CRISPR/Cas12a-Loaded intelligent DNA hydrogel for Universal and Ultrasensitive Exosome Assay

Jie Luo¹, Binpan Wang¹, Xiaoqi Tang¹, Ping Huang¹, Sha Yang¹, Shuang Zhao¹, Shuang Xie¹, Qiaofeng Li¹, Kai Chang¹, and Ming Chen¹

¹Third Military Medical University Southwest Hospital

September 21, 2023

Abstract

Tumor-derived exosomes are crucial for early non-invasive and accurate tumor diagnosis in clinical. The development of highly sensitive, simple and intuitive exosome assays has sparked a research upsurge in clinical diagnostics. Here, we develop a bioreponsive the intelligent DNA hydrogel loaded with CRISPR/Cas12a for universal and ultrasensitive detection of the exosomes. The aptamer serves as the target response unit and switch of the intelligent DNA hydrogel network. The target competitively disintegrates the region of the DNA linkers then Cas12a/crRNA encapsulated in the intelligent DNA hydrogel can be released and activated by the collateral sequence, resulting in a high fluorescent intensity for exosome detection at the detection limit of 119 particles/µL. Moreover, a prefabricated colorimetric tube is made by loading a colorimetric filter membrane on the tube lid and intelligent DNA hydrogel on the tube bottom, which enables one-pot portable colorimetric detection. Without the need for laboratory instruments and professionals, this strategy allows for naked eye detection with LOD as low as 10⁴ particles/µL, and shows great applicability in distinguishing between healthy individuals, pretreatment patients, and post treatment patients after obtaining a testable analyte. Collectively, this study constructs an ultra-sensitive detection platform for exosomes, which enables one-step sensing and dual signal output, making it a promising tool for the application of liquid biopsy based on exosomes in clinical diagnosis.

CRISPR/Cas12a-Loaded intelligent DNA hydrogel for Universal and Ultrasensitive Exosome Assay

Jie Luo¹#, Binpan Wang¹#, Xiaoqi Tang¹, Ping Huang¹, Sha Yang¹, Shuang Zhao¹, Shuang Xie¹, Qiaofeng Li¹*, Kai Chang¹*, Ming Chen¹

¹ Department of Clinical Laboratory Medicine, Southwest Hospital, Third Military Medical University (Army Medical University), 30 Gaotanyan, Shapingba District, Chongqing 400038, China

College of Pharmacy and Laboratory Medicine, Third Military Medical University (Army Medical University), 30 Gaotanyan, Shapingba District, Chongqing 400038, China

# These authors contributed equally to this work.

* Corresponding Author

Ming Chen
E-mail: chming1971@126.com

Kai Chang
Email: changkai0203@163.com

Qiaofeng Li
Email: qiaofengli001@foxmail.com

ABSTRACT

Tumor-derived exosomes are crucial for early non-invasive and accurate tumor diagnosis in clinical. The development of highly sensitive, simple and intuitive exosome assays has sparked a research upsurge in clinical diagnostics. Here, we develop a bio-responsive the intelligent DNA hydrogel loaded with CRISPR/Cas12a for universal and ultrasensitive detection of the exosomes. The aptamer serves as the target response unit and switch of the intelligent DNA hydrogel network. The target competitively disintegrates the region of the DNA linkers then Cas12a/crRNA encapsulated in the intelligent DNA hydrogel can be released and activated by the collateral sequence, resulting in a high fluorescent intensity for exosome detection at the detection limit of 119 particles/µL. Moreover, a prefabricated colorimetric tube is made by loading a colorimetric filter membrane on the tube lid and intelligent DNA hydrogel on the tube bottom, which enables one-pot portable colorimetric detection. Without the need for laboratory instruments and professionals, this strategy allows for naked eye detection with LOD as low as $10^4$ particles/µL, and shows great applicability in distinguishing between healthy individuals, pretreatment patients, and post treatment patients after obtaining a testable analyte. Collectively, this study constructs an ultra-sensitive detection platform for exosomes, which enables one-step sensing and dual signal output, making it a promising tool for the application of liquid biopsy based on exosomes in clinical diagnosis.

KEYWORDS

Exosomes, the intelligent DNA hydrogel, CRISPR/Cas, fluorescence, colorimetric

1 INTRODUCTION

As nanoscale liquid biopsy biomarker secreted by cells, exosomes are found in a variety of body fluids with high abundance and stability, and the minimal-invasive approach.1 By enabling intercellular communication, angiogenesis, and cancer metastasis,2,3 it is very suitable for early cancer screening, diagnosis, and disease surveillance.4 In this case, tumor-associated exosomes have been increasingly recognized as diagnostic biomarkers. Recently, many published literatures have been exploring the screening and detection methods of tumor-derived exosomes.5,6 However, less specific, less sensitive, and less practicable methods such as nanoparticle tracking analysis (NTA),7 flow cytometry8 and ultra-centrifugation (UC) for exosomes detection resulted in unideal clinical guidance during the early warning, cancer treatment, and prognosis, especially when cancer cells secrete fewer exosomes,9 making it more difficult to detect the exosomes, efficiently and sensitively. All these drawbacks indicate the need for a more straightforward and sensitive platform for exosome detection.

