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Development of a Chemiluminescent Optical Fiber Immunosensor to Detect *Streptococcus pneumoniae* Antipolysaccharide Antibodies

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Abstract

A chemiluminescent-based optical fiber immunosensor was developed for the detection of antipneumococcal antibodies. This was accomplished by developing a different chemical procedure utilizing 3-aminopropyl trimethoxysilane and cyanuric chloride to conjugate pneumococcal cell wall polysaccharides to the optical fiber tips, and by improving the sensitivity of the photodetection system. The lowest titer of antipneumococcal antibodies detected by the optical fiber was at a 1:819,200 dilution. The lowest corresponding value by standard enzyme-linked immunosorbent assay was at a 1:98,415 dilution. It was concluded that the optical immunosensor system is an accurate and sensitive method to detect antipneumococcal antibodies and may be an adequate tool to monitor antibodies in specimens such as saliva and urine.

Index Entries: Optical fiber; chemiluminescence; immunosensor; *Streptococcus pneumoniae*.

Introduction

Streptococcus pneumoniae (pneumococcus) is a bacterial pathogen associated with life-threatening invasive diseases such as meningitis,

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pneumonia, and sepsis *(1,2)*. This pathogen is the most common cause of bacterial pneumonia and is especially prevalent in the elderly and children *(3,4)*. Pneumococcal infections are traditionally treated with antibiotics. However, the recent emergence of antibiotic-resistant strains stresses the need for safe and efficacious vaccines *(5)*. To date, about 90 pneumococcal types have been identified using the serological properties of their capsular polysaccharides *(6)*. However, only 23 serotypes account for the majority of the known pathogenic cases. Several vaccine formulations against these serotypes have been developed and are currently either in use or in clinical trials *(1,7,8)*.

To assess the efficacy of a vaccine, it is essential to monitor the antipneumococcal IgG levels by multiple blood sampling. Multiple blood sampling is traumatic, especially in the pediatric population; therefore, it would be more appropriate to use noninvasive specimens such as urine or saliva. Unfortunately, both the urinary and salivary antibody titers are generally much lower than that of blood, and often cannot be adequately detected with classic enzyme-linked immunosorbent assay (ELISA) methods, despite the presence of humoral antibodies (*[9]*, R. Dagan, unpublished data). Thus, a new and more sensitive yet specific method would be a welcome improvement.

A new diagnostic tool has been developed based on optical fiber sensor technology for the detection of jejunal immunoglobulins for human cholera antitoxin *(10)*. Optical fibers are ideal transducers. Their silica composition enables macromolecular conjugation via silanization, they transmit optical signal with minimal loss, and they can be easily adapted from one antigen-antibody system to another. In addition, optical fibers are flexible, inexpensive, and amenable to mass production. Herein, we further improved the optical system so as to detect rabbit model antipneumococcal antibodies, as a first step toward the end goal of monitoring noninvasive clinical specimens. The optoelectronic detector system was made more sensitive than previously published *(10)*, and a new immobilization strategy was developed to conjugate bacterial capsular polysaccharide macromolecules to the optical fiber.

Materials and Methods

Preparation of Optical Fiber Surface

The multimode optical fiber used was PUV 400BN (CeramOptec, GmBH, Bonn, Germany) with an original numerical aperture of 0.40, a pure silica core diameter of 400 μm, and a refractive index of 1.4571 (at 633 nm); and a cladding diameter of 440 μm and a refractive index of 1.4011 (at 633 nm). The black Tefzel™ jacket was stripped away using a scalpel blade, and the silicone cladding was burned away by flame, leaving a 1-cm decladed optical fiber tip. The tips were cleaned by incubation for 5 min in 48% hydrogen fluoride (Merck, Darmstadt, Germany), rinsed three times with deionized water, once with absolute ethanol (Frutarom, Haifa,

Israel), and finally air-dried. Thereafter, the tips were treated with 5 *M* chromic acid (2 h, 90°C), rinsed five times with deionized water, and treated with a 7:3 (v/v) mixture of 96% H_2SO_4 (Frutarom) and 30% H_2O_2 (Aldrich, Milwaukee, WI) for 60 min at 90°C.

