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**Surface Plasmon Resonance and Latex Microparticle Methods
Employing Photothermal Detection for Chemical Analysis**

by

Victoria VanderNoot, B.Sc.

A Dissertation submitted to the Faculty of Graduate Studies
in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

Department of Chemistry
Carleton University
Ottawa, Ontario CANADA
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The undersigned recommend to the Faculty of Graduate Studies acceptance of the dissertation "*Surface Plasmon Resonance and Latex Microparticle Methods Employing Photothermal Detection for Chemical Analysis*" submitted by Victoria A. VanderNoot, B. Sc., in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Chairman, Department of Chemistry

Thesis Supervisor

External Examiner

Carleton University

ABSTRACT

A new immunosensor which is based on the optical phenomenon of surface plasmon resonance (SPR) and which employs photothermal deflection spectroscopy (PDS) as a means of detection is presented. Immunoglobulins adsorbed to a thin silver film which serves as the analysis surface can be detected in sub-monolayer quantities. Using an analysis surface coated with a monolayer of the antigen γ -immunoglobulin (IgG), quantities as small as 2×10^{-9} g or 1×10^{10} molecules of specific anti-human γ -immunoglobulin (anti-IgG) can be detected. Investigations into the high intensity domain revealed that above 40 W cm^{-2} the non-radiative decay of surface plasmons saturated and was accompanied by an increase in the radiative decay mode as evidenced by increased light scattered normal to the silver surface. The use of fluorescently-labelled antibodies was investigated as a means of enhancing the sensitivity of the SPR-PDS immunoassay. It doubled the total shift in resonance angle for a given quantity of IgG. A sandwich method employing fluorescently-labelled antibody as a second stage to develop the immunoresponse further served to increase the total shift in SPR resonance angle by a factor of 6 from 0.5° to 3° . SPR-PDS was also used to characterize thin polymer films and to quantify the film thickness. Preliminary work was carried out to characterize thin polymer films molecularly imprinted with theophylline as a template molecule. The uptake of theophylline into the polymer film was monitored by a 0.25° shift in the SPR resonance angle after exposure of the film to a 4 mg mL^{-1} aqueous solution of theophylline.

Photothermal detection of agglutination of IgG-coated latex microparticles (LMP) was also explored. The sensitivity of photoacoustic spectrometry (PAS) for quantifying particles close in diameter to the excitation wavelength was observed to be enhanced three-fold. Use of this phenomenon in an immunoassay for anti-IgG was evaluated. The agglutination immunoassay employed small LMP ($0.204\mu\text{m}$ in diameter) so that their clusters, formed by antigen-antibody interactions, were of comparable size to the excitation wavelength. A detection limit of 5 ppm of anti-IgG was achieved. A heterogeneous immunoassay employing dyed latex microparticles was also developed in which agglutinations are selectively collected on small-pore membrane filters for analysis by PAS. The sensitivity exhibited a better detection limit of 0.5 ppm or an equivalent of 2×10^{12} molecules of anti-IgG.

A novel method for the extraction and quantification of mercury(II) was successfully developed. Dithizone was first incorporated into the interior of LMP and this represents the first time an analytical reagent has been incorporated into LMP. Mercury (II) was then quantitatively extracted from aqueous samples by complexation with dithizone-impregnated LMP. Solid samples were prepared by filtration of these particles through small-pore membrane filters and analyzed for Hg(II) by photochromism-induced PAS. The method exhibited high selectivity for Hg(II) and a limit of detection of 50 pg.

PUBLICATIONS AND PRESENTATIONS

Publications

1. Victoria A. VanderNoot and Edward P. C. Lai, 1991. Detecting Surface Plasmon Resonance Using Photothermal Deflection Spectroscopy: Development of an Optical Immunosensor. *Spectroscopy*, (1991), **6**(7), 28-33.
2. Victoria A. VanderNoot and E. P. C. Lai, 1992. Determination of Mercury(II) in Dithizone-impregnated Latex Microparticles by Photochromism-induced Photoacoustic Spectroscopy. *Analytical Chemistry*, (1992), **64**, 3187-90.
3. E. P. C. Lai, Barbara Wong and Victoria A. VanderNoot, 1992. Preservation of Solid Mercuric Dithizonate Samples with Polyvinyl Chloride For Determination of Mercury (II) in Environmental Waters by Photochromism-induced Photoacoustic Spectrometry. *Talanta*, (1993), **70**, 1097-1105.
4. E. P. C. Lai, S. J. Whiticar and V. A. VanderNoot, 1993. Photochromism-induced Photoacoustic Spectrometry of Methylmercury Dithizonate. *The Analyst* (submitted 05/93).
5. D. A. Berg, Victoria A. VanderNoot, P. G. Barradas and E. P. C. Lai, 1993. In-Situ Photoacoustic Determination of Thallium Underpotential Deposition on Copper. *Journal of Electroanalytical Chemistry* (accepted 09/93).
6. Edward P.C. Lai, Victoria A. VanderNoot, Mari Kono and Ania Fafara. Surface Plasmon Resonance for Characterization of Polymeric Films and Molecularly Imprinted Sorbent Assay of Theophylline. *in preparation*

