A Visible and Colorimetric Aptasensor Based on DNA-capped Single-Walled Carbon Nanotubes for Detection of Exosomes

Yaokun Xia^a, Mengmeng Liu^a, Liangliang Wang^a, An Yan^a, Wenhui He^a, Mei Chen^a, Jianming Lan^a, Jiaoxing Xu^{b,c}, Lunhui Guan^{b,c,*}, Jinghua Chen^{a,*}

^a Department of Pharmaceutical Analysis, The School of Pharmacy, Fujian Medical University, Fuzhou, Fujian Province 350108 (P. R. China) E-mail: cjh_huaxue@126.com

^b Key Laboratory of Design and Assembly of Functional Nanostructures, Fujian Institute of Research on the Structure of Matter, Chinese Academy of Sciences, Fuzhou, Fujian Province 350108 (P. R. China) E-mail: guanlh@fjirsm.ac.cn

^c Fujian Provincial Key Laboratory of Nanomaterials, Fujian Institute of Research on the Structure of Matter, Chinese Academy of Sciences, Fuzhou, Fujian Province 350108 (P. R. China) E-mail: guanlh@fjirsm.ac.cn

*Corresponding authors: Dr. Jinghua Chen (cjh_huaxue@126.com), Dr. Lunhui Guan (guanlh@fjirsm.ac.cn)

Abstract

Recently, many studies have shown the potential use of circulating exosomes as novel biomarkers for monitoring and predicting a number of complex diseases, including cancer. However, reliable and cost-effective detection of exosomes in routine clinical settings, still remain a difficult task, mainly due to the lack of adequately easy and fast assay platforms. Therefore, we demonstrate here the development of a visible and simple method for the detection of exosomes by integrating single-walled carbon nanotubes that being excellent water solubility (s-SWCNTs) and aptamer. Aptamers, specific to exosomes transmembrane protein CD63, are absorbed onto the surface of s-SWCNTs and improve the mimic peroxidase activity of s-SWCNTs, which can efficiently catalyze H_2O_2 -mediated oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) and lead to a change from colorless to blue in solution. However, after adding exosomes, the aptamers are bound with CD63, leaving from the surface of s-SWCNTs through conformational changes, which results the color of solution from deep to moderate, and this can be observed by the naked eye and monitored by UV-vis spectrometry. Under optimal conditions, the linear range of exosomes is estimated to be 1.84×10^6 to 2.21×10^7 particles/ μL with a detection of limit (LOD) of 5.2×10^5 particles/ μL . Consequently, a visible and simple approach detecting exosomes is successfully constructed. Moreover, this proposed colorimetric aptasensor can be universally applicable for the detection of other targets by simple change the aptamer.

Keywords: Visible and colorimetric aptasensor; DNA-capped SWCNTs; Exosomes

1. Introduction

Exosomes have become a research focus in recent years because of their unique function with cell behavior and disease diagnosis ([Chiu et al., 2016](#)). As nano-sized extracellular vesicles with sizes of 30-100 nm, exosomes are secreted from various cells in the body, including tumor cells, neurons, immune cells and reticulocytes, and their pathological and physiological significance have been paid attention by researchers ([Lee et al., 2015](#); [Zhou, Y.G. et al., 2016](#); [Gajos-Michniewicz et al., 2014](#); [Hu et al., 2012](#); [Valadi et al., 2007](#); [Rajendran et al., 2014](#)). Currently, the exosomes that exist in bodily fluids are thought to carry abundant molecular information derived from their originating cells, including microRNA, carbohydrates and protein, and mediate intercellular communications by conveying information ([Lee et al., 2016](#); [Schorey and Bhatnagar, 2008](#)). More importantly, exosomes have been reported to involve in tumor antigens, anti-tumor immune response and tumor diagnostics, which is close related to public health ([Clark et al., 2015](#)). These unique properties make exosomes become a potential biological candidate for diseases therapy as well as diagnosis. Thus, detection of exosomes provides foundation for further relevant studies.

