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A surface modification strategy on silicon nitride for developing biosensors

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Abstract

A surface modification strategy for the use of giant magnetoresistive materials in the detection of protein–protein interactions is developed. This modification strategy is based on silanization of semiconductive materials. A native silicon nitride surface was treated with concentrated hydrofluoric acid to improve surface homogeneity. Nano-strip was used to oxidize silicon nitride to form a hydrophilic layer. Aminopropyltriethoxysilane was subsequently used to functionalize the treated surfaces to form amine groups, which were further activated with glutaraldehyde to introduce a layer of aldehyde groups. The effectiveness of this modification strategy was validated by chemiluminescence immunoassays of purified 6× His-HrpW of *Pseudomonas syringae* pv. *tomato* DC3000 and human transferrin. Signals with intensities related to concentrations of these two immobilized model proteins were observed. The modified surface was also validated by a more complex system: intercellular proteins secreted by DC3000. HrpW in these protein mixtures was successfully recognized by anti-HrpW antibodies when mixed proteins were immobilized onto activated surfaces. This surface modification strategy provides a platform onto which proteins can be directly immobilized for biosensor and protein array applications.

Keywords: GMR; Protein immobilization; Immunoassays; Biosensor; Silicon nitride

Biomolecular interaction detection based on magnetic sensing is an emerging technology in the past 10 years. Various magnetic sensors, e.g., superconducting quantum interference devices [1–3] and inductance interference magnetic permeability meters [4–6], have been developed to detect magnetically labeled biomolecules. Giant magnetoresistive (GMR)[1] sensors are a potential high-density sensing system for the high-throughput detection of nucleic acid (DNA) and protein interactions [7–10]. Biomolecules, e.g., DNA or protein, can be immobilized to the surface of GMR sensors to capture other biomolecules in solution through specific interactions. The captured molecules can either be directly labeled to magnetic particles or recognized by secondary molecules attached to magnetic labels. The specific presence of magnetic particles alters the magnetic field in the vicinity of the GMR sensor, resulting in electronic detection. The semiconductor materials industry has developed technologies for the fabrication of GMR sensors with high spatial density, at least one million sensors in one square centimeter [11], and magnetic particles with a size range from nanometer to micrometer are commercially available. Thus, GMR sensors can reach a higher degree of integration and compactness than conventional optical systems often used in biomolecular detections. Those are desirable features for many life science applications, e.g., field inspections and point-of-care testing.

The development of GMR sensors requires efficient surface modification techniques. GMR sensors are

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[1] *Abbreviations used:* GMR, giant magnetoresistive; APTES, aminopropyltriethoxysilane; IgG, immunoglobin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HF, hydrofluoric acid.

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covered by a layer of silicon nitride, which is electronically neutral and nonporous so that noncovalent immobilization techniques based on physical adsorption, ionic binding, and entrapment are not effective [12]. Hence, modification strategies are needed to facilitate the attachment of biomolecules to silicon nitride surfaces. Sheehan et al. [13] deposited a thin film of gold over the top of silicon nitride. DNA was modified with thiol linkers and immobilized to gold through thiol–gold chemistry. While the immobilization strategy based on thiol–gold chemistry is convenient and well studied, it is not simply extended to protein immobilization because of the chemistry of proteins. Additionally, theoretical considerations suggest that signal intensity decreases cubically with an increase in the distance between magnetic labels and GMR sensors [14]. Therefore, a technology for immobilizing proteins directly onto GMR sensors is desirable.

In this study, a direct surface modification approach based on silanization with aminopropyltriethoxysilane (APTES) and activation with glutaraldehyde is described. A complementary approach was studied by Williams and Blanch [12] with a focus on the covalent binding efficiency using different surface modifying processes and protein linkers. Similar approaches have been used to immobilize proteins onto silicon nitride in developing protein patterning technologies [15,16]. This study, however, focuses on the development of a silicon nitride modification strategy compatible with protein detection based on magnetic labels. The strategy developed in this study could be integrated with the development of GMR sensors for protein detection, although this integration could require substantially more effort.