Conference Presentations

1. 36th Canadian Spectroscopy Conference, Brock University, St. Catharines, ON, Canada. August 1-3, 1990. Photothermal Deflection Spectroscopy Detection in a Surface Plasmon Resonance Optical Sensor. V.A. VanderNoot and E.P.C. Lai.
2. 37th Canadian Spectroscopy Conference, Carleton University, Ottawa, ON, Canada. August 12-14, 1991. Investigations of Non-linear Absorption in a Surface Plasmon Resonance Optical Sensor. V.A. VanderNoot and E. P. C. Lai.
3. 38th Canadian Spectroscopy Conference, Trent University, Peterborough, ON,

Canada. August 10-12, 1992. Photochromism-Induced Photoacoustic Determination of Mercury Using Dithizone-Impregnated Latex Microparticles. V.A. VanderNoot and E.P.C. Lai.

4. 38th Canadian Spectroscopy Conference, Trent University, Peterborough, ON, Canada. August 10-12, 1992. Photoacoustic Determination of Underpotential Deposition of Thallium. V.A. VanderNoot, D.A. Berg, R.G. Barradas and E.P.C. Lai,
5. Varian Day Student Lecture, Carleton University, April 21, 1992. Preliminary Studies into the Use of Dithizone-Impregnated Latex Microparticles for Determination of Mercury(II) V.A. VanderNoot and E.P.C. Lai.

Poster Presentation

1. Gordon Conference on Photoacoustic and Photothermal Phenomena, Colby Sawyer College, New London, New Hampshire, U.S.A Application of PAS to the Characteristization of Thallium Underpotential Deposition. V.A. VanderNoot and E.P.C. Lai, D.A. Berg and R.G. Barradas.

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LIST OF SYMBOLS

Symbol	Description
Ab	antibody
Ag	antigen
a	$1/e^2$ radius of Gaussian beam (mm)
α	absorption coefficient (cm^{-1})
c	speed (of light in vacuum) (m s^{-1})
C_p	specific heat (constant pressure) ($\text{cal/g } ^\circ\text{C}$)
d	diameter (μm)
D	thickness (nm)
E	energy (mJ)
ϵ	dielectric permittivity
I	intensity (W cm^{-2})
K	wavevector
l	path length (cm)
λ	wavelength (nm)
μ	thermal diffusion length (μm)
η	refractive index
ρ	density (g cm^{-3})
θ	angle of incidence (degrees)
ω	frequency of oscillation
ϕ	probe beam deflection angle (rad)
T	temperature ($^\circ\text{C}$)
κ	thermal conductivity ($\text{cal/cm s } ^\circ\text{C}$)
k	thermal diffusivity ($\text{cm}^2 \text{s}^{-1}$)
Q_{ext}	extinction coefficient
f	frequency of modulation (Hz)
t	time (s)
P	pump beam power (mW)
m	relative refractive index
h	perpendicular displacement (of probe beam) (μm)
r	radial distance (mm)
x	propagation distance of probe beam (mm)
y	vertical distance between probe and pump beams (mm)
z	normal distance of probe beam to sample surface (mm)

GENERAL INTRODUCTION

Our bodies' immune response is what protects us from disease. Perhaps just as important is the realization that the immune response in a living system can occur well before symptoms evidence themselves. Since early diagnosis and treatment is the cornerstone of effective medical care, accurate and sensitive methods must be available to detect the onset of infection. The field of immunology has undergone some amazing transformations during the past decade. In the late 70's none but the largest and best equipped hospitals and laboratories were capable of performing routine immunological assays. By now, even modest hospitals have the means to perform immunoassay, especially with the development of pre-packaged assay kits.

Earliest commercial reagents involved the use of radioactive isotopes as labels, principally ^{125}I and ^3H . For these radioisotopic immunoassays (RIA) gamma or liquid scintillation counters were needed. These were not standard equipment for any but the best equipped labs in the early days and, even today, still represent a significant capital investment. RIA became the leader in the arena of immunological testing as a result of two important factors: firstly, the inherent high sensitivity of signals even when relatively few analyte molecules are present and secondly, general insensitivity to environmental interference since radioactive decay is unaffected by physical or chemical factors.

Even though researchers have occasionally been accused of indulging in methodological novelty for its own sake, compelling reasons for the development of

alternative techniques do exist. In spite of the excellent sensitivity that is available from RIA, there exists a real effort to find substitutes for radioisotopic labels. The potential biological hazards associated with the handling and disposal of radioactivity, the limited shelf-life of radioisotopic labels. The technology has become so standardized, however, that it would require compelling arguments to get many laboratories to switch away from RIA in favour of another technique in spite of the draw-backs mentioned. Ekins suggested that it is unlikely that any technique will ever be developed that exhibits comparable sensitivity while using significantly cheaper materials or more convenient (automatic) instrumentation than RIA [1], however the recent advances (after 1985) made to techniques such as enzyme immunosorbent assay (ELISA) may contradict this assertion [2]. Achieving the ultimate in sensitivity for immunoassays should not, however, be considered as the only criterion. It is generally more appropriate to target a method to the analyte. For general information, Table I lists some common biologically or therapeutically useful substances and their relative concentrations in the body.

