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ORIGINAL PAPER

Immunoassay-type biosensor based on magnetic nanoparticle capture and the fluorescence signal formed by horseradish peroxidase catalysis for tumor-related exosome determination

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Abstract

A sandwich-type fluorescent biosensor for the determination of tumor-related exosome was designed. It is based on magnetic nanoparticle (MNP) capture and horseradish peroxidase (HRP) catalysis. MNPs were used as the substrate to capture exosomes by modifying the CD63 antibody on MNPs surface. After that, the biotinylated epithelial cell adhesion molecule (EpCAM) antibody was used to capture the tumor-related exosomes, which specifically express EpCAM. A novel method for the fluorescence measurement of tumor-associated exosome was achieved, with a detection limit as low as 200 (± 9) particles mL^{-1}. The analytical range of this method is from 576 (± 15) particles mL^{-1} to 5.76×10^7 ($\pm 5.1 \times 10^5$) particles mL^{-1}. For the fluorescence measurement, the excitation wavelength was set to 320 nm. Fluorescent spectra were collected at emission wavelength in the range 370 to 550 nm; the data shown in the calibration plot were studied by using the fluorescence intensity at 406 nm. This sensor was also able to successfully detect the exosomes from the plasma of patients with hepatocellular carcinoma (HCC) and healthy humans.

Keywords Fluorescent biosensor · Cancer diagnosis · Sandwich structure · Biomarker · CD63 · EpCAM

Introduction

With the increase in the incidence of cancer, early detection is the most effective way to reduce cancer mortality in humans [1]. Tissue biopsy has always been the gold standard diagnostic procedure for tumors [2]. However, tissue biopsy has some limitations, such as their invasiveness and inability to reflect the spatial heterogeneity of tumors [3]. Liquid biopsy has gradually been considered as an approach for clinical diagnosis of cancers. It is a technique that utilizes circulating biomarkers in the body fluids of cancer patients to provide information about cancer [4]. It focuses on the detection of circulating tumor cells, circulating tumor DNA and exosomes [5]. Liquid biopsy can be used to analyze the molecular characterization of the tumor without the need for a tissue biopsy. It is vitally important to choose the specific biomarkers in liquid biopsies, and the exosome is one of the most potential detection objects in liquid biopsy [6].

Exosomes, membrane-bound extracellular vesicles (EVs) of 30–200 nm in diameter [7], have been reported existed in a wide number of biological fluids [8]. They are secreted by most types of cells into the extracellular space [8]. When exosomes are circulated in biofluids, they are served as important mediators of intercellular communication which has a close relationship with the development and progression of cancer [9]. In cancer, exosomes are associated with the crosstalk between cancer cells and normal cells, thus promoting malignant processes [9]. Exosomes have now been widely

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evaluated as the new generation biomarker in cancer because of its superiority: more homogeneous in size, higher content in biofluids, containing more specific markers (RNAs, DNAs, lipids, and proteins [10].

Exosome analysis has attracted great attention; many different analytical methods of exosome have been studied. Transmission electron microscopy (TEM) can provide a high-resolution image of exosome but requires extensive sample fixation, it may also destroy the samples [11]. In atomic force microscopy (AFM) [12], a mechanical cantilever is passed over a surface, with deflections indicating the presence of surface structures. With the possibility of sub-nanometer resolution, AFM is particularly suited to assessments of exosome morphology [13]. Cryo-electron microscopy can obtain the original state of exosomes but need high operating technology [14]. Dynamic light scattering (DLS) calculates the average size of relatively monodisperse populations of isolated particles, it has also been used to analyze exosomes [7]. Nanoparticle tracking analysis (NTA) can obtain information about the concentration and size distribution of exosome [15]. But NTA lacks the standardization for exosome analysis which undermines its compatibility under different conditions [15]. Resistive pulse sensing (RPS) can also be used to measure the concentration and diameter of exosome [16]. These methods are all about the characterization of the physical properties of exosomes. Western blot and enzyme-linked immunosorbent assay (ELISA) can quantitatively analyze the marker proteins of exosomes [11]. Flow cytometry realized characterizing the membrane proteins of exosome by binding micron-sized latex bead and stained fluorescent antibody respectively [17]. Mass spectrometry (MS) enables us to identify, quantify, and validate the proteins [18] and lipids [19] in exosome. Because of the size of exosome nanoscale, the determination of exosome remains a significant challenge. Although many methods for exosome analysis are mentioned above, each of these techniques can only give a certain aspect information of exosome. Therefore, we usually choose several methods combined for a comprehensive analysis of exosome.

