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Isoelectric BSA: Robust blocking agent for enhanced performances in optical-fiber based DNA sensing

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Abstract: Surface blocking is a well-known process for reducing unwanted nonspecific adsorption in sensor fabrication, especially important in the emerging field where DNA/RNA applied. Bovine serum albumin (BSA) is one of the most popular blocking agents with an isoelectric point at pH 4.6. Although it is widely recognized that the adsorption of a blocking agent is strongly affected by its net charge and the maximum adsorption is often observed under its isoelectric form, BSA has long been perfunctoril]y used for blocking merely in neutral solution, showing poor blocking performances in the optical-fiber evanescent wave (OFEW) based sensing toward DNA target. To meet this challenge, we first put forward the view that isoelectric BSA (iep-BSA) has the best blocking performance and use an OFEW sensor platform to demonstrate this concept. An optical-fiber was covalently modified with amino-DNA, and further coupled with the optical system to detect fluorophore labeled complementary DNA within the evanescent field. A dramatic improvement in the reusability of this DNA modified sensing surface was achieved with 120 stable detection cycles, which ensured accurate quantitative bioassay. As expected, iep-BSA blocked OFEW system showed enhanced sensing performances toward target DNA

with a detection limit of 125 pM. To our best knowledge, this is the highest regeneration cycles ever reported for a DNA immobilized optical-fiber surface. This study can also serve as a good reference and provide important implications for developing similar DNA-directed surface biosensors.

Keywords: isoelectric BSA; DNA hybridization; reusable biosensor; evanescent wave; optical-fiber biosensor.

As devices that use biological components for detection/quantification of targets of interest, biosensors are increasingly considered for applications including environmental, disease diagnosis, and other key health life-related fields.[1-3] Among those developed biosensors, optical-fiber evanescent wave (OFEW) based biosensing has become a hot topic in application-oriented analytical science due to its ability to be performed at inaccessible sites or in harsh environment with high sensitivity and reusability.[4-5] In fluorescence based OFEW systems, high sensitivity came from the intrinsic noise reduction feature of the exponential decayed evanescent field (usually half of wavelength of incident light from the optical-fiber's surface): Noises from bulk solution were shielded due to weak field strength, only fluorescent reporters within evanescent field could be excited.[6-11] Most successful cases involving fluorescence based OFEW platforms chose immunoagents (antigen/antibody) as core signal recognition elements. Such OFEW immunosensors usually utilize fluorescent labeled

antibody as the signal reporter, and have been applied to detect pesticide,[12] plasticizer, [13] illegal food additives,[14] bacterial toxins,[15] and other small analytes.[16]

Although OFEW immunosensors exhibited satisfying sensitivity and selectivity toward multiple targets, they might meet an insurmountable barrier toward sensing substances outside the target category of immunoreagents, such as heavy metals and important oligonucleotides (microRNA, antibiotic resistance genes, etc.).[17] Moreover, the protein nature of most antibodies may cause other problems such as poor batch stabilities, high prices, and stringent storage conditions.[18] As the nucleic acid counterpart of immunoagent, DNA based surface affinity recognition represents a powerful tool in terms of biosensing. Typically, surface immobilized DNA was designed to capture its complementary strands in solution.[19-20] Other functional DNA molecules (like aptamers or DNAzymes) could also be used for various targets, ranging from whole cell to metal ions, with lower price and less strict reaction/storage condition.[21] Therefore, it is of great importance to develop OFEW sensing systems using DNA as signal recognition element for the enrichment of biosensors.

However, blocking challenges appeared when it comes to DNA immobilized optical-fiber surface. The flexible chain structure and special charge distribution of single strand DNA (ssDNA) often led to strong nonspecific adsorption. To circumvent unwanted nonspecific adsorption, blocking is usually a necessity in biosensing. Bovine serum albumin (BSA) is one easy accessible and broadly used blocking agent which has been applied to block various biosensing surfaces, such as ELISA assay,[22]

SPR system,[23] planar waveguide surface,[24] evanescent wave all-fiber biosensing platform,[25] and highly dispersed nanomaterials.[26] In these studies, BSA was dissolved either in pure water or in near-neutral buffers like PBS. However, we found that such neutral BSA solutions exhibited poor blocking effect onto DNA immobilized optical-fiber surface. Strong nonspecific adsorption could be observed in a neutral BSA aqueous solution blocked OFEW platform (Figure 1c). High signals induced by non-target strand suggested the existence of active sites for nonspecific adsorption on the optical-fiber surface. Reliability of data generated from such surfaces is questionable (signal/noise <3).