This research work reflects the current desire to develop non-radioisotopic methods. In the first section of this thesis, an optical immuno-sensor based on surface plasmon resonance was developed. In the second section one homogeneous and one heterogeneous assay based on latex microparticle agglutination was examined. The third section represents the determination of mercury(II) by complexation and extraction into dithizone-impregnated latex microparticles. Throughout this thesis there is the common thread of photothermal spectrometry (PTS). PTS is a diverse group of techniques in

which the analytical signal results from the decay of absorbed light energy into heat. The techniques are sensitive and have frequently been used as truly non-destructive chemical probes. PTS has earned a genuine respect among researchers in analytical chemistry over the past decade and have been widely accepted as an excellent tool for solids, liquids and gases [3]. Immunoassay methods can generally benefit from the superior sensitivity of photothermal methods over traditional spectrometries.

A note should be made here concerning units. There exists a very real difficulty in comparing absolute sensitivities of various methods in the field of immunological analysis. Even a quick perusal of the literature reveals a plethora of units used, reflecting the varying backgrounds of researchers in the field. Perhaps the most common, and appropriate, unit encountered is the International Unit (IU) which has been defined by the World Health Organisation (WHO) as the potency of a given weight of an internationally accepted standard preparation of antiserum or antigen [4] and is approximately equal to 100 ng mL^{-1} of protein. Wherever possible throughout this thesis, protein quantities and detection limits will be reported in IU.

Table I. Approximate concentrations of several biologically and therapeutically important substances in serum [5].

Typical Blood Substance	Approximate Serum Concentration (moles/l)
ethanol, salicylate, acetaminophen	10^{-1}
glucose, urea, cholesterol	10^{-2}
gentamicin, theophylline	10^{-5}
cortisol, T4 ^a	10^{-6}
immunoglobulins	10^{-7}
human chorionic gonadotropin, insulin	10^{-8}
progesterone, T3 ^b ,	10^{-8}
CEA ^c , AFP ^d	10^{-11}

^a L-thyroxine, ^b3,3',5-triiodo-L-thyronine, ^ccarcinoembryonic antigen, ^d α -feto protein

CHAPTER 1: Surface Plasmon Resonance

INTRODUCTION

The search for alternative methods of immunoassay has developed along a variety of lines. Particle-based, enzyme-based and fluorescence-based techniques have previously been evaluated and all demonstrate advantages and disadvantages. In this chapter we will concentrate on new optical methods. Particle-based assays will be discussed more fully in the next chapter.

In the area of immunoassay, there is a desire to develop methods that can be incorporated into stand-alone biosensors which, ultimately, can make the leap from laboratory to a patient's bedside. These immunosensors, biosensors which incorporate immunological reactions, have gained popularity due to their very high selectivity. Of the direct immunosensor formats, Cullen and Lowe pointed out that those based on optical phenomena, and specifically surface plasmon resonance, are most promising [6]. Surface plasmon resonance (SPR) is a sensitive optical phenomenon that responds to optical changes at the sensor surface. It has been exploited for a variety of applications: surface plasmon microscopy (SPM) for high contrast imaging of thin dielectric films [7],[8], surface enhanced Raman scattering (SERS) and coherent anti-Stokes Raman scattering (CARS) [9], SPR enhanced holography [10], fibre optic chemical sensing [11] and SPR enhanced laser desorption [12].

SPR has been used widely in the area of immunoassay [13],[14]. SPR-based immunoassays depend on the fact that the resonance condition is sensitive to

changes in dielectric permittivity very close to the sensor surface. It has gained popularity since it requires no labelled species [15],[16], and also because it can be used to monitor the binding process kinetically, something that RIA cannot do, and to obtain information about binding capacity and specificity [17]. Changes in the resonance condition have traditionally been probed by measuring a loss in reflectance [18]. Photothermal techniques are ideal for probing radiationless processes such as SPR [19]. Inagaki *et al* employed photoacoustic spectrometry (PAS) [20] which is much more sensitive mode of detection. This work takes the further step by replacing the gas-microphone PAS detection with photothermal deflection spectrometry, another photothermal technique which has higher sensitivity than even gas-microphone PAS and is more amenable to incorporation into an immunosensor [21].