Several new methods for exosome detection have been reported in the literature, including fluorescence resonance energy transfer (FRET) platform [20], fluorescence polarization technique [21], hybridization chain reaction [22], electrochemical biosensor [14, 23], surface plasmon resonance biosensor [24] and plasmonic biosensor [25]. Among them, we designed a novel biosensor that can determine the concentration of exosomes based on the measurements of fluorescence intensity. In this design, horseradish peroxidase (HRP) plays an extremely important role. HRP is a monomeric heme-containing redox enzyme [26]; it has many ideal properties such as high stability, monomeric structure, and high turnover rate [27]. Depending on the categories of substrates, different signals such as optical signals and electrochemistry signals can be produced under the HRP catalysis [28]. Tyramine is a phenolic derivative that can serve as a substrate of HRP [29] and itself has no fluorescence. In the presence of hydrogen peroxide (H_2O_2), HRP can catalyze tyramine to form a dimer and generate fluorescent signals [30]. Based on the above description, we have designed an ultrasensitive biosensor that can detect cancer-sourced exosomes by the determination of the fluorescent signals produced by HRP catalyzed tyramine.

Therefore, we report a specific and sensitive approach for the determination of cancer-derived exosomes, which combined HRP with magnetic nanoparticles (MNPs). There are enriched specific proteins expressed on the surface of exosomes, including CD63 and epithelial cell adhesion molecules (EpCAM) [31]. CD63 is a characteristic membrane tetraspanin protein present in exosomes [32]. EpCAM is a transmembrane glycoprotein that is viewed as an important biomarker of cancers; it is also a cancer-associated antigen [32]. MNPs were used as the substrate to capture exosomes, CD63 protein that on exosome surface can bind to CD63 antibody on MNPs. And the biotinylated EpCAM antibody can bind to the EpCAM proteins on the surface of cancer-derived exosomes specifically. One molecule of streptavidin can bind to four-molecule biotin with high specificity [33]. HRP-modified antibodies were introduced by forming a sandwich structure of "antibody-exosome-antibody." In the presence of H_2O_2, HRP can catalyze tyramine to form a dimer and produce fluorescent signals. We selected two antibodies to enhance the specificity by forming the sandwich structures. MNPs have good collecting ability and can be separated from matrix by an external magnetic field to reduce matrix signal interference. Thus, a novel, high sensitivity, and specificity biosensor is designed successfully and it is suitable for detection of exosomes from various biological samples, especially the tumor-sourced exosomes. The strategy of this sensor may become a promising and potential method for the ultrasensitive determination of exosome in the early clinical diagnosis of cancers and medical research.

Experimental

Materials and reagents

Bovine serum albumin (BSA, ≥ 96%), streptavidin-modified HRP (SA-HRP), N-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA; www.sigmaaldrich.com). Anti-CD63 antibody and biotinylated anti-EpCAM antibody were purchased from Abcam (Cambridge, MA, UK; www.abcam.cn). Carboxylated MNPs were bought from XFNANO Co., Ltd. (Nanjing, China; www.xfnano.com); the catalog number of MNPs is xfj69. The human liver cancer HepG2 cell line was from American Type Culture Collection. The other reagents

were of analytical grade and used without further purification. Ultrapure water prepared from a Millipore water purification system (a resistivity of 18 MΩ·cm, Milli-Q Direct 8) was used in all runs. All experiments of this study were carried out in strict accordance with the institutional regulations and relevant laws (National Health Commission of the People's Republic of China, ethical review of biomedical research involving human beings). The biological samples such as the plasma of patients with hepatocellular carcinoma and the healthy human plasma were acquired from Affiliated Dongfeng General Hospital, Hubei University of Medicine. This study was approved by the Dongfeng General Hospital's Ethics Committee (Shiyan, China). Informed consent was obtained for any experiment performed on patients with hepatocellular carcinoma and healthy human subjects.