The intelligent DNA hydrogel can serve as a candidate to improve detection sensitivity because of its target-stimulated responsiveness and stable controllability of DNA sequences.10,11 As a functional material that perfectly integrates programmable skeletal structures with the biological functions of DNA, the intelligent DNA hydrogels encapsulates functional nucleic acids (such as aptamers or DNAzyme) and signal probes in a polymer skeleton.12-14 forming a three-dimensional network structure through cross-linking, greatly improving the binding efficiency of analytes and their receptors. This colloidal structure to some extent contributes to high sensitivity, which is superior to homogeneous solution detection.15 Currently, various target-responsive DNA hydrogels have been established for the detection of biomolecules, chemicals, metal ions, etc.16-18 However, some of these responses rely on indirect signal transduction, for example, an extra introduction of electroactive substances may have an impact on the result readout.19 As for other methods of releasing signal molecules directly, the signal molecules (e.g. fluorophores) are simply encapsulated in the hydrogel matrix, resulting in a low loading capacity and the high signal background, which in turn makes low sensitive and unstable sensing systems.20 Such research gaps inspire us to develop the intelligent DNA hydrogel with one-step response, which will have considerable prospects for facilitating highly sensitive and bio-friendly detection of exosomes.

CRISPR-Cas systems (clustered regularly interspaced short palindromic repeat and CRISPR-associated
protein) have aroused a research upsurge in diagnostic biosensing because of their versatility and programmability.\textsuperscript{21,22} Especially, Cas12a requires only one CRISPR RNA (crRNA) to be activated by the target sequence to exert strong side-branch cleavage activity.\textsuperscript{23} Upon selective targeting of homologous sequences, Cas12a undergoes conformational changes that cleave ssDNA reporter sequences or result in programmable degradation of the material and release of signal load, converting the target information into a measurable signal.\textsuperscript{24-26} At present, the detection strategies of various biomarkers (such as N-gene,\textsuperscript{27} transcription factor,\textsuperscript{28} nucleic acids\textsuperscript{29}) have been reported based on intelligent hydrogel sensing platforms and Cas12a. In principle, these methods rely mainly on introducing the Cas12a and reporter probes after the upstream recognition.\textsuperscript{30,31} The tedious procedures only allow the signal substance to be released first, then start their second function as a trigger in the downstream response.\textsuperscript{32} Therefore, developing an intelligent program with "recognition-response" guided by Cas12a will simplify the analysis protocol and has potential application value to be a versatile platform for other targets.

Thus motivated, an intelligent DNA hydrogel that integrates "Programming, Recognition, Response, and Dual Signal Output" has been explored for the ultrasensitive detection of exosomes. To be specific, two stages were included in this assay. First, the specific identification stage, the aptamer of human hepatocellular carcinoma HepG2 cell-derived exosome was functionalized as the molecular recognizer, which can separate the cross-linked sequences in the intelligent hydrogel when the exosome exists.\textsuperscript{33} In the meantime, the encapsulated Cas12a/crRNA was released, and the branched ssDNAs (S1, S2) attached to the polyacrylamide backbone were exposed. The second stage named intelligent response of signal self-amplifying, the released S1 and S2 served as activator of Cas12a/crRNA and signal reporter, respectively. Guided by single crRNA, the Cas12a/crRNA recognized S1, then its indiscriminate ssDNA cleavage activity was activated to completely degraded ssDNA (S2), enabling the releasing fluorescence signal.\textsuperscript{34} The more targets in the system, the more the intelligent DNA hydrogel collapsed. In this case, the Cas12a/crRNA was continuously released and activated, thus producing a highly amplified fluorescent signal. Except for one-step fluorescence analysis, the different statuses of the intelligent DNA hydrogel textures before and after the reaction provided us with a colorimetric approach to detect the tumor-derived exosomes with the naked eye or by a smartphone with the assistance of lyophilized gold nanoparticles.\textsuperscript{35} This the intelligent DNA hydrogel involves no separate reporting probes and no pre-amplification stage but only one-step assay from molecular recognition to signal response, and to the final visual readout, minimum the reliance on expensive instruments, making it more considerable promise for sensitive bioanalysis.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

All custom-synthetic, HPLC-purified oligonucleotides (Table S1) employed in this work were purchased from Shanghai Sangon Biotechnology Co., Ltd. Acrylamide was obtained from GENERAL-REAGENT Shanghai Titan Technology Co., Ltd. N, N, N', N'-Tetramethylethylenediamine (TEMED), ammonium persulfate (APS), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Cas12a was purchased from Guangzhou BIO-LIEE Biotechnology Co., Ltd. RPMI 1640 medium and Fetal Bovine Serum (FBS) were acquired from Viva Cell (Shanghai, China), and penicillin/streptomycin solution and PBS were purchased from Gibco (NY, USA). Anti-CD63 antibody, Anti-TSG101 antibody, Cytochrome C, Gold Colloid, and 1× TE Buffer were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). FILTER PAPERS 1 was bought from Whatman (UK). 5× TBE buffer were purchased from Solarbio Life Sciences (Beijing China). Human hepatocellular carcinoma HepG2 cells were obtained from the American Type Culture Collection (ATCC, USA). Super GelRed nuclear staining was acquired from Biotium (Fremont, CA, USA). Glucose and Sodium chloride (NaCl) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. All solutions were made with the ultrapure water (resistivity of 18.2 MΩ/cm) procured from the Millipore ultrapure water system. Other reagents were at least analytical grade.