Silanization and Chemical Modifications of Optical Fiber Tips

The optical fiber tips were silanized with 96% 3-aminopropyl trimethoxysilane (Sigma, St. Louis, MO) (1 h, 90°C), rinsed with deionized water, and air-dried. The tips were then exposed to a solution of 3% 1,3,5-trichlorotriazine (cyanuric chloride) (Sigma) in acetonitrile (Frutarom), to which 30 µL of triethylamine (Sigma) was added for a catalytic purpose and allowed to react for 2 h at 0°C. The tips were then washed three times in deionized water, and once with acetone. The unreacted amine moieties of the 3-aminopropyltrimethoxysilane were blocked with 10% formaldehyde (1 h, 20°C), and the Schiff base was stabilized with 0.3 m*M* sodium cyanoborohydride (Aldrich) (1 h, 20°C).

Conjugation of Pneumococcus Polysaccharide to Optical Fiber Tips

The tips were incubated with pneumococcus polysaccharide type 14 (PnPs-14, ATCC 23-X; American Type Culture Collection, Rockville, MD) dissolved in PBS (pH 7.2), at a 125 µg/mL solution (1 h, 0°C), and rinsed twice with phosphate-buffered saline (PBS). The unreacted chlorine moieties of cyanuric chloride were blocked by excess 1% (v/v) glucose solution for 30 min at room temperature. The PnPs-14 conjugated fibers could then be stored in PBS for up to 7 d at 4°C.

Immunoassay Sensor Design

The immunological principles of the assay are similar to those of an indirect ELISA. The conjugated fiber tips were incubated with rabbit antipneumococcal serotype 14 polysaccharide (anti-PnPs-14, HT 94-0050; Wyeth-Lederle, West Henrietta, NY) at different concentrations for 30 min, and washed twice with wash buffer (PBS plus 2% [v/v] Tween-20 and 1% [w/v] bovine serum albumin [BSA] [Sigma]). The tips were then incubated with horseradish peroxidase (HRP)-conjugated goat antirabbit IgG (Sigma) for 30 min at a working dilution of 1:1000 and rinsed four times with wash buffer. To measure the chemiluminescent reaction, each fiber was mounted into the optoelectronic device (Fig. 1), and its tip was dipped into 100 µL of enhanced luminol reagent (Renaissance™; NEN, Boston, MA) within a 0.5-mL conical tube pierced to allow the insertion of a 0.8-mm-diameter Teflon tube (Supelco, Bellefonte), connected to a 1-mL syringe containing 100 µL of oxidizing reagent (Renaissance; NEN). The sample tube was placed into a lightproof, polyvinyl chloride container of 7 mm id and 7 cm in height to exclude environmental light. The chemiluminescent reaction was initiated once the H_2O_2 solution was injected into the tube.