In this work, one possible reason for the poor blocking performance of neutral BSA solution toward the optical-fiber surface was inferred to be inadequately blocking, in other words, inadequately adsorption toward active sites. BSA was reported to be an ellipsoidal protein with a molecular weight around 66.5 kDa and a pI value (isoelectric point, iep) of 4.6.[27] Many researchers have studied the adsorption properties of BSA. It has been reported that the maximum adsorption of BSA occurred at its isoelectric point on various surfaces, including magnetic particles,[28] nanostructured TiO_2 thin films,[29] mesoporous material SBA-15,[30] ordered mesoporous silica,[27] and ultrafine silica particles.[31] Most of these studies mentioned that small lateral repulsion could be the main reason for the maximum adsorption. Although numerous studies have investigated the adsorption properties of BSA, the linkage between adsorption behavior and blocking performance is overlooked. After poking the mist, we found

that the blocking performance of BSA molecules could be enhanced by simply adjusting pH of the blocking buffer to its isoelectric point. For the first time, isoelectric BSA (designated as iep-BSA in the following context) is reported as a robust blocking agent for improved performances in optical-fiber based DNA sensing. Iep-BSA blocked optical-fiber showed sensitive detection ability toward 125 pM-100 nM of target DNA. Besides, the iep-BSA blocked optical-fiber can be reused for 120 cycles.

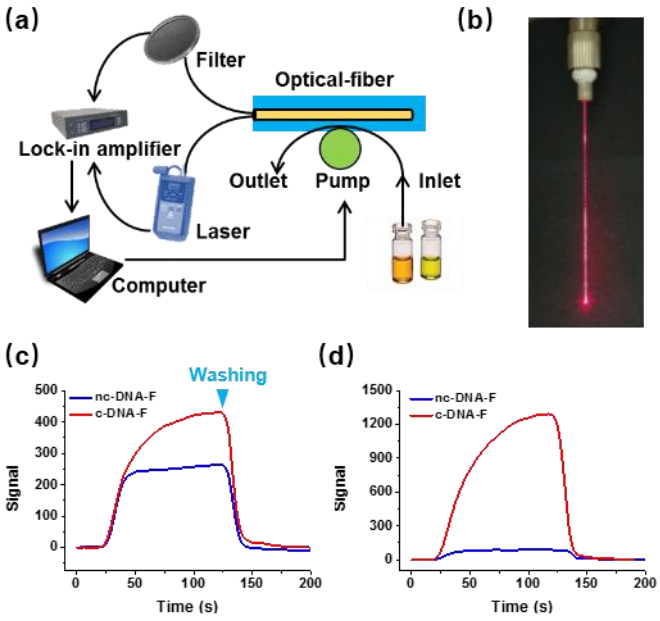

Figure 1. (a) Scheme of the optical-fiber evanescent wave (OFEW) biosensing system used in this work. **(b)** Photo of an optical-fiber in coupler with the laser turned on. **(c)** Signals induced by 10 nM complementary DNA-Cy5.5 (c-DNA-F) and 10 nM noncomplementary DNA-Cy5.5 (nc-DNA-F) from an optical-fiber blocked with 2 mg/mL neutral BSA aqueous solution (pH 6.8). **(d)** Signals induced by 10 nM c-DNA-F and 10 nM nc-DNA-F from an optical-fiber blocked with 2 mg/mL isoelectric BSA solution (pH 4.6).