2.2 Apparatus

The fluorescence spectra assay was analyzed via Thermo Scientific Varioskan Flash (MA, USA). The flow
cytometry (FCM) analysis was performed using a BD FACSCanto II flow cytometer (BD, USA). Transmission electron microscopy (TEM) images were acquired on a JEM-1200EX instrument (Japan). Scanning electron microscopy (SEM) images were recorded on a ZEISS Sigma 300 instrument (Germany). Nanoparticle tracking analysis (NTA) was conducted utilizing the Malvern NanoSight NS300 (UK). Rheological test was carried out on the Anton Paar MCR 302 (Austria). Rheological experiments were performed on the Anton Paar MCR 302 (Austria). The Laser confocal microscope (CLSM) imaging was performed utilizing a ZEISS LSM780 (Zeiss, Germany).

2.3 Cell culture and exosome isolation

HepG2 cells were cultured in 1640 medium containing 1% (v/v) penicillin-streptomycin and 10% (v/v) fetal bovine serum (FBS) in a humid atmosphere of 5% CO$_2$. The supernatant was removed and the serum-free medium was added until a 70% enrichment of HepG2 cells was obtained. After 48 h, the supernatant was collected to extract the exosomes. To be specific, the collected supernatant was centrifuged at 3000 g for 15 min to remove cell debris and large particles. Then the large granular vesicles were discarded after filtration (0.22 µm pore size) and ultracentrifugation (11,000 g, 30 min). Subsequently, the supernatant was ultracentrifuged at 120,000 g for 2 h to obtain exosome particles. Finally, the exosome particles were resuspended with PBS and stored at -80°C prior to the experiment. The morphology and the concentration of exosomes were measured by TEM, and NTA, respectively. Besides, western blotting analysis with CD63 antibody was used to confirm the expression of surface proteins of exosomes, and the binding efficiency of CD63 aptamer (AP) and exosomes was analyzed by the NanoFCM platform.

2.4 Fabrication of functional DNA hydrogel

Before the fabrication, the strand S1 was first hybridized with CD 63 aptamer (AP) heated at 95°C for 5 minutes and then cooled down to 4°C at a rate of 4°C per minute to obtain a pre-hybridized solution (S1-AP). At the same time, 150 nM Cas12a was incubated with 180 nM crRNA in 1× reaction buffer (10 mM Tris-HCl, 5 mM MgCl$_2$) at 25°C for 20 min.

Then, the intelligent DNA hydrogel was constructed by the following steps. First, DNA-grafted polyacrylamide chains were synthesized. The pre-hybridized solution (S1-AP) and S2 separately were mixed with 4% acrylamide, 1×Tris-EDTA buffer (TEB, pH 8.0), 200 mM NaCl and ultrapure water, and degassed at 37 for 10 min in a vacuum oven to form S1-AP-acr and S2-acr. The polyacrylamide-DNA (PA-S1-AP and PA-S2) was obtained after the polymerization by adding the freshly prepared APS (0.06%, m/v) and TEMED (0.6%, v/v) were introduced into the above solution and degassing for 6 min at 37°C to initiate polymerization to obtain polyacrylamide-DNA (PS1-AP, PS2). Finally, the prepared PS1-AP (14 µM) and PS2 (14 µM) were mixed and incubated at 65°C for 5 min, then the Cas12a/crRNA (150 nM) was added and incubated at 45°C for 10 min to ensure homogeneity. Then the mixture was cooled slowly down to 25°C to form the intelligent DNA hydrogel.

2.5 Optimization of the detection

The key parameters of DNA hydrogel construction were explored to obtain an optimized performance of this assay. To be specific, for the cross-linking density of DNA hydrogel, the concentration of the acrylamide (4, 6, 8, 10, and 12 wt%) and the concentration of DNA probes (S1, S2 and AP) (6, 8, 10, 12, and 14 µL) were determined. Besides, the optimum DNA probe concentration and acrylamide concentration was also by the thermogram by combining two single factors. For the different reaction time for exosomes detection (30, 40, 50, 60, and 70 min) and temperature (21, 29, 37, 45, 53) were carried out to gain the optimal results. In addition, since the embedding amount of Cas12a/crRNA in DNA hydrogel can significantly affect the sensitivity of the analysis, the concentration of Cas12 (25, 50, 100, 150, and 200 nM) was optimized as well.

