Journal of Biomedical Optics 5(3), 350–354 (July 2000)

Antibody-based biosensor for breast cancer with ultrasonic regeneration

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Abstract. We describe a novel method and instrumental setup for regenerating antibodies immobilized on a fiberoptic probe of an immunosensor using ultrasonic irradiation with broadband imaging transducers. The instrumental setup and irradiation conditions for antibody regeneration using ultrasound are described. The results of the measurements with antibody against breast cancer antigen illustrate the effectiveness and potential of the regenerable immunosensor. A 65% removal of the antigens bound to the Mab immobilized on the fiber surface is attained after ultrasound regeneration. [S1083-3668(00)00703-6]

Keywords: immunosensor; antibody; regenerable biosensor; ultrasound; fiberoptic.

Paper 90024 JBO received Apr. 16, 1999; revised manuscript received Feb. 6, 2000; accepted for publication Mar. 8, 2000.

1 Introduction

Fiberoptic biosensors using immobilized antibodies as recognition elements have been extensively applied for the determination of various analytes such as pesticides, explosives, drugs, toxins, damaged DNA, or pathogenic organisms.[1,2] For the past 10 years, our laboratory has been developing a wide variety of antibody-based fiberoptic sensors for environmental and biomedical applications. The first fiberoptic fluoroimmunosensor for environmental monitoring was developed to detect the carcinogenic pollutant, benzo[a]pyrene (BaP).[3] An immunosensor to monitor health effects (e.g., DNA damage) due to chemical exposure has also been developed.[4] Techniques to improve selectivity using phase-resolved detection have been applied to an antibody-based sensor.[5] An antibody-based fiberoptic nanosensor to detect BaP has also been reported.[6] Removal of antigens bound to antibodies covalently attached to optical fiber surfaces is one of the limiting factors in the development of reusable, inexpensive, and reliable optical fiber immunosensors for environmental and clinical analysis. In a previous study, we have used chemical reagents to cleave the binding between antibody and antigen, thus regenerating the biosensor.[7] This chemical procedure is simple but ineffective after multiple regeneration operations (less than five cycles), due to possible denaturation of the antibody. Another approach in the development of regenerable biosensors involves the design of microcapillary systems capable of delivering and removing reagents and antibody-coated microbeads into the sensing chamber without removing the sensor from the sample.[8] Several investigators have searched for fast dissociation protocols that are able to regenerate immobilized antibodies while maintaining their stability for use in routine analysis, commercial immunosorbents,[9] or optical fiber sensors.[10]

Antigen–antibody interactions can be classified in three different groups:[11] hydrophobic interactions, electrostatic (or Coulombic) interactions, and interactions due to a combination of both forces. Hydrophobic interactions are due to the propensity of nonpolar groups and chains to aggregate when immersed in water. This type of interaction is maximized between the hydrophobic complementary determining regions of the antibody parotopes and the predominantly hydrophilic groups found in the antigen epitope. Electrostatic interactions between antigen and antibody are caused by one or more ionized sites of the epitope and ions of opposite charge on the parotope. After primary binding has occurred through hydrophobic and electrostatic interactions, the epitope and the parotope will be close enough to allow van der Waals and hydrogen bonds to become operative. In order to dissociate the antigen–antibody complexes, the strength of these forces may be reduced by changing the *p*H, ionic strength, or temperature; or through the addition of dehydrating agents or organics. In this sense, strong acids such as HCl or H_2SO_4, mixtures such as glycine–HCl, or basic solutions of tetraethylamine, for example, have been used when the primary attractive forces in the bond can be considered as electrostatic interactions.[2] Lu et al.[12] pointed out that the use of organic solutions such as ethylene glycol could improve the washing efficiency by reducing both the van der Waals and the Coulombic forces maintaining the bond. Ligler et al.[13] made a similar observation. Nevertheless, every antigen–antibody pair may differ with respect to the nature of the forces implicated in the binding site.