The APTES–glutaraldehyde modification approach has been previously used on silica and silicon dioxide surfaces for the attachment of antibodies for the detection of antigens [17–19]. In these previous studies, native silica surfaces were modified and used to study small molecule drugs [17] or to probe protein G–IgG interactions [18]. In the present strategy, the APTES–glutaraldehyde modification strategy is extended to silicon nitride surfaces and used to probe protein–protein interactions that include the presence of a magnetic bead and to determine the effectiveness of this approach in the detection of specific interactions in complex biological samples. APTES dissolved in ethanol was used to silanize silicon nitride surfaces to generate surface amine groups. The amine groups were then activated into aldehyde groups by glutaraldehyde. Proteins were immobilized on these surfaces through Schiff's base condensation between free amine groups on proteins and aldehyde groups on the modified surfaces. The modification of surfaces after each step was confirmed by static contact angle measurements. To test this surface modification strategy with immunoassays, we chose human transferrin and HrpW of a plant pathogenic bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (henceforth DC3000) as model systems. Human transferrin is an important protein for ion transfer in the peripheral blood system [20]. HrpW is a virulence factor secreted by DC3000 during infection [21] and the detection is performed in a complex mixture. The modified surfaces were used successfully to detect the protein–protein interaction between the HrpW and the anti-HrpW and between transferrin and anti-transferrin by chemiluminescent immunoassays. The presence of an aldehyde group offers the opportunity to react with multiple proteins due to the abundance of amine groups on protein surfaces [22].

Materials and methods

Surface modification of silicon nitride (Si_3N_4)

The Si_3N_4 surface was modified as described previously [12] with the following changes. The untreated native Si_3N_4 surfaces were obtained from Silicon Quest (Santa Clara, CA) as 200-μm-thick Si_3N_4 layers deposited on ⟨100⟩ silicon wafer by low-pressure chemical vapor deposition (refractive index 2.00 ± 0.05). The native Si_3N_4 surfaces were etched with concentrated (49%) hydrofluoric acid (HF) (Mallinckrodt Baker, Phillipsburg, NJ) for 1 min to remove oxides and other surface contaminants. The etched surfaces were oxidized in Nano-strip (Cyantek, Fremont, CA) at 70 °C for 30 min to form a new hydrophilic oxide layer. Pretreated surfaces were immersed in 2% APTES (Gelest, Morrisville, PA) dissolved in anhydrous ethanol and incubated in a glovebox (M. Braun, Stratham, NH) with humidity below 1 ppm for 24 h. After incubation, surfaces were sonicated in anhydrous ethanol for 1 min using a sonicator (Model SC-50T, Sonicor, Copiague, NY) and cured in an oven (Fisher Scientific International, Hampton, NH) at 90 °C for 30 min. These surfaces were then immersed in phosphate-buffered saline (PBS), pH 9.0, supplemented with 25% glutaraldehyde for 1 h at room temperature. After incubation, surfaces were washed in PBS twice for 2 min each to remove excess glutaraldehyde, dried by filtered air flow, and stored at room temperature until experiments.

Static contact angle measurement

A static contact angle measurement was carried out after each step to evaluate the effectiveness of surface modification and to monitor process reproducibility. The contact angles were determined by an NRC contact angle goniometer (Model 100-00; Ramé-Hart, Mountain Lakes, NJ) at room temperature. A droplet of deionized water was gently placed onto the surface. The angle between the edge of the droplet and the surface was

measured. Measurements were made in different regions on the surface with six replicates.