Experimental section

Materials

Quartz optical-fiber with a length of 8.5 cm and a diameter of 600 μm was purchased from Chunhui Science & Technology Industrial Co., China. Bovine serum albumin (BSA), glutaraldehyde (25% aqueous solution), 3-aminopropyl-triethoxysilane (APTS), sodium citrate ($Na_3C_5H_5O_7 \cdot 3H_2O$), citric acid ($C_6H_8O_7 \cdot H_2O$), and sodium chloride were purchased from Sigma-Aldrich, Inc. Glycine, sodium and cyanoborohydride ($NaCNBH_3$) were purchased from AMRESCO LLC. Phosphate buffered saline packs were purchased from Thermo Fisher Scientific Inc. Cy5.5 NHS ester was purchased from GE Healthcare Life Sciences. Polymers including polystyrene sulfonate (PSS, MW 500,000), polyacrylic acid (PAA, MW 3,000), and poly(allylamine hydrochloride) (PAH, MW 56,000) were bought from Energy Chemical Company. BSA-Cy5 conjugate was bought from Beijing Biosynthesis Biotechnology Co. Ltd. All acids and organic solvents were purchased from Beijing Chemical Works. All aqueous solutions were prepared using molecular biology grade USP sterile purified water (Corning Cellgro, NY, USA). All oligonucleotides (HPLC-purified) were obtained from Sangon Biotechnology Co. Ltd. (Shanghai, China).

DNA used in this work (5'-3')[①]:

NH_2-DNA: 5'- NH_2-$(CH_2)_6$-AGAGAGAGAGGGAGAGAGAGAGGG

NH_2-A10: 5'- NH_2-$(CH_2)_6$-AAAAAAAAAA

[①] We chose the sequence based on the work of Razumovitch and coworkers (reference 32). In practice, the selection of strand based on the downstream application and the impact of the sequence and length of the DNA should be considered (Li, X., He, Z., Zhou, J., 2005.Nucleic Acids Res. 33(19), 6114-6123.).

c-DNA-F: Cy5.5-CCCTCTCTCTCTCCCTCTCTCTCT (complementary DNA)

nc-DNA-F: Cy5.5-AAAAAAAAAAAAAAAAAAAA (non-complementary DNA)

Isoelectric BSA (iep-BSA) blocking solution:

2 mg/mL BSA dissolved in 0.1 M sodium citrate/citric acid buffer (pH 4.6).

Instrumentation

The surface morphologies of different substrates were observed by atomic force microscopy (AFM, SPA-300HV, Seiko Instruments, Japan) and scanning electron microscopy (SEM, JEOL, JSM-700AF). Fluorescent spectra were obtained by a fluorescent spectrometer (Hitachi F-7000, Japan). The optical-fiber diameter was monitored with a microelectrode polisher (model 2002-C, Inbio Life Science Instrument Co., Ltd., Hubei, China).

Preparation of a DNA modified optical-fiber

First, 3.5 cm of cladding was stripped away at one end of the optical-fiber. Then the optical-fibers were perpendicularly dipped in hydrofluoric acid (HF) for around 2.5 h to form a tapered structure with a final diameter of 220 μm. The etched optical-fibers were thoughtfully washed with ultra-pure water and dried with nitrogen for long time storage.

Before DNA immobilization, the optical-fibers were immersed in freshly prepared piranha solution (3:1 mixture of H_2SO_4 and 30% H_2O_2) for 1 h at 110 ^oC to remove surface residues. Cleaned optical-fibers were animosilanized by immersing them in a 1% v/v solution of APTS in dry toluene for 2 h at room temperature (25 ^oC). The

APTS-modified optical-fibers were thoroughly washed with toluene, sonicated for 15 min in toluene, dried under nitrogen, and fixed by heating in a 200 ^{o}C oven for 1 h. After silanization, the optical-fibers were immersed in 1% v/v glutaraldehyde aqueous solution overnight using a 37 ^{o}C shaker to allow the formation of Schiff base.[32] Aldehyde-actived optical-fibers were immersed in 300 nM NH_2-DNA and 100 nM NH_2-A10 (prepared in 50 mM NaCl aqueous solution, NH_2-A10 was used as a short strand mixer) for 3 h at room temperature with gentle shaking. After that, 20 mM of glycine aqueous solution was used to react with excess aldehyde groups. Next, the optical-fibers were reduced in 20 mg/mL of $NaCNBH_3$ aqueous solution for 1h. Before use, the optical-fibers were blocked using several blocking agents.