Haga et al.[14] studied the effect of 28 kHz ultrasonic radiation with an intensity of 0.83 W/cm^2 on the dissociation of antigen–antibody complexes immobilized on CH–Sepharose gels. They observed that the percent of dissociation increased with irradiation time and input wattage obtaining 22% of dissociation after 20 min, whereas the immune reactivity decreased 8% without degradation of the dissolved antibody upon exposure to the ultrasound for times of up to 120 min. Higher frequency ultrasound (high kHz to low MHz range) is used to remove small (micrometer to submicrometer) particles from the surfaces of silicon wafers.[15–18] This so-called mega-

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sonic cleaning process does not damage the surfaces, suggesting that its mode of action does not depend on the strong effects of inertial cavitation that occur with lower frequency (e.g., 20 kHz) ultrasonic horns and baths. The mechanisms of megasonic cleaning likely involve nonlinear effects such as acoustic streaming[15–18] and radiation pressure as well as the oscillatory linear forces.[16] Stable cavitation (a less intense process than inertial cavitation) may also play an important role,[15,17] particularly in enhancing the streaming effects. The success of the gentle but effective megasonic cleaning process suggests that MHz range ultrasound may have some use in regeneration of biosensor surfaces.

In this paper we describe a novel procedure for regenerating antibodies immobilized on a fiberoptic surface with ultrasonic irradiation using a broadband imaging transducer operating near 5 MHz. This type of ultrasound device is commonly used for the detection of flaws in the nondestructive evaluation of engineering materials and measurement of the mechanical properties of various media[19] including biological tissues.[20] The use of ultrasound for the regeneration of optical fiber immunosensors could be an important advance in the application of these devices for *in vivo* and *in situ* measurements since it would no longer be necessary to supply a regeneration solution to the sensor system that could lead to the denaturation of the immobilized antibody.

2 Experiment

2.1 Materials

Monoclonal antibody to cancer breast antigen CA15-3, and human cancer antigen CA15-3, antigen grade, were purchased from Biodesign International (Kennebunk, ME). (3-Glycidoxypropyl)trimethoxysilane (GOPS) was obtained from Fluka (Buchs, Switzerland). 1,1′-carbonyldiimidazole, phosphate buffered saline, *p*H 7.4, and all other reagents were supplied by Sigma Chemical Co. (St. Louis, MO). Water was purified using a Milli-Q system from Millipore. Multimode fused-silica fibers, 600 μm core, silica/silica cladding were from Fiberguide Industries (Stirling, NJ). Fibers were cut into 0.5 m lengths and one of the ends was hand polished with aluminum oxide grit paper with 15, 3, and 0.5 μm grit size, using an Ultra Tec (Santa Ana, CA) polisher. The sensing tips of the fibers were tapered under computer control using a Sutter Industries P-2000 instrument.

2.2 Instrumentation

2.2.1 *Fluorescence Sensing System*

Figure 1(a) shows a schematic of the fiberoptic immunosensor detection system. The 632.8 nm line of a HeNe laser (6.4 mW) was passed through a laser bandpass filter, through a Uniblitz shutter and onto a holographic beamsplitter (Kaiser, Ann Arbor, MI). The shutter permits the delivery of controlled duration pulses from the laser in order to reduce the effects of photobleaching. The beam is then focused onto the optical fiber and delivered to the tapered end. The fluorescence is collected at the tip of the fiber and returned through the optical fiber to the collimating optics. The light then passes though the holographic beamsplitter and a holographic Raman filter to reduce the background due to the laser. The fluorescence is then focused onto the active area of a photomultiplier tube (Hamamatsu Model E849-35) with a 650 nm long-pass filter to further reduce the background due to the laser scatter. Data were collected on a strip chart recorder.

