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Carbon Nanofiber-based Luminol-biotin Probe for Sensitive Chemiluminescence Detection of Protein

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A carbon nanofiber-based luminol-biotin probe was synthesized for the sensitive chemiluminescence (CL) detection of a target protein by grafting luminol and biotin onto an oxidized carbon nanofiber. This carbon nanofiber was prepared by chemical vapor-deposition with methane in the presence of the Ni–Cu–MgO catalyst, which was followed by oxidization with HNO_3–H_2SO_4 to produce a carboxyl group on the surface of the nanofiber. The material was grafted with luminol and biotin by means of a standard carbodiimide activation of COOH groups to produce corresponding amides. The substance was water-soluble and thus could be utilized as a sensitive CL probe for a protein assay. The probe showed highly specific affinity towards the biotin-labeled antibody *via* a streptavidin–biotin interaction. The detection limit for this model assay was approximately 0.2 pmol of the biotinized IgG spotted on a polyvinylidene fluoride (PVDF) membrane. Nonspecific binding to other proteins was not observed. Therefore, the synthesized carbon nanofiber-based CL probe may be useful for a sensitive and specific analysis of the target protein.

Keywords Chemiluminescence, carbon nanofiber, luminol, proteins, streptavidin

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Introduction

Carbon nanofibers are cylindrical nanostructures in which graphene layers are arranged as stacked cones, cups or plates.[1] As well as other carbon nanomaterials, carbon nanofibers are attractive for a wide range of biosensing applications. Their key advantages are: low cost, chemical inertness, biocompatibility, possibility of functionalization, high electrical conductivity, and mechanical strength. Compared with carbon nanotubes, they have a larger number of plane defects which facilitate chemical modification.[2] Until now carbon nanofibers have found wide applications in chemical sensing, especially in electroanalysis. Furthermore, they have been found ideal for immobilization of antigens and antibodies to develop immunosensors,[3] which rely on specific redox reactions of antigens or on other changes of carbon nanofiber-based film properties that could be traced electrochemically.[4]

Immunoaffinity-based protein assays rely on high specificity of antibodies to antigens; however, the sensitivity of the assay depends entirely on the detection principle of the antibody–antigen complex. In the assays a recognition partner is generally labeled with a radioactive, fluorescent, chemiluminescent or enzymatic tag. All those tags could be easily detected using relatively simple and sensitive instrumentations. One of the constraints of a classical immunoassay is that only one reporter molecule (*i.e.* an enzyme, a fluorophore or chemiluminophore) is associated with one antibody–antigen recognition event. In order to improve the potential sensitivity of detection, various strategies of signal amplification have been developed and this field is still an important and active area of research.[5] One of the most efficient ways of signal amplification is the application of tags that are not directly detected but can catalyze a reaction that yields an easily detectable product such as a dye.[6] Another method for increasing the sensitivity of immunoassays utilizes a multilabeling technique,[7] where an antibody is labeled not at a single NH_2 or COOH site, but a number of such functional groups are modified with a tag.[8] This strategy has a serious limitation because a high degree of substitution affects the specificity of antibodies. A more interesting technique utilizes multicarrier labels connected to a single functional site of the antibody.[9] Several types of multi-functional carriers have been used in the tags synthesis, such as dendrimers,[10] natural polysaccharides,[11] gold nanoparticles,[12] viral nanoparticles,[13] nanotubes,[14] nanocrystals,[15] liposomes[16] and polymers.[17]

The strategy investigated by our and other groups is the application of polysaccharides as carriers of multiple enzymatic or chemiluminescent labels.[11,18–22] These natural polymers allow for the straightforward covalent attachment of tags and the tuning of such chemical properties as solubility and the molecule size. Nevertheless, polysaccharides possess a strong affinity

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towards proteins driven by electrostatic interactions.[23] Such property could be used for direct detection of proteins, but in the case of specific antibody-based assays it is a potential source of background signal and requires an appropriate washing protocol.[18,21]