Preparation of purified HrpW

To purify the DC3000 HrpW for subsequent immunoassays, the *hrpW* gene from DC3000 was cloned in the pET-DEST42 vector (Invitrogen, Carlsbad, CA) under the T7 promoter and upstream of the gene producing a histidine tag (6× His). The plasmid was electroporated into *Escherichia coli* BL21. The strain was grown in LB medium [23] supplemented with 100 μg/ml ampicillin in an incubator–shaker (37 °C, 250 rpm) overnight to reach the stationary phase. The overnight culture was diluted in fresh LB medium at a ratio of 1:60 and incubated in an incubator–shaker (250 rpm). Cells were grown at 37 °C until OD_{600} reached 0.6. Isopropyl-β-D-thiogalactopyranoside was then added at 0.05 mM to induce the expression of 6× His-HrpW, and the temperature was reduced to 30 °C. Cells were incubated for 5 h and then harvested by centrifuging at 4000*g* for 15 min at 4 °C. Cell pellets were stored at −70 °C until protein purification. 6× His-HrpW was purified by following the protocol of the Qiagen Ni–NTA–agarose purification kit (Qiagen, Valencia, CA). The purified 6× His-HrpW solution was concentrated and desalted with filter columns (Centricon Plus-20 and Microncon; Millipore, Billerica, MA). The retentate was collected and stored at −20 °C until experiments.

Harvest of supernatants of DC3000 and its isogenic ΔhrpL mutant

DC3000 was first grown in King's medium B (KB medium) [24] supplemented with 50 μg/ml rifampicin in an incubator–shaker (30 °C, 250 rpm). The isogenic *ΔhrpL* mutant was first grown in KB medium supplemented with 50 μg/ml rifampicin and 50 μg/ml spectinomycin. The cells were harvested when the OD_{600} reached a value of 6. Cells were then washed once with Hrp minimal medium [25], transferred to Hrp minimal medium, and incubated in an incubator–shaker (25 °C, 250 rpm). When the OD_{600} reached 0.6, the culture was centrifuged and the supernatant was collected and stored at −70 °C. Before use, the supernatant was thawed and concentrated with filter columns (Amicon Ultra MWCO 50KDa; Millipore). The retentate was collected and stored at −20 °C.

Chemiluminescence detection of proteins immobilized on Si_3N_4

The detection was performed by following the protocol developed by Dune et al. [26] with modifications. Human transferrin (Sigma, St. Louis, MO) and 6× His-HrpW were diluted in 10-fold series in PBS (pH 7.4) to give concentrations from 1.0 ng/μl to 1.0 μg/μl (transferrin) and 7.3 ng/μl to 7.3 μg/μl (6× His-HrpW). Protein solutions were then spotted onto activated surfaces at 0.3 μl/spot. The spotted surfaces were incubated in a closed humid chamber for 2 h at room temperature. After the incubation, the surfaces were inverted gently to contact a blocking solution (1.5% BSA, 5% low-fat dry milk in PBS, pH 7.4) for 1 min. Then the surface was turned face up, covered with the blocking solution, and incubated for 1 h at room temperature. After incubation, blocking solutions supplemented with 0.1% Tween 20 and primary antibodies were applied to the top surfaces. Goat anti-human transferrin (Sigma) was diluted 16,000-fold and rabbit anti-HrpW was diluted 100-fold to detect the spotted protein. Surfaces covered with primary antibodies were incubated in a humid chamber for 1 h at room temperature. After the incubation, the surfaces were washed four times for 5 min each with washing solutions (PBS, pH 7.4, 0.1% Tween 20). Secondary antibodies were also diluted in blocking solution supplemented with 0.1% Tween 20. Anti-goat IgG-HRP (Santa Cruz Biotech, Santa Cruz, CA) was diluted 6000-fold. Anti-rabbit IgG-HRP (Promega, Madison WI) was diluted 600-fold. Surfaces covered with secondary antibody solutions were incubated for 1 h at room temperature. After the incubation, surfaces were washed four times for 5 min each with washing solutions. Probed surfaces were developed in premixed SuperSignal working solutions (Pierce, Rockford, IL). Emitted signals were recorded with film (Eastman Kodak, Rochester, NY). The recorded images were scanned with Fujifilm FLA-3000 scanner (Fuji, Tokyo, Japan). The absorbance wavelength was set at 473 nm, the filter wavelength was set at 520 nm, and the sensitivity was set at F1000. The scanned image was analyzed by Image Gauge V3.46 (Fuji Photo Film, Elmsford, NY).