To date, the exosomes should be purified by ultracentrifugation before analysis. Afterwards, exosomes are analyzed by various methods, including western blot ([Zhou et al., 2006](#)), flow cytometry ([Clayton et al., 2001](#)) or enzyme-linked immunosorbent ([Ueda et al., 2014](#)). Although these methods are effective and robust, the apparent shortcomings such as time-consuming operations, expensive instruments, and rely

heavily on the sample handling skills severely limit their widely applications. Hence, the development of a simple strategy to detect exosomes is significant importance, at best as high sensitivity and low cost. Recently, Jeong et al. reported a rapid, on-site exosomes screening assay based on an integrated magnetic-electrochemical coupling with antigen-antibody reaction (Jeong et al., 2016). Beside electrochemistry, surface plasmon resonance (SPR) was also applied to exosomes analysis (Zhu et al., 2014). However, most assays involved in antibody microarrays specific to the exosomes membrane proteins, the stable source of antibody required expensive, laborious, and time consuming preparation process. And the results also were acquired by expensive instruments and complicated operations. In order to overcome the disadvantages of antibody, Zhou et al. reported an electrochemical aptasensor that took advantage of aptamer specific to CD63 (a transmembrane protein commonly present in exosomes) to analysis exosomes. The signal probes that hybridization with aptamer were pre-labeled with redox moieties. And the signal probes were released in the presence of exosomes, causing change of electrochemical signal (Zhou, Q. et al., 2016). This method indeed introduced aptamer, rather than antibody, which improved the stability of this system. However, it needed laborious labeling procedures, which was complicated. Luckily, in the past decades, much research has concentrated at the peroxidase that was a ubiquitous oxidative enzyme due to its efficient catalytic activity for electron donor substrate in the presence of H_2O_2 , causing obvious color change, and the colorimetric assay showed promise to solve the concerned issues, with theoretical and technical simplicity (Rodríguez-López et al., 2001).

As a common peroxidase, because of high substrate specificity and catalytic activity, horseradish peroxidase (HRP) has been commercialized for biochemical applications ([Xianyu et al., 2013](#); [Zeng et al., 2015](#); [Zhao et al., 2016](#)). Although the benefits of natural enzyme are already apparent, some disadvantages still limit their widespread use, such as complicated preparation, purification, and storage, instability of structure ([He, S.B. et al., 2014](#)). Therefore, the development of natural enzyme mimics and/or artificial enzyme with unique properties is importance. The investigation on artificial enzymes has recently gained considerable interest because of inherent advantages over their counterparts. A wide range of nanomaterials such as gold nanoparticles ([Weerathunge et al., 2014](#)), iron oxide nanoparticles ([Liu and Liu, 2015](#)), V₂O₅ nanowires ([André et al., 2011](#)), carboxyl-modified graphene oxide ([Song et al., 2010a](#)), graphene oxide-platinum/gold nanoparticle ([Zhang et al., 2015b](#)), carbon nanodots ([Shi et al., 2011a](#)), Co₃O₄ NPs ([Mu et al., 2012](#)), CuO NPs ([Chen et al., 2011](#)), CoFe₂O₄ magnetic NPs ([Shi et al., 2011b](#)), Au@Pt nanostructures ([He et al., 2011](#)), carbon nanotubes ([Cui et al., 2011](#)), Ag/Pt bimetallic nanoclusters ([Zheng et al., 2014](#)), Fe doped CeO₂ nanorods ([Jampaiah et al., 2016](#)), have been reported having intrinsic enzyme mimetic activity (nanozymes), which is similar to that found in natural peroxidase. Compared with HRP, these nanozymes are interesting because of low cost, bulk preparation, resistance to pH and temperature, which makes them attractive for various applications, particular for biomedicine ([Lu et al., 2015](#); [Zuo et al., 2009](#); [Chen et al., 2013](#)).

SWCNTs, a tubular nanomaterial, were considered as ideal candidates for

nanodevice applications over the past several decades (Li et al., 2013; Iverson et al., 2013; Zhang et al., 2015a). Intriguingly, the catalytic activity of SWCNTs has been studied in the presence of H_2O_2 (Heller et al., 2009). Song et al. reported that SWCNTs possessed intrinsic peroxidase-like activity and detected single-nucleotide polymorphism (SNP) based on TMB- H_2O_2 (Song et al., 2010b). Although the resulting SWCNTs could catalyze TMB- H_2O_2 with high catalytic activity, they were needed to treat by sulfuric and nitric acid, which was time-consuming, sophisticated, and even destroyed the integrity of SWCNTs. Besides, the SWCNTs were centrifuged during detection process due to their poor water solubility, which made the result of detection is not accurate enough.