2.6 The fluorescence detection of exosomes

Under the optimized conditions, the tumor-derived exosomes with certain concentrations were added into the reaction 10× buffer (100 mM NaCl, 150 mM MgCl$_2$, 100 mM Tris-HCl (pH 9.0), 0.5% Tween-20, 10 mM DTT). After adding the above solution into the intelligent DNA hydrogel, the reaction was activated and
then incubated at 37°C, 300 rpm for 1 h, under the dark condition. After that, the fluorescence intensity at 520 nm of the reaction mixture was measured by a Thermo Scientific Varioskan Flash (MA, USA). The fluorescence intensity of different groups of responses was utilized for calculating the sensitivity of the intelligent DNA hydrogel. LOD was defined as the minimum detectable concentration that had a fluorescence intensity greater than the negative control.

In addition, polyacrylamide gel electrophoresis (PAGE) was performed to verify the binding of different DNA strands. Specifically, all samples were incubated at 25 and then mixed with loading buffer (5:1 volume ratio), then a 12% gel electrophoresis was performed at 125 V for 40 min in 1 x TAE buffer. After being stained with gel red, the obtained gels were imaged with GenoSens (1860).

To assess the specificity, the exosomes (1x10^5 particles/µL) and three interferents including glucose, cytochrome C and BSA with a concentration of 6 µg/mL, were tested under the optimized fluorescence detection. Finally, 6 parallel tests for exosomes (1x10^5 particles/µL) were conducted using the proposed fluorescence assay and the tests’ relative standard deviation (RSD) was calculated to evaluate the repeatability.

2.7 The colorimetric detection of exosomes

Preparation of freeze-dried colorimetric filter paper: a workable filter paper was cut into a disc shape (5 mm in diameter), 20 µL of NaCl (500 mM) solution was added dropwise, and NaCl loaded colorimetric filter paper was obtained by freeze-drying. In the same way, the cut filter paper was soaked in nano-gold solution (9x10^11 particles/mL), and then freeze-dried to obtain the colorimetric filter paper loaded with gold nanoparticles. The two filter papers loaded with NaCl and loaded with gold nanoparticles were fixed on the lid of centrifugal tube.

Optimizing the conditions for color development: Firstly, the reaction of gold nanoparticles with different concentrations of NaCl (25, 50, 75, 100, 200 mM) was explored in solution state. Secondly, the DNA loading capacity of different filter paper specifications (Filter paper, Whatman paper, Non-woven fabrics, Glass fiber membrane) were tested to screen the most suitable filter paper substrate.

As mentioned in the section 2.4 and 2.5, the intelligent DNA hydrogel was constructed in a 100 µL centrifuge tube. After adding the target exosomes into this tube, the mixture was incubated at 37°C for 60 min. During the above process, the prepared colorimetric paper was always fixed in the lid of the centrifuge tube with upright state. However, the centrifugal tube was inverted at the end of the reaction and the colorimetric paper in the lid absorbed the sol-liquid in the tube, then the tube was placed back to upright state with a visible color displayed on the lid after 5 min.

Moreover, the lid image of the test tube was taken by smartphone and the colorimetric detection was conducted by analyzing the RGB information of the region of interest (ROI) on the colored lid, by using the Colorpicker App in a smartphone. The sensitivity of the proposed method was determined by investigating the relationship between the diluted exosomes and the relative color change. The visual LOD was defined as the lowest exosome concentration to produce an observable pale purple color by naked eye evaluation by a random group of 30 people. The R/B value was calculated according to the equation below:

\[
R/B(\%) = \frac{Rx}{Bx} \times 100\%
\]

(Rx and Bx respectively represent the R value and B value obtained from the image of colorimetric paper with different concentrations of exosomes input in the detection system.)

2.8 The applicability for testing clinical samples

In addition, since this sensing platform is used for the detection of biological samples, the real serum samples, including healthy individuals (H), patients (P), and post-treatment patients with HCC (PT), with 20 people in each group, were randomly collected from the volunteers at the Southwestern Hospital, and were
tested to confirm the applicability in clinical field. To be specific, the above serums were centrifuged at 5000 g for 30 min. The upper plasma was collected and continued to be centrifuged at 500 g for 10 min. Then the supernatant was collected and centrifuged at 15000 g for another 20 min. Finally, the exosomes solution was obtained by resuspension precipitation with PBS for subsequent detection analysis (referring to the above fluorescence detection and colorimetric detection schemes). The p-value represents the difference in fluorescence intensity of exosomes responses between different groups. The colorimetric results were analyzed using algorithmic clustering analysis. This research proposal was approved by Southwest Hospital. Participants provided written informed consent to participate in this study.

2.9 Statistical Analysis

Error bar displayed in the figures in this work was obtained by performing triplicate experiments except for special statements and the data was reported as mean ± standard deviation. Comparison between group P and group PT was performed using independent-sample T-tests. Ap-value less than 0.05 or 0.001 was considered to be statistically significant of the observed difference, with the mark by asterisks * or ****, respectively. Statistical analysis was carried out by SPSS (version 20.0). The limit of detection was estimated based on 3σ/ s (σ represents the standard deviation of blank sample (without exosomes), and s is the slope of linear calibration). Different groups (P, PT, H) of clustering analysis were conducted using the K-means algorithm in Python.