Probing with magnetic bead-labeled proteins

To test the compatibility of these modified surfaces with magnetic labels, activated surfaces were functionalized with a serial 10-fold dilution of human IgG (Sigma; 0.1–0.001 μg/μl) and anti-transferrin (10–0.1 μg/μl). Protein A beads (Dynabeads Protein A) were obtained from DynalBiotech (Brown Deer, WI). Transferrin was conjugated with epoxy-functionalized magnetic beads (Dynabeads M-270 Epoxy; DynalBiotech) by following the manufacturer's instructions. Bead-labeled protein A and bead-labeled transferrin were applied to the functionalized surfaces and incubated in a humid chamber at room temperature overnight. After incubation, surfaces were washed in PBS, pH 7.4, with gentle shaking for 1 min to remove unbound beads. The bound beads were detected with an optical microscope (Model BX51W1; Olympus American, Melville, NY). Images were acquired with a charge-coupled device camera (Retiga 1300; Q Imaging;

Southern Micro Instruments, Marietta, GA) and analyzed with ImageJ Ver1.32 (http://rsb.info.nih.gov/ij/).

Results and discussion

In this study, a direct surface modification strategy for Si_3N_4 was developed. The modification process was monitored by static contact angle measurements. The standard deviation of static contact angles decreased after the HF etching step (Table 1). This change indicates improved surface homogeneity. The contact angle decreased from 33 ± 1 to $5 \pm 1°$ after the Nano-strip oxidation, suggesting increased surface hydrophilicity. This is consistent with a high surface density of silanol groups. The value increased from 5 ± 1 to $53 \pm 3°$ after APTES silanization, indicating increased surface hydrophobicity. This increase is likely due to the presence of amine groups, and the carbon backbone of APTES, which are more hydrophobic than silanol groups. There is no significant difference between the contact angles before and after activation. This observation is consistent with the results of Lee et al. [15] showing no significant change of contact angle after modifying aminosiloxanes-silanized surfaces with glutaraldehyde. Anhydrous ethanol was used for silanization because water in solvent has been reported to cause significant precipitation of APTES oligomers [27].

Tests using a model system of transferrin were performed next. Transferrin at concentrations of 0.001–1 μg/μl was immobilized on the activated surfaces of Si_3N_4. Goat anti-transferrin antibody was used to probe the immobilized transferrin. These antibodies were detected by chemiluminescence. As expected, the immunoassay showed signals with intensity related to spotted protein concentration as shown in Fig. 1. No signals were measured when immobilized transferrin was probed by anti-HrpW as a negative control (data not shown). The compatibility of this surface modification strategy with magnetic labels was also verified using magnetic beads as shown in Fig. 2. As expected, bead-labeled protein A (Fig. 2A) and bead-labeled transferrin (Fig. 2B) were able to bind to surfaces functionalized with IgG and anti-transferrin, respectively, with a particle density related to immobilized protein concentration. In contrast, the beads did not bind to surfaces functionalized with BSA as a negative control (Figs. 3A and B). Additionally, bead-labeled protein A and bead-labeled transferrin pre-incubated in IgG and anti-transferrin solutions did not bind to surfaces functionalized with IgG and anti-transferrin (Figs. 3C and D).

In addition, purified HrpW was studied as a model system. The 6× His-HrpW produced by *E. coli* was purified, immobilized on Si_3N_4, and detected by rabbit anti-HrpW antibody. As expected, signals with intensity proportional to the concentration of immobilized 6× His-HrpW (0.27–7.3 ng/μl) were observed (Fig. 4A). As a

Table 1
Contact angle measurement of surfaces after each step in the modification process

Surfaces	Native Si_3N_4	HF etching	Oxidation	Silanization with APTES	Activation with glutaraldehyde
Contact angle	$32 \pm 12°$	$33 \pm 1°$	$5 \pm 1°$	$53 \pm 3°$	$54 \pm 2°$

The angle is expressed as means ± SD.