Cell culture

HepG2 cells were cultured in Dulbecco's modified essential medium (DMEM, Cellgro). The culture medium contained about 10% fetal bovine serum, 1% 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. HepG2 cells were cultured at 37 °C in a 5% CO_2 humidified incubator.

Extraction and purification of exosome

The exosomes were collected and purified from the conditional medium after cells were cultured in the exosome-depleted medium for 48 h. Then, we use standard ultracentrifugation to distinguish the exosomes from the conditional medium. Firstly, the medium was centrifuged at 300*g* at 4 °C for 10 min to remove the cells. Then, to remove the cell debris and apoptotic blebs, a 20 min centrifugation with 2000*g* was used. The supernatant was then filtered through a 0.22-μm pore filter (Millipore). After that, the filtrate was further centrifuged at 10,000*g* for 30 min to avoid the cell vesicles and biomacromolecules. The supernatant again centrifuged at 100,000*g* at 4 °C for 70 min, and the sediment was resuspended in 20 mL sterile phosphate-buffered saline (PBS). Then, the resuspending was pelleted again by ultracentrifugation for 2 h at 100, 000*g* at 4 °C. Finally, we discard the supernatant and the residual sediment was the exosomes. The pelleted exosomes that were resuspended in 200 μL of PBS were stored at − 80 °C and ready to be used.

Characterization of exosome

TEM

Exosome preparations were analyzed by negative staining transmission electron microscopy (TEM) as previously described. Four hundred–mesh carbon-coated copper grid was floated (carbon side down) on top of a 20 μL droplet of exosome suspensions for 5 min. The samples then fixed on the copper grid for 20 min with 2% glutaraldehyde (Sigma-Aldrich; www.sigmaaldrich.com), followed by drying in the room temperature. Grids were then dipped in 2% phosphotungstic acid for dyeing purposes. Images were obtained in a transmission electron microscope (TEM) (Hitachi H-7000 NAR) operating at 80 kV soon after the samples were rinsed with PBS and dried at room temperature.

NTA

Nanoparticle tracking analysis (NTA) can be used to analyze the size distribution and concentration of exosome particles. Briefly, suspensions were diluted in PBS until an optimum concentration of particles was obtained (1/400) after filtrating through a 0.22-μm pore filter. After that, samples were injected into the equipment and illuminated by the NTA laser beam. The typical Brownian motion of exosomes can scatter lights, and be observed by a microscope equipped with a 405 nm laser and sCMOS camera (Zeta view Ltd., Germany). With the optimal settings, the individual exosomes in the suspensions can be visualized. All tests were performed at room temperature. Each sample was tested 3 times.

Western blot

Western blot can be used to demonstrate the presence of marker proteins reportedly associated with exosomes in preparations. We selected two proteins to characterize exosomes, including CD63 and EpCAM. The purified exosome preparations were mixed with loading buffer directly (99 °C, 5 min), and the proteins can be separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis was suspended after the protein of exosomes electrophoretic to the bottom of SDS-PAGE. After that, we transferred the proteins from SDS-PAGE gels to the polyvinylidene difluoride (PVDF) membranes. Then, we incubated it with milk for 1 h, added CD63 and EpCAM, and shook slowly overnight at 4 °C. Next, we added the goat anti-rabbit antibody and incubated for 2 h at room temperature. Finally, we analyzed the images of gels.

