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**CHEMICAL
RESEARCH,
DEVELOPMENT &
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CENTER**

CRDEC-TR-303

**RECOGNITION USING BIOSPECIFIC
INTERACTION ANALYSIS**

Sheila J. Wood
RESEARCH DIRECTORATE

Russ Granzow
PHARMACIA BIOSENSOR
Piscataway, NJ 08854

William Petri, Jr.
UNIVERSITY OF VIRGINIA
Charlottesville, VA 22904

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PREFACE

The work described in this report was authorized under Project No. 1FJ1-2-R PEW. This work was started in January 1991 and completed in January 1991. This work was performed at the research applications laboratory, Pharmacia Biosensor, Piscataway, NJ. Experimental results are on file at the U.S. Army Chemical Research, Development and Engineering Center, Aberdeen Proving Ground, MD.

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This report has been approved for release to the public.

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1. INTRODUCTION

Real-time biospecific interaction analysis (BIA) provides a myriad of ways to study macromolecular interactions without chemically modifying or labeling the interactants. Based on advanced surface plasmon resonance technology, changes in the surface concentration of biomolecules are followed over time. BIAcore™ response reflects analyte accumulation as a result of its binding to an immobilized capture reactant. This capture reactant is immobilized to a dextran matrix which is positioned to interact with complementary reagents introduced within the fluid stream.

Derivation (esterification) of carboxyl groups nestled within the hydrophilic dextran matrix creates an activated surface to which ligands can be coupled. Any biological material, with a free amino terminus, can be immobilized to the layer using conventional covalent coupling techniques. The immobilized materials are then available to react in three dimensions with moieties introduced in the sample stream. A sophisticated fluid channeling system directs analytes of interest to the immobilized capture reagent. Instrument response directly reflects increase in mass due to interaction of immobilized components with complementary ligand. Changes in reflectance minima are transcribed graphically to display changes in mass within the matrix as reactions proceed.

Purified reagents are used within this system to study molecular interactions. Small volumes, 1-50 microliters, handled at 5-10 minutes per sample, are standard use parameters. The instrument can determine analyte concentration, characterize antibodies, assess structural characteristics, and monitor receptor-adhesin interactions. It was used in this study to determine the possibility of detecting antigen in preparations of whole, solubilized amoeba. Monoclonal antibodies (mAbs), reactive against surface, membrane-associated, protein, were used as capture reagents. These monoclonal antibodies distinguish pathogenic from nonpathogenic strains on the basis of epitope specificity.(1) The objective of the study was to evaluate the possibilities of detecting antigens within a mixture of lipopolysaccharides and other proteins.

Pathogenic determinants, found on the outer surface of Entamoeba histolytica, determine their ability to adhere to and/or invade mammalian tissues. The lectins produced by E. histolytica provide the basis for development of an assay for detection of adherence and invasive properties harbored by this potential threat agent. The galactose-binding lectin is a 260 kilodalton (kDa) heterodimeric glycoprotein consisting of a 170 kDa heavy subunit linked by disulfide bonds to a 35 kDa light subunit. This lectin mediates in vitro adherence to human colonic mucin glycoproteins suggesting its involvement in

colonization and invasion of the colon. Six antigenically and functionally distinct epitopes have been mapped on the heavy subunit with monoclonal antibodies (mAb). The epitope specificities and designations of the mAbs, respectively, are as follows: epitope 1, 3F4; epitope 2, 8A3; epitope 3, 7F4; epitope 4, 8C12; epitope 5, 1G7; epitope 6, H85. Epitopes 1 through 6 are present on pathogenic zymodemes but only epitopes 1 and 2 are found on nonpathogenic isolates. This specificity provides the basis for distinguishing pathogenic from nonpathogenic strains.(1,2)

2. MATERIALS AND METHODS

The carboxymethylated surface of the sensor chip was derived using N-ethyl-N'-(dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). The esters formed as a result of this derivation (see Figure 1) were then available to react with the primary amino terminal groups of the rabbit anti-mouse immunoglobulin (RAMFc), attached as the primary, reusable surface. After coupling the RAMFc antibody to the matrix, remaining esters were deactivated with ethanolamine, resulting in hydroxyethyl amide groups.

Two standard buffers were used in the experiments. Rabbit anti-mouse Fc (RAMFc) was suspended in sodium acetate buffer, 10 mM, pH 5.0. Rinses and monoclonal antibody suspensions were made using HBS buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant). Amoeba were solubilized initially in 150 mM NaCl, 50 mM Tris pH 8.3, 0.5% Triton X 100, 5 mM EDTA (ethylene diamine trichloroacetic acid), 2 mM PMSF (poly-methyl-sulfonyl-fluoride), 2 mM PHMB (parahydroxymercuribenzoic acid), and diluted in HBS.

Initially, the sensor chip was washed with HBS buffer for 5 min to equilibrate the read. EDC at a concentration of 400 mM in water and NHS at 100 mM in water were allowed to mix and 30 uL of this mixture was passed over the sensor chip surface to activate it. Next, 35 uL of 100 ug/mL RAMFc was injected and immobilized to the surface. To deactivate excess reactive groups, 35 uL of 1.0 M ethanolamine was injected. To remove noncovalently bound material, 15 uL of 100 mM HCl was used to rinse the chip.

After a baseline read was established, 10 uL of a 20 ug/mL preparation of mAb 8A3 in HBS was injected. Monoclonal antibody 8A3 is directed against epitope two of the adherence lectin on the surface of Entamoeba histolytica. This portion of the adherence lectin is found on both pathogenic and nonpathogenic strains. Antibody 8A3 bound to the RAMFc and resulted in an increase in baseline of 923 resonance units (RU). Ten microliters of 100 mM HCl was then used to regenerate the RAMFc surface to test for nonspecific binding. After the baseline was reestablished, mAb 8A3 was injected again at 10 uL of 20 ug/mL

followed by solubilized amebic antigen from a pathogenic strain. Ten uL of 1:100 dilution of the equivalent of 1×10^4 /mL amoeba in HBS was injected. After this was allowed to react, 10 uL of a 1:100 dilution of the nonpathogenic strain in HBS was injected and allowed to react with the immobilized mAb 8A3.