Bearing this in mind, our group has prepared s-SWCNTs that are rich in carboxyl groups and good water solubility in the present study, which overcomes the poor water solubility and simplifies experimental procedure, and makes the s-SWCNTs are more suitable in analysis fields. At the same time, it is worth noting that our s-SWCNTs have weak peroxidase-like activity, catalyzing TMB- H_2O_2 from colorless to moderate blue, but DNA is able to increase the peroxidase activity of s-SWCNTs significantly, which is similar to other nanomaterials, such as Fe_3O_4 NPs and AuNPs (Liu and Liu, 2015; Hizir et al., 2016; Chen et al., 2016). More importantly, the peroxidase activity of s-SWCNTs can be regulated through altering DNA sequence, length and concentration. In addition, we utilized aptamer technology coupled with s-SWCNTs to demonstrate the possible application for exosomes detection in the present paper. The developed aptasensor detects exosomes through visual inspection

or UV-vis spectrophotometry, using TMB and aptamer-capped s-SWCNTs as substrate and catalyst of catalytic reaction, respectively, which is simple, rapid and intuitive. To the best of our knowledge, this is the first study to use DNA-capped s-SWCNTs for label-free detection of exosomes that from MCF-7 cells and breast cancer patient's serum. Furthermore, we envision that the proposed aptasensor holds excellent potential to be used in a wide target only by changing the aptamer.

2. Experimental Section

2.1. Reagents and apparatus

All oligonucleotides were synthesized by TaKaRa (Dalian, China), and their base sequences were illustrated in Table S1. The concentrations were quantified by OD260 based on their individual absorption coefficients. The sequence of CD 63 aptamer was designed according to relevant reference ([Zhou, Q. et al., 2016](#)). The TMB was purchased from Tokyo Chemical Industry (TCI, Shanghai). The 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) was purchased from Sangong Biotechnology Co. Ltd. (Shanghai, China). Fetal bovine serum (FBS) and roswell park memorial institute (RPMI) were purchased from Hyclone. MEGMTM BulletKit was purchased from Lonza. Exosomes Antibodies, Array & ELISA Kits were purchased from System Biosciences (SBI). The commercial single-walled carbon nanotubes (Hipco) were purchased from Carbon Nanotechnologies Incorporated Company (CNI[®]). All other chemicals were analytical grade and all aqueous solutions were prepared with Milli-Q water (18 M Ω).

The fourier transform infrared (FT-IR) experiment was carried out on a Thermo

Nicolet iS50 FT-IR spectrometer, scanning from 4000 to 500 cm^{-1} at room temperature. The fluorescence spectra were recorded with an F-4600 fluorescence spectrophotometer (HITACHI, Japan). UV-Vis absorbance spectra were measured with a Cary-60 spectrophotometer (Agilent Technologies) using a quartz cell with 1.0 cm optical pathway. The transmission electron microscopy (TEM) images were carried out on a JEM-2010 microscope (JEOL Ltd., Japan) at an acceleration voltage of 150 kV. The ultracentrifuge was Beckman Optima XPN-100 (U.S.A.).

2.2. The preparation of water-soluble SWCNTs

The high-purity pre-functionalized SWCNTs (p-SWCNTs) samples were provided by our group, and they prepared from high-temperature arc-discharged method were intact and poor functionalization on surface ([Wu et al., 2009](#)). The related FT-IR characterization was shown in Figure S1A (curve a), which indicated that there was little oxygen-containing group on their surface. To improve their water solubility, p-SWCNTs were functionalized with carboxylic groups according to the references ([Huang et al., 2014](#)). Briefly, (1) p-SWCNTs (50 mg) were first refluxed in toluene solution containing 3.2 g azodiisobutyronitrile (AIBN) at 75 °C for 4 h. (2) The products were treated in refluxing in the mixture of sodium hydroxide and methanol at 60 °C for 48 h. (3) The precipitates were neutralized with hydrochloric acid, and filtered, washed with deionized water and dried at 50 °C overnight, then the water-soluble SWCNTs (s-SWCNTs) were obtained. After treatment by above steps, the obtained s-SWCNTs beared with a large amount of carboxylic groups on surface (Figure S1A, curve b). At the same time, as shown in Figure S1B, transmission

electron microscopy (TEM) was performed to examine the morphologies of the s-SWCNTs, and all the s-SWCNTs showed bundle-like. Consequently, the difference between the p-SWCNTs and the s-SWCNTs was that the s-SWCNTs were more functionalization with carboxylic groups more soluble in water than the p-SWCNTs.

2.3. Cell culture and exosomes preparation

MCF-7 cells were cultured in RPMI containing 10 % FBS, 1 % penicillin and streptomycin. MCF-7 cells (1×10^8 cells) were cultured in RPMI containing 5 % FBS (exosomes depleted) for 48 h before ultracentrifugation. MCF-10A cells were cultured in minimal essential growth medium (MEGM) containing cholera toxin. All cell lines were maintained in a humidified atmosphere of 5 % CO₂ at 37 °C. Exosomes were isolated from above cell culture medium using the standard ultracentrifugation method with some slight modifications ([Clark et al., 2015](#)). Briefly, the cell culture medium was centrifuged at 300 g for 10 min, and then was centrifuged at 2000 g for 20 min and 11000 g for 45 min to remove intact cells, cells debris and protein. Subsequently, the supernatant was centrifuged at 100000 g for 2 h to acquire sediment exosomes. Last, the sediment exosomes were resuspended in PBS. The number of exosomes was quantified by Exosomes Antibodies, Array & ELISA Kits.