Procedures for exosome determination

Fluorescent biosensing processes of exosomes determination include the following steps. Firstly, the MNPs were carboxylated and dissolved in PBS to prepare the MNPs mixed solution with the concentration from 20 to 200 μg mL^{-1}. Secondly, the EDC/NHS mixed solution was added into the MNPs solution to activate the carboxyl group for 30 min. After magnetic field separation, the supernatant was discarded and the product was dissolved in 1 mL PBS to prepare the mixed solution of carboxylated MNPs (concentration of

100 μg mL^{-1}). Then, we added CD63 antibody; the carboxylated MNPs were modified with the CD63 antibody and sealed with BAS. For exosome determination, exosomes and biotinylated EpCAM antibody were added to the above MNPs at room temperature. Subsequently, HRP-modified streptavidin was added, and a sandwich structure of "antibody-exosome-antibody" was formed by combining MNPs modified with antibodies, SA-HRP, and exosomes. Finally, the MNPs complexes were added to the solution containing tyramine and H_2O_2, and to quartz sample cell for fluorescence measurements. Wash MNPs with PBS after each step of the above reaction of modified antibody.

Fluorescence measurements

The fluorescence measurement was carried out at room temperature on Hitachi F-4600 spectrophotometer (Hitachi Co. Ltd. Japan, https://www.hitachi.co.jp) equipped with a xenon lamp excitation source. The excitation wavelength was set at 320 nm; fluorescent spectra were collected at emission wavelength from 370 to 550 nm. The optimum experimental parameters were studied by using the fluorescence intensity at 406 nm, and the performance of this designed detection system was also evaluated. The excitation and emission intervals were both set at 5 nm. Excepted for the special groups, the fluorescence intensity of each group was measured three times and calculated the standard deviation. The quantitative determination of exosomes was expressed by the final fluorescence intensity.

Results and discussion

Determination mechanism

Figure 1 shows a fluorescence method for determination of exosomes using MNPs as a substrate and using tyramine to form a dimer in the presence of HRP. Due to both EpCAM and CD63 protein being expressed on the surface of the exosome, CD63-modified MNPs was designed as the substrate to capture exosomes. After that, the biotinylated EpCAM antibody was added to capture tumor-associated exosomes that have bound with MNPs, which rely on the specific binding between the EpCAM antibody and EpCAM protein. Finally, streptavidin-modified HRP (SA-HRP) was introduced and formed the sandwich structure of "antibody-exosome-antibody." Add tyramine as substrate and H_2O_2 as catalyzer after the sandwich structure has been formed. Tyramine is a phenolic derivative that can serve as a substrate of HRP. In the presence of H_2O_2, SA-HRP can catalyze tyramine to form a dimer and generate fluorescent signals. As a result, the concentration of tumor-related exosome can be quantified by measuring the fluorescence intensity. There is a linear relationship between the fluorescence intensity and the content of tumor-related exosomes in a certain concentration range. Thus, the content of tumor-associated exosomes can be quantified specifically by the measurement of the final fluorescence intensity.

Characterization of exosomes

To obtain the hepatic carcinoma-specific exosomes, we isolated exosomes from the culture medium of HepG2 cell lines by ultracentrifugation. The extracted exosomes were characterized by the TEM and NTA. As shown in Fig. 2a, a prototypical double-membrane morphology was observed for the majority of exosomes and the diameter of exosomes was about 150 nm in the TEM. As seen in Fig. 2b, nanoparticle tracking analysis (NTA) measurement revealed that the size distribution is within the range of 50 to 200 nm and the concentration is about 4.7×10^{10} particles mL^{-1}. These characterization results reveal that a large number of exosomes with narrow and uniform size can be extracted according to our strategy. The results of the western blot are shown in Fig. 2c; two clear bands can be seen that represent the expression of CD63 and EpCAM on exosome surface. This is the key part of the assay mechanism that our sensor can detect exosomes specifically.

Optimization of experimental parameters

To achieve the higher sensitivity performance and better results for exosome determination, the following parameters were optimized: (a) concentration of CD63 antibody; (b) concentration of biotin-EpCAM antibody; (c) concentration of MNPs; (d) the reaction time; (e) concentration of SA-HRP; (f) concentration of tyramine. Respective text and figures on

