via* agarose gel electrophoresis.

5 μL of the BRCA product was mixed with different concentrations of SYBR Green I and diluted to a final volume of 150 μL using DEPC-treated water. The mixture was incubated for 5 min and the fluorescence signal was measured using a Synergy™ HT Multi-Mode Microplate Reader. The excitation wavelength was 485 nm, while the emission wavelength was 530 nm. Subsequently, 5 μL of the BRCA product was mixed with an optimal concentration of SYBR Green I and diluted to a final volume of 4 mL using DEPC-treated water. The fluorescence spectra were recorded in a quartz cuvette using a Hitachi F-4500 spectrofluorometer (Tokyo, Japan). The excitation wavelength was 494 nm, and fluorescence spectra were recorded between 500 nm and 650 nm. Fluorescence emission intensity was measured at 530 nm.

Target exosome detection

Different concentrations of exosomes were incubated with the MUC1 aptamer (100 nM) at 37 °C for 1 h. The mixture was filtered using a 22 nm pore size filter and washed 5 times with PBS to remove unbound aptamer. The formed exosome–aptamer complex was resuspended with ddH_2O and heated at 95 °C for 10 min to release the aptamer. One μL of the padlock probe was incubated with the aptamer at 55 °C for 5 min and annealed at 37 °C for 30 min and slowly cooled to room temperature. The hybridization product was added to the ligation mixture containing 2 μL 10× T4 DNA ligase reaction buffer and 3 μL T4 DNA ligase. The ligation process was performed at 37 °C for 90 min. Subsequently, 0.2 μM of the second primer, 5 U Phi29 DNA polymerase, 2.5 μL 10× Phi29 DNA polymerase buffer, and 12.5 μL SYBR Green I were added to produce a total solution of 25 μL. The BRCA reaction was conducted at 37 °C and monitored *via* real-time fluorescence measurement (Applied Biosystems StepOnePlus Real-Time PCR System) over a 5 h period.

Results and discussion

Exosome characterization

To confirm exosomal purification, isolated samples were examined using TEM and Nanosight. Exosomes stained with phosphotungstic acid had a typical saucer-like shape (Fig. 2A). Nanosight results confirmed the size distribution of collected exosomes (Fig. 2B). We also analyzed exosomes based on their surface protein markers using western blot and immunogold labeling (Fig. 2C and D).

BRCA reaction

Gel electrophoresis was used to verify whether the aptamer could trigger circularization of the padlock probe and to initiate the BRCA reaction. The bright bands with the same mobility in lane 1 (before exonuclease I and exonuclease III treatment) and lane 2 (after exonuclease I and exonuclease III treatment) indicated successful preparation of the circular template and its resistance to enzymatic digestion (Fig. 3A). By contrast, no band was observed in lane 3, to which no T4 DNA ligase was added during incubation and both single-stranded DNA and double-stranded DNA were digested by exonuclease. Simultaneously, DEPC water and ssDNA library were also incubated with a padlock probe as the control. The obtained products were then used as BRCA templates. A bright band indicating a large-molecular-weight product was observed only in lane 3, demonstrating that only the aptamer could be used for ligation (Fig. 3B).

We optimized SG dye concentration, in order to improve the sensitivity of fluorescence quantification of cancer exosomes. Variance in the fluorescence signal with SG dye concentration is shown (Fig. 3C). The signal reached a maximum level at 1-fold and decreased when the concentration rose from 1-fold to 12-fold. Thus, 1 fold was chosen for subsequent assays. Fig. 3D shows the fluorescence signal of different RCA products when DEPC water, ssDNA library, and aptamer, respectively, were used for ligation following incubation with