In this study, we developed a carbon nanofiber-based luminol-biotin probe that possessed a strong affinity towards biotinylated proteins and simultaneously was capable of CL. Therefore, the probe could be applied to an immunoassay and to similar analytical techniques. CL-based sensing was selected as it has many advantages, such as low cost and simplicity, as well as measurement against a low background.[24]

The desirable key features of this probe were: good solubility, low nonspecific affinity towards proteins, and a great amount of luminol and biotin that could be covalently attached to the nanofiber surface. Well defined compact geometry of the carbon nanofiber potentially minimized the steric effects.

Experimental

Reagents and materials

All chemicals were of analytical reagent grade, and used as received without any further purification. Carbon nanofibers were synthesized in compliance with a published procedure.[25] Tetra-*n*-propylammonium hydroxide (TPA) (1.0 M aqueous solution), bovine serum albumin (BSA), and biotin-conjugated anti-mouse IgG antibody (b-IgG) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Anti-human IgG rabbit antibody was obtained from Oxford Biochemical Research (Oxford, MI). Hydrogen peroxide (30% aqueous solution) and streptavidin were supplied by Wako Pure Chemical Industries (Osaka, Japan). Hemin (>98%) was supplied by Fluka (Buchs, Switzerland). D(+)Biotin 98%, *N*,*N*′-diisopropylcarbodiimide (DIC) and *N*-hydroxysuccinimide (NHS) were supplied by Acros Organics (Geel, Belgium). D(+)Biotin hydrazide was prepared according to the procedure specified in literature from D(+)biotin.[26] Nylon and polyvinylidene fluoride (PVDF) membranes (0.45 μm pore size) were purchased from Atto Co. (Tokyo, Japan). A supply of 4′-hydroxyazobenzene-2-carboxylic acid (HABA) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Avidin was purchased from Nacalai Tesque (Kyoto, Japan). Milli-Q water was used for all the experiments.

Instruments

The structure and morphology of carbon nanofibers and products of their modification were examined on a high-resolution scanning electron microscope ZEISS SUPRA 35 (Carl Zeiss, Jena, Germany). Transmission electron microscopy (TEM) investigations were performed with the application of an S/TEM TITAN 80-300 (FEI, Hillsboro, OR) instrument. Chemiluminescence intensities on the membranes were detected using a Light Capture AE-6971/2 device (Atto Co., Tokyo, Japan). The diameter of the probe particles was measured on a High Performance Particle Sizer (Malvern Instruments, Worcestershire, UK).

Probe synthesis

The carbon nanofiber was obtained by a catalytic decomposition of methane over a quartz-supported Ni–Cu–MgO catalyst.[25] The resulting raw product (3 g) was stirred with concentrated HCl (100 mL) overnight at room temperature. The carbon nanofiber was separated from quartz particles, centrifuged and washed with water to give 1.4 g of a dry product. The product (225 mg) was oxidized for 5 h with 40 mL of the HNO_3-H_2SO_4 mixture (1:3 v/v) in an ultrasonic bath at 50°C. The resulting black viscous suspension of oxidized carbon nanofiber was diluted with water, centrifuged and washed thoroughly with water and dried. The yield of the dry product was 206 mg (elemental analysis gave: C, 48.12%; H, 0.87%; N, 0.39%). To attach a number of biotin and luminol moieties to carboxyl groups formed during the oxidation step, 56 mg of oxidized carbon nanofiber and 34 mg of NHS was dispersed in 25 mL of dry DMF, followed by the addition of 63 μL of DIC. After stirring for 50 min under the N_2 atmosphere, 33 mg of luminol and 10 mg of biotin hydrazide was added. The mixture was stirred for 12 h, then centrifuged, washed successively with methanol, 5% sodium carbonate and water, and finally dried to give approximately 50 mg of the carbon nanofiber-based luminol-biotin probe in the form of a black powder (elemental analysis gave: C, 57.53%; H, 2.24%; N, 6.82%).