Baseline was again established, and 10 uL of 20 ug/mL mAb 8A3 was injected, followed by 10 uL of pathogenic solubilized amoeba at 1:10 in HBS. Following return to baseline, 30 uL of 75 ug/mL mAb 8A3 was injected and challenged with 30 uL of pathogenic solubilized antigen at 1:10 in HBS. Baseline was reestablished, 30 uL of 20 ug/mL mAb 8C12 added, and a 30 uL challenge of a 1:10 dilution of pathogenic antigen followed. Monoclonal antibody 8C12 is directed against epitope 4 of the adherence lectin and reacts with only pathogenic strains as this epitope is present only on pathogenic strains. Next, mAb 8C12 and mAb 8A3 (10 ug/mL) were each incubated with solubilized pathogenic and nonpathogenic antigen (undiluted). Controls of mAb 8C12 and mAb 8A3 alone were compared to the incubated mixtures.

3. RESULTS

Figure 2 represents a baseline read for RAMFc antibody bound to the matrix. Figure 3 shows the successful capture of mAb 8A3 with RAMFc and regeneration shows lack of nonspecific binding. An increase of 923 resonance units (RU) was observed. Figure 4 shows return to baseline, recapture of mAb 8A3 and challenge with first pathogenic, then nonpathogenic solubilized amebic antigen. Although mAb 8A3 was captured consistently with ease, it did not react with either antigenic preparation. Figure 5 shows another attempt to react mAb 8A3 with pathogenic antigen at a lower dilution (1:10). This proved unsuccessful. Figure 6 represents the use of mAb 8A3 at a larger volume and higher concentration challenged with pathogenic antigen at 1:10, larger volume. Also mAb 8C12 was challenged with pathogenic antigen. Results show good binding of both antibodies, but no interaction with either antigen preparation. Equivalents of 100, 1000, and 3000 amoeba were tried.

Finally, Figure 7 shows analysis of pre-incubated mixtures of 10 ug/mL antibody and undiluted antigen (to achieve antigen excess). Little difference in the level of response was observed among these mixtures. Signals from antibody alone are only slightly lower than those of the mixtures.

4. DISCUSSION

Inclusive within this discussion will be descriptions of portions of technology used within BIAcore™. The objective is

to correlate each step of the process to interpretive molecular interaction during sample processing. The recognition process, specifically a receptor/ligand coupling, will be discussed.

The recognition step assesses molecular function without addressing structural features of the interactants. To perform this type of analysis, the first interactant must be successfully immobilized to the sensing surface or sensor chip. In order to do this, reagents, buffers, and samples are supplied to the sensor chip surface through delivery pumps, a sample injector, and a liquid control unit or integrated micro-fluidics cartridge. In our example, RAMFc immunoglobulin served as the mode by which purified antibody was fixed to the sensor chip. The success of this step is exemplified (Figures 2 and 3) by the increase in baseline resonance units. Successful regeneration and lack of nonspecific binding was determined directly from the graphic printouts. Advantages of being able to observe this process as it occurs include the ability to trouble shoot and quantify each step of a procedure, from least to most complicated, fewest interactants to several stacking layers. This cannot be accomplished with ease using conventional techniques such as enzyme linked immunoabsorbant assay (ELISA).

Prior to detailing the analytical process, theoretical consideration will be given to the physical parameters governing surface plasmon resonance (SPR), illustrated in Figure 8. The optical phenomenon of surface plasmon resonance can best be described as a shadowing or absence in reflected light at a specific angle of incidence. This approach is based on total internal reflectance circumscribed by an electromagnetic field component of light called an evanescent wave. To create an evanescent wave, specific conditions are required. The light must be monochromatic and polarized. It must hit a metal surface situated between two transparent media of differing refractive indices. Due to the differences in refractive indices of the transparent layers, the light is internally reflected and in addition, an electromagnetic field emanating from the metal separating layer, penetrates into the medium having the lower refractive index. This electromagnetic field is called an evanescent wave. The evanescent wave, moving into the solution from within the thin metal layer, manifests itself as an absence of reflected light at a specific angle of incidence. The phenomenon is called "surface plasmon resonance," specifically describing the activity of the plasmons (electrons in the metal surface) with the evanescent wave. The allowable measuring distance for penetration of the evanescent wave into the matrix/solution is approximately 300 nm. However, the refractive index of the surface layer of solution is probed by the evanescent wave to a depth of 1 μ m, with the dominant contribution coming from the first several 100 nm.(3,4)

The resonance response is a result of several complementary parts of the instrument. Instrument response directly reflects the concentration of analyte accumulating at the sensor chip surface as a result of biospecific interaction of the immobilized reagent. The resultant signal comes from the contributions of the sensor chip itself, the optical system, liquid handling, software, optics, and interpretation of SPR response.(5)

The sensor chip consists of an optically flat glass slide with a 50 nm gold film on one side. The gold is covered with 100 nm of covalently bound hydrophilic matrix, modified to allow immobilization of biomolecules. The advantages of this dextran matrix include its provisions for a surface environment favorable for optimal interactions to occur. Considering the fact that fixation to a flat metal surface denatures proteins, the ability to covalently immobilize biomolecules to an expanded binding capacity surface, rendering the biomolecules capable of reacting in three dimensions within a liquid medium, is a definite technological breakthrough. Carboxyl groups within the dextran matrix are treated using well-defined covalent coupling chemistry. Activated groups in the matrix then couple to free primary amines.(5) Our example covalently coupled RAMFc antibody to the matrix to react with and hold the mAbs tested. This surface was regenerated after each response and used throughout the set of experiments. A resonance unit increase of 7017 RU showed that approximately 7 ng/mm^2 (for an average mL wt antibody, 150,000), of RAMFc antibody was successfully immobilized. The resilience and reproducibility of the active surface was noted as it was regenerated and used throughout, with minimal variations in resonance response (87 resonance units range). After this response was set to correspond to a relative response of 0, hence baseline, the responses of reactants were compared to this.

