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ORIGINAL PAPER

Chemiluminescence assay for the glycoprotein tenascin-C based on aptamer-modified carboxylated magnetic carbon nanoparticles

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Abstract
We report on a method for chemiluminescence (CL) determination of the glycoprotein tenascin-C. Carboxylated carbon nanoparticles (cCNPs) were prepared from activated carbon. Next, the cCNPs were conjugated to magnetic beads (MBs) with a diameter of ~1 μm by linking its carboxy groups to the amino groups of the MBs. The assay involves the following steps: (a) An aptamer labeled with the CL reagent N-(4-aminobutyl)-N-ethylisoluminol (ABEI) (the labeled aptamer) was adsorbed onto the surface of the carboxy-modified magnetic carbon nanoparticles to form labeled aptamer modified cCNPs-MBs. (b) On addition of sample tenascin-C, it will interact with the labeled aptamer to form a complex with the labeled aptamer. (c) This tenascin-aptamer complex is then dissociated from the surface of the particles and detected by CL whose intensity is linearly related to the concentration of tenascin-C in the 1 pM to 1 nM range. The detection limit is as low as 0.4 pM, and the RSD is 4.2 % at a 50 pM level (for n= 7). The method has been successfully applied to the determination of tenascin-C in human serum samples and holds promise as a widely applicable general platform for aptamer-based CL detection of proteins.

Keywords Tenascin-C · Carboxylic carbon nanoparticles · Magnetic beads · Chemiluminescence

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Introduction

Carbon-based nanomaterials, which include carbon nanotubes, fullerenes, nanofibers, graphene, nanodiamonds, carbon-based thin films and molecules, etc., have been widely studied and applied in nanotechnology, biosensing, and drug delivery, etc. [1].

Very recently, carbon nanoparticles, a new kind of carbon-based nanomaterial has been developed and researched. Carbon nanoparticles (also known as, carbon dots, carbon nanodots) are discrete nanoparticles of near spherical geometry with sizes below 10 nm [2]. Carbon nanoparticles are the newly emerged member of the nanocarbon material family [3]. But carbon nanoparticles have attracted much attention because they have many advantages, such as chemical inertness, lack of blinking, inherently low cytotoxicity, excellent biocompatibility, and amphiphilic characteristics depending on the surface capping materials [4–13]. Up to now, a few aptamer-based biosensors coupling with carbon nanoparticles have been exploited [14–17].

Tenascin-C is a large extracellular matrix protein newly expressed during tissue remodeling processes including angiogenesis, inflammation, and tumor growth. Tenascin-C is especially overexpressed in tumor tissues [18, 19]. Western blot was usually been used for Tenascin-C detection [20, 21]. Since the advantages of aptamers, such as the absence of risk, ease of production and chemical modification, and high affinity to targets, aptamers have attracted extensive attention. Kim's group has reported a method based on QD-conjugated aptamer for expression research of Tenascin-C in cancer cell. As far as we know, there are no other reports about Tenascin-C detection [22].

This work focuses on researching and developing a novel CL method for the determination of protein. The main idea in our work is that adsorption of CL reagent labeled aptamer on carbon nanoparticles results from competition of the

hydrophobic and electrostatic repulsion interactions. As a model system, the Tenascin-C was used as the interest target. In the presence of Tenascin-C, Tenascin-C aptamer preferentially binds with Tenascin-C and the Tenascin-C aptamer complex dissociates from the carbon nanoparticles. The released Tenascin-C aptamer complex was detected with CL. To the best of our knowledge, it is the first time that carbon nanoparticles were used for Tenascin-C detection with the merits of being simple and sensitive.

Experimental

Reagents and apparatus

Human Tenascin-C was purchased from EMD Millipore Corporation (Billerica, MA, USA, http://www.millipore.com/index.do). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA. www.sigmaaldrich.com). ABEI were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan. http://www.tcichemicals.com/). Amino groups modified magnetic beads (about 1 μm, 10 mg mL^{-1}) and magnetic racks were obtained from BaseLine ChromTech Research Centre (Tianjin, China. http://www.qiuhuan.com/). All the reagents were analytical grade and used without further purification.