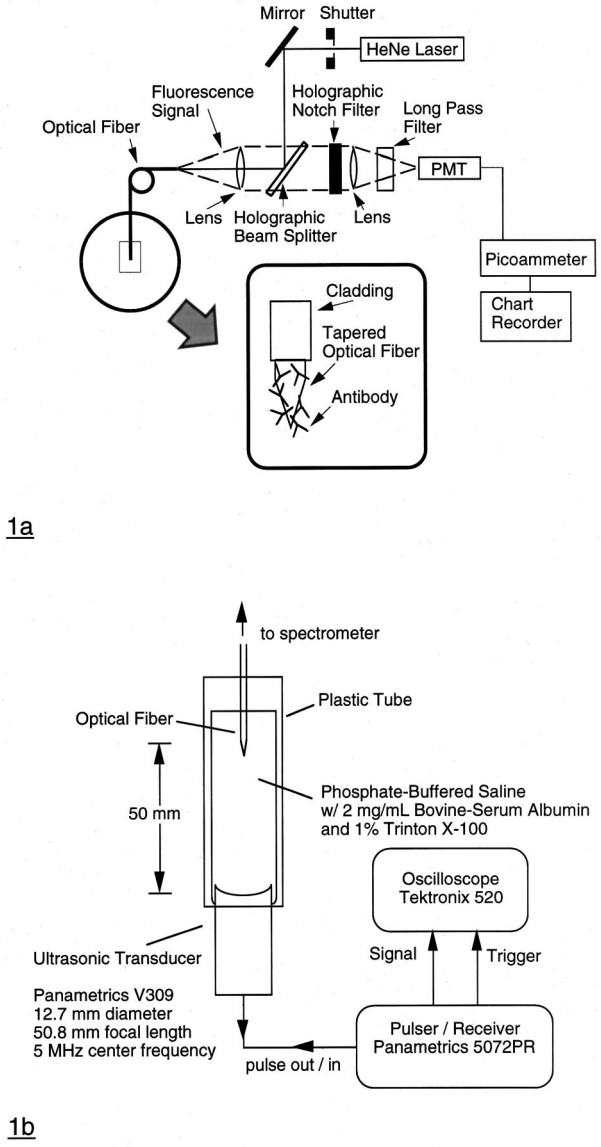

Fig. 1 (a) Diagram of the optical setup. (b) Diagram of the ultrasound system.

2.2.2 *Ultrasound Generation System*

The transducer (Panametrics V309) used in this study was a 12.7 mm diam aperture with an acoustic lens to focus the sound beam on axis at a distance of 50.4 mm (2 in.) from the face of the device. It is optimized to produce short pulses a few μs wide with center frequencies near 5 MHz. The pulse repetition rate was 5000/s (one pulse every 200 μs). The setup for the ultrasonic irradiation of the fiber tip is schematically illustrated in Figure 1(b). The transducer was placed at the end of a plastic tube just large enough to fit over the transducer aperture. The tube was mounted vertically and the transducer face served as the bottom edge of a cylindrical bath containing phosphate buffered saline (PBS), Triton X-10, and

BSA. In all cases the fiber was lowered into the bath oriented such that its tip pointed toward the transducer along its central axis. The fiber tip was positioned approximately 50 mm (2 in.) from the transducer. The proper placement of the fiber tip was verified using the oscilloscope to monitor the time interval between the excitation pulse and the echo returned from the tip. The transducer was excited by a broadband pulser (Panametrics 5072PR). Since the ultrasonic effects relevant to regeneration increase with sonic power, we configured the pulser to deliver the maximum possible amount of energy to the transducer. This was judged by finding the largest signal on the oscilloscope from the echo returned from the surface of the PBS at the top of the tube.

2.3 Experimental Procedures

2.3.1 *Optical Fiber Surface Silanization and Activation*

The tapered end of the optical fiber was activated for protein immobilization according to a procedure previously described[21] with the modifications indicated below. The fiber was successively washed for 30 min with HNO_3, HCl: absolute ethanol (1:1) and H_2SO_4. After the wash, the fiber was rinsed with distilled water and ethanol (100%) and dried for 15 min at 80°C. Following the last wash, the fiber remained overnight in the oven at that temperature. Afterward the tip of the tapered end of the fiber was submerged in 100 ml of 100 mM acetic acid/sodium acetate buffer, *p*H 5.5 and 1 ml of GOPS was added. The solution was stirred for 7 h at 90°C. The diol phase fibers were activated with CDI (0.35 g) by heating under reflux for 20 h in anhydrous dioxane (30 ml), and washed several times with the same solvent before drying overnight at 80°C under vacuum.

2.3.2 *Protein Attachment*

The activated fiber was incubated for 3 h, at room temperature, in 300 μl of a solution containing 0.05 mg/ml monoclonal antibody (Mab) to cancer breast antigen CA15-3 in PBS and rinsed with PBS several times. Nonspecific binding sites on the optical fiber were blocked afterwards, by treating the tapered end with PBS/BSA (2 mg/ml) for 2 h. The fiber was stored in PBS at 4°C.