While interactions occur on the bottom surface of the sensor chip (within the matrix), optical measurements originate from the upper surface of the chip. To insure an integrated optical interface, a light-directing prism coated with silicone polymer and matched to the refractive index of the glass, butts against the glass (top surface), coupling with the sensor chip. Liquid is channeled to four independent areas on the sensor surface, allowing immobilization of four different ligands if desired.(5) One channel was used for this set of evaluations. Concentrations of purified monoclonal antibody in the ranges of 200 ng, 300 ng and 2.25 ug total protein were used. Reads of approximately 900 to 1800 RU indicated that from 1.0 to 1.8 ng/mm^2 was captured by the RAMFc and available to react with antigen challenge.

Reads for each of the four liquid channel areas are accomplished by optics and liquid handling. A high efficiency near-infrared light source, 760 nm, is focused at fixed incident

angles designed to measure surface concentrations of 0.1 ng/mm² to 30 ng/mm². (5) Our surface concentrations ranged from approximately 7 ng/mm² (anti-Fc) to approximately 1.0 ng/mm² (mAb 8A3 and mAb 8C12).

A fixed array of light-sensitive diodes monitors the surface plasmon resonance response over the entire wedge of reflected light. Each of the four channels on the sensor chip is measured by a unique linear detector array. Spacing between diodes in the array correspond to a difference of 0.05 degrees in light angle. The angle at which minimum reflection occurs is resolved by computer interpolation at a sensitivity of 10⁻⁴ degrees. (5) We were looking for a change in at least 800 RU to indicate binding of amebic antigen to captured antibody. The entire adhesin protein has a molecular weight of 260 kDa which breaks down in to an inactive 35 kDa portion and an active 170 kDa portion. This corresponds closely to the mL wt of an antibody, 150 kDa. Therefore, if complementary antigen coupled to the 1.0 to 1.8 ng/mm² concentration of mAb, a stacking response of approximately 1,000 RU would be expected. The implications of the negative results will be discussed later in this section.

A stationary wedge of incident light and a fixed array of detectors, eliminates the need for physical movement of optics, detector, or sensor chip. The advantages this imparts to accuracy and reproducibility of measurements in real time are obvious. For each flow cell, the area monitored covers 1.4 x 0.16 mm. The response is averaged over this area to compensate for microscopic irregularities in the chip surface or uneven adsorption of analyte. The natural nature of the dextran layer allows placement of carboxyl groups at every aldehyde group. To compensate for any uneven distribution of biological material during immobilization, or steric interferences in read due to proteins directly adjacent to one another, an entire area is monitored and reads are averaged. This inherently invisible control, harbored within the design, imparts obvious advantages for overall reproducibility. (5) Recent evidence supports the assumption that homogeneity is obtained when immobilizing within the matrix. Experiments are under way to confirm this evidence.

Surface plasmon resonance measures displacement of reflectance minima caused by changes in surface concentration of biomolecules. This can be seen as a change in the absence of reflected light at a specific angle of incidence, which is a direct measure of the change in refractive index of the solution close to the metal film, as biomolecular coupling occurs. Changes in mass on the surface of the sensor chip are measured as they occur. (3,4) From the data, Figures 3 through 7, this absence of reflected light is graphically presented in the form of change in incident angle over time. It is clear from the data in Figure 2 that immobilization of RAMFc antibody was successful,

as the quantity of protein bound to the matrix was indicated by the degree of change in light angle. Also, each of the mAb's was successfully bound, as seen in Figures 3 through 6, providing an surface ready to react with incoming sample containing amebic antigen.

Shift in incidence angle is recorded as a function of time on a sensorgram. (Figures 2 through 7) Resonance units (RU) are recorded and correspond as follows: 1000 RU corresponds to a shift of 0.1 degree in resonance angle or 1 ng/mm^2 , or refractive index of 10^{-3} . Typical responses range from 100-20,000 RU. Response tends to be linear over a range of 2-40 ng/mm^2 for most proteins. Glycoproteins and lipoproteins will give slightly lower SPR response due to their lower specific refractive index increments (change in bulk refractive index per unit change in protein concentration).(5) The mAbs used in this experiment were approximately 150 kDa. Although their exact structure is not entirely clear, it is useful to note that each of the six monoclonals previously reacted uniquely (following immobilization) in methods requiring differing ionic environments.(6) This could result from varying glycogen contents of each mAb.

Interpretation of the data suggests that perhaps proteins within the amebic preparation are too large to get into the matrix. It is known that the adhesion antigens are membrane associated, and since microsphere agglutination assays have specifically agglutinated these solubilized forms,(6) the lack of response in this analysis could be size related or a direct result of inaccessibility of binding sites. Another possibility is that micelles form, due to different detergents in each buffer. As a result, circular micelles with surface repulsive charge, deny antigen access to antibody within the matrix.

In the preincubated samples, it is unlikely, but possible, that excess unbound antibody is present, is binding initially, and preventing complex from binding. Either steric factors alone, or a combination of events, such as antibody binding (slight excess), partial antigen/antibody complex binding could be occurring.

Although a sensitivity problem is unlikely, the possibility exists that a threshold amount of adherence lectin (within the total protein) is required to generate a positive signal. The smallest response that can be measured in BIAcore is 10 RU, corresponding to a potential lower limit of detection of 0.1 to 17 pg/mm^2 . The flow cell surface corresponds to 1.5 mm^2 . Assuming 40% of analyte at 1 ng/mL in solution binds, this corresponds to a concentration of 13 pg/mm^2 , a response of 13 RU. In general, the response is determined by the weight of the analyte and the number of immobilized capturing molecules.