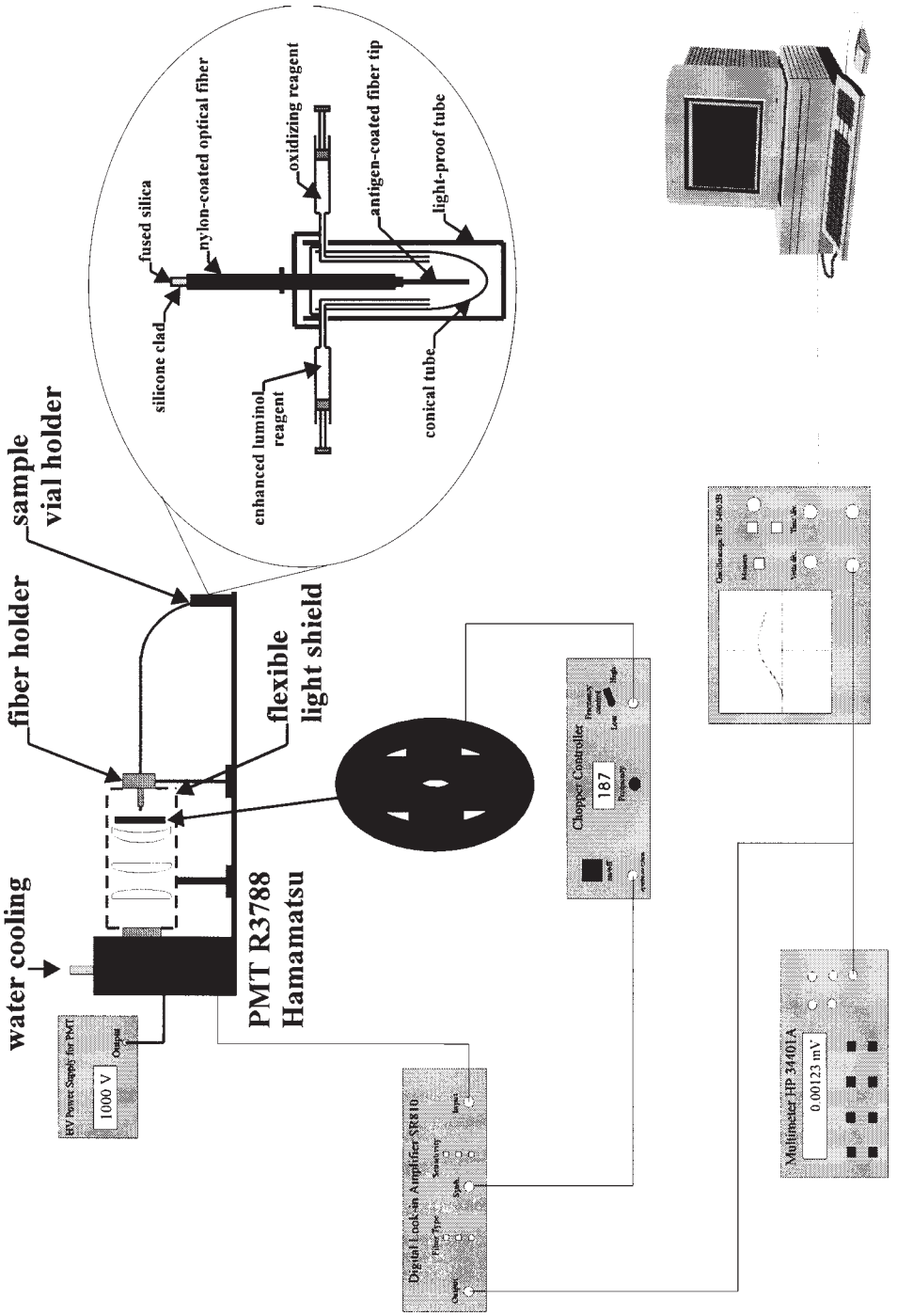
water cooling
PMT R3788
Hamamatsu
fiber holder
sample
vial holder
flexible
light shield
fused silica
nylon-coated optical fiber
oxidizing reagent
antigen-coated fiber tip
light-proof tube
silicone clad
enhanced luminol
reagent
conical tube
1000 V
Chopper Controller
187
0.00123 mV

Fig. 1

Instrument Setup

The optical fiber was mounted via an adjustable single fiber mount (77833; Oriel, Stratford) into a precision optical fiber holder (77837; Oriel). The chemiluminescent signal was focused onto a photomultiplier tube window (R3788; Hamamatsu, Hamamatsu City) via a condenser, composed of an f = 67 mm meniscus lens (Eksma, Lithuania), which fits the aperture of the optical fiber, a collimating plano-convex lens with a focal distance at 50 mm (Eksma), and an achromatic lens (f = 100 mm) (Newport). The photomultiplier tube, with a sensitivity peak at 430 nm, was mounted into a water-cooled system (Hamamatsu). Because the chemiluminescent light is a continuous signal (i.e., its spectrum is found in a low-frequency range at about 0.03–1 Hz), an optical chopper (75152; Oriel) was added into the light pass. The chopper modulated the useful signal to move it into a range of higher frequencies at about 4000 Hz, while noise levels were about 0.03–1 Hz. The signal was then filtered and demodulated by a lock-in amplifier (Stanford Research Systems, Sunnyvale, CA) into a corresponding amplified voltage signal. The electrical signal was then transferred to an oscilloscope (HP54602B; Hewlett Packard, Engelwood) to monitor the type of signal and its real-time behavior. A multimeter (HP34401A; Hewlett Packard) converted the signal from analog to digital data. The data were collected by a 133-MHz pentium personal computer and analyzed by HP 34820 Benchlink Suite version A.01.10 software (Hewlett Packard). The signal was measured as the time (milliseconds) required by the system to reach a 50-mV level. It assumes here that the enzymatic reaction at the fiber-silane layer interface behaves initially according to a typical Michaelis-Menten kinetic law. Therefore, the time required to reach a constant level was proportional to the enzyme concentration in the presence of an excess of substrate. Figure 1 illustrates the instrument setup.