Measurement of the diameter of the probe particles

The probe was dispersed in water to make 1 mg mL^{-1} suspension, and 100, 50 and 10 μg mL^{-1} suspension were prepared by serial dilution. The diameter of the particles was measured using 1.2 mL of the above samples in a 10 × 10 × 45 mm cuvette at 25°C. The measurement was taken 13 times for each concentration, and the mean values of the diameter obtained with three different concentrations were further used for calculation of the average diameter ± standard deviation.

Typical procedure for the detection of biotin-labeled proteins

CL assays were performed using the procedure developed previously.[18] Briefly, protein spots (1 μL) were placed on a PVDF membrane (2 – 5 cm^2) with an automatic micropipette immediately after the membrane was spotted with ethanol (1 μL). The membrane was dried in vacuo at 37°C for 15 min and then incubated in 2.0 mL of an aqueous mixture (pH 3.5) containing 200 μg of the carbon nanofiber-based probe that possessed luminol and biotin moieties, 200 μg streptavidin, 90 mg of boric acid and 50 μg of Triton X-100 (TX100) at 37°C for 1 h with gentle shaking. The membrane was then washed consecutively with water for 5 s, 15% acetic acid for 3 min, water for 5 s and 75% methanol for 3 min (4 mL each). After drying the membrane for 5 min, the CL reaction was initiated using a mixture of 685 μL of acetonitrile, 285 μL of 1 M TPA, 30 μL of 2 mg mL^{-1} of hemin (in 1 M TPA) and 50 μL of 30% H_2O_2. Immediately after the addition of H_2O_2 to the mixture, the membrane was immersed in this solution for 5 s and then the chemiluminescence detection with the CCD camera with 2-min exposure time was carried out.

HABA assay[27]

Avidin (10 mg) was dissolved in approximately 8 mL of PBS, to which was added 100 μL of 10 mg mL^{-1} HABA solution in dimethylsulfoxide. PBS was then added to the mixture to the final volume of 10 mL. The probe (10 mg) was suspended in 1 mL of PBS and 100 μL of this solution was mixed with 900 μL of the above HABA-avidin solution. After gentle shaking of the mixture for 10 min, the supernatant was applied to the measurement of absorbance at 500 nm. The biotin content in the probe was calculated according to Green's publication.[27]