Attempts will be made to control micelle formation and ensure the use of adequate antigenic material. Since the lectin comprises 0.1 of 2% of total protein of the membrane of E. histolytica, and approximately 4 ug of lectin in 1 mL of solubilization buffer was injected, a maximum of 40 ng of lectin was present in the injection. Preliminary follow-up experiments show that at least 100 ng of this protein must be injected to achieve a recognizably positive signal.

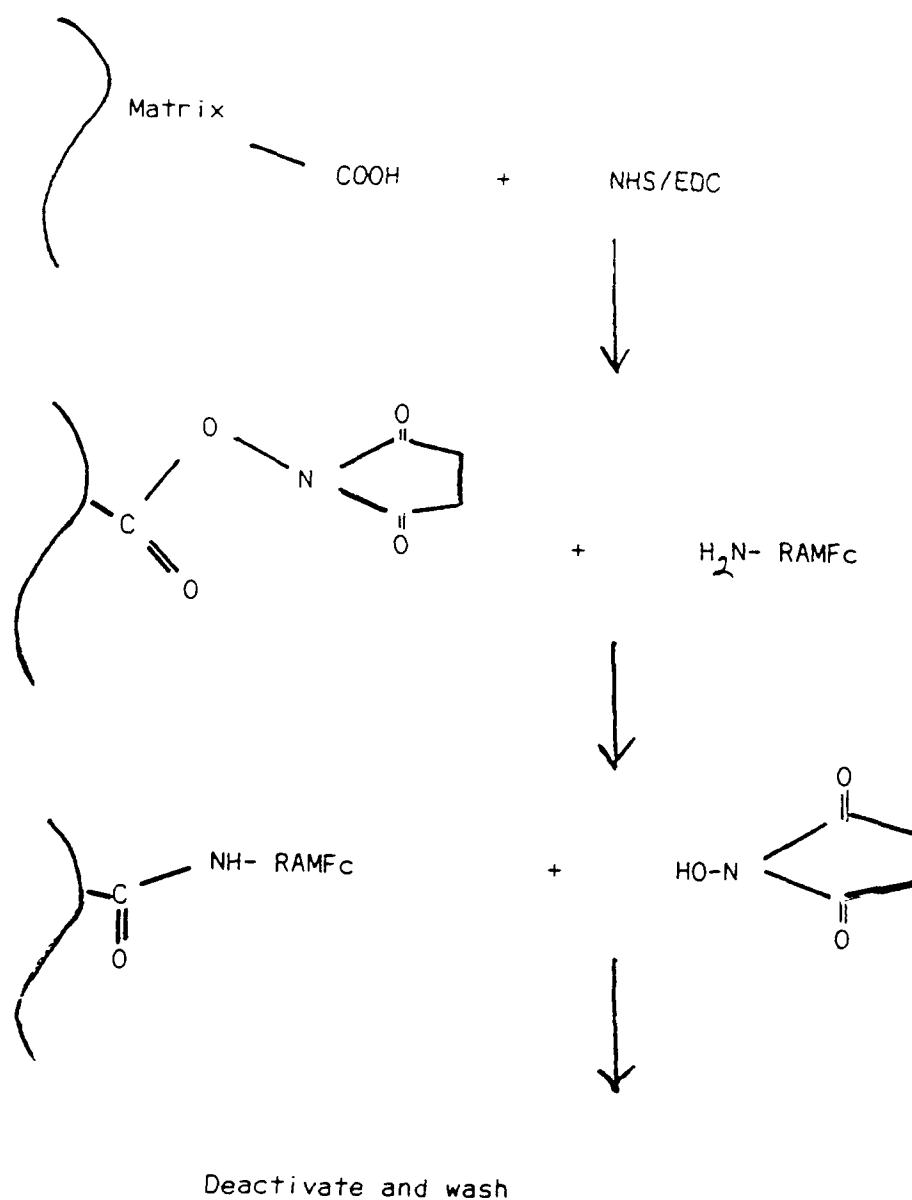
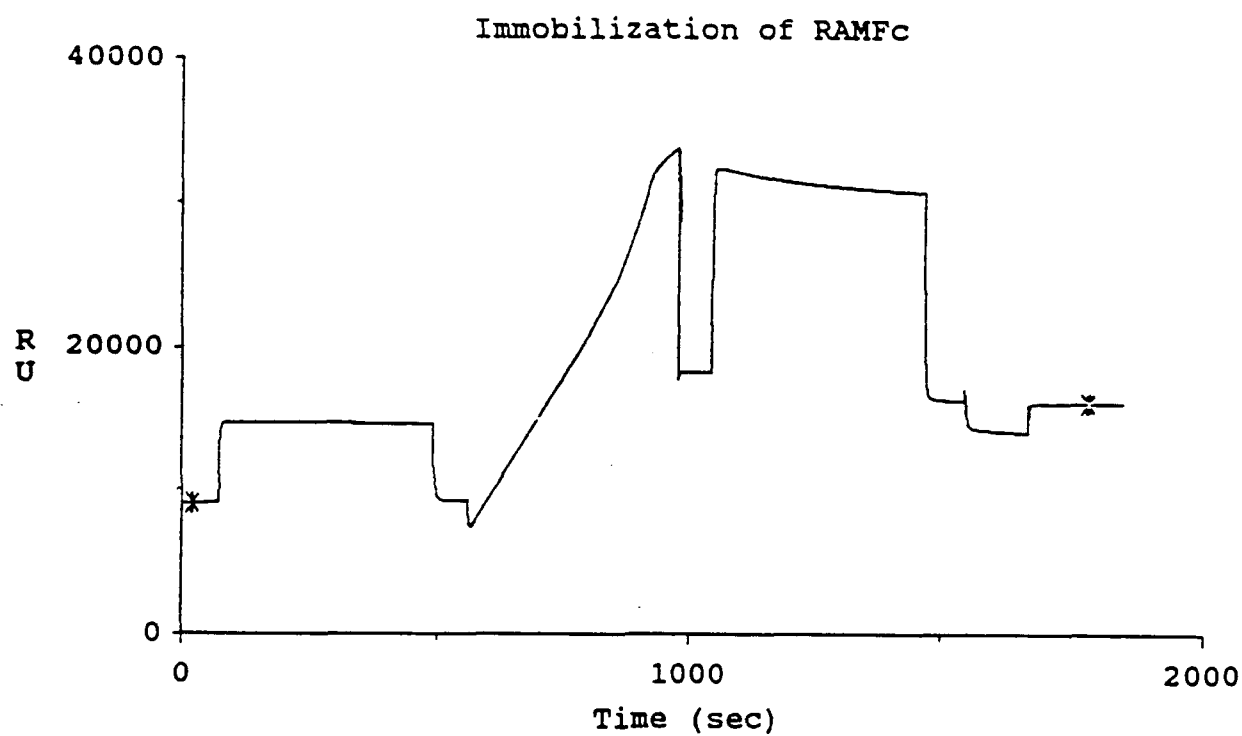