2.4. Exosomes detection

The system containing CD63-aptamer (0.8 μL, 100 μM) and 4 μL 0.1 mg/mL s-SWCNTs was mixed by vortex for a certain time. Then the different concentrations of exosomes solution were added into the mixtures and mixed by vortex. After 10 min, 5.0 μL TMB (10 mM) and 3.0 μL H₂O₂ (100 mM) were added into the above mixtures

until the final volume of 100 μ L with HAc-NaAc buffer. The resulting solutions were kept at 35 $^{\circ}$ C for 30 min in dark and measured by Cary-60 UV-vis spectrophotometer using a 1.0 cm quartz cell.

3. Results and discussion

3.1. The principle of the aptasensor

Herein, TMB is acted as peroxidase substrate. We find DNA-capped s-SWCNTs have higher peroxidase activity in solution than naked s-SWCNTs, catalyzing TMB from colorless to deep blue in the presence of H_2O_2 (Figure S2A). And the catalytic activity is the strongest if and only if all components (DNA, s-SWCNTs, TMB and H_2O_2) are presented in the system (Figure S2B). Therefore, in this manuscript, we constructed an aptasensor based on aptamer technology and s-SWCNTs to detect exosomes. Scheme 1 demonstrated the mechanism of this aptasensor. The aptamer was absorbed onto the surface of s-SWCNTs through noncovalent reactions. Due to higher affinity between aptamer-capped s-SWCNTs and TMB, the peroxidase activity could be improved. However, in the presence of exosomes, the aptamer could be bound with CD63, undergoing target-responsive structural changes followed by desorption from the s-SWCNTs surface, which made s-SWCNTs turn into their origin state, resulting in a decrease of catalytic activity. Thus, there were the decreased color change and absorbance to different degrees depending on the concentrations of exosomes. The results could be observed by either the naked eye or read by UV-vis spectrometer.

Scheme 1. should be inserted in here

3.2. The characterization and quantification of exosomes

Exosomes were purified from the cell culture supernatant of MCF-7 cells by ultracentrifugation. TEM was performed to examine the morphologies of the purified exosomes, showing a mean diameter of ~50 nm (Figure 1A), and the size was consistent with reported exosomes (Lee et al., 2015). In order to further validate the efficacy of the isolation of the exosomes, we analyzed CD63 protein by Western blotting, and observed obvious bandings (Figure 1B). To quantify the number of purified exosomes, we first established corresponding standard curve through commercial immunoassays (Exosomes Antibodies, Array & ELISA Kits, System Biosciences), and has been fitted ($A=0.07639N+0.06401$, $r=0.993$) (Figure 1C). According to above standard curve, the concentration of acquired exosomes was about 3.68×10^8 particles/ μL .

Figure 1. should be inserted in here

3.3. Investigation of the feasibility of our aptasensor by spectroscopy

The feasibility of our aptasensor was firstly studied. As shown in Figure 2A, the naked s-SWCNTs showed weak peroxidase activity (curve a, photo a), while the exosomes could not improve the catalytic ability of s-SWCNTs (curve b, photo b). However, when the CD63-aptamer was adsorbed on the surface of s-SWCNTs, and as a result, it had strong catalytic activity, leading to strong UV-vis absorbance and deep blue of solution (curve c, photo c). In contrary, in the presence of exosomes, the CD63-aptamer was desorbed from the surface of s-SWCNTs due to combination of CD63 and its aptamer. Thus, the catalytic activity was decreased, producing lower

UV-vis absorbance and moderate blue (curve d, photo d). According to literature, the fluorescence of fluorophore (FAM) labeled aptamer was quenched through fluorescence resonance energy transfer (FRET) when aptamer was absorbed by s-SWCNTs, while the fluorescence recovered in the presence of target, such as thrombin (Yang et al., 2008). Similarly, we also introduced the FAM labeled CD63-aptamer further validates our mechanism. As shown in Figure 2B, FAM labeled CD63-aptamer emitted strong fluorescence when s-SWCNTs and exosomes both were absent (curve a). However, the fluorescence was quenched by FRET upon the addition of s-SWCNTs (curve b). In the presence of exosomes, the increase of fluorescence signal was observed due to forming of exosomes-aptamer complex, causing CD63-aptamer desorption from s-SWCNTs surface (curve c). According to the results, we believed that the s-SWCNTs reverted in its original form, which resulted in a decrease of catalytic activity of s-SWCNTs and decrease color change.