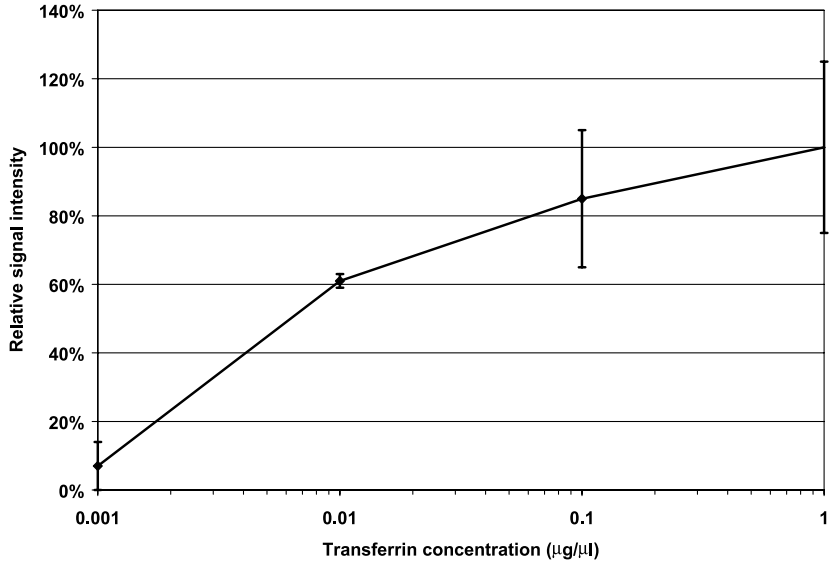

Fig. 1. Chemiluminescence detection of transferrin immobilized on Si_3N_4. Error bar represents ±1 SD.

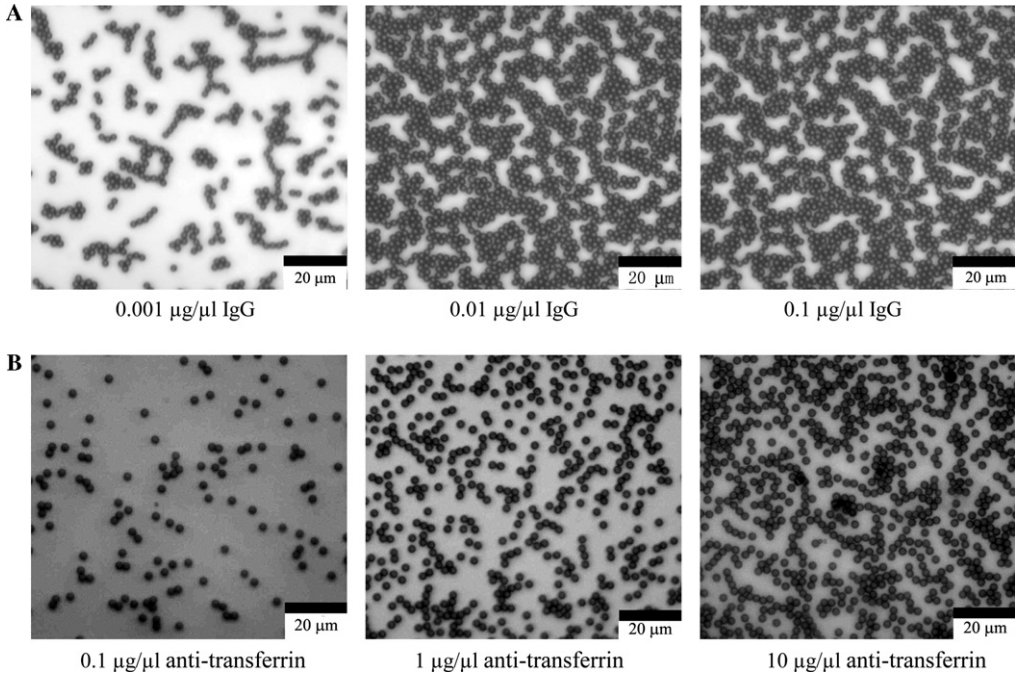

Fig. 2. Images of surfaces functionalized with serial dilutions of IgG and anti-transferrin probed with bead-labeled protein A and bead-labeled transferrin. (A) Surfaces functionalized with 0.1–0.001 μg/μl IgG probed with bead-labeled protein A. (B) Surfaces functionalized with 10–0.1 μg/μl anti-transferrin probed with bead-labeled transferrin.