3 DISCUSSION

3.1 Design of the dual-mode detection

Previously reported strategies for exosome detection often involved multiple steps and required specialized instruments for result interpretation. Therefore, we designed the fluorescence and colorimetric dual-mode strategy to detect the exosomes based on the programmable CRISPR-Cas12a and DNA hydrogels. As represented in Figure 1, in the fluorescence detection, the functional DNA hydrogel was first constructed wrapping with Cas12a/crRNA and cross-linked nuclide acid sequences inside. At this stage, a soft-solid functional DNA hydrogel with less liquid and low fluorescence intensity was formed. When the target exosome exists, the dissociation of the functional DNA hydrogel was triggered by exosomes recognizing the AP due to its function as a bridge linker of the cross-linked nuclide acid sequence. Meanwhile, the above change led to the transformation of the soft-solid texture to a more sol-liquid and high fluorescence intensity mixture. This one-step response assay ensured the fluorescence signal trapped inside unless the appearance of the target, avoiding the false positive result and amplifying the fluorescence intensity, and can finally improve the sensitivity. In the colorimetric detection, the freeze-dried gold nanoparticles and NaCl was loaded on the filter paper fixed on the lid on the tube. After the previous reaction, the tube was inverted until the liquid wet the filter paper, then the color transition from blue to red could be observed by naked eye with the increase of the concentration of the target exosome. Besides, a semi-quantitative detection was established by analyzing the RGB information with a smart phone. This rapid and portable colorimetric assay ensured a secondary confirmation of test results without extra laboratory instruments, providing a simple and portable way to interpret the results.

3.2 Characterization of the exosomes and functional DNA hydrogel

The polymerization process of the intelligent DNA hydrogel is shown in Figure 2A. Under the catalysis of APS and TEMED, S1-AP and S2 can rapidly react with acrylamide to form polyacrylamide-DNA (PS1-AP, PS2), respectively, and then co-incubated with Cas12a/crRNA to form an intelligent DNA hydrogel. The successful synthesis of DNA hydrogels was verified by observing the morphology via SEM and testing its rheological properties. As shown in Figure 2B, comparing the storage modulus (G") and loss modulus (G') of the optimized DNA hydrogels, G" was always smaller than G', indicating the presence of gelation behavior in the DNA hydrogels. And the DNA hydrogels were seen to be multi-cross-linked 3D reticular structures in SEM images (Figure 2C, D).

In this work, exosomes purified from the supernatants of HepG2 cell culture were used as the analytes.
The concentration of exosomes analyzed by NTA was $2.2 \times 10^{10}$ particles/mL and the average hydrodynamic diameter was approximately 127 nm (Figure 2E). TEM image demonstrated that the small vesicles were wrapped in a bilayer of lipid molecules in exosomes (Figure 2F), consistent with previous reports. In addition, significant CD63 bands and TSG101 bands were observed by western blotting (Figure 2G), but the exosome negative marker calnexin was not expressed, which indicates that CD63 can serve as an effective biomarker for detecting exosomes.

The optimal formation of the intelligent DNA hydrogel is a prerequisite for the reaction. The factors during the synthesis of the intelligent DNA hydrogel were optimized, including acrylamide concentration and the concentration of DNA probes (S1, S2 and AP), to stabilize the multi-dimensional extension of polymeric chains. As shown in Figure S1A, the fluorescence intensity continued decreasing with the increase of acrylamide concentration, due to the high acrylamide concentration increases the stiffness of DNA agarose gel, resulting in a decrease in the efficiency of gel-to-sol transition. DNA concentration is not only the key factor of cross-linking density of DNA hydrogel but also the direct source of fluorescence signal of the detection platform. Figure S1B shows that the fluorescence intensity increased with increasing DNA concentration. Moreover, Figure S1C displays the results of the interaction of the above two parameters, which yielded the same results as the single-factor experiment. Therefore, the 14 $\mu$M of DNA hydrogel and 4% wt of acrylamide were chosen in the subsequent experiments.

### 3.3 Feasibility analysis of the working principle

Once the intelligent DNA hydrogels were established, it would keep a stable gel-solid state. One of the highlights of our design is that the AP sequences are not only functions as linkers of the DNA hydrogels, but also specific recognition elements for exosomes, which means that the more exosomes present in the system, the more AP sequences will be combined, thus leading to the less hydrogels formation. Meanwhile, the presence of exosomes witnesses the dissociation of the 3D crosslinked structure, exposing the branching sequences, releasing the Cas12a, which then serves as scissors to shear the lateral branches. Theoretically, nearly no fluorescence signal can be determined before adding the exosomes, while strong fluorescence signal will be generated with the exosome’s existence. Therefore, a certain correlation between fluorescence changes and the concentration of exosomes can be observed in this one-step response system.