Figure 2. should be inserted in here

3.4. Optimization of experimental conditions

Above results indicated that DNA improved peroxidase activity of s-SWCNTs. However, we speculated there were many factors to influence this effect. Therefore, these potential factors were investigated as following.

3.4.1 Optimization of pH value

The peroxidase activity of s-SWCNTs in the presence and absence of DNA at different pH (from pH 2.0 to 8.0) is studied (Figure S3A). The results illustrated that the catalytic activity of naked and DNA-capped s-SWCNTs was pH-dependent, and

the reaction rate was enhanced by DNA at each pH, and exhibited the strongest peroxidase activity at pH 4.0, so 4.0 was chosen as optimal pH value for further studies. We found the zeta potential of DNA-capped s-SWCNTs (-19.83 ± 3.65 mV) is more negative than naked s-SWCNTs (-11.32 ± 2.43 mV) at pH 4.0 (Figure S3B), which may facilitate the adsorption of positively charge substrate TMB ($pK_a=4.2$) (Liu and Liu, 2015), resulting more efficient catalytic activity.

3.4.2 Studying of peroxidase activity of DNA-capped different SWCNTs

As shown in Figure S4, the s-SWCNTs (tube a), carboxyl SWCNTs (c-SWCNTs, from XianFeng Nano, No: XFS21) (tube b), p-SWCNTs (tube c) and commercial Hipco SWCNTs (tube d) all possessed intrinsic peroxidase-like activity. Meanwhile, the p-SWCNTs had strong catalytic activity that may be due to their electronic structure and their interactions with H_2O_2 and TMB (Song et al., 2010b; Ma et al., 2008). However, due to poor water-solubility, the peroxidase activity of p-SWCNTs could not be enhanced by DNA (Figure S5). Interestingly, we found the catalytic activity of s-SWCNTs showed largest enhancement after adding DNA comparing with other SWCNTs (Table S2 and Figure S4), which depended on their good water solubility. Therefore, we compared the water solubility of above SWCNTs. From the Figure S6, the s-SWCNTs displayed homogeneous solution after being treated by ultrasound for 8.0 h. However, other SWCNTs all exhibited poor water solubility even agglomeration under the same conditions. It was worth noting that the c-SWCNTs and s-SWCNTs both had carboxyl groups, but difference in carboxyl groups' content may result different water solubility and distinct catalytic activity (Figure S1A). As far as

we know, the acidity may influence carboxyl groups. So we studied the zeta potential of s-SWCNTs at different pH. As shown in Figure S3B (left inset), the zeta potential became less negative with decreasing of pH value (from 9.0 to 3.0), even approximately to 0 mV at pH 3.0, which may attributed to a part of carboxyl groups being protonated at low pH value (Novio et al., 2014). At the same time, we found the s-SWCNTs had highly negative surface charge ($\text{Zeta}\zeta=-33\text{ mV}$) in neutral water solution (Figure S3B, left inset). The highly negative charge was an indication of the repulsive force and caused long term stability of s-SWCNTs, which was in accordance with the previous works of increased dispersion of SWCNTs (Alpatova et al., 2010). In contrast, the p-SWCNTs have poor water solubility due to less negative charge ($\text{Zeta}\zeta=-2.49\text{ mV}$), even through adding DNA (-8.60 mV) (Figure S3B, right inset). Consequently, the water solubility was an important factor for catalytic activity. Moreover, we believe with the emergence of various commercial water-soluble SWCNTs in the future, it will simplify the experimental steps and improve detection efficiency of our methods.

Under physiological condition, there is definite salt concentration in human body fluid. Good salt tolerance is an important property in this experiment. Therefore, we compared salt tolerance of s-SWCNTs and AuNPs with and without DNA, respectively. When the DNA-capped s-SWCNTs were dispersed in different concentration NaCl solutions, the solutions were still displaying uniformity (Figure S7A). Interestingly, when TMB- H_2O_2 was catalyzed by above DNA-capped s-SWCNTs, the UV-vis absorbance at 652 nm was a little change even treated by 150

mM NaCl (decreased 27.19 %) (Figure S7B). In contrary, the initial DNA-capped AuNPs was wine-red and stable (Figure S7C). However, with the increasing of concentration of NaCl, the color of solution become purplish red, bluish, and even gray, as depicted in Figure S7C (From a to f). The color changes attribute to aggregation of AuNPs. At the same time, the UV-vis absorbance of oxidized TMB at 652 nm is decreased 87.36 % after DNA-capped AuNPs being mixed with 150 mM NaCl (Figure S7D). Next, we also studied the salt tolerance of naked s-SWCNTs and naked AuNPs. As shown in Figure S7E, the s-SWCNTs exhibit uniformity even in the high concentration NaCl solution. However, the AuNPs display obvious color change with the increasing of concentration of NaCl (Figure S7F). These works evidenced the s-SWCNTs possess good salt tolerance. Based on above results, the s-SWCNTs were chosen for following experiments.