THEORY

Surface Plasmon Resonance (SPR)

Surface charge density oscillations, or surface plasmons, in a metal film exist as transverse magnetic (TM) waves of the electron plasma along the flat metal surface [22],[23]. The wave vector of the oscillations, K_{sp} , is given by

$$K_{sp} = \frac{\omega}{c} \sqrt{\frac{\epsilon_m \epsilon_s}{\epsilon_m + \epsilon_s}} \quad (1)$$

where ω is the frequency of the oscillations, c is the speed of light, ϵ_s the dielectric function of the sample medium adjacent to the metal surface and ϵ_m is the dielectric function of the metal [24]. The oscillations are thus dependent on ϵ_m which is a complex function of the form $\epsilon_m = \epsilon' + i\epsilon''$ (storage and loss moduli). The real term, ϵ' , governs the propagation of plasmons in the metal and must be a negative value. The imaginary component, ϵ'' , governs the damping of the surface plasmons which results from internal absorption by the metal. This term, which is the smallest for silver, often makes Ag the metal of choice for SPR investigations. As the magnitude of this imaginary term determines the width of the angular resonance profile, silver exhibits very narrow resonances which are roughly Lorentzian throughout the visible region [23]. Sharp resonances are desirable in immunosensing applications since the width of the angular resonance profile will ultimately determine the sensitivity of the assay. Gold, although it is chemically more inert and would for that reason be preferable to silver, displays much broader resonances throughout the visible region and is essentially useless

below 500 nm where it absorbs strongly.

It is not possible to excite the surface plasmons (SP) directly with incident radiation since the dispersion curve of light in air ($K_a = \omega/c$) and that of plasmons in the metal (K_{sp}) do not intersect as can be seen from Figure 1. Hence, there is no possibility of matching simultaneously both frequency and wave vector [25]. However, the coupling of light to surface plasmons in a metal becomes possible through the use of a dielectric medium interposed between the exciting radiation and the metal plasma. When light enters a dielectric medium such as glass, its wave vector is modified by a factor equal to its refractive index or the square root of its dielectric function and becomes

$$K_g = \frac{\omega}{c} \sqrt{\epsilon_g} \quad (2)$$

As can be seen in Figure 1, this allows for a simultaneous match in both K and ω at the intersection of the K_{sp} and K_g curves.

Under conditions of total internal reflection in glass, the electromagnetic field of the radiation extends beyond the boundary of the glass and into the medium beyond (Fig. 2). This field extension is termed the evanescent wave. The field strength of the evanescent wave exhibits an exponential dependence on the distance z from the glass

$$e^{-\frac{\omega}{c} \sqrt{n_p^2 \sin^2 \theta - n_s^2} z} \quad (3)$$

boundary where n_p is the refractive index of the prism and n_s is the refractive index of the medium adjacent to the interface. The exponential decay of the field through the silver film into the sample medium is an exploitable property of the SPR system and one

Figure 1: Dispersion curves in air, glass and silver.

Figure 2: Total internal reflection at a dielectric boundary.

that allows it to behave as a sensor. Dielectric changes at the silver/sample interface drastically affect the propagation of the surface wave and dramatically alter the dispersion properties. Only the lateral component of K_g contributes to the evanescent wave and this is represented by the $\sin \theta$ function,

$$K_{ev} = K_g \sin \theta = \frac{\omega}{c} \sqrt{\epsilon_g} \sin \theta \quad (4)$$

where θ is the angle of incidence. When the wave vectors, K_{sp} and K_{ev} match at $\theta = \theta_0$, as shown in the following equation,

$$\frac{\omega}{c} \sqrt{\epsilon_g} \sin \theta_0 = \frac{\omega}{c} \sqrt{\frac{\epsilon_m \epsilon_s}{\epsilon_m + \epsilon_s}} \quad (5)$$

there is a resonant transfer of energy from the incident light to the plasmon modes along the surface. This has been traditionally observed as a decrease in the reflected beam intensity [18].

SPR has been carried out in a number of standard configurations, the two most common of which are illustrated in Figure 3. The Otto geometry, sometimes known as the frustrated total reflectance (FTR) mode, uses a prism with a spacer layer of air. The Kretschmann geometry, known as the attenuated total reflectance (ATR) mode, employs a thin metal film attached to the prism base [26]. This geometry, which will be used for this work, is much more amenable to sensor applications than the FTR geometry as the sample medium may conveniently be placed in contact with the silver surface, either as a solution or as a thin solid deposit. A third configuration, direct coupling to a diffraction grating is also possible [27] but the theory is at present less well developed

Figure 3: a) The Otto geometry b) the Kretschmann geometry

for gratings. Prism coupling also has the advantage of being cheaper and more readily available. [28]

Typical metal film thicknesses (D) used in the Kretschmann geometry for SPR work are of the order of 600 Å. Films much thinner than this are problematic for two reasons. Firstly, the two SP modes propagating on the faces are coupled in the interior of very thin films, since the decay length of the SP field is as large as 200 Å. Secondly, very thin films can exhibit island structures on the surface leading to increased direct scattering of the coupled light [29]. In practice, any moderate thickness above 400 Å is suitable.

Non-radiative plasmons propagate along the metal surface and are eventually damped as a result of internal absorption in the metal film, resulting in Joule heating of the surface which can be probed via a photothermal technique or by measuring the increase in metal temperature [30]. Intrinsic damping of the plasmon wave is due to energy absorption in the metal, is proportional to the imaginary part of the dielectric function of the metal, ϵ'' and determines the fundamental width of the angular resonance profile. Radiation damping can also take place in which a light wave is emitted into the dielectric medium [31]. Momentum is conserved during radiation damping; the photons are emitted only in the direction of the specular reflection of the incident light and are superimposed on the reflected beam. Radiation damping increases the width of the angular resonance profile and is strongly dependent on the metal film thickness. For thin films (< 400 Å), radiation damping becomes the predominant factor that determines the halfwidth of the SPR spectral profile [32].