Enzyme-Linked Immunosorbent Assay

The ELISA procedure to measure rabbit anti-PnPs-14 was essentially as described by the Centers for Disease Control (Atlanta, GA) and by Roth et al. *(11)*. Briefly, flat-bottomed 96-well polystyrene microtiter plates (Nunc, PGC Scientific, Gaithersburg, MD) were coated for 5 h at 37°C with 100 μL of a solution of 20 μg/mL of PnPs-14 in PBS (pH 7.4) per well. Excess antigen was removed by washing five times with PBS+Tween (0.1%). The anti-PnPs-14 polyclonal antibody was neutralized with pneumococcal cell wall polysaccharides (Statens Seruminstitute, Copenhagen, Denmark) at a

Fig. 1. *(previous page)* Block diagram of the instrument setup, displaying the optical components used in the chemiluminescent measurements of the optical fiber immunosensors. The light is transferred via an optical system to a photomultiplier, which converts it into an electrical signal. A lock-in amplifier demodulates and amplifies the signal, which is then monitored by an oscilloscope and processed by a computer.

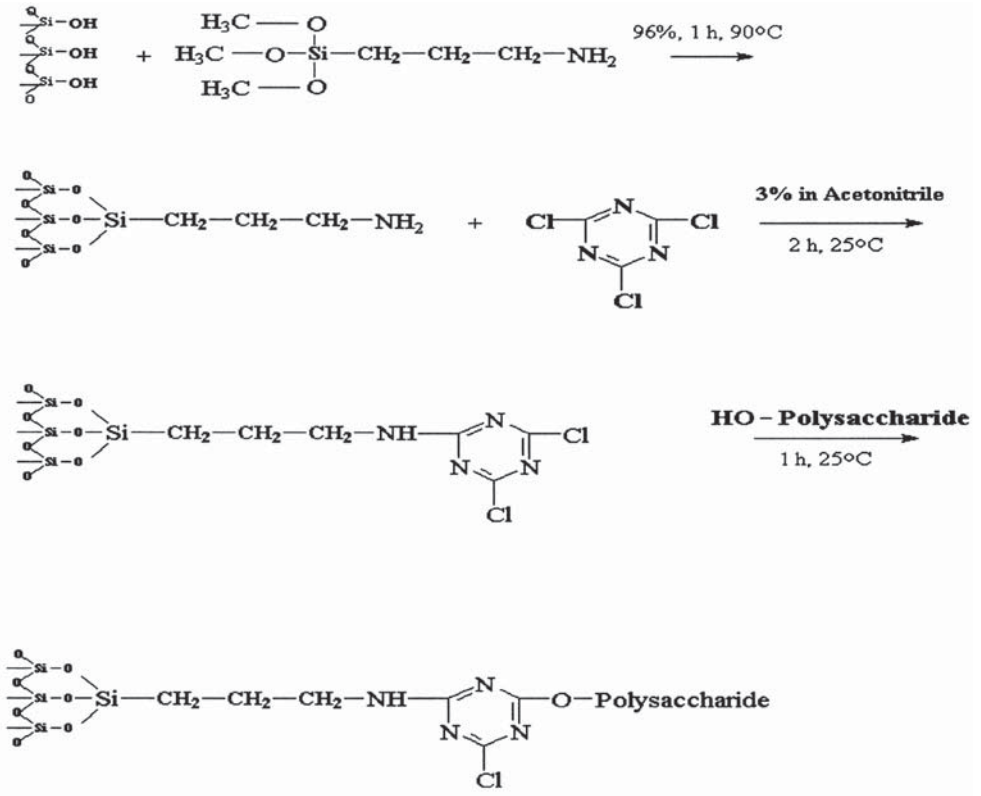

Fig. 2. The chemical modification steps to bind the pneumococcal polysaccharide PnPs-14 onto the optical fiber silica tip sensor. The 3-aminopropyl trimethoxysilane is coupled to the silica surface followed by cyanuric chloride. The cyanuric chloride reacted first via its most active chlorine atom to the amine moiety of the 3-aminopropyltrimethoxysilane, and then one of its lesser active chlorine atoms reacted with the hydroxy moieties of the polysaccharide antigen.

final concentration of 10 μg/mL. The antibody was diluted 1:50 with PBS plus 0.05% (v/v) Tween-20 and 1% (w/v) BSA (PBS-Tween), and titers were made by threefold dilutions. A 50-μL aliquot of the antibody titers was applied in duplicate and incubated for 120 min at room temperature, followed by five washes with PBS-Tween (0.1%) to remove unbound antibodies. Then, 50 μL of HRP-conjugated anti-IgG at a 1:3000 dilution in PBS-Tween-BSA was added and incubated for 120 min at room temperature. Tetramethylbenzidine (KLP, Gaithersburg, MD) was added to each well (100 μL). The enzyme reaction was stopped after 15 min with 100 μL of 0.18 *M* H_2SO_4. All incubations were done at 25°C. For all buffers and dilutions, endotoxin-free, sterile, double-distilled water was used. The color reaction was quantitated at an optical density of 450 nm with a V_{max} ELISA plate reader (Molecular Devices, Palo Alto, CA). Background signals were obtained from wells treated with PBS-Tween-20 without antibody.