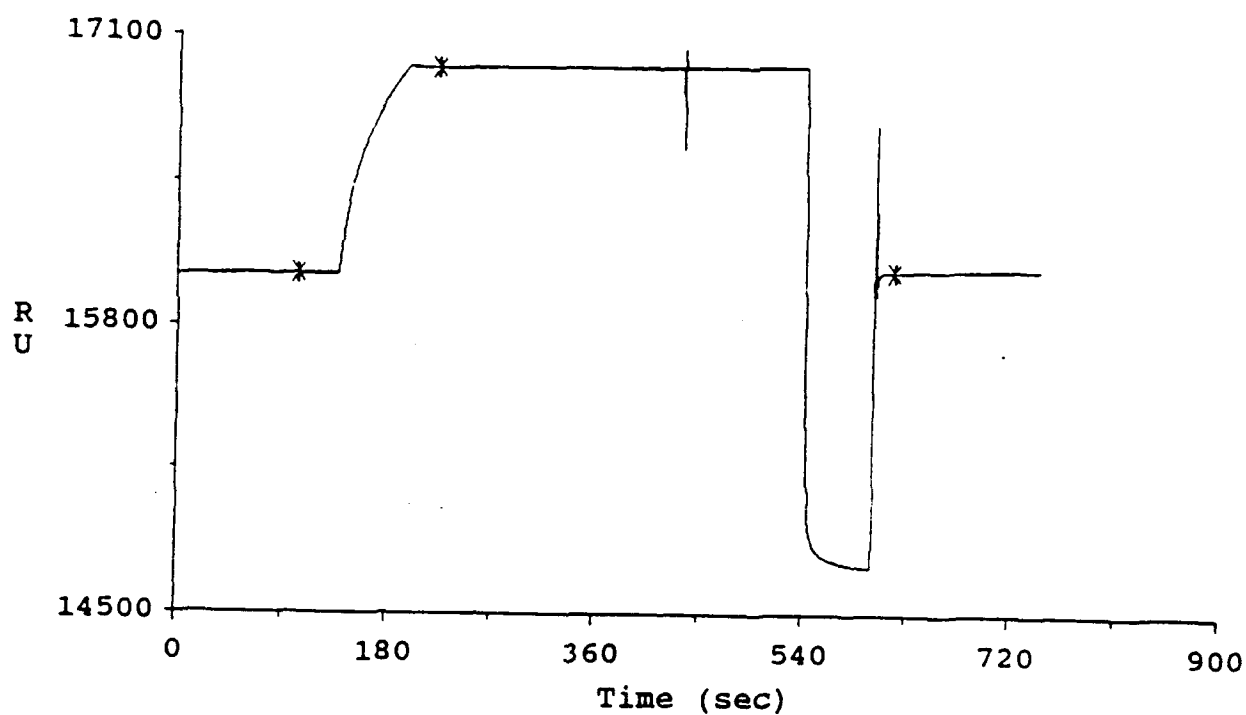
Figure 1. Covalent coupling chemistry.



<u>APROG</u>	<u>FC</u>	<u>Time</u>	<u>Window</u>	<u>AbsResp</u>	<u>Slope</u>	<u>Baseline</u>	<u>RelResp</u>	<u>Id</u>
immob	4	19.0	4.0	9080	0.23177	YES	0	Baseline
immob	4	1783.5	4.0	16097	-0.16247	NO	7017	RAMFc

Figure 2. Rabbit anti-mouse Fc immobilization to the sensor chip.

Nonspecific Binding Control, mAb 8A3



<u>Time</u>	<u>AbsResp</u>	<u>Slope</u>	<u>Baseline</u>	<u>RelResp</u>	<u>Id</u>
103.5	16026	-2.85156	YES	0	base1
223.5	16949	1.21875	NO	923	8A3 20ug/ml, 10ul
619.5	16049	-0.10156	NO	23	100 mM HCl, 10ul

Figure 3. Capture of Monoclonal Antibody (mAb) 8A3 by RAMFc and regeneration of the sensor chip.