Surface plasmons are bound to the metal/sample interface because their momenta are always greater than those of the light in the sample medium (or air). Direct conversion of plasmons into photons, or the radiative decay of surface plasmons, is then not allowed. At an ideally smooth interface no light emission into the sample medium should be observed; in real systems there is always some random surface roughness. Extra momentum required to convert the non-radiative plasmons into radiative plasmons which decay by emission of light can be obtained from the roughness profile [23]. Examination of the light emitted can give information about the root-mean-squared roughness of the metal surface. In practice, as long as reasonable care is taken in preparing the metal films, roughness may be essentially ignored [23].

Photothermal Beam Deflection Spectroscopy (PDS)

Photothermal beam deflection spectroscopy, sometimes known as probe beam refraction (PBR), can be carried out in a number of standard configurations. In the most common configuration, as investigated by Jackson *et al* among others, the probe beam is passed through the sample medium collinear or parallel with the excitation or pump beam, offset by a small distance r (see Fig. 4a) [33]. The excitation beam is assumed to have a Gaussian energy profile [34]:

$$E(r) = \frac{E_i}{\pi a^2} \exp\left(-\frac{r^2}{a^2}\right), \quad r \leq a \quad (6)$$

where E is the energy and a is the pump beam radius. In this collinear configuration, the refractive index (RI) gradient is both created and probed in the sample. For a variety

Figure 4: Configurations for PDS a) collinear b) transverse

of samples, such as solids or opaque liquids, the collinear configuration is just not feasible and the transverse configuration is more appropriate. In a transverse configuration (Fig. 4b), the RI gradient is probed external to the sample in the fluid (air) layer in thermal contact with the sample. For our experiments carried out using the SPR cell, the transverse geometry is used.

For the transverse system, it is assumed that the fluid in contact with the sample medium does not absorb the excitation radiation. Additionally, it is assumed that the dimensions of the media extend radially to infinity, although in practice all that is necessary is that the pump beam be focused to a smaller spot than the sample dimensions. The amount of heat deposited in the absorbing sample is proportional to the incident power, P_c , the absorption coefficient of the sample, α , and inversely proportional to a . The temperature rise in the sample medium is dependent on the thermal properties of the sample medium, specifically the conductivity κ , the diffusivity k ($=\kappa/\rho C_p$, where ρ is the density and C_p is the heat capacity), and the amount of heat deposited. Diffusion of the heat deposited in the sample medium sets up a temperature gradient which in turn develops a refractive index gradient normal to the sample surface contacting the fluid layer. Note that there is also an acoustic wave generated in this process but that its effect on the deflection angle due to pressure variations is negligible, at moderate modulation frequencies f , since the ratio of acoustic to thermal components is $\approx 10^{-11} f \exp(z_0/l_t)$ where z_0 is the distance between the region of heat deposition and the probe beam and l_t is the thermal length [35].

In the transverse PDS configuration, a Gaussian probe beam propagating in the

x-direction through a spatially changing refractive index will obey [33]

$$\frac{d}{dx} \left(\eta_0 \frac{dh}{dx} \right) = \nabla_{\perp} \eta(h, t) \quad (7)$$

where η_0 is the uniform index of refraction, h is the perpendicular displacement of the beam from its original direction, and $\nabla_{\perp} \eta(h, t)$ is the gradient of the refractive index perpendicular to the probe beam path. Angular deflections are typically 10^{-5} rad over a propagation distance, x , of 1 cm, leading to a spatial deviation of $0.1 \mu\text{m}$, which is very much smaller than the typical probe beam spot size. Since the deviation is small, it is possible to approximate the deflection angle, ϕ , by the expression derived from Eq. 7:

$$\phi = \frac{dh}{dx} = \frac{1}{\eta_0} \frac{\partial \eta}{\partial T} \int_{\text{path}} \nabla_{\perp} T(h, t) dx \quad (8)$$

The probe beam will be deflected both in a direction parallel to and perpendicular to the surface according to

$$\phi_{\parallel} = \frac{1}{\eta_0} \frac{\partial \eta}{\partial T} \int_{-\infty}^{\infty} \frac{\partial T}{\partial y} dx \quad (9)$$

$$\phi_{\perp} = \frac{1}{\eta_0} \frac{\partial \eta}{\partial T} \int_{-\infty}^{\infty} \frac{\partial T}{\partial z} dx \quad (10)$$

In this work, the deflection normal to the surface will be measured and the deflection angle reaches a maximum when there is no offset between the excitation beam and the probe beam axes [36].