2.3.3 *Preparation of the Cy-5-labeled Breast Cancer Antigen*

One vial (50 KU) of Human Breast Cancer Antigen CA 15-3 (Biodesign International, Kennebunk, ME) was incubated with one vial of Cy-5 (Fluorolink Cy-5 Reactive Dye Pack, Biological Detection Systems, Inc., Pittsburgh, PA) for 30 min at room temperature. The breast cancer antigen solution was dialyzed overnight at 4°C against 100 ml of 0.1 M sodium carbonate buffer (*p*H 9.3) to adjust the *p*H of the protein solution before reaction with the Cy-5. Following the dye conjugation step, the labeled cancer antigen was separated from the dye using a Sephadex G-15 column and eluting with phosphate buffered saline. Fractions eluting in the void volume of the column were collected and pooled. As a further precaution to ensure complete removal of the free dye, the pooled fractions were dialyzed against PBS (100:1 volume of dialysis buffer to fraction volume for 24 h at 4°C).

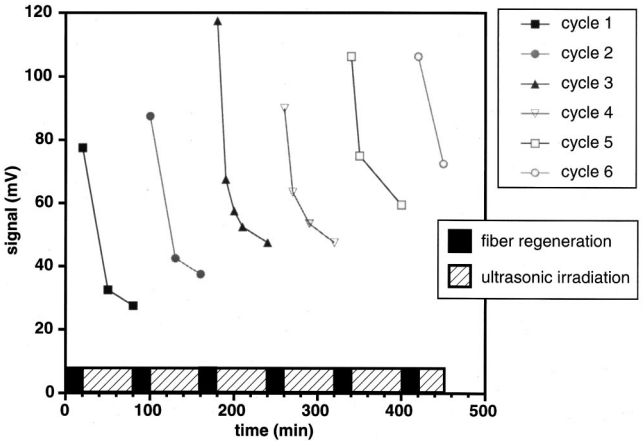

Fig. 2 Antibody regeneration assay using ultrasound. The sensitive tip of the fiber was exposed to 600 ng/ml of Cy-5-CA15-3 in PBS/BSA (2 mg/ml)/Triton X-100 (1%) for 20 min. The tip was removed from the labeled antibody and exposed to the ultrasound irradiation with the 5 MHz focused transducer. Next the fiber was reincubated with the antibody labeled solution for another 20 min. The fluorescent signal was recorded at the end of each incubation and at various times during irradiation. The cycle was repeated five times.

2.3.4 *Measurement and Regeneration Procedure*

To regenerate the immunosensors, the fiber coated with the antibody was immersed, with the aid of a fiber micropositioner, in PBS/BSA (2 mg/ml)/Triton X-100 (1%) (to prevent nonspecific binding to the fibers),[22] in a special plastic chamber attached to the ultrasonic transducer. The approximate volume of the chamber was 10 ml. After a stable baseline reading from the PMT was obtained, the buffer was removed and the fibers were incubated in 400 μl of a solution containing 600 ng/ml of Cy-5-CA15-3 antigen in blocking solution at room temperature. After 20 min, the fiber was rinsed several times with the blocking solution and dipped again in the plastic chamber mentioned above. The fluorescence signal for the sample was collected during the 1 s period that the shutter was open. The ultrasonic transducer was then connected and the fluorescence signal of the fiber was measured as a function of the sonication time, blocking the laser excitation with the shutter between data acquisitions to avoid photobleaching.

3 Results and Discussion

The fluorescence signals from a labeled fiber over six cycles of antigen exposure/regeneration and ultrasonic irradiation are shown in Figure 2. During the first cycle, the signal fell 45% after 30 min of irradiation and the signal continued to fall throughout the cycle. A similar pattern held for subsequent cycles. Figure 3 displays the percentage decrease in signal at 10, 30, and 60 min averaged over all the cycles.