The synthetic oligonucleotide was purchased from SBS Genetech Co. Ltd. (Beijing, China. http://www.sbsbio.com/default.asp). The sequence was as following [22, 23]:

5′- PO_4^3- CCTGCACTTGGCTTG GATTTCAGAAGG GAGACCC-3′

Chemiluminescence emission was detected with a FI-CL instrument (IFFM-E, Remex Analytical Instrument Co. Ltd., Xian, China. http://www.xaremex.com). Transmission electron microscopy (TEM) image was taken with a JEM-2000EX instrument (JEOL Ltd, Japan. http://www.jeol.com/). A TGL-16G centrifuge (Shanghai Anting Science Instrument Co., China. http://www.centrifuge.com.cn/) was used for centrifugation.

The Preparation of carboxylated carbon nanoparticles

Carboxylated carbon nanoparticles were prepared by adopting a procedure reported in the literature [17, 24]. In a typical procedure, 100 mg activated carbon was mixed with 20 mL 5 M HNO_3 and refluxed for 12 h. After cooled down to room temperature, the solution was neutralized by NaOH. Then the solution was collected by centrifugation (16,000 g for 30 min). The collected precipitates were prepared as carboxylic carbon nanoparticles solution of the concentration of 2.0 mg mL^{-1}. The formation of carbon nanoparticles was confirmed by TEM measurements. The diameter of the carbon nanoparticles was found to be 2~3 nm (Figure. S1, ESM).

Preparation of carboxylated carbon nanoparticles modified magnetic beads

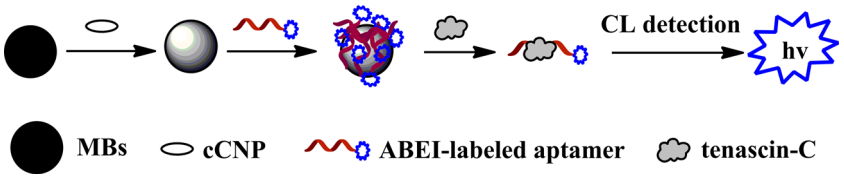
The carboxylic carbon nanoparticles modified magnetic beads were prepared as following [25, 26]. Briefly, 100 μL suspension of aminated MBs was placed in a 1.5 mL Eppendorf tube and separated from the solution on a magnetic rack. After washing three times with 200 μL of phosphate buffer solution, 20 mg EDC and 10 mg NHS in 1.0 mL water were added. And the mixture kept at room temperature for 30 min with continuous mixing. Then the resulting solution was washed three times with 200 μL of 0.1 M phosphate buffer solution and resuspended to a final volume of 100 μL. And then 100 μL carboxylic carbon nanoparticles was added to the above MBs, and incubated at 37 °C overnight. Finally, the resulting carboxylic carbon nanoparticles modified magnetic beads (cCNPs-MBs) were washed with 200 μL of 0.1 M phosphate buffer solution for three times, and resuspended in 200 μL phosphate buffer solution and stored at 4 °C for further use.

Preparation of labeled aptamer

Labeled aptamer were prepared according to the reference with a slight modification [26, 27]. Briefly, 200 μL of a 0.1 mol L^{-1} imidazole solution (pH 6.8) was added to the 2 OD (about 66 μg of DNA) 5′-phosphate terminal of aptamer for the activation of the phosphate group for 30 min, then 100 μL of 0.1 mol L^{-1} EDC and 100 μL of 1 mmol L^{-1} ABEI were added. The labeling reaction was incubated at room temperature for 12 h with shaking. Finally the solution was transferred to a 5 mL centrifuge tube, and 100 μL (1/5 volumes) of 3 mol L^{-1} sodium acetate and 2.0 mL (4 volumes) of 100 % cold ethanol was added. The solution was chilled for 8 h at −16 °C and then centrifuged for 20 min (18,000 rpm min^{-1}). The precipitate was washed with 200 μL of cold 70 % ethanol several times to remove any free ABEI. Finally, the resulting labeled aptamer was dissolved in 200 μL water and stored at −16 °C for futher use without dilution.