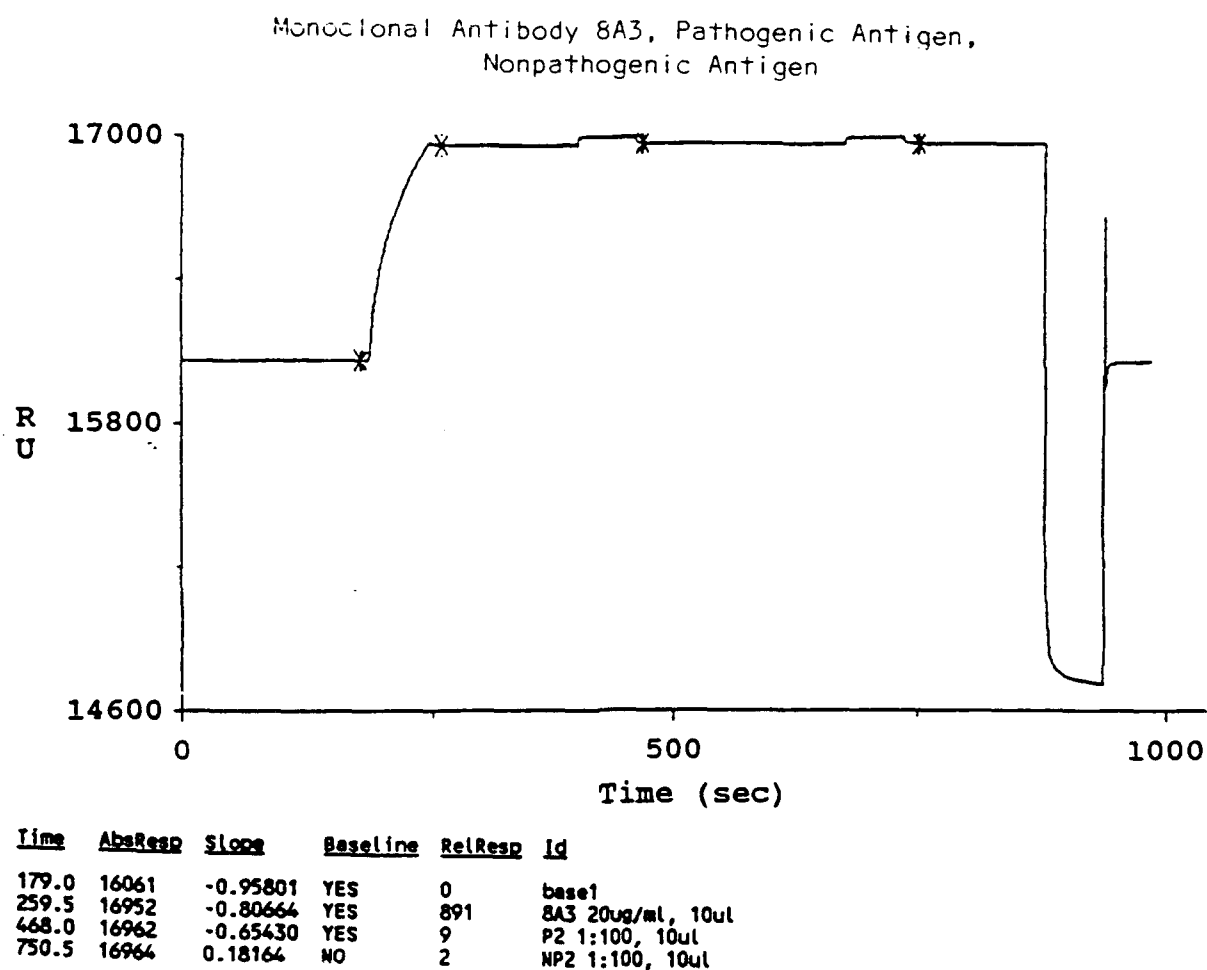
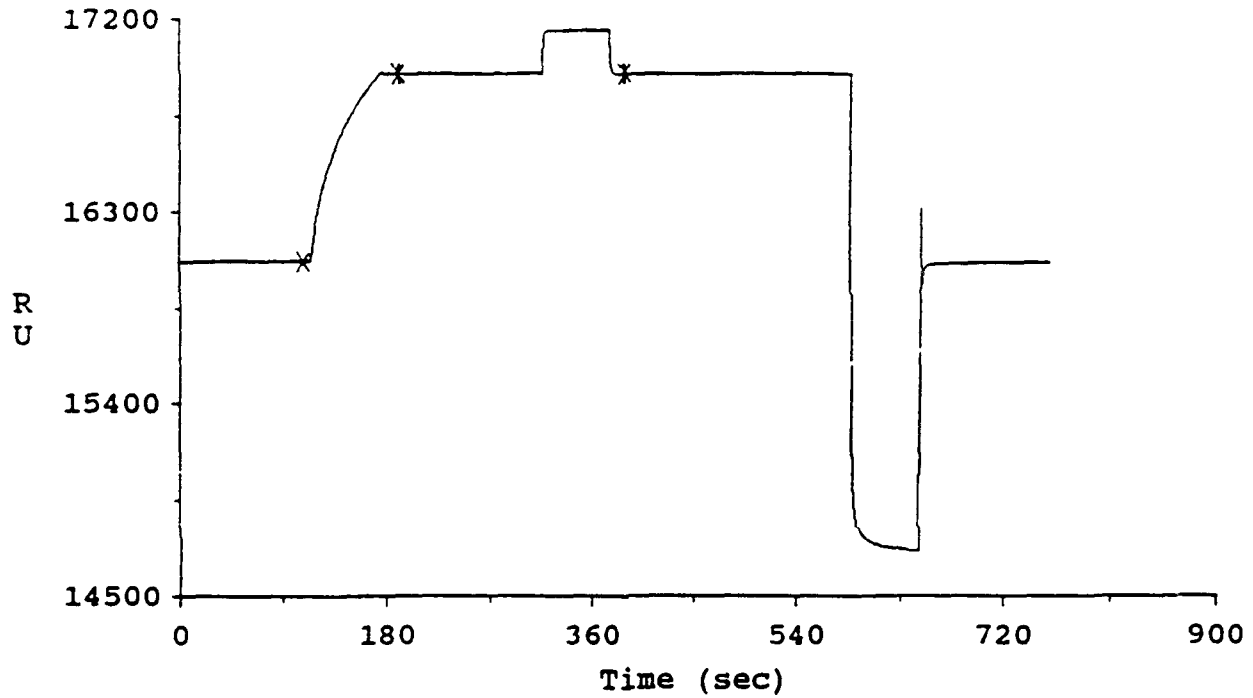