Safety considerations

1. Freshly prepared piranha solutions are unstable and extremely energetic, careless handling may cause explosion or injury resulting from chemical and thermal burns. Please use glass containers and do NOT keep freshly prepared piranha solution in a closed container. After use, please keep piranha solution in a labeled, open container to react overnight before final neutralization or professional waste disposal.
2. After use as a selective reducing agent, sodium cyanoborohydride solution should be kept in a labeled container prior to professional waste disposal. Do NOT mix it with strong acids or oxidizing agents.

Results and Discussion

Iep-BSA for blocking nonspecific adsorption of ssDNA on optical-fiber surface

Figure 1a showed the components and construction of the OFEW biosensing platform used in this work. Samples containing fluorescent labeled DNA strands were pumped into the flow cell, where the fluorophores (in this work, Cy 5.5) would be excited within the evanescent field around the optical-fiber. Afterwards, fluorescent emission signals were coupled to the optical fiber and could be quantitatively recorded. Here, the degree of nonspecific adsorption of ssDNA on the optical-fiber surface was judged by noncomplementary DNA-F (nc-DNA-F) induced signals. In order to investigate possible reasons for the poor blocking performance of neutral BSA solution toward the optical-fiber surface, free fluorescent molecules (Cy5.5) induced signals were analyzed as another control group. As shown in Figure S1, signal induced by 10 nM free Cy5.5 molecules (blue solid curve) exhibited a long washing time compare with the ideal rapid washing curve (red short dash).

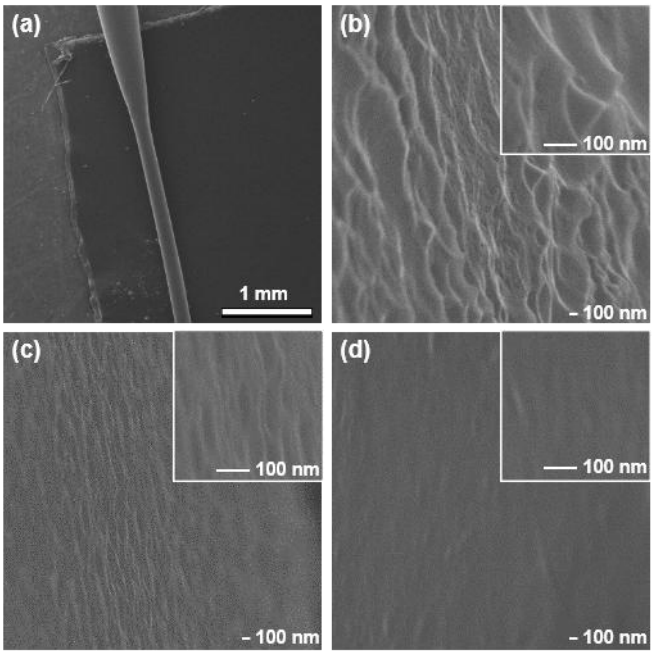

Figure 2. SEM images of tapered optical-fiber surfaces. **(a)** Tapered structure of the optical-fiber. Optical-fiber surfaces with different treatment: **(b)** unblocked, **(c)** neutral BSA blocked, and **(d)** iep-BSA blocked. (b)-(d): 50,000 X magnification; (b)-(d) insert: 200,000 X magnification. All blockings were done under 150 rpm shaking for 4 h at 37 ^{o}C, the BSA concentration was 2 mg/mL.

Slow washing speed might suggest for the existence of physical barriers on the surface,[33] therefore we investigated the morphologic changes in the modification and blocking process of the optical-fibers. It is noteworthy that before DNA immobilization, the optical-fiber was first etched by HF to form a tapered structure for improved signal acquisition.[34-35] However, facile HF etching might induce irreversible structural damage, resulting in a coarse surface. SEM images further proved this conjecture. As shown in Figure 2a, a tapered structure could be observed. When the etched portion was 50,000X magnified, irregular holes appeared on the optical-fiber surface (Figure 2b). After blocking with neutral BSA solution, holes on the surface of the optical-fiber shallowed to a certain, as shown in Figure 2c. Inspired by these

observations, we assumed the poor blocking performance of neutral BSA solution was generated from inadequately blocking.