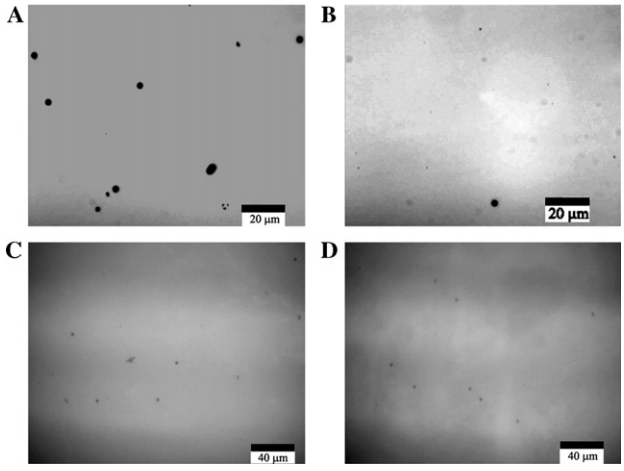

Fig. 3. Immunoassay of the functionalized surfaces (negative controls). (A) Surfaces functionalized with 2% BSA probed with bead-labeled protein A. (B) Surfaces functionalized with 2% BSA probed with bead-labeled transferrin. (C) Surfaces functionalized with 1 mg/ml IgG probed with bead-labeled protein A preincubated in a solution containing 1 mg/ml IgG. (D) Surfaces functionalized with 10 mg/ml anti-transferrin probed with bead-labeled transferrin preincubated in a solution containing 10 mg/ml anti-transferrin.

negative control, immobilized transferrin (1 μg/μl) did not give any signal when probed with anti-HrpW (data not shown). Hence, these modified surfaces may be useful to detect specific protein–protein interactions using magnetic beads.

Although the study of model systems such as the ones above and others [17–19] provides a basis for new technology, it is also important to demonstrate an analysis of a complex biological sample. Thus, we also studied the intercellular proteins secreted by DC3000 and *ΔhrpL* mutant, which, without purification, were spotted on modified surfaces and probed with anti-HrpW. In this case, we expect HrpW to be present in the DC3000 sample, but not present in the sample of isogenic *ΔhrpL* strain [28]. The immunoassay using secreted protein from DC3000 showed strong signals, again with intensities related to the spotted concentration within the tested range, as shown in Fig. 4B. In contrast, the sample from the *ΔhrpL* mutant did not show any observable signals (data not shown).

The results discussed above suggest that these modified surfaces can be used to detect protein–protein interactions. Further, the attachment chemistry relies on the presence of aldehyde groups and may be useful for a wide variety of proteins. The signal intensity of chemiluminescence immunoassays reached a maximum at 100 ng/μl immobilized transferrin and 7.3 ng/μl immobilized 6× His-HrpW. The volume of each spot was 0.3 μl and the size of each spot was 2 mm in diameter. Though the signal intensity is limited by the quality of antibodies and subsequent detection techniques in addition to the amount of immobilized proteins, it is a reasonable estimation that 955 ng/cm^2 transferrin and 70 ng/cm^2 6× His-HrpW can be immobilized and detected in these experiments (assuming all proteins in each spot were immobilized onto surfaces). The reason for different saturating protein loads may be due to different surface densities of amine groups on various proteins.