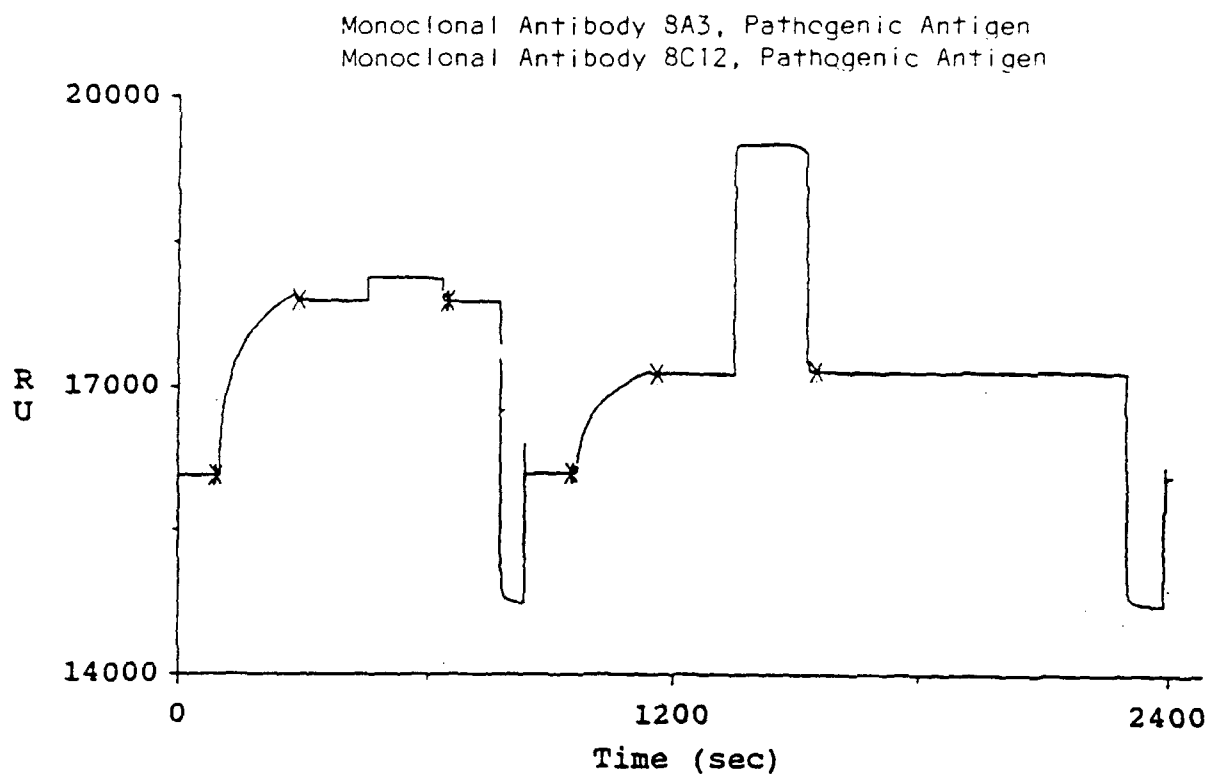
Figure 4. Capture of mAb 8A3 and challenge with Entamoeba histolytica pathogenic (P2) and nonpathogenic (NP2) antigenic preparations.

Monoclonal Antibody 8A3, Pathogenic Antigen



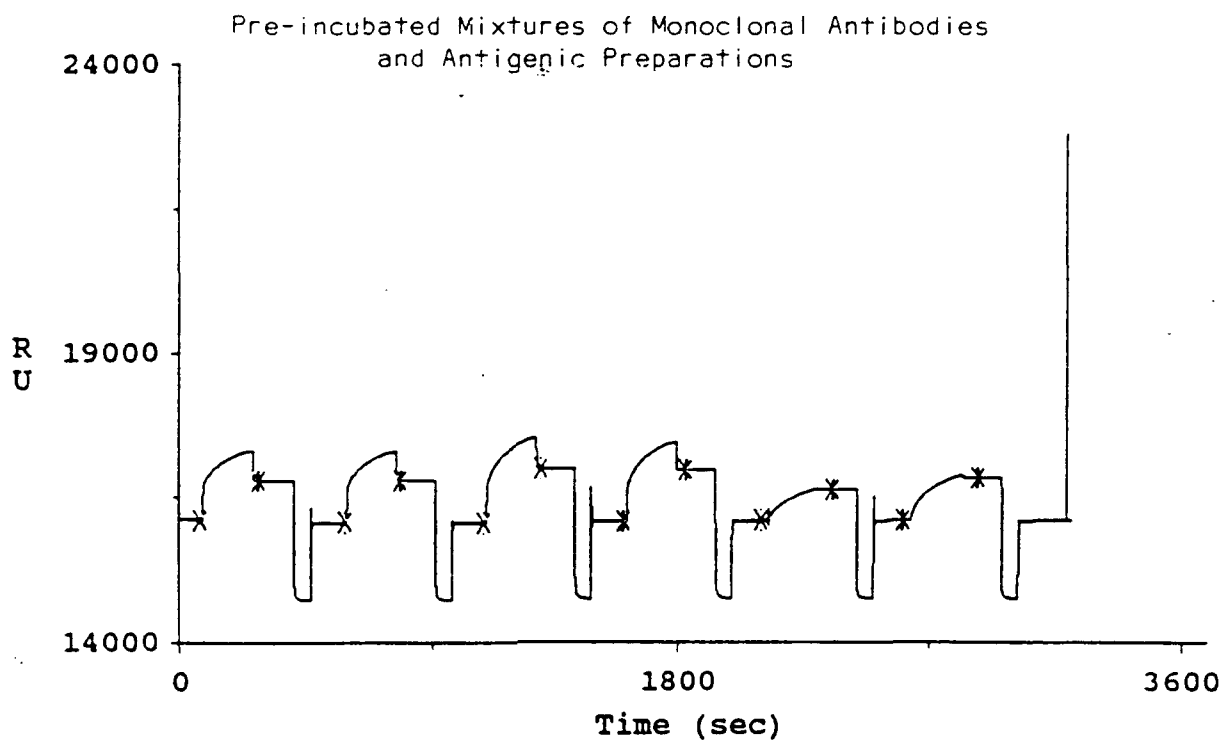
Time	AbsResp	Slope	Baseline	RelResp	Id
108.5	16067	-1.50098	YES	0	base1
190.5	16942	-1.02148	YES	875	8A3 20ug/ml, 10ul
389.0	16938	-0.55469	NO	-4	p2 1:10, 10ul

Figure 5. Capture of mAb 8A3 and challenge with Entamoeba histolytica pathogenic antigen (P2) at a minimal dilution.