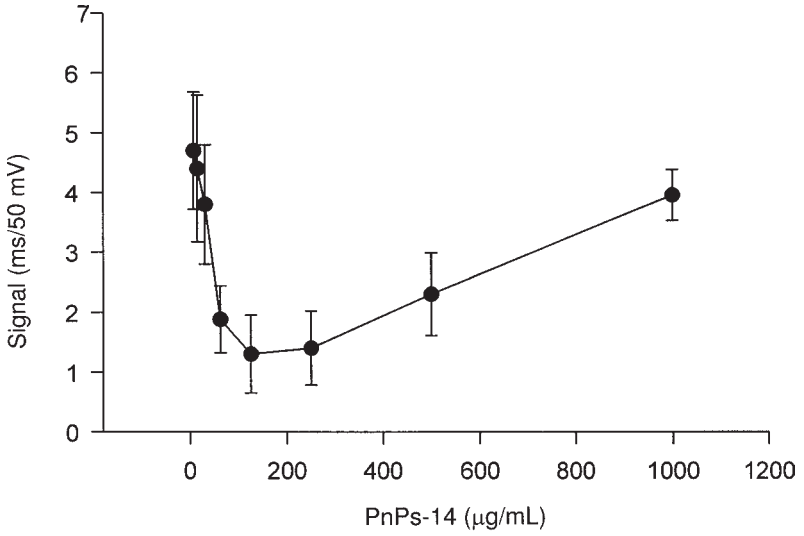

Fig. 3. The chemiluminescent signal (time in milliseconds to reach 50 mV) as a function of the pneumococcal polysaccharide PnPs-14 concentration in the solution used to conjugate the antigen to the fiber. The anti-PnPs-14 antibody and the antirabbit HRP were used at a 1:1000 dilution.

Results and Discussion

Conjugation of Pneumococcal Polysaccharide to an Optical Fiber

Protein antigen is conventionally and stably covalently linked to the optical fiber by 3-glycidoxytrimethoxysilane *(10,12–14)*. Although this method was used in the past to couple polysaccharide to silicon and silicon rubber *(15)*, it could not be adopted by our laboratory, because very low signals were continually obtained, suggesting an unstable conjugation. To alleviate this problem, we silanized the fibers with 3-aminopropyltrimethoxysilane *(16,17)* and chemically modified the amine moiety by the linker using cyanuric chloride *(18)*. Cyanuric chloride may react with either amino or hydroxy moieties. In an aqueous solution, the first chlorine atom is displaced at 0°C, whereas the other two are displaced at 30–50°C and at 90–100°C, respectively. Hence, cyanuric chloride reacted initially with the amine moieties of the silanized fibers, and subsequently with one hydroxy moiety on the polysaccharide, although we do not presently know which. As demonstrated subsequently, this novel conjugation method resulted in a greater covalent coupling yield of the pneumococcal polysaccharide to the optical fiber tips (Fig. 2).

Optimal Polysaccharide Immobilization on Optical Fiber

To assess the optimal and useful quantitative polysaccharide immobilization, the silanized fibers were derivatized with increasing concentrations of PnPs-14, and immunoassays were run with a rabbit anti-PnPs-14 at a titer of 1:1000 (*see* "Immunoassay Sensor Design"). The results shown in Fig. 3 indicate an optimal reaction concentration of PnPs-14 in solution in