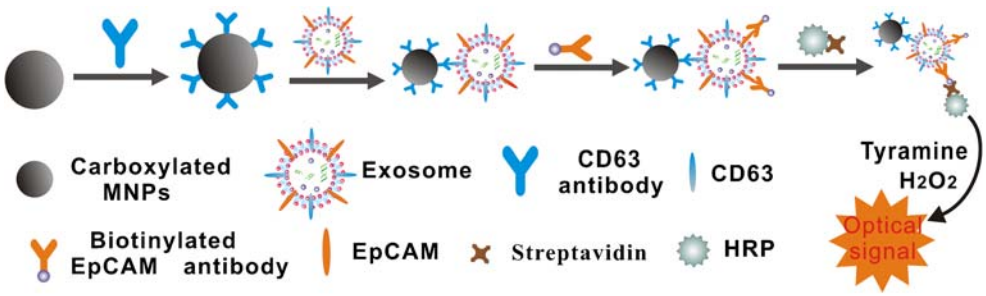

Fig. 1 Schematic illustration of the immunoassay-type fluorescent biosensor for exosome detection based on combining magnetic nanoparticle capture and horseradish peroxidase (HRP) catalysis

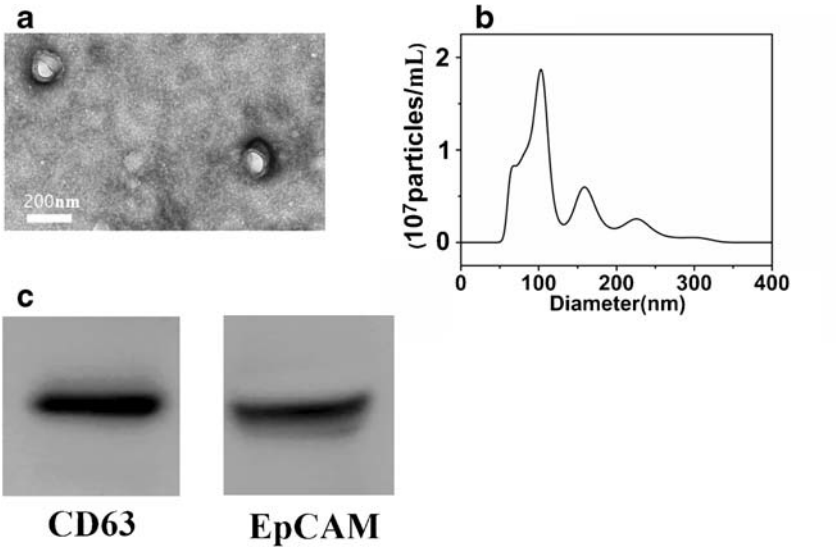

Fig. 2 Characterization of exosomes. **a** The TEM imagine of exosome. **b** The NTA analysis of exosome. **c** The western blot of exosome

optimizations are given in the Electronic Supporting Material. The fluorescence intensity was chosen to evaluate the effects of the experimental parameters on the sensing performance of our biosensor strategy. Each experiment was repeated three times ($n = 3$), and the result is the average of three times. In short, the following experimental conditions were found to give best results: (a) best concentration of CD63 antibody, 10 μg mL^{-1}; (b) best concentration of biotin-EpCAM antibody, 6 μg mL^{-1}; (c) best concentration of MNPs, 100 μg mL^{-1}; (d) best reaction time, 15 min; (e) best concentration of SA-HRP, 15 μg mL^{-1}; (f) best concentration of tyramine, 0.6 mg mL^{-1}.

Sensitivity for exosome determination

In addition to optimizing the parameters of the reaction, the sensitivity of the proposed biosensor strategy for exosome determination was also evaluated at different concentrations. Each experiment was repeated three times ($n = 3$), and the result is the average of three times. As shown in Fig. 3a, we find that the fluorescence intensity increased with the increasing concentration of exosome, from 0 to 5.76×10^8 ($\pm 5.2 \times 10^6$)particles mL^{-1}. It can be seen from Fig. 3a that there is a strong correlation between the concentration of exosome and the fluorescence intensity. Figure 3 b displays a good linear relationship between the fluorescence intensity and the logarithm of exosome concentration, in the range from 576 (± 15) particles mL^{-1} to 5.76×10^7 ($\pm 5.1 \times 10^5$) particles mL^{-1}. After the calculation of the test data, the regression equation of lg C and the fluorescence intensity was expressed as $y = 91.7$ lg C – 127.3 ($R^2 = 0.9898$), where lg C is the logarithm of exosome concentration (particles mL^{-1}). The detection limit of this sensor for exosome was calculated to be 200 (± 9) particles mL^{-1} (according to the 3S rule). As shown in Table 1, the exosome determination