In this study, we were not able to achieve 100% removal of the antigens bound to the Mab immobilized on the surface of the optical fiber. The percent decreases for the respective incubation/irradiation cycles (as measured by the maximum and minimum signals for each respective cycle) exhibited a downward trend with cycle number. Although the signal decreased by 65% over the first cycle, in the fifth cycle the decrease was only 44%. This could indicate that the ultra-

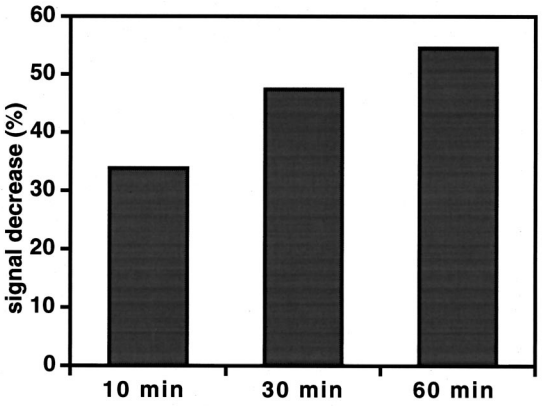

Fig. 3 The percentage decrease in the fluorescence signal (averaged over the cycles in Fig. 2) for 10, 30, and 60 min of ultrasonic irradiation. The decrease was calculated for each cycle relative to the initial value for the cycle.

sound is less effective in the dissociation of the antigen–antibody complexes as the number of cycles increases. Although more work is needed to gain a clear understanding, this feature could arise due to the fact that some of the antigen–antibody complexes are more tightly bound than others. The ultrasonic irradiation could dissociate the more weakly bound complexes, freeing up these sites to possibly form more tightly bound complexes upon subsequent reincubation.

Of the possible ultrasonic mechanisms contributing to the observed dissociation, it is likely that acoustic streaming is the dominant effect as is believed to be the case with megasonic cleaning.[15,17,18] Acoustic streaming, a consequence of the nonlinear propagation of sound in fluids, refers to the establishment of steady (i.e., not oscillating) currents of circulating fluid in an oscillatory ultrasonic field. It is likely that these types of flows are present near the tip of the optical fiber as the ultrasound passes through and could play a role in the dissociation. These flows can also be a secondary effect of the oscillation of bubbles in the fluid. Stable cavitation, the oscillation of bubbles with little change in size over a few acoustic cycles, can also occur and enhance the effects of streaming. Inertial cavitation, the rapid growth and violent collapse of bubbles, is most likely not occurring in our system. This type of cavitation is most often associated with the ultrasonic cleaning and sonochemical phenomena produced with higher-power and/or lower-frequency devices than those used here. A common test for inertial cavitation is to look for damage in a thin foil of aluminum placed in the sound field. After 1 h of ultrasonic irradiation with our transducer, there were no signs of cavitation-induced damage or wear on a sample foil. Both the relatively high-frequency content (~5 MHz) and short duration (a few μs wide emitted every 200 μs) of the ultrasonic pulses used in this work are unfavorable for the generation of strong cavitation effects. Because inertial cavitation is not occurring and the water and glass fiber are not strong attenuators of ultrasound, it is unlikely that the sensor is absorbing any significant heat from the sonic waves. The propagation of ultrasound is considered to be adiabatic and so without sizeable local attenuation there should be no net heat flow due to the passing wavepacket. The temperature, however, will vary with the rise and fall of the pressure in the passing waves. Since there is no significant heat deposited at the sensor, the temperature will return to ambient after the short ultrasonic pulse has passed. It is not clear at this time what effects, if any, will stem from this transient temperature variation. Currently we are conducting studies to more fully develop this ultrasonic technique by using various transducers/frequencies and further examining its applicability and reproducibility.

4 Conclusion

In this study, we have demonstrated the possibility of using ultrasound from broadband imaging transducers to regenerate antibody-based biosensors. The ultrasound regeneration scheme is a nondestructive approach that has a great potential to be applied to *in situ* monitoring systems. The results demonstrate the effectiveness of this innovative approach for the antibody-based biosensor to remote biosensing applications.

Acknowledgments

This research is sponsored by the Office of Biological and Environmental Research, U.S. Department of Energy under Contract No. DE-AC05-960R22464 with Lockheed Martin Energy Research Corporation, and by the ORNL Laboratory Directed Research and Development Program (Advanced Nanosystems). Dr. Moreno-Bondi gratefully acknowledges the Spanish Ministry of Education and Culture research grant for a short stay at ORNL. Dr. Mobley acknowledges the support of Oak Ridge Institute for Science and Education for his postdoctoral appointment at ORNL.

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