Although blocking is known to be a complex comprehensive effect of a series of weak forces (hydrophobic interaction, electrostatic interaction, and Van del Waals forces) and sometimes covalent interaction, the physical driving force of incipient blocking was precisely similar to that of adsorption. Thus the above mentioned inadequately blocking could be first viewed as challenges in insufficient adsorption. Luckily, BSA is a soft protein with low conformational stability[36] and may change its adsorption properties under different conditions. Therefore we focused on investigating the optimal blocking condition of BSA solution instead of searching for another fancy blocking agent. Valle-Delgado and coworkers reported that the electrostatic interaction was negligible between BSA molecules at pH near its iep, a small attraction even appeared before the overlap of BSA molecules.[37] Both reduced repulsion and the small attraction could help BSA molecules to pack more closely on the surface, which may lead to a smoother surface. Moreover, having 60 surface lysine groups, BSA could act as a polycation when protonated at its iep,[38] which might accelerate its adsorption toward negatively charged DNA modified surface.

Inspired by these valuable studies, we applied iep-BSA solution as blocking agent in the OFEW system. Indeed, blocking efficiencies of BSA to the optical-fiber surface changed with pH values of the solution, and were calculated to be 70%, 50% and 57%

at pH 4.6, 6.8 and 8.0, respectively (Figure S2). Therefore, the maximum blocking amount of BSA occurred at its isoelectric point. Besides, as shown in Figure 2d, no apparent hollows could be seen after isoelectric BSA blocking.