It is worth mentioning that the switch element of the whole reaction is exosomes, which can be combined with the AP sequence competitively. Then the crosslink structure of that intelligent hydrogel is dissociated to release Cas12a activated by the exposed branching sequence and shears the lateral branches to generate a fluorescence signal, which realizes the one-step response of exosome detection (Figure 3A).

The synthesis of the intelligent DNA hydrogels and whether it can be successfully dissociated in the presence of exosomes is critical for the entire assay. First, PAGE analysis was performed to check the feasibility. As shown in Figure 3B, lanes 2, 3 and 4 represent S1, S2 and AP bands, respectively. Lanes 5, 6 display the bands of S1-AP duplexes and S2-AP duplexes, which demonstrates that S1 and S2 could cross-link with AP. Lane 7 shows the triple-stranded complex of S1 and S2 hybridized with AP (AP-S1-S2). While in the presence of exosomes, the bands significantly reduced in lane 8, indicating the dissociation of cross-linked structures. Whether the AP can bind to exosomes is a key step for whether the 3D network structure of the intelligent DNA hydrogels can be finally destructed. As shown in Figure 3C, the results of NanoFCM showed that about 99.6% of the exosomes were successfully recognized and bound by AP, indicating that the surface exosomes expressed abundant CD63 and were able to bind well to AP. In addition, the response process triggered by exosomes was also verified by fluorescence analysis. As shown in Figure 3D, nearly no fluorescence was observed without the presence of exosomes, proving that the disorderly cleavage activity of Cas12a/crRNA was not activated in this case, thus leading to a low fluorescence background of the intelligent DNA hydrogel. Oppositely, in the presence of exosomes, activated Cas12a/crRNA cleavage reaction yielded a significant fluorescent signal, which proved that exosomes could competitively dissociate AP sequences and expose S1 and S2 sequence. Besides, this phenomenon was illustrated more intuitively when the reaction system was exposed to UV irradiation (Figure 3E). As expected, the above results proved that the established the intelligent DNA hydrogel could collapse after the triggering of exosomes. At the same time, the
Cas12a/crRNA could be successfully activated and finally the fluorescence signal was released, indicating the designed assay is capable of detecting exosomes.

### 3.4 Detection performance of the fluorescence assay

Then to obtain the best performance of this biosensing method, parameters such as reaction time, temperature and concentration of Cas12a were optimized. As shown in Figure S2A, with the increase of reaction time, the number of APs competing with exosomes on the constructed functional DNA hydrogel was increasing, leading to the release of more Cas12a/crRNA. Subsequently, the activated CRISPR-Cas12a system performed its cleavage function, resulting in the restoration of fluorescence in the solution that was previously quenched. After 60 min, a stable and remarkable fluorescence intensity was observed, indicating that the reaction reached saturation in 60 min. A proper biological activity temperature is important for detection. As shown in Figure S2B, a low temperature resulted in weak fluorescence signal, which might be due to the low temperature led to the poor biological activity of exosomes and the stable structure of DNA hydrogel. However, a higher temperature above 37 caused a lower fluorescence response, which was attributed to the inactivation of exosomes and the inactivation of Cas12a protein at high temperature. Figure S2C depicts that the fluorescence intensity increased continuously until the concentration of Cas12a reached 150 nM. It is possible that the inactive Cas12a in the intelligent hydrogel binds to the substrate after reaching super-saturation concentration, thereby protecting the substrate from being cleaved by the inactive Cas12a. The above optimized conditions were performed for the subsequent test.

The fabricated the intelligent DNA hydrogel was employed for exosome detection under the optimized conditions. First, the sensitivity of the intelligent DNA hydrogel was investigated to evaluate its analytical performance. As shown in Figure 4A, the fluorescence intensity of DNA hydrogels increased with the increase of the concentration of exosomes and a good linear relationship ($R^2=0.9705$) was obtained between the logarithm of the target concentration ranging from $1\times10^3$ to $1\times10^7$ particles/$\mu$L and the fluorescence intensity (Figure 4B). The detection limit of exosome was 119 particles/$\mu$L, which was in accordance with the $3\sigma$ rule.

### 3.5 Specificity and reproducibility

To evaluate the specificity of the constructed method, the possible coexisting substances in biological samples were taken as control, such as Cyt C, Glucose, and BSA. The results shown in Figure 4D demonstrated the proposed intelligent hydrogel has good specificity for exosomes in distinguishing different biological samples, since only a considerable fluorescence intensity could be determined when exosomes were tested in intelligent hydrogel, which was significantly higher than that of the other three interferents. Then, the repeatability test of the detection system was carried out by constructing 6 parallel tests of exosomes with the concentration of $1\times10^5$ particles/$\mu$L (Figure 4E). The obtained 2.4% of RSD indicates a stable signal output 6 parallel tests, which is particularly important for the reliability and robustness of our established assay.