EXPERIMENTAL

System Design

Figure 5 illustrates the SPR-PDS experimental apparatus. A rotation stage was designed and manufactured by the Science and Technology Centre at Carleton University [37]. It had an angular resolution of 0.09° . The stage was large enough that the essential optical equipment could be mounted on the stage. Components being fixed relative to each other eliminated the need for re-optimization after the angle of incidence θ was changed. At the centre of the stage was placed a right-angled glass prism such that the axis of rotation coincided with the front surface of a glass slide (which would ultimately support the silver film) when it was attached to the prism's hypotenuse side. Alignment of the incident beam to the prism was made such that the incident beam intersected the prism vertex at 0.00° . The prism was mounted on a support which had a close-fitting depression in the base and a spring-clip to allow rapid and accurate repositioning of the prism when changing samples. The PDS detection system consisted of a 0.5-mW HeNe laser beam (Uniphase Corp., Sunnyvale, CA, model 1508-p) directed onto a photodiode and running close and parallel to the silver surface. The optical components (photodiode and probe laser) were mounted on x-y translation stages to allow independent optimization of position with respect to the PDS signal amplitude. The signal from the photodiode, which measured the absolute change in voltage resulting from the deflection of the probe beam, was sent to a lock-in amplifier with the associated

Figure 5: Schematic diagram of the SPR-PDS apparatus.

data acquisition software (Stanford Research Systems, Sunnyvale, CA, models SR510 and SR565). Signal filters (60 and 120 Hz) were employed and a time constant of 3 s was typically selected. Autophase calculations ensured that the SPR-PDS signal was maximized with respect to phase angle.

Laser excitation was provided by an argon ion pumped dye laser system (Spectra-Physics, Mountain Valley, CA, models 164 and 375B). The dye, R6G (Exciton, Dayton, OH), provided a usable wavelength range of 570 to 630 nm. Tuning of the wavelength was accomplished by means of standard tuning wedge and an ultra fine etalon which provided a resolution of 0.01 nm. For tunability in the 600 to 660 nm range, the laser dye was replaced with DCM. For higher intensities and for shorter wavelengths (488 and 514.5 nm), the dye laser could be bypassed and the pump beam taken directly from the argon ion laser. In either case, the excitation beam was chopped mechanically at 200 Hz and then rotated to the p-polarised orientation [38] through the use of three mutually perpendicular mirrors in order to excite surface plasmons.

Since silver tarnishes readily in air, it cannot be used under ambient conditions indefinitely. All of the films were stored under vacuum until they were needed and fresh silver films were used for each study. In addition, once the metal film is exposed to the immunoproteins, it cannot be cleaned after use and must be discarded. Hence, the silver films were prepared onto disposable glass slides that were coupled to the prism using a refractive-index-matching oil (dibutyl phthalate) between the glass faces to minimize reflectance losses [13].

Silver films of 600 Å thickness were prepared initially by the Optical Components

Laboratory of the National Research Council (NRC) of Canada (Ottawa, ON) by vacuum evaporation of 99.99% pure silver and deposition on 25mm x 25mm pyrex microscope slides (thickness = 1mm). Sloan Dektak readouts verified the deposited silver film thickness. Silver films were subsequently prepared at Interoptics (Nepean, ON) as a result of personnel changes at NRC. At Interoptics, a $1/4 \lambda$ (1500 Å) thick layer of aluminum oxide was laid down on the slide prior to deposition of the silver. This was necessary in order to improve adhesion of the silver to the glass; otherwise, the silver would tend to lift from the glass on exposure to aqueous sample solutions. Why this was necessary only with the Interoptics films is unclear but is presumably due to a subtle variation in the substrate surface preparation between the two facilities. Regardless, the application of the aluminum oxide layer proved to be quite effective and did not interfere with the SPR phenomenon since the oxide has a similar refractive index to that of the glass slides. The rate of deposition of silver to form a thin film affects the roughness. Rapidly deposited films reproduce the surface smoothness of the substrate and form homogenous surfaces. Slower deposited films can lead to discontinuous agglomerated structures [39]. In this work, films (Interoptics) were deposited at 10 Å/sec. This rate was found to generate high quality films which yielded sharp resonance peaks and which exhibited very low intensity of light scattered off the silver surface, which was taken as an indication of minimal surface roughness.

Figure 6 illustrates the prism, probe and pump beam configuration for PDS detection. The detection system was optimized by monitoring the SPR-PDS signal from bare silver films. The beam deflection was monitored by means of a photodiode

Figure 6: Prism, probe and pump beam configuration for PDS detection.

Figure 7: PDS signal amplitude mapped over the probe beam profile.

equipped with a pin-hole ($\sim 50 \mu\text{m}$). The pin-hole served to localize a given spot on the probe beam intensity profile such that when the probe beam was deflected, a new spot on the beam profile was brought to fall on the photodiode. An absolute difference in the amount of light falling on the photodiode then gave rise to the PDS signal. This is analogous to a knife edge or a slit which has been reported before [40],[41]. The PDS signal was monitored with the pin-hole fixed at various points throughout the two-dimensional (yz) probe beam profile (Fig. 7). Two maxima in the z-direction, were discernible and would correspond to the two points of inflection of the Gaussian probe beam profile. The nearer one to the silver surface, and thus the heat source, was much larger as would be expected. The pin-hole (and photodiode) was fixed in place at this point of inflection of the probe beam profile where the PDS signal sensitivity was the highest and the response with respect to beam deflection was linear. Since the analysis surface was vertical, it was also necessary to establish whether there was any vertical contribution (y) to the thermal refractive index gradient. There were also two small maxima in like positions along the y direction. Since the two y-direction maxima exhibited very small PDS signal amplitudes relative to the z direction maxima, the heat flow could be considered to be directly out from the silver film surface, to a first approximation.