Changes of nonspecific adsorption signals

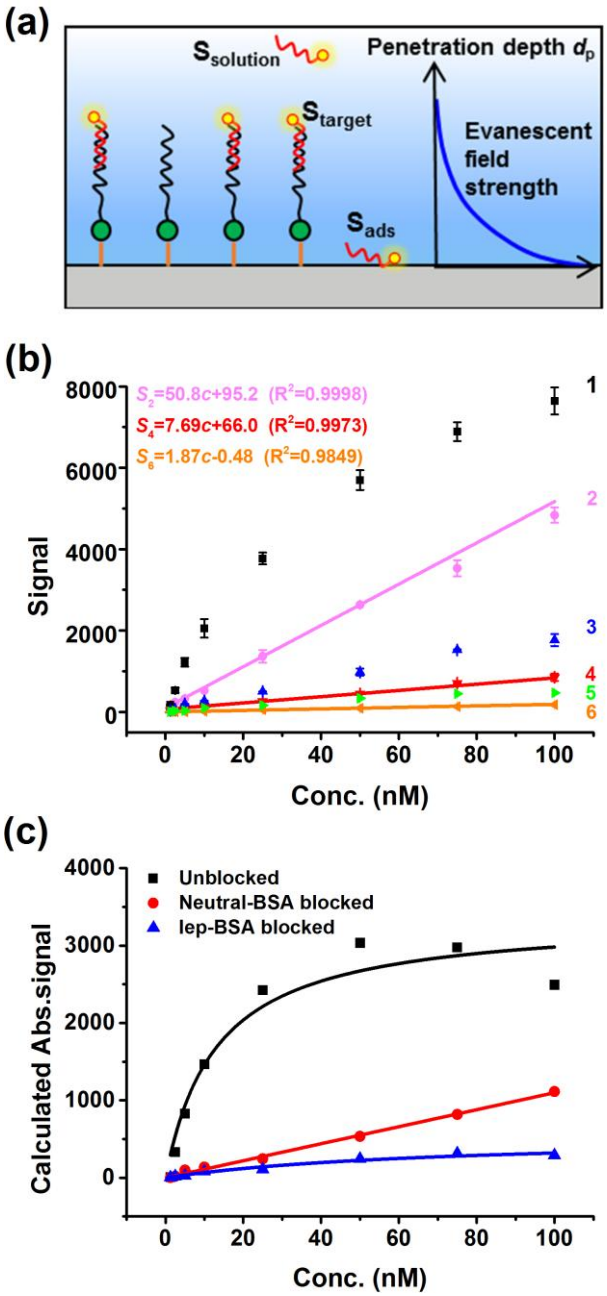

Figure 3. (a) Scheme of three types of signal sources contributing to the total signal collected using a OFEW system. **(b)** $S_{Cy5.5}$ from unblocked (2), neutral BSA blocked (4), and iep-BSA blocked (6) optical-fibers; $S_{nc\text{-}DNA\text{-}F}$ from unblocked (1), neutral BSA blocked (3), and iep-BSA blocked (5) optical-fibers. S_2, S_4 and S_6 are the linear fitting equations corresponding to curve 2, 4 and 6. **(c)** Calculated S_{ads} signals from unblocked (black), neutral-BSA blocked (red), and iep-BSA blocked (blue) optical-fibers and corresponding fittings using Langmuir isotherm model (supplementary information, section 4).

Thinking in a signal decomposition way, peaks appeared on the OFEW display panel represent comprehensive fluorescent contribution (S_{tot}) generated from three types of fluorescent sources (Figure 3a), including fluorescent labeled molecules fully dissolved in solution ($S_{solution}$), adsorbed onto the sensing surface (S_{ads}), and hybridized with surface tethered ssDNA strands (S_{target}), as described by Equation (1) in a simple symbolic way. To determine the relative proportions of each signal, we chose free Cy5.5 dye and nc-DNA-F as signal indicators for $S_{solution}$, $S_{solution}$+ S_{ads}, respectively (Equation (2)—(3)).

$$(1)\ S_{tot} = S_{ads}+S_{target}+S_{solution}$$

$$(2)\ S_{Cy5.5} = S_{solution}$$

$$(3)\ S_{nc\text{-}DNA\text{-}F} = S_{ads}+S_{solution}$$

$S_{Cy5.5}$ and $S_{nc\text{-}DNA\text{-}F}$ data obtained from unblocked, neutral BSA blocked, and iep-BSA blocked optical-fibers were plotted in Figure 3b, and $S_{Cy5.5}$ data (series 2, 4 and 6) were fitted using linear equation. Obviously, both $S_{Cy5.5}$ and $S_{nc\text{-}DNA\text{-}F}$ reduced significantly on the two blocked optical-fibers. Compared with unblocked optical-fiber, the slope of linear fitted c-$S_{Cy5.5}$ relationship decreased 84.9% and 96.3% on neutral BSA blocked and iep-BSA blocked optical-fibers, respectively. The improvement in signal suppression for nonspecific adsorptions indicated that the effective blocking of iep-BSA began to take shape. Next, S_{ads} values were calculated from the difference of Equation (3) and Equation (2) and were further plotted in Figure 3c.

Data in Figure 3c were fitted by Langmuir isothermal adsorption model.[39] The fitting results are listed in Table S1. After iep-BSA blocking, the adsorption isotherm obeyed equations of the Langmuir type with reasonable parameters (R^2=0.96122), which was consistent with previous observation that isoelectric BSA blocking smoothed the sensing surface.

Probing target hybridization signals

After iep-BSA blocking, hybridization signals could be clearly discriminated with nonspecific signals (Figure 1d). The signal/noise ratio induced by 10 nM c-DNA-F and 10 nM nc-DNA-F reached 14.125, which was 8.7 times greater than that of neutral BSA blocked optical-fiber (signal/noise ratio was only 1.63, Figure 1c). Besides, net signals induced by c-DNA-F was 3 times greater on the iep-BSA blocked optical-fiber, which implied that iep-BSA blocking also ensured higher hybridization efficiency. We also tried polymer blockers including PSS, PAH and PAA, no significant hybridization signals could be recognized, and the nonspecific adsorption signals are 8.7-12.4 times higher than that on an iep-BSA blocked optical-fiber (Figure S5).To probe target hybridization signals, c-DNA-F of different concentrations were prepared in hybridization buffer (0.1 M phosphate solution, 0.15M NaCl, pH 7.4). After optical-fiber installation and basic parameter settings, c-DNA-F solutions were pumped into the flow cell of the iep-blocked OFEW platform. Then the pump would stop for a period of time, allowing c-DNA-F to hybridize with tethered DNA on the

optical-fiber surface. Fluorescent signals were collected by the inset computer software and displayed on an electronic touch screen.