### 3.6 Detection performance of the colorimetric assay

In addition, to better expand the applicability and portability of detection method, a one-pot visual test strategy was proposed by loading a colorimetric filter paper in the lid of the detection tube. After the experimental operation in section 2.7, as illustrated in Figure 4A, the color of the tube lid will change from blue to red with the increase of exosome concentration. The main theoretical support for this inference is that more exosomes trigger more gel to sol transition, during which more DNA single strands will be released to avoid the aggregation of gold nanoparticles induced by NaCl, thereby achieving significantly different colors.

To verify the above scheme, the colorimetric filter paper was prepared and loaded in the tube lid (Figure S3A). Figure S3B showed the principle and the result of confirmatory experiment, when the target existed, the intelligent DNA hydrogel underwent a gel-to-sol transition. The sol contains a large amount of single-stranded DNA to protect the gold nanoparticles from aggregation, resulting in a red color on the colorimetric paper. Conversely, in the absence of the target, the double-stranded DNA encapsulated in the gel remains integrity state with less liquid. When the test tube was inverted, nearly no single-stranded DNA in the
liquid dissolved the colorimetric filter membrane, leading to the aggregation of gold nanoparticles into blue color. Such phenomenon demonstrated the successful design of this colorimetric assay. For a maximum color intensity of colorimetric filter paper, different concentrations of NaCl solution were checked to further determine the aggregation degree of gold nanoparticles and a remarkable blue color was observed at a concentration of 100 mM NaCl solution, while more input of NaCl resulted in less chroma (Figure S3C). Moreover, the adsorption capacity of the colorimetric filter paper for DNA and gold nanoparticles is crucial for the output of the results. Therefore, first, four kinds of colorimetric filter papers were chosen and their microscopic structure were observed under the electron microscope (Figure S4). Then, the DNA adsorption efficiency of each filter paper was studied under LSCM and results showed that the non-woven fabric membrane exhibits satisfactory DNA adsorption efficiency (Figure S5A).

Under the optimized conditions, the developed assay can be used to determine the exosomes by the naked eye. As shown in the Figure 4A and Figure S5B, the color of the colorimetric filter paper exhibited a blue-red transition as the concentration of exosomes increased from $1 \times 10^3$ to $1 \times 10^7$ particles/µL, whether viewed from the side or above. Besides, we noticed that less exosomes hardly caused color changes (Figure S5C), which might be attributed to the DNA hydrogel barely dissolves with excessively low exosome concentration. The color change could be recognized by the naked eye until the exosomes concentration reached $1 \times 10^5$ particles/µL, indicating the colorimetric detection performance is basically comparable to fluorescence assay. Still, to eliminate the influence of different individuals’ color sensitivity, we utilized smartphones to identify color information and generate a standard curve. This allows for semi-quantitative detection of the test samples, making the results more objective and traceable. The R and B values were identified using a smartphone and a good linear relationship ($R^2=0.976$) was calculated in the range of $1 \times 10^3$-$1 \times 10^7$ particles/µL (Figure 4C), and LOD was set as $1 \times 10^4$ particles/µL based on the visual recognition results of 30 people surveyed (Figure S6).

### 3.7 The applicability for testing clinical samples

To further evaluate the feasibility of the proposed intelligent DNA hydrogel in the real clinical diagnosis scenario (Figure 5A), 60 serum samples from healthy individuals, patients, and post-intervention patients with HCC (20 samples per each group) were randomly selected and analyzed by the proposed dual-mode detection (Figure S7). The pre-experiments for detection in serum samples were first performed. As shown in Figure 5B, the fluorescence intensities in buffer and serum were almost the same level, which indicates that the intelligent DNA hydrogel is applicable in complex matrix. In the fluorescence assay, as shown in Figure 5C, the fluorescence signal of healthy human samples was significantly lower than that of the patient group, which was consistent with previous reports.\(^{39}\) In the patient group, there was a significant difference ($p$ value $<0.0001$) in fluorescence signal between preoperative and post-interventional patient samples. In the colorimetric assay, after the K-means algorithm, 60 samples were scattered obviously into three clusters, which was turned out to be the group of patients, post-interventional patients and healthy individuals (Figure 5D), showing the considerable capability of colorimetric assay.

Finally, the comparison of fluorescence and colorimetric methods for the detection of exosomes was summarized in Table S2. Compared to other fluorescence methods, the LOD obtained by intelligent DNA hydrogel “one-step method” is equivalent to other single colorimetric methods and better than other fluorescence methods. Compared with other colorimetric methods, the operation process of our prefabricated colorimetric tube is simple, and naked-eye detection can be realized without large instruments. In addition, the results of dual-mode detection in a reaction tube can be mutually verified, which ensures the reliability of the results. Overall, with the advantages of high sensitivity, easy operation, rapid readout, and applicable in real serum samples, this developed dual-mold strategy could discriminate the cancer in different stages and might possess promising application in tumor outcome monitoring and prognostic assessment.