The probe beam distance from the silver film surface was varied and found to be a maximum at approximately 0.2-0.3 mm as is shown in Figure 8. The probe beam was aligned above the pump beam spot centre where the transverse PDS deflection was found to be at a maximum as seen in Figure 9.

Figure 8: Effect of the probe beam distance from the silver surface, z.

The tilt angle of the probe beam with respect to the sample surface was estimated to be less than 0.3° . While the tilt angle had been shown to affect PDS sensitivity, it would only become a problem for large probe beam radii if the beam actually intersects the sample. At any rate, significant sensitivity loss would only be expected for tilt angles an order of magnitude greater than those involved in this experimental system [33].

Generally the PDS signal is known to increase inversely with the pump beam radius [33]. In this experimental system, since the lateral position of the pump spot on the sample surface necessarily would move somewhat as the angle of incidence changes, it was decided to eliminate focusing of the probe beam. The pump beam spot for all experiments was 2 mm in diameter [42]. Ideally, it is important that the probe is

Figure 9: Effect of the distance between the pump and probe beam centers on the PDS signal amplitude, y .

significantly smaller than the pump beam [33]. This was satisfied by the inherent probe beam radius of 0.5 mm.

The pump beam spot also spread out laterally as the angle of incidence θ was increased. Since this expansion of the beam spot was in the direction of propagation of the probe beam, it would be expected to have little effect on the PDS signal. To ensure that this was a valid assumption, the PDS signal amplitude was monitored for a neutral density filter as a function of angle of incidence. The results, shown in Figure 10, show that except for very large angles of incidence, the PDS signal amplitude is insensitive to the changes in incident angle.

Figure 10: Effect of changing the angle of incidence on the PDS signal amplitude for a 0.4 neutral density filter.

Immunoassay by SPR-PDS

Immunoglobulins were obtained in lyophilized form from Sigma Chemical (St. Louis, MO). Solutions were prepared in normal saline (0.85% w/v) and divided into small aliquots that were stored frozen. Fresh solutions were thawed for use each day to ensure no bacterial contamination.

A monolayer of antigen (Ag), typically IgG, was made by exposing a silver film to a 0.1 mL aliquot of $200 \mu\text{g mL}^{-1}$ for a 5 minute period. This time was sufficiently long with respect to the adsorption process to ensure full coverage [14]. Immunoproteins are known to adsorb readily to metal surfaces to form a monolayer [43]. The loaded film was then rinsed with several millilitres of distilled water and allowed to air dry before mounting onto a prism for SPR-PDS analysis. The SPR-PDS signal amplitude was measured for each incidence angle used to generate an SPR angular profile. In order to obtain maximum SPR sensitivity, an inflection-point angle was chosen halfway along the sharp slope portion of the SPR angular profile obtained for the film coated with a monolayer of IgG. The rotation stage was then locked in place at this angle θ . The film sample on the glass slide was then demounted and exposed to a 50-ppm solution of anti-IgG for 30 minutes. The film was then rinsed with several millilitres of distilled water and allowed to air dry before re-mounting for analysis. The new PDS signal amplitude at the previously chosen angle θ was recorded. The difference in signal amplitude relative to the film coated only with IgG was then plotted versus the anti-IgG concentration, diluted from the original 50 ppm stock, to generate standard calibration curve using solutions of different anti-IgG concentrations exposed to individual IgG-

Figure 11: Schematic representation of the IgG monolayer after exposure to a solution of specific antibody.

loaded silver films (Fig. 11). To account for variations in laser intensity, the signal difference was normalized by the total PDS signal observed at the resonance angle θ_0 .

When working with more dilute solutions (< 5 ppm), it became apparent that there were considerable losses experienced since proteins are known to physically adsorb to solid surfaces. As a result it became necessary to prepare all standards in the presence of a supporting protein. Bovine serum albumin (BSA) was chosen as the protein as it showed no interference with any of the antibodies used in this work. Unless otherwise noted, the supporting protein concentration was maintained at 1 mg mL^{-1} [21].

Saturation Studies

In order to explore the high pump laser intensity domain, the dye laser was bypassed and the beam taken directly from the argon ion laser. All of the laser lines (primarily 488.0 nm and 514.5 nm) contributed to the total output intensity. A variable interference band-pass filter (Oriel, Stratford, CT) was employed to separate the lines.