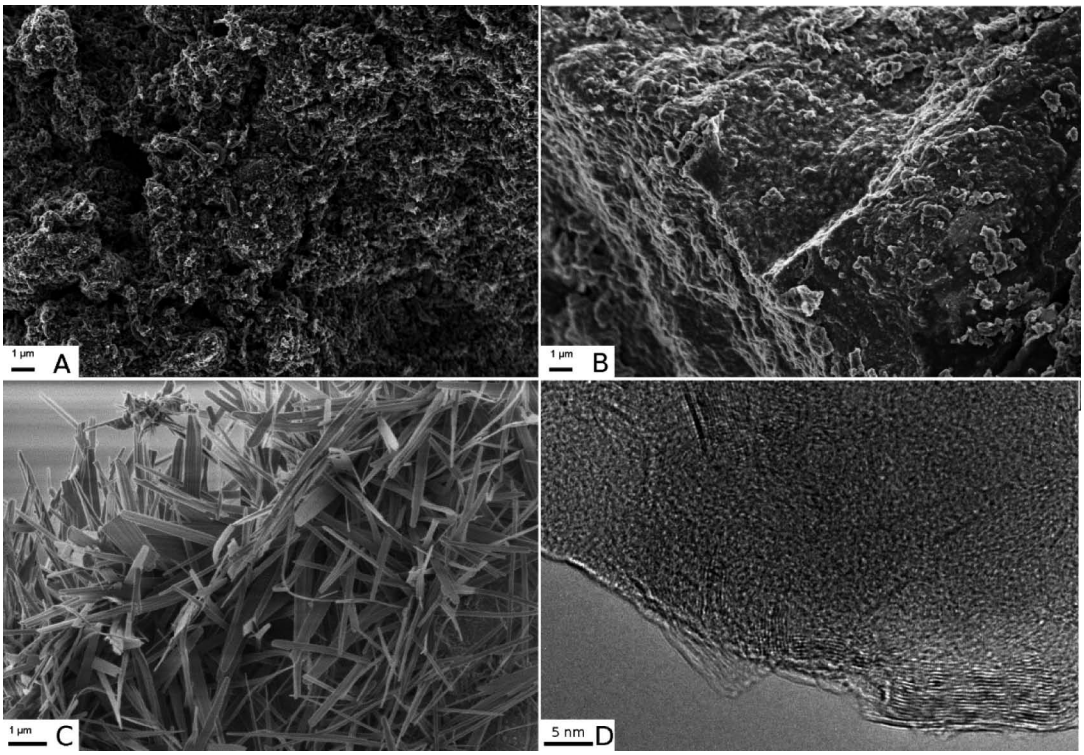
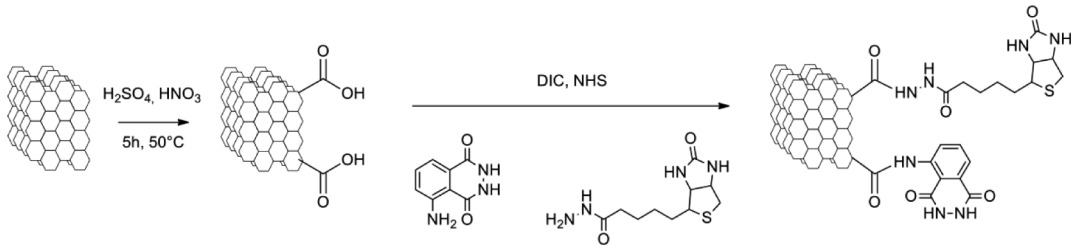


Fig. 1 Microscopic images of carbon-based materials used in this study. A: carbon nanofiber; B: oxidized by HNO_3–H_2SO_4; C and D: luminol-biotin probe.



Scheme 1 Synthesis of carbon nanofiber-based probe.

Results and Discussion

Synthesis of the probe

Carbon nanofiber for the synthesis of a chemiluminescent probe was obtained using the procedure specified in the literature.[25] The morphology of the product was shown on a microscopic image (Fig. 1A). The length of nanofibers was approximately 50 – 200 nm. Further treatment of the material included oxidation with the HNO_3–H_2SO_4 mixture, which led to the formation of carboxylic groups on the surface of carbon nanofiber. The content of oxygen in the oxidized material was 53%, which corresponds to the maximal content of 16.6 mmol g^{-1} of COOH groups. A microscopic image of oxidized material is shown in Fig. 1B. It is visible that the nanofibers were highly shortened during oxidation. Due to that fact and due to high oxygen content the oxidized product was quite (> 1 mg mL^{-1}) soluble in water and DMF.

Luminol and biotin were grafted on the surface of oxidized material using the DIC/NHS chemistry as shown in Scheme 1. The ratio of luminol to biotin hydrazide was 5:1 in order to introduce a large number of chemiluminescent moieties compared with biotin moieties.

Characterization of the probe

An elemental analysis of the final product gave 57.53% C, 2.24% H and 6.82% N, which indicates a successful introduction of luminol and biotin. The resulting probe was quite soluble in water. The solution was indefinitely stable and did not show any signs of sedimentation. As presented in Figs. 1C and 1D, the final material possessed a grass-like structure formed by small, non-aligned graphite layers.