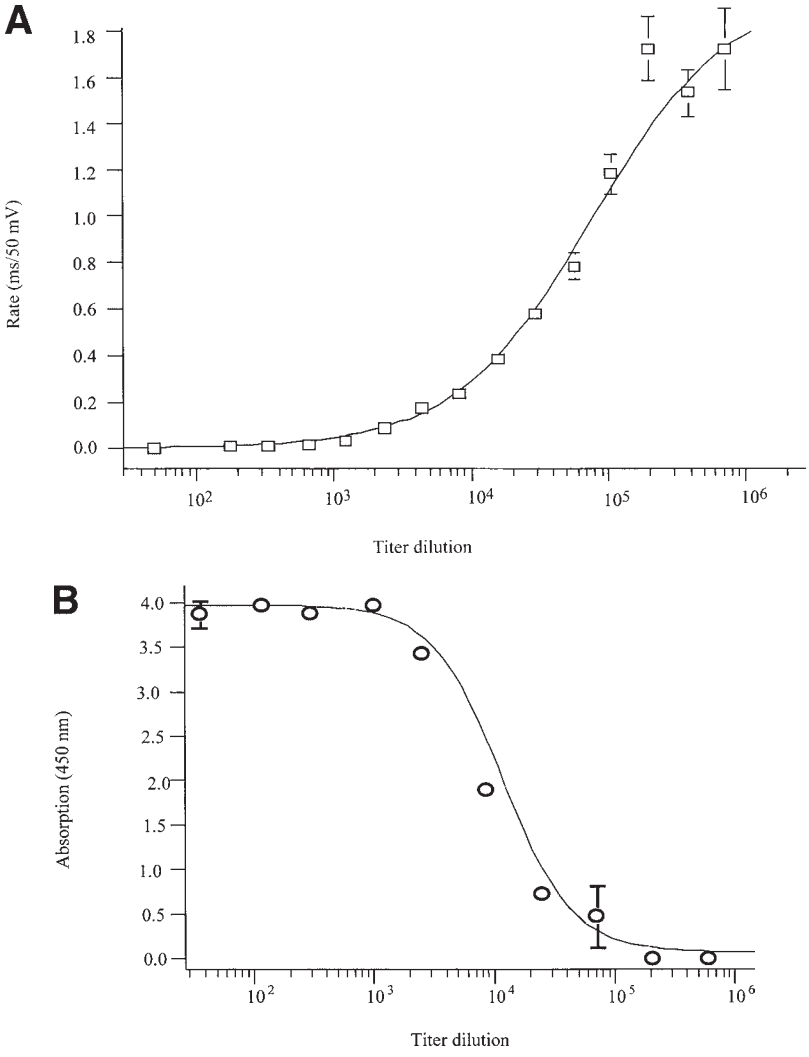

Fig. 4. Calibration curves generated by the chemiluminescent immunosensor **(A)** or standard microtiter ELISA method **(B)**. The signals are time (milliseconds) to reach 50 mV for the chemiluminescent immunosensor and absorption at 450 nm for the ELISA assay.

the range of 125–250 μg/mL. Below and above these concentrations, the time required to reach a 50-mV level was increased. The explanation for this phenomenon is currently unknown and was not further investigated in this study. To generate a calibration curve and assess the optical immunosensor sensitivity, a constant PnPs-14 concentration of 125 μg/mL was used.

Generation of Calibration Curve

The marker HRP, conjugated to the secondary antibody, was the indicator in our experiment that a recognition event had taken place between

the antigen-coated fiber and the immunoglobulin analyte. Its existence and quantification was determined by measuring the photons at λ = 428 nm, emitted when high-energy products of oxidized luminol return to the ground state *(19)*. The rate was calculated as the time in milliseconds required to reach a constant level of 50 mV. The calibration curve was generated from an initial dilution of the anti-PnPs-14 antibodies at 1:50, and a serial twofold dilution up to 1:1,638,840 was tested. Figure 4 shows the results. The fitted curve was calculated by UltraFit™ software (Biosoft, Cambridge, UK) using an asymmetrical sygmoid equation. The lowest titer detected in our assay was at 1:819,200. The lowest titer detected by ELISA was 1:98,415 (Fig. 4B). The results demonstrated that the immunosensor was about one order of magnitude more sensitive than ELISA. Currently, we are exploring several procedures to increase the sensitivity further.

Conclusion

A sensitive optical fiber immunosensor was developed to detect anti–*S. pneumoniae* antibodies. This required the elaboration of a more sophisticated photodetection system, as well as a novel surface chemistry for the immobilization of the polysaccharide. In the future, we intend to create a calibration curve using human antipneumococcal IgG antibodies and to compare it to ELISA, as well as to adapt the optical fiber immunosensor diagnostic power to various human physiological fluids such as serum, urine, and saliva.

Acknowledgments

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