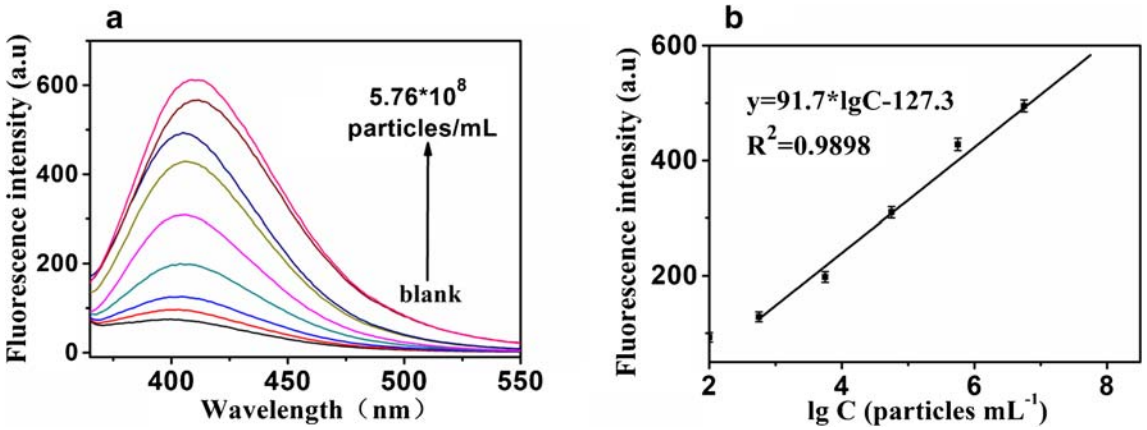

Fig.3 **a** Fluorescence emission spectra of the biosensor in the presence of exosome with different concentrations: from bottom to top, 0 to 5.76×10^8 particles mL^{-1}. **b** The fluorescence intensity as a function of exosome concentration. It shows the strong correlation between fluorescence intensity and exosome concentration and the emission wavelength of 519 nm. Error bars: SD, $n = 3$

Table 1 The comparison between our method and other reported biosensors for the detection of exosomes.

Analytical method	Detection limit	Linear range	Ref.
Fluorescence resonance energy transfer (FRET) platform	1.4×10^3 particles mL^{-1}	10^4 to 10^9 particles mL^{-1}	[20]
Fluorescence polarization biosensor	17.5×10^8 particles mL^{-1}	5×10^8 to 7.6×10^9 particles mL^{-1}	[21]
Hybridization chain reaction	100 particles mL^{-1}	1000 to 10^7 particles mL^{-1}	[22]
Electrochemical biosensor	1.72×10^4 particles mL^{-1}	1×10^5 to 5×10^7 particles mL^{-1}	[23]
Electrochemical biosensor	2.09×10^4 particles mL^{-1}	10^5 to 10^{12} particles mL^{-1}	[14]
Surface plasmon resonance biosensor	8.28×10^6 particles mL^{-1}	1.0×10^8 to 3.0×10^{12} particles mL^{-1}	[24]
Plasmonic biosensor	1 ng mL^{-1}	1 to 10^3 ng mL^{-1}	[25]
Sandwich-type fluorescent biosensor	200 particles mL^{-1}	576 to 5.76×10^7 particles mL^{-1}	This method

performance of our strategy was also compared with that reported in most other literature.