In order to evaluate the contents of luminol and biotin in the probe, HABA assay was first conducted. HABA shows the change in absorbance maximum upon binding to avidin, and this change can be traced by measuring the absorbance at 500 nm.[27] The moderate affinity between HABA-avidin is easily disrupted by the addition of biotin, resulting in a decrease of absorbance at 500 nm. The result indicated that 1 mg of the probe contains 0.17 μmol of biotin, which is equal to 9.63 μg of nitrogen. Since elemental analysis of the probe exhibited the nitrogen content of 6.82%, 1 mg of the probe theoretically

contains 68.2 μg of nitrogen. The difference in these nitrogen amounts (58.57 μg) could come only from luminol since the skeleton of carbon nonofiber contains no nitrogen atoms. The mole of luminol in 1 mg of the probe was then calculated to be 1.39 μmol, which is about 8 times higher than the biotin content. This result matches the amounts of biotin and luminol used in the coupling reaction.

The molecular weight of the probe was next presumed from the volume of the probe. The diameter of the probe was measured to be 57 ± 4 nm on a particle sizer. Assuming the density of the probe is equal to the density of graphite (2.23 g cm^{-3}), the molecular weight of the probe can be estimated to be 1.3×10^8 Da. Taking these results into consideration, the number of biotin and luminol molecules incorporated into one molecule of carbon skeleton were determined to be 22000 and 181000 respectively. The fact that one molecule of the probe contains multiple biotin moieties would be a benefit for the formation of higher order macromolecular assembly *via* biotin–avidin interaction. Furthermore, a large number of luminol molecules in one probe molecule should offer effective signal amplification in combination with the assembly formation.

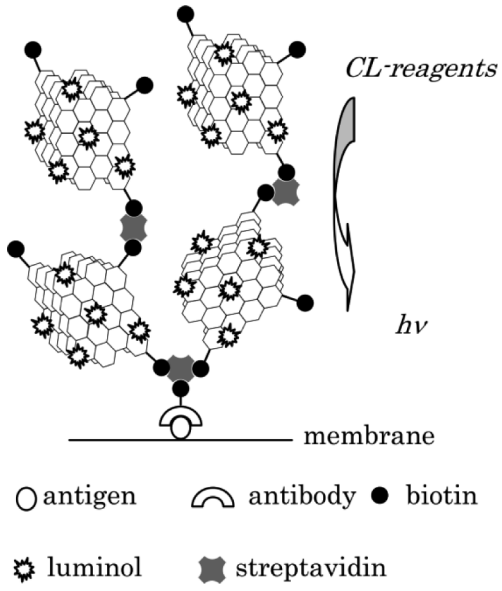


Fig. 2 Application of carbon nanofiber-based CL probe in a biotin-streptavidin-enchanced immunoassay.

Binding of the carbon nanofiber-based luminol-biotin probe to biotin-labeled IgG on a PVDF membrane

The aim of this study was also to achieve a highly efficient chemiluminescent probe suitable for labeling antibodies for further use in the immunoassay. The possible routes included a covalent attachment of luminol-derivatized carbon nanofiber containing free COOH groups to an antibody *via* free NH_2 functional sites of the antibody. A more convenient way was exploiting the biotin-streptavidin chemistry. Streptavidin possesses four binding sites towards the biotin molecule, and thus multiple biotinylated tags have the capacity to create a supramolecular assembly connected together *via* streptavidin links (Fig. 2).[28] Moreover, biotinylated antibodies are commercially available; therefore a luminol-biotin probe could be used in a range of assays without the requirement of antibody modification.

The synthesized probe was examined for the presence of chemically bonded luminol in the probe by spotting a PVDF or nylon membrane with various amounts of the probe (20, 2, 0.2, 0.02 μg). On the same membrane, free luminol (2, 0.2, 0.02 ng) was spotted as well. The spots were visualized by immersion of the membrane in a solution of hemin, TPA and H_2O_2, in compliance with our previous paper.[18] The CL signal was especially strong in the case of probe spots of 0.2 μg or more on a PVDF membrane, while luminol spots were invisible even with no use of a washing buffer. The results explain that luminol was chemically bonded to the carbon nanofiber and furthermore that the probe was strongly retained by the physical spotting on the PVDF membrane and not released out from the membrane during detection, although free luminol was readily released. Therefore it was important whether the probe was naturally adsorbed or not on the membrane in the protocol for the protein assay using the PVDF membrane.