The calibration curve was plotted using net hybridization signals collected from the inset software of OFEW system. In the presence of 125 pM-100 nM c-DNA-F, higher concentrations of c-DNA-F resulted in larger hybridization signals until reaching a plateau when the concentration of c-DNA-F was higher than 100 nM (Figure 4a). According to the *signal/noise>3* principle in analytical chemistry,[40] this OFEW system provided a measured detection limit (LOD) of 125 pM. A linear relationship between the net hybridization signals and c-DNA-F concentrations could be observed in the range of 5 nM-100 nM (S=22.23c/nM+1131.7, R=0.998) (Figure 4b). Sensing performances of several DNA based optical-fiber sensors were listed in Table 1, this OFEW sensing platform was shown to be rapid and sensitive in target detection.

Table 1. Sensing performances of several DNA based optical-fiber sensors

No	Detection time	LOD	Reusable cycles	Ref
1	3 min	125 pM	120	This work
2	15-20 min	200 nM	Not mentioned	20
3	70 sec	5 nM	Not mentioned	41
4	45 min	16 nM	At least 5	42
5	25 min	1.1 nM	A few repeated assay cycles	43
6	3 min	1.1 nM [a] / 24 fM [b]	Single-exponential signal loss during consecutive hybridization assays	44

a. LOD toward unlabelled target DNA;

b. LOD toward a fluorescein-labelled target DNA;

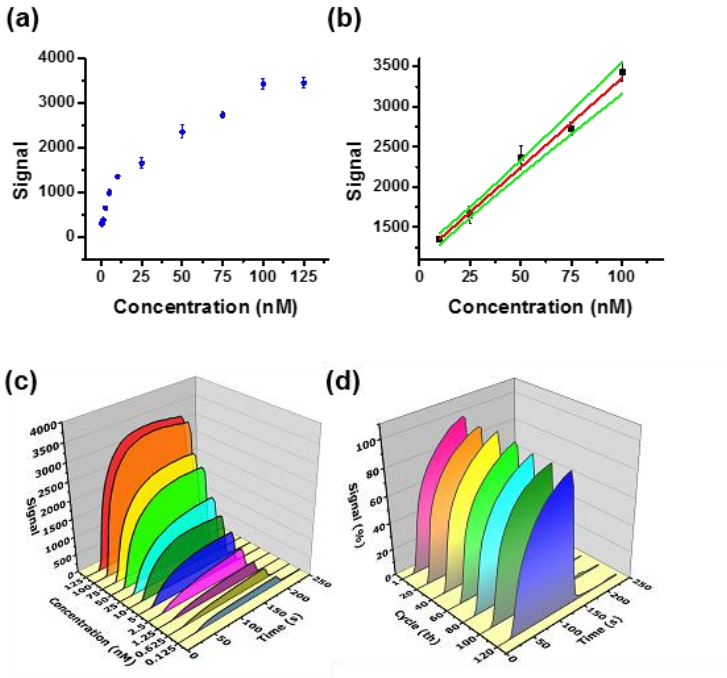

Figure 4. (a) Calibration curve of the DNA based OFEW system toward target complementary DNA. **(b)** Linear range 10-100 nM with a 95% confidence band (shown in green). **(c)** Original signal traces

induced by c-DNA-F at different concentrations. **(d)** 7 representative cycles from 120 detections using one optical-fiber. 10 nM c-DNA-F samples were used for all these cycles.