<u>Time</u>	<u>AbsResp</u>	<u>Slope</u>	<u>Baseline</u>	<u>RelResp</u>	<u>Id</u>
89.0	16066	0.20996	YES	0	base1
289.0	17890	-0.19824	YES	1823	8A3 75ug/ml, 30ul
646.0	17876	-0.09570	NO	-14	p2 1:10, 30ul
953.0	16096	-0.36328	YES	-1794	base2
1160.0	17137	-0.62012	YES	1041	8C12 20ug/ml, 30ul
1549.0	17165	-0.72168	NO	28	P2 neat, 30ul

Figure 6. Capture of mAb 8A3 and 8C12. Challenge with Entamoeba histolytica pathogenic antigen (P2).



Time	AbsResp	Slope	Baseline	RelResp	Id
73.0	16113	-0.82520	YES	0	base1
280.0	16793	-0.11328	NO	679	8C12-P2
588.0	16069	0.59277	YES	-44	base2
788.0	16797	-0.10254	NO	728	8C12-NP2
1093.0	16069	-0.20850	YES	-1	base3
1299.0	17002	-0.97266	NO	934	8A3-P2
1604.0	16083	0.72998	YES	15	base4
1834.0	16980	0.22168	NO	897	8A3-NP2
2105.0	16093	0.29150	YES	10	base5
2366.0	16633	-0.56055	NO	540	8C12
2612.0	16097	0.71533	YES	4	base6
2876.0	16850	0.50879	NO	753	8A3

Figure 7. Pre-incubated mixtures of mAb 8A3 and 8C12 with pathogenic antigen and nonpathogenic antigen.

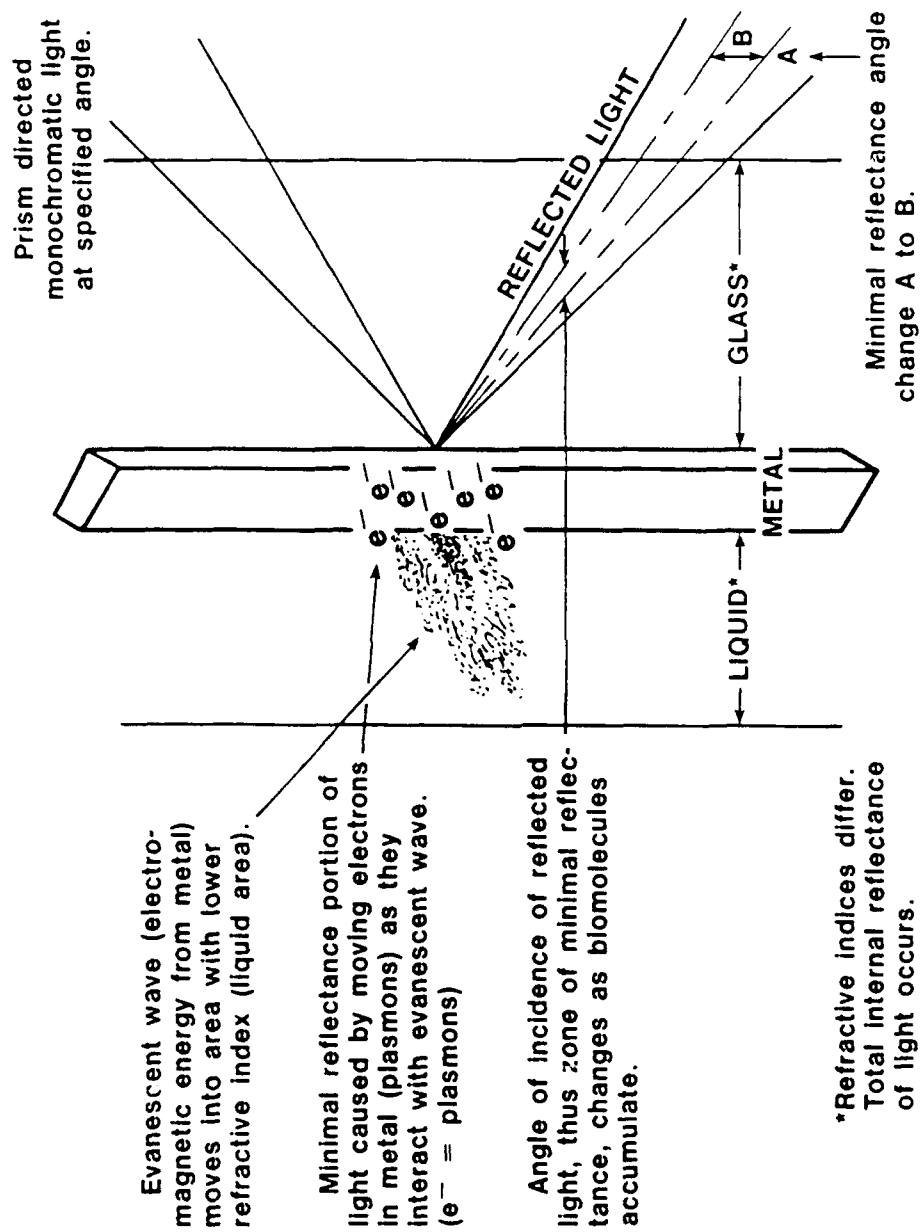


Figure 8. Physical depiction of the principle of surface plasmon resonance.

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