We attempted to optimize the binding conditions of the

Table 1 Optimization of immunoassay protocol using carbon nanofiber-based CL probe

Entry	Step 2	Step 3					Step 4	b-IgG
	Blocking[a] Yes/No	Surfactant	Buffer[b]	Streptavidin/μg	Probe/μg	Time/h	Washing agent	CL level
1	N	—	PBS	100	50	0.5	0.25% SDS	0
2	Y	—	PBS	200	200	0.5	0.25% SDS	1
3	Y	—	PBS	400	400	0.5	0.25% SDS	0
4	N	0.0025% TX100	H_3BO_4	200	200	0.5	0.25% SDS	0
5	N	0.0025% TX100	H_3BO_4	200	200	0.5	CH_3COOH, CH_3OH	0
6	N	0.0025% TX100	H_3BO_4	—	200	0.5	CH_3COOH, CH_3OH	0
7	Y	0.0025% TX100	H_3BO_4	200	200	0.5	CH_3COOH, CH_3OH	0
8	N	0.1% SDS	PBS	200	200	0.5	0.1% SDS	0
9	N	—	PBS	150	120	0.5	0.1% SDS	0
10	N	0.0025% TX100	H_3BO_4	200	200	0.5	0.2% TX100, CH_3OH	0
11	N	0.0025% TX100	H_3BO_4	200	300	0.5	0.2% TX100	0
12	N	0.0025% TX100	H_3BO_4	200	200	1	CH_3COOH, CH_3OH	3
13	N	0.0025% TX100	PBS	200	200	2	CH_3COOH, CH_3OH	1
14	N	0.0025% TX100	PBS	200	200	3	0.1% SDS	2
15	N	0.0025% TX100	H_3BO_4	200	200	3	CH_3COOH, CH_3OH	2

a. 5% skimmed milk in 10 mM PBS, b. 0.7 M PBS (pH 7.0) or 0.7 M H_3BO_4 (pH 3.5); Each operation was carried out in 2 mL (step 2, 3) or 4 mL (step 4) of solution.

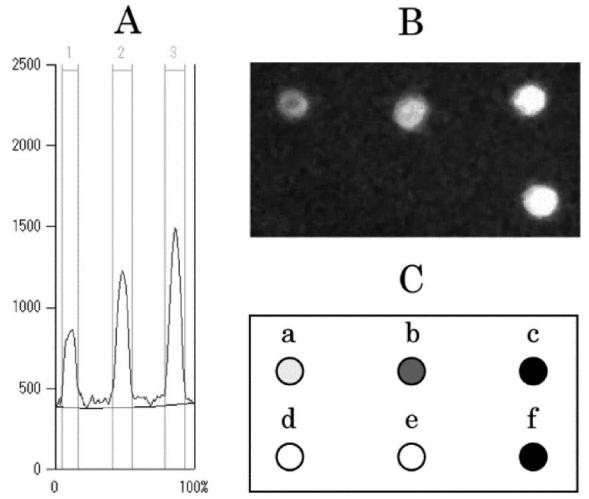


Fig. 3 CL detection of proteins with a carbon nanofiber-based probe. (A) CL integrals for the three upper spots, (B) visualized PVDF membrane with six spotted samples, and (C) spotting scheme for B: a, b, c and f for b-IgG (2.5, 5, 10, 10 pmol); d for BSA (5 pmol); e for IgG (10 pmol).

synthesized probe to a biotinized IgG protein. The procedure was divided into five key steps: step 1) spotting of samples and drying of the membrane; step 2) blocking of the membrane for 30 min; step 3) binding of the probe and streptavidin in a solution; step 4) final double washing of the membrane for 5 min; step 5) drying of the membrane and then CL detection. Steps 1 and 5 were performed in compliance with our previous publication.[18] Steps 2 – 4 were investigated simultaneously in compliance with Table 1.