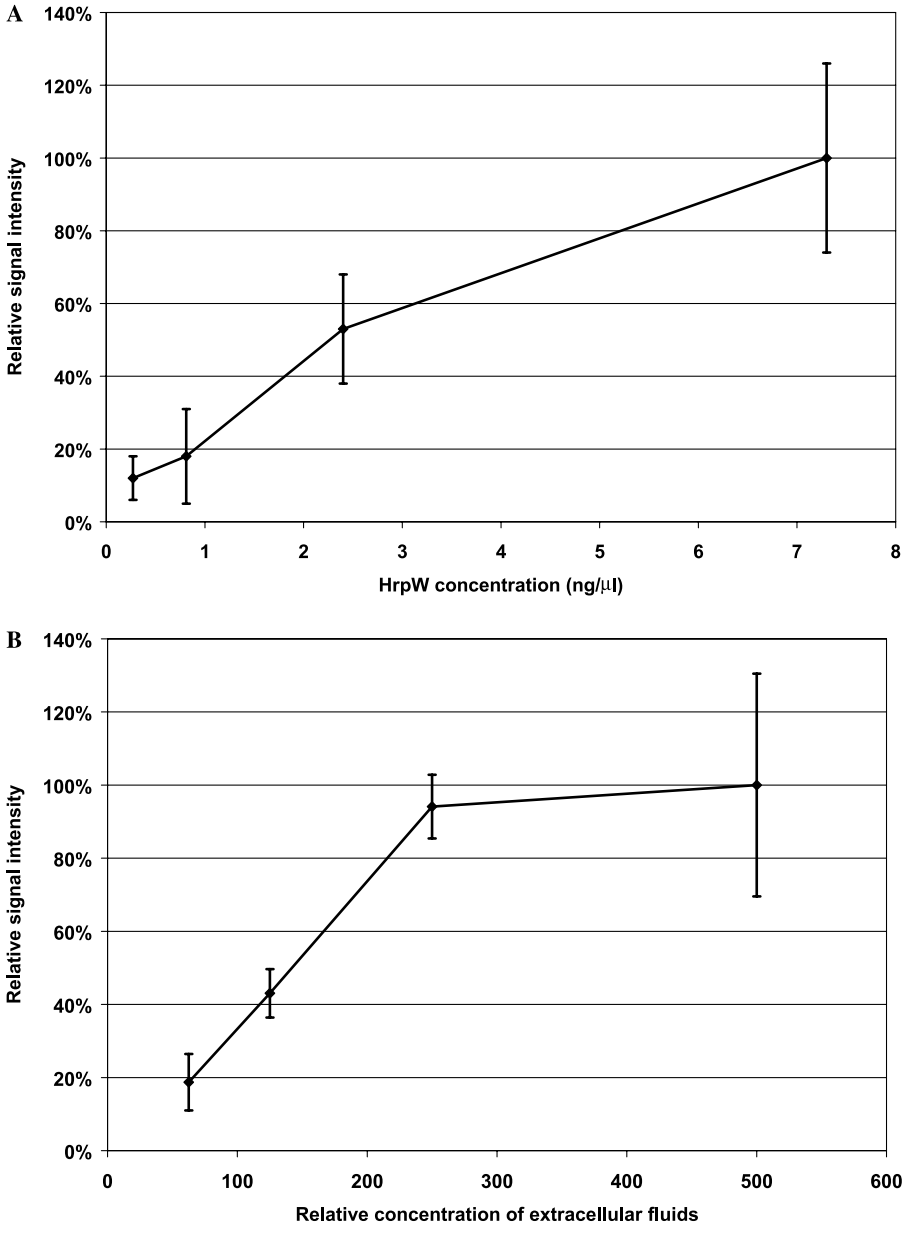

Fig. 4. Chemiluminescence detection of HrpW immobilized on Si_3N_4. (A) Purified 6× His-HrpW immobilized on Si_3N_4. (B) Quantitative results of chemiluminescence detection of HrpW in proteins secreted by DC3000. Initial extracellular fluid was concentrated 62.5, 125, 250, and 500 times, respectively.

The surface modification strategy has been applied to silicon nitride surfaces which have applications in the use of GMR magnetic sensors and which rely on immobilized proteins that may now be extended to applications beyond biosensors, e.g., protein arrays. In this study, as little as 80 pg 6× His-HrpW was successfully detected by chemiluminescence immunoassays. In contrast, approximately 73 ng 6× His-HrpW was required to show a significant signal in Western blots (data not shown). The detection limit may be further improved by using microfluidic liquid handling systems to reduce the volume of each spot. This is a desirable feature for high-throughput assays and precious samples.

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