### 4 DISCUSSION

In summary, CRISPR/Cas12a signal amplification system was embedded in the spatial confinement based on DNA hydrogel, creating a novel and user-friendly the intelligent DNA hydrogel detection platform. In this
detection platform, the output of the results includes fluorescence signal and colorimetric signal, which enable both accurate quantification and rapid visual detection. By utilizing target recognition based on aptamers, the target signal was converted into the cutting efficiency of Cas12a/crRNA on the hydrogel skeleton chain. It is worth noting that the Cas12a/crRNA system was pre-encapsulated in the DNA hydrogel, enabling one-step completion of target input - signal output. The 3D mesh structure of the DNA hydrogel increases the chances of aptamer-target binding and provides a rich surface area, thereby enhancing the sensitivity of detection. A detection limit of 119 particles/µL was achieved, and good specificity was maintained even in the presence of other interfering substances. Additionally, this reaction system enables rapid and convenient visual detection with LOD of $10^4$ particles/µL. In the detection of clinical samples, both fluorescence and colorimetric detection have demonstrated good ability to identify differences in the expression levels of exosomes between healthy individuals and patients. However, further research still needs to be done to advance its applicability in testing the real clinical samples since the sample pool of this study is not big enough. Although that may be the case, our approach still provides some guidance in exosomes detection, and the detection strategy provided by this the intelligent DNA hydrogel can be extended to detect other biological targets by changing its adapter sequence, which will lay the foundation for the combination detection of disease biomarkers and improve the accuracy of disease diagnosis.

ACKNOWLEDGEMENTS

The authors would like to thank all participants who were willing to participate in this study. The authors would also like to thank all the medical and experimental staff who provided technical support and assistance. This study was supported by the National Key Research and Development Program of China (No. 2022YFC2603800), the National Natural Science Foundation of China (No. 82122042, 82030066, 81972027, 82372352), Chongqing Science Fund for Distinguished Young Scholars (No. CSTB2022NSCQ-JQX0007, CSTB2022NSCQ-MSX0862) and Chongqing Medical Scientific Research Project (No. 2023ZDXM021).

CONFLICT OF INTERESTS

The authors declare that no competing interests exist.

REFERENCES


38. R. T. Fuchs, J. L. Curcuru, M. Mabuchi, A. Noireterre, P. R. Weigele, Z. Sun, G. B. Robb, *Communications biology* 2022, 5, 325.


**Figure S**

**FIGURE 1** Overview of the dual-mode detection of exosomes based on the intelligent DNA hydrogel loaded with CRISPR/Cas system
FIGURE 2 Characterizations of the intelligent DNA hydrogel and exosomes. (A) The process of synthesizing the intelligent DNA hydrogel loaded with CRISPR/Cas system. (B) The rheological parameters of the synthesized the intelligent DNA hydrogel. (C) and (D) Establishment of the 3D network structure of the intelligent DNA hydrogel under the SEM microscope. (Scale bar: 5 µm and 10 µm). (E) Results of the average size of exosomes tested by NTA. (F) The TEM images of exosomes. (Scale bar: 100 nm). (G) Western blot analysis of CD63 and TSG101 expression on exosomes.
Hosted file

FIGURE 3 Feasibility of the working principle. (A) Schematic illustration of exosomes triggering dissociation, activation and cleavage of the intelligent DNA hydrogel. (B) 12% PAGE analysis of functional DNA synthesis process. Lane 1: 500 DNA ladder; Lane 2: S1; Lane 3: S2; Lane 4: AP; Lane 5: S1 and AP; Lane 6: S2 and AP; Lane 7: S1, S2 and AP; Lane 8: Exosomes, S1, S2 and AP (C) The banding efficiency of AP to exosomes analyzed by nano flow cytometry. (D) Fluorescence spectra with response to the presence of target and absence of target from 560 to 650 nm. (E) Results of differential fluorescence of target (-) and target (+) under UV light.

FIGURE 4 The performance of the intelligent DNA hydrogel. (A) Illustration of the schematic diagram of dual-mode (fluorescence and colorimetry) of signals. Inset: Fluorescence spectra and images of color filter paper with response to different concentrations of exosomes from 505 nm to 580 nm. a-f: Blank (without exosomes), $1 \times 10^3$ particles/µL, $1 \times 10^4$ particles/µL, $1 \times 10^5$ particles/µL, $1 \times 10^6$ particles/µL, $1 \times 10^7$ particles/µL. (B) The linear relationship between fluorescence intensities and the logarithmic values of different exosomes concentrations. (C) The linear relationship between R/B values and the logarithmic values of different exosomes concentrations. (D) Result of the specificity. (E) Repeatability of the intelligent DNA hydrogel.
FIGURE 5 Applicability of the detection of exosomes in real samples by dual-mode detection. (A) The extraction of exosomes from serum samples. (B) Fluorescence response in different environments without (1) and with (2) exosomes, data are presented as mean ± SD, and significance is determined using independent-samples T-tests. *p < 0.05. (C) Scatter plot corresponding to fluorescence intensity of clinical samples (healthy individuals (H), patients (P), and post-treatment patients with HCC (PT), with 20 people in each group) analyzed by the proposed method. Significance is determined using ANOVA with post hoc multiple comparisons (LSD). ****p < 0.0001. (D) Cluster analysis diagram. Dots with different colors represent colorimetric values of different samples.