Fabrication of tenascin-C biosensor

The procedure of the fabrication of tenascin-C biosensor based on aptamer noncovalent assembly on cCNPs is illustrated in Scheme 1. Briefly, 10 μL labeled aptamer was added to 50 μL cCNPs-MBs solution. After incubate for 30 min., the



Scheme 1 Schematic diagram for the tenascin-C biosensor fabrication based on carboxylic carbon nanoparticles modified magnetic beads and labeled aptamer

mixture was separated with a magnetic rack. The precipitate was transferred to the quartz cuvette containing 50 μL tenascin-C sample sulution (containing 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$) and incubated at 4 °C about 30 min. After separation with a magnetic rack at 4 °C, supernatant was taken for CL detection.

Flow injection-chemiluminescence detection

The flow injection system used in this work is shown in Scheme S1. The sodium hydroxide solution delivered the samples in the sample loop solution to react with the mixture of hydrogen peroxide solution and cobalt ions solution in the flow cell to produce CL. And the CL signal was detected by IFFM-E FI-CL analyzer. The CL reaction was triggered by injecting supernatant with a syringe through a septum. The peak heights of the emission curves were measured by means of a photon counting unit. The peak height of intensities was used for quantification.

Preparation of blood samples

Fresh blood was collected in clean plastic tubes; no anticoagulant was added to the blood samples allowing the blood coagulation naturally and then centrifuged at 3,500 rpm for 5 min. The supernatant part was extracted as serum with a mini sample collector. Serums were stored at −20 °C before using. The serum was properly diluted in assay buffer before using.

Results and discussion

Fabrication of biosensor and detection process

It has been validated that ssDNA can absorb strongly to carboxylic carbon nanoparticles surfaces [28]. ssDNA containing hydrophobic aromatic structures in the nucleobases can be adsorbed on the hydrophobic regions of the cCNPs surfaces through the π-stacking. Milewhile, the electrostatic repulsion between the –COOH groups of cCNPs and phosphate groups of the oligonucleotides weaks the adsorption of the nucleobases on the cCNPs surfaces. These two competitive interactions result in a total decrease in the adsorption of oligonucleotides on cCNPs. Under the conditions that the electrostatic repulsion is less than the π-stacking interaction, the oligonucleotides will be adsorbed on cCNPs surfaces (Scheme 1).

For the fabrication of the tenascin-C sensor based on the interaction between carboxylic carbon nanoparticles and ssDNA, cCNPs-MBs was first prepared with the cCNPs and MBs. Then the labeled aptamer was added into cCNPs-MBs to formed labeled aptamer modified cCNPs-MBs. Then the sample solution was added to the labeled aptamer modified cCNPs-MBs solution to make tenascin-C bind to labeled aptamer. Then the formed tenascin-C and labeled aptamer complex dissociated from the cCNPs-MBs. After separation on a magnetic rack, the supernatant was taken for CL detection.

To examine the successful adsorption of labeled aptamer to cCNPs-MBs mentioned above, we tested the CL intensities of labeled aptamer and cCNPs-MBs to investigate experimentally if the labeled aptamer was absorbed to cCNPs-MBs. The experimental results were show in Fig. 1. The cCNPs-MBs showed weak CL signal. And the labeled aptamer gave strong CL signal. After separation with a magnetic rack when labeled aptamer being added into cCNPs-MBs, the cCNPs-MBs gave strong CL signal. And the supernatant which contained labeled aptamer gave weaker CL signal than the labeled aptamer. This contributed to the decreasing of concentration of labeled aptamer in supernatant. Whereas, using ABEI to substitute labeled aptamer, at this time cCNPs-MBs produced weak CL signal. This change largely originated from adsorption of labeled

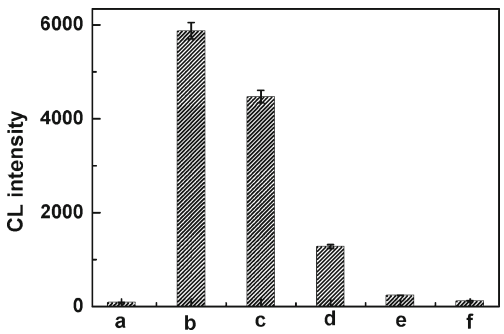


Fig. 1 CL responses of different solutions: cCNPs-MBs solution (**a**), labeled aptamer solution (**b**), cCNPs-MBs solution after treated with labeled aptamer (**c**), the supernatant of cCNPs-MBs after treated with labeled aptamer (**d**), cCNPs-MBs solution after treated with ABEI (**e**), MBs solution after treated with labeled aptamer (**f**) respectively