Each experiment was performed on a PVDF membrane spotted with b-IgG (2.5, 5, 10 pmol), PBS (10 nmol), carbon nanofiber-based probe (4 μg), and luminol (1 μg). The membrane was washed with 4 mL of an aqueous solution of a surfactant or with an organic solvent. In the case of entries 5 – 7, 12, 13, 15 the first washing was done with the acetic acid solution followed by a methanol wash. After each membrane was visualized, the light intensity was quantified and each result was marked based on a relative four-level scale where 0 is no signal and 3 is a strong signal.

The most important factor that influenced the signal was the binding time. If it was shorter than 0.5 h, b-IgG spots were not clearly visible. When it was increased to 1 – 3 h (entries 12 – 15), b-IgG spots were easily distinguishable. The washing conditions had a low impact, but lack of organic solvent during step 5 resulted in a high signal of free luminol or the probe directly spotted on the membrane. The blocking step did not improve the CL signal probably because the BSA layer hampered the contact between the probe and b-IgG. Low background level even without the blocking step was most likely due to very low concentration of the probe in the binding solution. The recommended conditions according to the results were as follows (entry 12 in Table 1): no blocking step, binding of the probe (200 μg) with streptavidin in a solution containing 0.0025% TX100 and 0.7 M H_3BO_4 for 1 h of binding time, and final washing with 15% acetic acid followed by 75% methanol.

Under the optimized binding conditions, the selectivity of the carbon nanofiber-based probe was investigated by spotting of b-IgG (2.5, 5, 10, 10 pmol), IgG (5 pmol), and BSA (5 pmol) on the membrane (Fig. 3). It is clear that the probe was fairly selective to the b-IgG, without nonspecific affinity towards other proteins. The brightness of the spots on the PVDF membrane was logarithmically correlated with b-IgG amount. The visible spots were those of b-IgG, which could be recognized by streptavidin followed by binding of the probe to the complex of streptavidin and b-IgG on the membrane.

Compared with our previous results the carbon nanofiber-based probe was as specific as other biotinized polysaccharide-based probes[11,19,20] or commercial biotin-HRP probe[19] and more specific than protein probes that do not contain biotin[18,21] or luminol-biotin-dextran probe.[11] With respect to the sensitivity of the assay, the probe allowed detection of b-IgG at a pmol level that is approximately 100 times worse compared with polysaccharide-based probes containing enzymatic tags[19,20] or 10 times worse than a commercial biotin-HRP probe[19] but at a similar level as luminol-biotin-dextran probes.[11] The advantage of the carbon nanofiber-based probe was simple synthesis and purification and less complicated protocol of the assay.

Conclusions

This research showed that carbon nanofibers can be easily chemically modified and used as a structural support for the synthesis of probes. Our synthesized carbon nanofiber-based CL probe indicated negligible nonspecific affinity towards proteins, while it was strongly bound to target biotinized antibody *via* streptavidin. An important property of the proposed tag is its versatility as it can potentially recognize every commercially available biotinylated antibody. Therefore, it may find applications in various analyses by use of commercially available biotinylated antibodies.

The advantage of our synthesized probe is its inherent signal amplification ability, because a single graphite particle can be labeled with a great number of luminescent and biotin units. Therefore, it allowed the detection limit of approximately 0.2 pmol, which could be further improved. One way would be increasing the number of functional sites by prolonging the oxidation time of carbon nanofibers. Replacing luminol with isoluminol or its derivatives would lead to a more quantum efficient emission.[29] Another option is to change the solid support from the membrane to glass slides or microtiter plates, which should result in a lower background signal caused by non-specific adsorption of the probe.

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