Specificity and stability

There are a lot of proteins in real biological samples, so we test whether these proteins will interfere with the determination of exosomes. The specificity of this strategy for the determination of exosome was also reviewed by adding five different kinds of protein, which include CD63 and the other four different types of proteins, BSA, Tn C, D-dimer, and PSA. At the same time, we also added normal cells as a blank control group to study whether it will affect the determination of exosomes. Testing these five different kinds of protein under the same conditions evaluates the specificity of this method for exosome determination. The results are shown in Fig. 4; by comparing with the blank control group, the fluorescence signal can be neglected in the presence of BSA, Tn C, D-dimer, and PSA. However, a non-negligible enhancement of fluorescence signal emerged in the presence of CD63 by comparing it with the other five groups. These results indicated there is an excellent specificity of this proposed biosensor for the exosome determination. The excellent specificity of this biosensor is mainly ascribed to the specific recognition and high affinity between the specific antibodies and the targets. The carboxylated MNPs of unmodified CD63 antibody has good stability, and the modified MNPs with CD63 antibody are also stable within 24 h. One week later, their activity is only about 50% of the original state. The corresponding figures and data are presented in the Electronic Supporting Material.

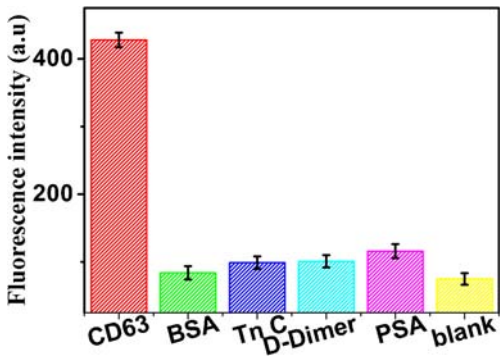

Fig. 4 Fluorescence intensity (at the emission wavelength of 519 nm) of the sensor in the presence of CD63 (10 ng mL^{-1}), BSA (50 ng mL^{-1}), TnC (50 ng mL^{-1}), D-dimer (50 ng mL^{-1}), PSA (50 ng mL^{-1}), and blank, respectively. Error bars: SD, $n = 3$

Determination of exosome in real samples

To further test the feasibility and potential application value of this strategy in biological samples, the plasma of patients with hepatocellular carcinoma (HCC) and healthy humans were prepared respectively. Firstly, exosomes were extracted from the plasma of patients with HCC and healthy humans. The fluorescence intensity of exosomes from patients with HCC and healthy human were measured by adding them to the

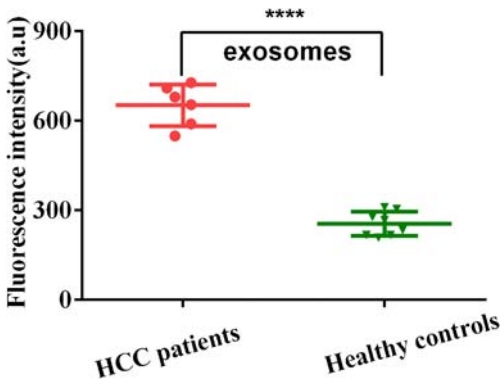

Fig. 5 Fluorescence intensity of the sensor for the detection of exosome from HCC patients and healthy controls. (****$P < 0.0001$) Error bars: SD, $n = 3$

mixture solution. As shown in Fig. 5, a significant increase in fluorescence intensity of HCC patients' exosome was observed by comparing with the group of healthy controls. Besides, the results also showed that there was an obvious statistical difference in the determination of exosomes between patients with HCC and normal human ($P < 0.0001$). It can be seen from the above description that using the proposed sensor to analyze tumor-related exosome in biological samples was feasible. This sensor can be a potential analytical method to detect tumor-related exosomes from the real biological samples sensitively.

Conclusions

In conclusion, we have successfully designed a fluorescent biosensor for the determination of tumor-derived exosome. Due to the specific recognition between the exosome surface antibodies and the targets, the sensitive and specific detection of tumor-derived exosome was achieved. This sensor is suitable for the determination of tumor-related exosomes. For long-term consideration, the sensor also has some limitations. If it is to be used in clinical, it needs a lot of tests and analysis; it also needs to eliminate the differences between the clinical samples. The activity of the sensor decreases by the day, the determination of exosomes needs to be completed in 1 day. Based on the different biomarkers expressed in different tumors, we believe that the sandwich structure of the "antibody-target-antibody" biosensor may be a potential strategy for the determination of other biomarkers by selecting the corresponding antibody in the early clinical diagnosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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