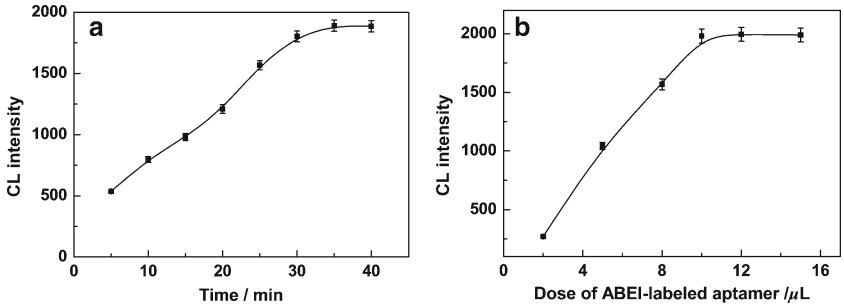


Fig. 2 The effect of binding time of tenascin-C with labeled aptamer modified cCNPs-MBs (**a**) and dose of labeled aptamer (**b**) on CL intensity

aptamer on the cCNPs-MBs. Furthermore, we also studied if the labeled aptamer was absorbed onto cCNPs-MBs through the interaction between ssDNA and cCNPs. cCNPs-MBs was replaced by MBs. After treated MBs with labeled aptamer, the MBs gave no obviously CL intensity. It can be concluded that the labeled aptamer was absorbed onto cCNPs-MBs through the interaction between ssDNA and cCNPs [28].

The effect of binding time of tenascin-C with labeled aptamer modified cCNPs-MBs on chemiluminescence intensity

In order to get the optimal analytical parameters, the relative experimental conditions such as binding time of tenascin-C with labeled aptamer modified cCNPs-MBs and dose of labeled aptamer were investigated.

The optimal binding time of the tenascin-C with labeled aptamer modified cCNPs-MBs was first studied. Figure 2a shows the effect of the binding time of tenascin-C with labeled aptamer modified cCNPs-MBs on CL intensity. It is apparent that CL intensity obviously increases with increasing the binding time from 5 to 30 min and then reaches a plateau in 35 min. This suggested that 35 min was enough for the tenascin-C to bind aptamer and induce the tenascin-C labeled aptamer complex displacement from the surface of cCNPs-MBs. Thus, 35 min was the binding time chosen in the following experiments.

The effect of dose of labeled aptamer on chemiluminescence intensity

The experimental results showed that the dose of labeled aptamer added into cCNPs-MBs solution greatly affected the CL response. Figure 2b shows the effect of dose of labeled aptamer on CL intensity. The cCNPs-MBs solution was keep 50 μL. It was found that the CL intensity increased with an increase in dose of labeled aptamer in the range from 2 to 15 μL, and reaches a plateau over the dose of 10 μL. The maximal CL signal was obtained at 12 μL labeled aptamer. Therefore, the dose of labeled aptamer was chosen 12 μL as the optimal concentration.

The effect of parameters of Flow injection-chemiluminescence system on chemiluminescence intensity

The parameters of FI-CL system were also investigated systematically to obtain the optimal conditions for the CL reaction. Considering both the CL intensity and the signal/background ratio, supernatant which contained tenascin-C and labeled aptamer complex was first mixed with H_2O_2 and then with Co^{2+}. Furthermore, the pH values of the sodium hydroxide solution and the concentrations of H_2O_2, Co^{2+} standard solutions were also studied [29]. The optimal pH values of the sodium hydroxide solution and concentrations of H_2O_2, Co^{2+} were 11.5, 1.5 mM, 0.2 mM, respectively.