3.4.3 Optimization of DNA base

In order to illustrate the effect of DNA on the peroxidase property of s-SWCNTs, we tested the relationship between catalytic activity and different DNA sequences while keeping the total DNA concentration constant. Results demonstrated that the order of reaction rate was: C₁₅>G₁₅>A₁₅>T₁₅>No DNA (Figure S8A). C₁₅-capped s-SWCNTs showed the most remarkable enhancement compare with other polynucleotide sequences. The base of C₁₅ was protonated at pH 4.0 (the pK_a of cytosine is 4.5) (Liu and Liu, 2015), which resulted the C₁₅ being adsorbed more tightly due to highly negative charge of surface of s-SWCNTs and bind more TMB with DNA-capped s-SWCNTs, accelerating the color reaction. Above experiments

showed electrostatic reaction between DNA-capped s-SWCNTs and substrate (TMB) was the other important factor for catalytic activity. In order to further validate this hypothesis, we chose ABTS, a negatively charge substrate, to act as color reagent. As shown in Figure S8B, the naked s-SWCNTs had stronger catalytic activity than DNA-capped s-SWCNTs for ABTS. To further confirm this mechanism, we added different concentrations Na^+ in the presence of DNA. We found that the activity of peroxidase was improved with increasing of Na^+ (Figure S8C), which demonstrated that charge plays a vital role in catalytic activity in this system.

3.4.4 Optimization of DNA length and concentration

The catalytic activity in the presence of different length DNA (C_5 , C_{10} and C_{15}) was evaluated. The reaction rate was DNA length-dependent ($C_{15} > C_{10} > C_5 > \text{No DNA}$) (Figure S9A), and C_{15} displayed the strongest enhancement (we also found that there was stronger catalytic ability with the longer DNA. We tested catalytic ability of C_{15} -capped s-SWCNTs in this paper considering the CD63 aptamer containing 15 poly-cytosines). This phenomenon was ascribed to the longer DNA possessing more binding sites, leading to higher affinity with the s-SWCNTs and adsorbing more TMB on the surface of s-SWCNTs. The further study demonstrated that the reaction rate was also related to DNA concentration (Figure S9B). These experiments strongly indicated that electrostatic reaction between DNA-capped s-SWCNTs and substrate (TMB) was the other important factor for catalytic activity.

To understand the catalytic activity of DNA-capped s-SWCNTs, we acquired the corresponding K_m value through Michaelis-menten equation. The K_m was 55.52 μM

and 2.02 mM for TMB and H₂O₂, respectively (Figure S10). This phenomenon suggested that the DNA-capped s-SWCNTs had a strong peroxidase-like activity, which made it become a potential candidate for detection. The detailed information was shown in Support Information section 2.

3.4.5 Optimization of exosomes detection conditions

In this system, some experimental variables, including color-reaction temperature, s-SWCNTs concentration, H₂O₂ concentration, aptamer concentration, affecting the whole sensing process were investigated. The optimal conditions of this assay were as follows: 35 °C was used as the optimal reaction temperature for whole sensing process (Figure S11A). 3 mM H₂O₂ (Figure S11B), 4 µg/mL s-SWCNTs (Figure S11C), 0.8 µM aptamer (Figure S11D) were chosen in this work, respectively.

3.5. Analytical performance in determination of exosomes

3.5.1 The sensitivity of the aptasensor

To test the performance of this aptasensor, our method was used to detect exosomes under the optimum conditions. It could be found that the UV-vis absorbance at 652 nm decreased with the concentrations of exosomes from 1.84×10^6 particles/µL to 2.21×10^7 particles/µL (Figure 3A). The difference in color of solutions was significant with the change of concentration (Figure 3A, inset). And the absorbance was linearly dependent on the concentration of exosomes (Figure 3B). The correlation equation could be expressed as $\Delta A = 0.14135C$ (particles/µL) + 0.03682 ($r=0.999$), where the ΔA was defined as $A_0 - A$ (A_0 and A were the absorbance intensities at 652 nm in the absence and presence of exosomes, respectively). The