Reusability of the DNA modified optical-fiber

Reusability is an important parameter for biosensors. Usually, reusable biosensors are cost effective, time-saving and also exhibit automation potential comparing with disposable counterparts. Regeneration of the DNA surface could be achieved by thermal or chemical denaturation. However, it has been reported that some chemical regeneration strategies like formamide, guanidine hydrochloride, HCl, even hot water might cause irreversible conformational damages to immobilized DNA strands, leading to less successful detections after a few repeated cycles.[42-43, 45-46] In this work, surface regeneration could be achieved by sequential flow of acid surfactant solution (0.5% SDS, pH ~1.9) as washing buffer and 10 mM PBS as stabilizing buffer. This strategy shortened the cycle time to 3 min per sample (Figure 4c), which ensured a rapid detection for emergency monitoring applications. Moreover, the optical-fiber maintained its sensing ability after being used for at least 120 times. Seven representative signal traces were plotted in Figure 4d. Signals in good parallelism indicated that no significant loss of surface blocked BSA could be observed during the reusable cycles, thus it was very likely that strong forces like covalent bonds formed after incipient adsorption. This obtained reusability was higher than other reported DNA immobilized optical-fiber sensors (Table 1). Besides, the optical-fiber kept its sensitivity after sonication in 10 mM PBS in a 100 W bath-type sonicator for 5 min

(Figure S5). Only 3% signal attunation appeared after sonication, which suggested that iep-BSA was a robust blocking agent for this reusable and sensitive OFEW system.

Conclusions

In conclusion, the light of routinely used neutral BSA as blocking agent dimmed when it was applied to the DNA immobilized OFEW sensing system, once again proved the consensus that no single blocking agent could be ideal for all systems.[33, 47] ② After morphologic studies, inadequate adsorption of the blocker protein toward the porous HF-etched optical-fiber surface might explain the poor blocking performance. Instead of isolating the blocking issue from the adsorption behavior, we applied research fruits on adsorption studies of BSA to the biosensing field. In this work, possibly due to large blocking amount and close lateral alignment of BSA molecules, iep-BSA blocking efficiently prevented the adsorption of non-target DNA on the optical-fiber sensing surface and increased the portion of active target DNA for surface hybridization. After iep-BSA blocking, complementary DNA strand could be detected at the range of 125 pM-100 nM. Moreover, one optical-fiber could be used for around 120 times. This work is the first to utilized iep-BSA as a robust blocking agent for OFEW system. We believe that this work will provide inspiration for other research, and may widen the application of OFEW system as a potent tool in environmental study as well as health-related research.

Associated Content

② Thus the authors suggest that necessary verifications should be conducted when applying isoelectric BSA as blocking agent for other surfaces like QCM and SPR. For immunoassays using OFEW system, netrual BSA showed good blocking performances, see reference 12-15, possibly due to the large molecular sizes and dispersed charges of antibodies.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. This supplementary information (PDF file) includes (1) signal trace of free Cy5.5 molecules, (2) BSA adsorption to optical-fiber surface at different pH values, (3) AFM images of DNA-modified chips, (4) isotherm adsorption models used in this work, (5) signal changes after sonication, (6) nonspecific signals using other surface blocking agents and (7) original signal traces of the 1st and 120th cycle.

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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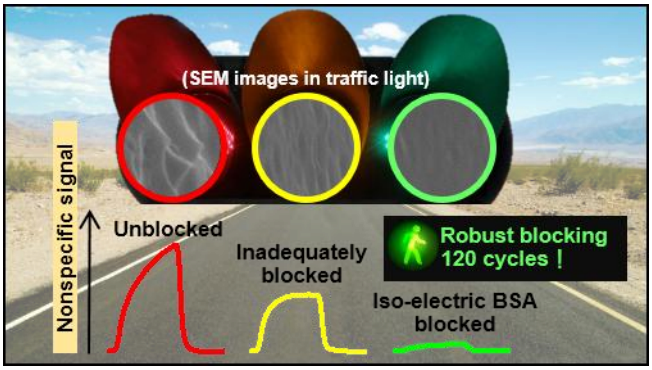
(SEM images in traffic light)
Nonspecific signal
Unblocked
Inadequately blocked
Robust blocking 120 cycles !
Iso-electric BSA blocked

For TOC only