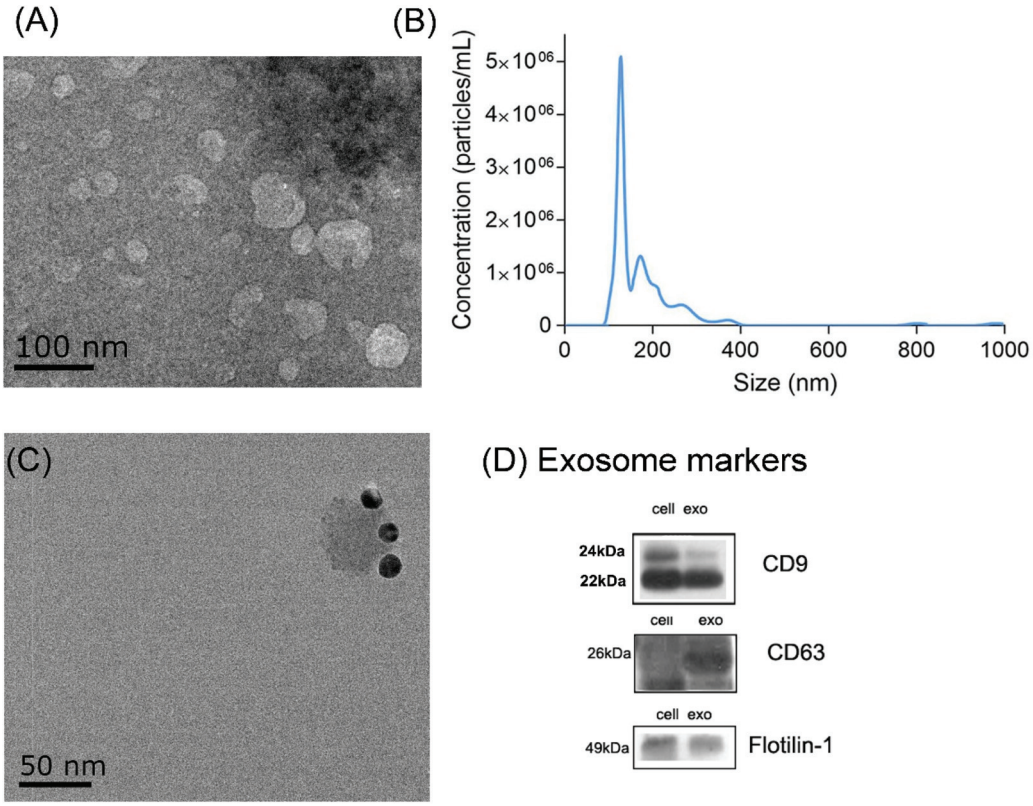

Fig. 2 (A) TEM image of exosomes. (B) Exosome concentration and size distribution based on Nanosight analysis. Exosomal concentration showed a peak at 122.8 ± 3.8 nm. (C) Immunoelectron microscopy following anti-CD63 gold staining showing surface markers on the exosomes. (D) Western blot analysis of cell lysis (cell) and exosomes (exo).

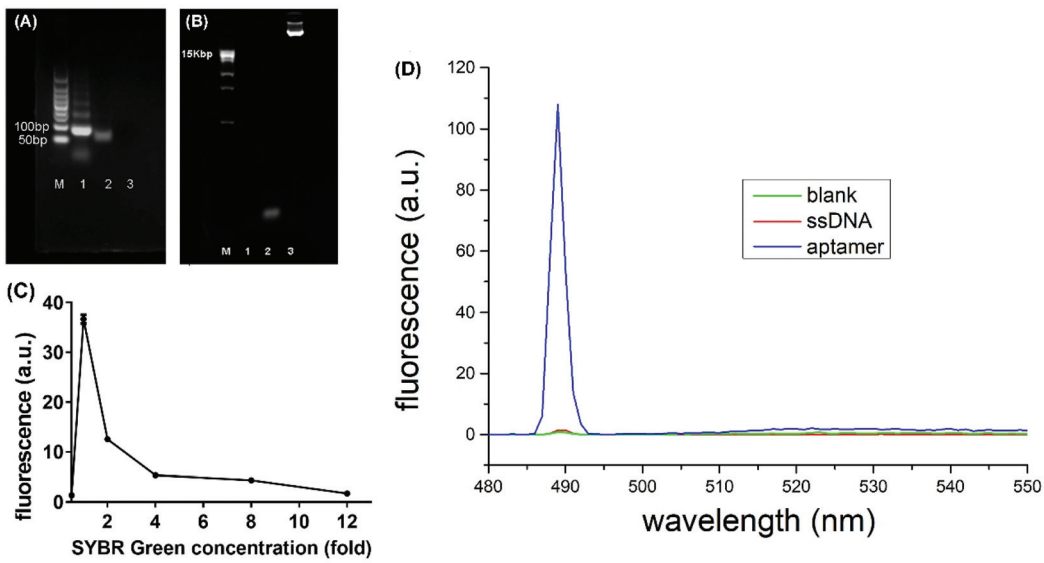

Fig. 3 (A) Gel electrophoresis of different samples. M, DNA marker; lane 1, padlock probe and aptamer incubated with T4 DNA ligase; lane 2, padlock probe and aptamer incubated with T4 DNA ligase, exonuclease I (Exo I) and exonuclease III (Exo III); lane 3, padlock probe and aptamer incubated with Exo I and Exo III; (B) Gel electrophoresis of different BRCA products. M, DNA marker; lane 1, DEPC water used for ligation, lane 2, ssDNA library used for ligation, and lane 3, aptamer used for ligation. (C) BRCA product incubated with different concentrations of SG dye. (D) Fluorescence-emission spectra of BRCA products using DEPC water, ssDNA library, and aptamer, respectively, for ligation.