limit of detection (LOD) of designed aptasensor based on 3σ method was 5.2×10^5 particles/ μ L, which was 10 times lower than commercial immunoassays relying on anti-CD63 antibody for detection in buffer (Immunoassays from Exosomes Antibodies, Array & ELISA Kits, System Biosciences). We had compared the sensitivity of our method with currently available methods firstly. From the [Table 1](#), we found that the detection limit of our assay was almost equivalent to some method ([Yadav et al., 2016](#); [Daaboul et al., 2016](#); [Oliveira-Rodríguez et al., 2016](#)). Although other methods had higher sensitivity than our strategy, these methods involved antibody ([Doldán et al., 2016](#); [Jeong et al., 2016](#); [Zong et al., 2016](#); [Vaidyanathan et al., 2014](#); [Im et al., 2014](#)) and complicated labeling technology and unstable electrochemical technology ([Zhou, Q. et al., 2016](#); [Zhou, Y. G. et al., 2016](#)), which improved detection cost, and interfered the detection accuracy. Moreover, the whole detection process took 40 min from adding into exosomes to generating signals in our method. Meanwhile, we also compared the detection time of currently available assays and our method (listed in [Table 1](#)). The detection time of some assays was similar and even better than this manuscript ([Jeong et al., 2016](#); [Zhou, Q. et al., 2016](#); [Oliveira-Rodríguez et al., 2016](#)). Nevertheless, these experiments often needed antibody, which influenced the stability of detection. In addition, some of them needed complicated electrode pretreatment and multiple rinse procedure when detected exosomes, which was tedious. Based on above facts, our method was more efficient to detect exosomes.

3.5.2 The selectivity of the aptasensor

The specificity was a pivotal issue for this developed aptasensor. Consequently, we studied selectivity of the aptasensor for exosomes that from breast tumor cells (MCF-7 cells) and non-tumorigenic cells (MCF-10A cells). According to related literature, the CD63 expression of MCF-10A cells-derived exosomes was lower than MCF-7 cells-derived exosomes (Etayash et al., 2016). As shown in Figure 3C, the strong signal was observed when MCF-7 cells-derived exosomes were existed in this system. In contrast, the signal was much weak in the presence of MCF-10A cells-derived exosomes. The results were in accordance with that reported in the previous paper (Etayash et al., 2016), which demonstrated that our method had good selectivity due to the specificity of aptamer.

Figure 3. should be inserted in here

3.6. Determination of exosomes in real sample

To establish clinical utility of this aptasensor in testing the levels of exosomes, we quantified exosomes in serum from breast cancer patients (B) and healthy individuals (H). All experiments were performed in accordance with relevant guidelines and regulations and were approved by the Ethics Committee of Fujian Medical University Union Hospital (Ethical certification NO. E2014021). The serum was centrifuged with 3000 g for 45 min firstly. Then, the supernatant was filtered with 0.22 μ m filter member after ultracentrifuged with 110000 g for 90 min. Subsequently, the solution was centrifuged at 110000 g for 70 min to acquire sediment exosomes. Last, the sediment exosomes were resuspended in PBS for further study. According to relevant diluting factor, we calculated that this aptasensor quantification results

approximately 1.5 fold increased presence of exosomes in the breast cancer patients' serum compared to healthy people (Figure 4, the red columns). Due to the CD63 may also exist on surface of the non-tumorigenic cells-derived exosomes, which results the exosomes also can be detected in serum of healthy individuals. However, the CD63 of non-tumorigenic cells-derived exosomes is less than tumor cells-derived exosomes. Consequently, the relatively low signal is acquired when detect exosomes in healthy individuals serum, and the data corroborates previous literatures of increased secretion of exosomes in cancer patients, which demonstrates its close relationship with cancer (Kanwar et al., 2014). In order to investigate the accuracy of our results; we compared our method with commercial immunoassays as the reference (the green columns). As shown in Figure 4, the results obtained from this aptasensor are agreement with those obtained by commercial immunoassays with the same samples. This assay displayed that a visible rapid-quantification of exosomes could be achieved in an easy way. In addition, the design of this aptasensor also provided an advantage to detect other exosomes through change corresponding aptamer.