Table 1 Comparison between the current method and other reported techniques for analysis of tenascin-C

Technology	Detection method	Detection range	Detection limit	Ref.
Electrode Array [a]	EC	–[b]	7 μg·mL^{-1}	[30]
ELISA	UV–vis	0.2 to 12.5 μg·mL^{-1}	–	[31]
EIS	BI1A	–	2 ng·mL^{-1}	[32]
StEIS	UV–vis	–	2 ng·mL^{-1}	[33]
Peroxidase-labeled	UV–vis	–	1.5 ng·mL^{-1}	[34]
CNPs absorption	CL	1.0×10^{-12} to 1.0×10^{-9} g·mL^{-1}	4.0×10^{-13} g·mL^{-1}	This method

[a] *EC*, electrochemical; *ELISA*, enzyme-linked immunosorbent assay; *EIS*, Enzyme immunoassay; *BI1A*, Bayer Immuno 1 analyzer; *StEIS*, sandwich-type enzyme immunoassay; *CNPs*, carbon nanoparticles

[b] not reported

Table 2 Accuracy, measured as recovery

Original [a] (pg mL^{-1})	Added (pg mL^{-1})	Found (pg mL^{-1})	Recovery ratio (%)
230.7	10.0	240.6	99.0
292.3	50.0	343.7	102.8
317.9	100.0	420.1	102.2
338.2	100.0	435.9	97.7
357.5	200.0	557.2	99.9

[a] n=9

Sensitivity of the biosensor

Based on the combination of the adsorption of aptamer on cCNPs with sensitivity of the FI-CL detection method, under the optimized experimental conditions, a linear function range for tenascin-C was achieved from 1.0×10^{-12}~1.0×10^{-9} g mL^{-1} with the equation of I_{CL}=3.56 C+31.61 (I_{CL} is the CL intensity; C is the concentration of tenascin-C, 10^{-12} g mL^{-1}; n=7, R^2=0.9997). And the detection limit of tenascin-C is 4.0×10^{-13} g mL^{-1} estimated using 3σ. A series of seven repetitive measurements of 5.0×10^{-11} g mL^{-1} tenascin-C were used for estimating the precision, and the relative standard deviation (RSD) was 4.2 %. It was shown that the protein biosensor had good reproducibility. This method and some other detection techniques, which can sensitively detect the tenascin-C, are listed in Table 1. Comparing to other methods listed in Table 1, the current protocol is one of the most sensitive assay for the determination of tenascin-C [30–34].

Selectivity of the biosensor

To assess the specificity of the method for the detection of tenascin-C, experiments were conducted on some relative substance such as thrombin, lysozyme, IgG, BAS and ATP. Different CL signals for the detection of 1.0×10^{-7} g mL^{-1} thrombin, lysozyme, IgG, BAS and ATP, were recorded under the same experimental conditions according to the protocol described in Section 2.5, respectively. Thrombin, lysozyme, IgG, BAS and ATP did not induce any significant changes in the CL signal as compared to that of tenascin-C, which suggests that the developed strategy has a sufficient selectivity and tenascin-C could be detected selectively. This may be explained by the fact that the tenascin-C can bind to labeled aptamer and induce the formed the tenascin-C labeled aptamer complex to divorce from the surfaces of the cCNPs-MBs.

Determination of tenascin-C in the human serum samples

The feasibility of applying the developed method to measure tenascin-C in a complex matrix was studied. This was conducted by adding various levels of tenascin-C into human serum samples. After adding the sample into the labeled aptamer modified cCNPs-MBs, the mixtures were subjected to incubation for 30 min to ensure the full reaction between the labeled aptamer and tenascin-C. Then CL intensity of each sample of the supernatant was applied to the calibration curve of the standard tenascin-C solution to obtain the tenascin-C level of the sample. The results of recovery test were shown in Table 2. The results indicate that the developed method is highly reliable.

Conclusions

In conclusion, a new strategy for the simple and sensitive detection of protein based on protein-aptamer recognition action and absorption interaction between the ssDNA and cCNPs was developed. The detection limit of 4.0×10^{-13} g mL^{-1} of target protein was obtained. The resulting protein biosensor exhibited ultrasensitivity, high selectivity, and good reusability as a promise alternative technique for other bioassays. It holds great promise for protein diagnostics, microarrays, and microchips, as well as for bioanalysis in general.

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