1× SG dye. The fluorescent response of the aptamer was clearly much stronger than those of the 2 control groups.

Standard curve for exosome detection

We also investigated the relationship between fluorescent response and the concentration of gastric cancer exosomes.

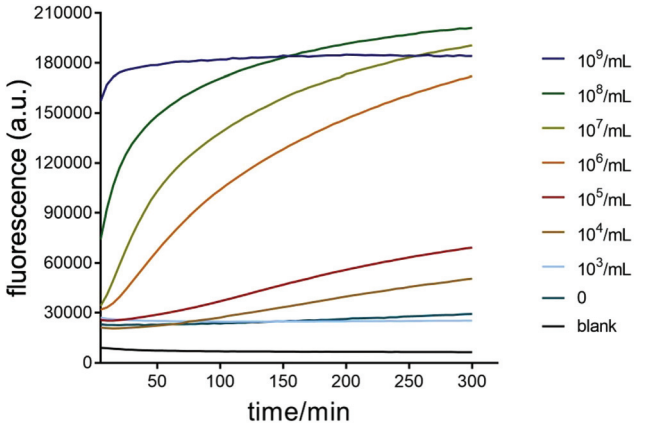

Fig. 4 Fluorescence intensities during the BRCA reaction.

Fluorescence intensities during the BRCA reaction were monitored *via* real-time fluorescence measurement (Fig. 4).

Results showed that fluorescence intensities increased remarkably with reaction time as well as with gastric cancer exosome concentration (Fig. 4 and 5A–F). The phenomenon that fluorescence intensities corresponding to an exosome concentration of 10^9 mL^{-1} were weaker than those corresponding to lower concentration of exosomes (10^8 mL^{-1}) might result from the precipitation of large BRCA products. A good linear correlation was observed between fluorescence intensity and gastric cancer exosome concentrations ranging from 10^5 to 10^9 exosomes per mL (Fig. 6). The detection limit for gastric cancer exosomes was defined as 3 times the standard deviation over the blank response (4.27×10^4 exosomes per mL).

Specificity of the aptasensor and real sample detection

The specificity of the aptasensor for gastric cancer exosomes was examined using exosomes secreted by a gastric epithelial mucosa cell line as the control (Fig. 7A). Results showed that the presence of normal exosomes caused a weaker fluorescence response than that caused by cancer exosomes. Finally, to demonstrate the validity of this aptasensor, an assay

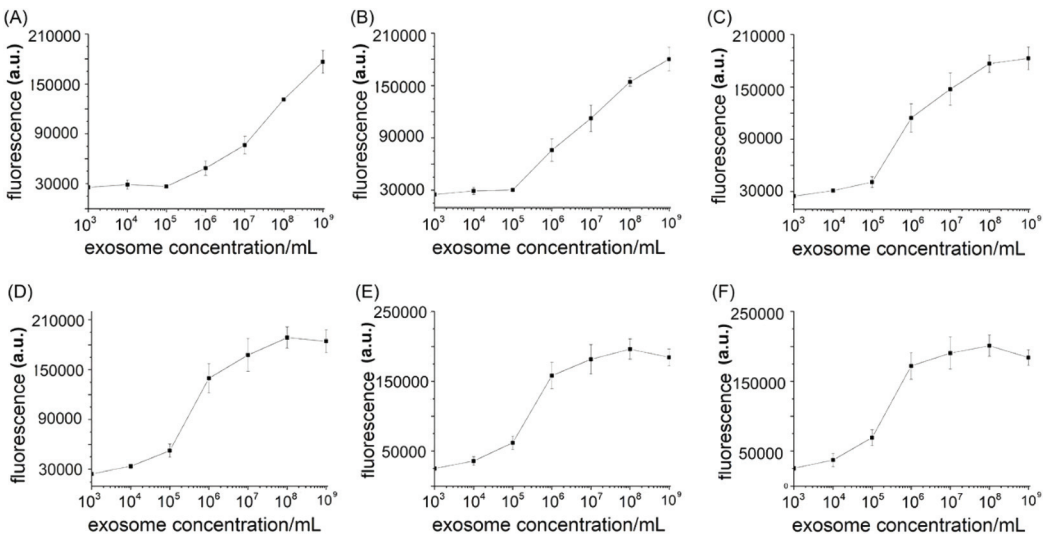

Fig. 5 (A)–(F) The relationship between fluorescence intensity and gastric cancer exosome concentration, for BRCA reaction times of 0.5, 1, 2, 3, 4, and 5 h, respectively.