Figure 4. should be inserted in here

4. Conclusion

To conclude, water-soluble SWCNTs were prepared and the peroxidase activity of s-SWCNTs was increased by DNA for TMB oxidation, and the related mechanism was also studied. Moreover, we constructed a label-free, fast and visible aptasensor to detect exosomes based on this phenomenon, leading to low technical and instrumental demands. Interestingly, comparing with other strategies, our method had following

advantages: (1) This aptasensor utilized aptamer to instead of antibody, which improved the stability of system, resulting the better sensitivity than commercial immunoassays for exosomes detection. (2) Our strategy used label-free technology to detect exosomes, thus reducing the cost. (3) The aptasensor detected exosomes in homogeneous system, avoiding complicated rinse procedure, simplifying the experimental process. At the same time, the results were easy readout by naked eye. Above merits showed the possibility of developing portable Point of Care Testing (POCT) devices for exosomes detection. Meanwhile, we also realized that our method still had some disadvantages, for example, we are trying our best to develop a “signal-on” strategy to replace “signal-off” strategy that is susceptible to interference. In addition, with the development of biotechnology, more and more specific markers on the surface of exosomes and corresponding aptamer will be discovered. We envision that our approach is able to achieve the goal of detecting exosomes specifically, which leads to it have extensive applications in the future.

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Figure Captions

Scheme 1. Schematic representation of the detection mechanism of the proposed method for exosomes.

Figure 1. (A) TEM image of exosomes. (B) The CD63 proteins of MCF-7 cells and exosomes are treated by Western blotting. (C) The relationship between concentration of exosomes and UV-vis absorbance. The standard curve was acquired through Exosomes Antibodies, Array & ELISA Kits. The error bar represents three independent assays.

Figure 2. (A) Typical UV-vis absorption spectra of TMB-H₂O₂ + s-SWCNTs (curve a), TMB-H₂O₂ + s-SWCNTs + exosomes (curve b), TMB-H₂O₂ + s-SWCNTs + aptamer (curve c), TMB-H₂O₂ + s-SWCNTs + aptamer + exosomes (curve d). The inset shows the corresponding digital images. (B) Fluorescence spectra of an analogic labeled fluorescence approach for verification of mechanism of exosomes detection. (a) 0.8 μ M FAM labeled aptamer, (b) 0.8 μ M FAM labeled aptamer + 4 μ g/mL s-SWCNTs, (c) 0.8 μ M FAM labeled aptamer + 4 μ g/mL s-SWCNTs + 2.21×10^7 particles/ μ L exosomes. (Excitation wavelength: 470 nm, Excitation Slit: 2.5 nm, Emission slit: 5.0 nm, Voltage: 600 V)

Figure 3. (A) UV-vis absorption spectra of the proposed strategy in the absence and presence of different concentrations of exosomes in HAc-NaAc buffer (from a to m: the concentrations of exosomes are 0, 0.184, 0.368, 0.736, 1.104, 1.472, 1.84, 2.21, 2.58, 2.94, 3.31, 3.68, 4.05×10^7 particles/ μ L). The inset shows the corresponding digital images. (B) The linear relationship between ΔA against concentration of

exosomes in HAc-NaAc buffer. (C) The histogram of selectivity. Each data point represents the average value of three independent experiments with error bars indicated.

Figure 4. The exosomes concentrations in the serum obtained from breast cancer patients and healthy individuals.

Scheme 1.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Table 1. Comparison of the sensitivity and detection time of currently available methods for the detection of exosomes

No	Method	LOD (particles/ μ L)	Time	Reference
1	Electrochemical detection method	4.7×10^5	~60 min	Yadav et al., 2016
2	Electrochemical sandwich immunosensor	2×10^2	~60 min	Doldán et al., 2016
3	Aptamer-based electrochemical biosensor	1×10^3	~15 min	Zhou, Q. et al., 2016
4	Single particle interferometric reflectance imaging sensor	3.94×10^6	> 12 h	Daaboul et al., 2016
5	Lateral flow immunoassay	8.54×10^5	~15 min	Oliveira-Rodríguez et al., 2016
6	Electrochemical sensor	50	~130 min	Zhou, Y.G. et al., 2016
7	Microfluidic and fluorescence	Not given	~70 min	Kanwar et al., 2014
8	Integrated magneto-electrochemical sensor	3×10^4	~48 min	Jeong et al., 2016
9	Microfluidic and fluorescence	Not given	~100 min	He, M. et al., 2014
10	Surface-enhanced Raman scattering (SERS) based on SERS nanoprobes and magnetic nanobeads	1.2×10^3	~120 min	Zong et al., 2016
11	Alternating current electrohydro- dynamic methodology	2.76×10^3	~120 min	Vaidyanathan et al., 2014
12	Nano-plasmonic sensor	3×10^3	~60 min	Im et al., 2014
13	Aptasensor based on DNA-capped s-SWCNTs	5.2×10^5	~40 min	This work