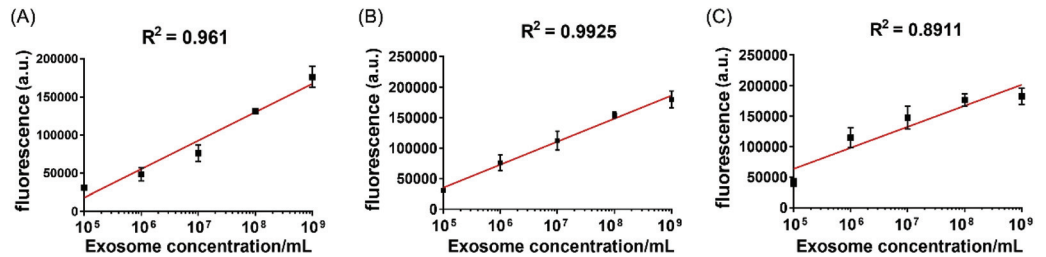

Fig. 6 (A)–(C) The relationship between fluorescence intensity and gastric cancer exosome concentration between 10^5 and 10^9 exosomes per mL, for BRCA reaction times of 0.5, 1, and 2, h, respectively.

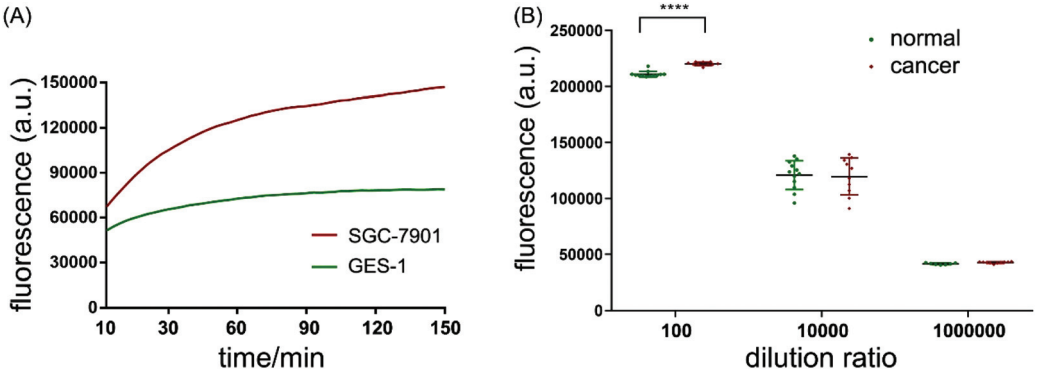

Fig. 7 (A) Fluorescence intensities associated with exosomes secreted by different cell lines. Exosome concentration was 10^7 exosomes per mL. (B) Fluorescence intensity of the normal group (n = 12) compared to that of the gastric cancer group (n = 10) (****, $P < 0.0001$).

was conducted on plasma samples collected from healthy donors and gastric cancer patients. Plasma samples were extracted from serum immediately by the usual means and could be stored up to 5 days at 4 °C before use. Prior to detection, the plasma samples were diluted 100, 10 000, and 1 000 000 fold, respectively. The results are shown in Fig. 7B. Due to the detection limit of the aptasensor, there was no obvious difference between highly concentrated groups. However, statistical significance was observed in the one-hundred-fold dilution group. The significance of signal suppression between two groups was analyzed using the Mann–Whitney U test ($P < 0.0001$). These results proved the application potential of the aptasensor.

Conclusion

In this study, a fluorescence aptasensor was developed to detect gastric cancer exosomes. The MUC1 specific aptamer was applied as a detection probe wherein BRCA was used to achieve signal amplification. The aptamer was used as a ligation probe for the preparation of a circular probe. BRCA was triggered by adding the second primer and the resulting long tandem double-stranded DNA product was detected using SYBR Green I as a fluorescent dye. The aptasensor exhibited specificity towards target exosomes with a detection limit of 4.27×10^4 exosomes per mL. Finally, the plasma samples from gastric cancer patients were tested using this aptasensor to verify the potential of this aptasensor for clinical use. Compared with that of our previous article, the present detection procedure was enormously simplified (previous assay needed more than ten steps and a constant temperature and humid environment was needed in most of the steps, while only four steps were included in the current method), which drastically reduced the time and cost (from more than one day to less than three hours). The need for equipment is also less stringent, besides a fluorescence spectrophotometer or real-time PCR system, gel electrophoresis could also reflect the concentration of the target exosome to a certain extent. In conclusion, this aptasensor provides a simple, rapid, and cheap detection method for gastric cancer and shows potential for the diagnosis and prognosis of gastric cancer.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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