Using a bare silver film, the rotation stage was fixed in place at the resonance angle θ_0 giving the maximum SPR-PDS signal amplitude. The SPR-PDS signal amplitude was recorded for a range of laser intensities. The light scattered normally from the air side of the silver surface was also collected via a fibre optic bundle (5 mm in diameter) and sent to a photomultiplier tube.

Fluorescent Tag and Particle Enhanced SPR-PDS Immunoassay

In order to significantly improve the detection limit of the SPR-PDS immunoassay

it would be necessary to increase the shift in the SPR resonance angle for a given quantity of anti-IgG. Since the angular shift is a function of the refractive index of the dielectric sample, it would be necessary to increase the refractive index of the antibody. One possible approach would be to employ a fluorescently labelled antibody raised against the analyte protein that has already been immobilized onto the silver surface. The fluorescent tag, fluorescein isothiocyanate (FITC) in this study, is not used in the traditional manner where fluorescence from the tag is measured; in actual fact, its light-absorbing properties would constitute a drawback since an absorbing layer on the silver can broaden the resonance peak (see Appendix A). Rather, it is the conjugation of, on average, three FITC molecules per protein molecule that is expected to alter the dielectric properties of the antibody sufficiently to generate a larger overall angular shift. Certainly, the presence of three FITC molecules (total molecular weight $\sim 1000 \text{ g mol}^{-1}$) is insignificant relative to the protein itself (146 000 daltons) and can not be expected to alter the molecular dimensions appreciably to account for a change in resonance angle on account of an increase in the film thickness. Other aspects of the immunoassay procedure used was identical to that already discussed.

Alternatively, a sandwich assay method can be adopted, in which a developing step employing the FITC-labelled antibody is used to enhance the resonance shift (Figure 12a). In this case, the silver was first exposed to the analyte antibody, then to a concentrated solution of antigen (200 ppm IgG), and finally to a concentrated solution of FITC-labelled antibody to the antigen molecule (50 ppm FITC-labelled anti-IgG).

A second approach to increase the shift in SPR resonance angle would be to

Figure 12: a) Sandwich method and b) particle enhancement of SPR-PDS immunoassay.

employ polystyrene latex microparticles, discussed fully in the next chapter, as a substrate for the proteins. The polystyrene latex has a refractive index of 1.5905, which is significantly higher than the 1.47 estimated for the proteins. In this assay, the silver film was exposed to the analyte antibody. The antibody layer adsorbed on the silver film as an incomplete monolayer was then exposed to antigens coated onto latex microparticles (see Experimental section of Chapter 2, page 87, for details of the coating process). The coated latex particles would then be allowed to bind to the antibody on the silver surface (Figure 12b). The presence of the higher-refractive-index coated latex can give rise to a larger change in SPR resonance angle. The efficiency of the assay technique was examined using different particle diameters.

SPR-PDS Evaluation of Thin Polymer Films

One of the driving forces in immunoassay research is for ways to incorporate the inherent specificity and sensitivity of immunoassay methods to the chemical analysis of drugs and haptens of lower molecular weights (typically < 2500 daltons)[44]. Haptens do not generally induce an immune response on their own and require conjugation to larger macromolecules prior to injection into the host animal in order to develop antibodies [45]. The future of immunoassay may include synthetic alternatives to antibodies that are simpler to prepare. The preparation of a synthetic alternative has been reported in the literature [46]. In the work, molecular imprinting (MI) was used to create selective binding sites for the drugs theophylline and diazepam. The first stage in the work was to prepare a polymer cast by reacting

functional monomers in the presence of the "print molecule" or template, theophylline. Removal of the print molecule by extraction left sites that complemented the template in shape and chemical functionality. This work represented the first demonstration of a completely synthetic approach to creation of functional binding sites. The goal would be to produce selectivities and binding constants comparable to those attainable in biological antibody systems.

Preliminary work was undertaken. The model drug, theophylline, was polymer cast using methyl acrylic acid (MAA) as the functional monomer and ethylene glycol dimethacrylate (EDMA) as the cross-linking monomer. The carboxylic acid functional group of the MAA interacted with the amino groups and the polar groups of the theophylline creating sites throughout the polymer matrix that have affinity and selectivity for the target antigen to rival those of the natural anti-IgG. Thin films of the polymer casts were evaluated by SPR-PDS to measure theophylline levels. The development of synthetic imprinted polymers should offer a number of advantages including easier and faster preparation, stability for storage, and potential use in solvents and under high-temperature conditions that would have destroyed antibodies.

The first stage of our study to apply the molecular imprinting technology to our SPR-PDS system was to examine simple thin polymer films by SPR-PDS to gain insight into the characterization of more complicated MI films. SPR has already proven to be a valuable tool for the imaging of thin polymer films [47]. There are a variety of techniques for preparing very thin polymer films and many of the them are based on Langmuir-Blodgett (LB) or self-assembly methods [48]. While these methods are

well developed and produce highly reproducible films, the apparatus required (especially with LB films) tend to be elaborate and limited in the amount of manipulation possible without damaging the delicate SPR silver films. For this reason, polymer films were prepared on the silver simply by spotting the surface with a small volume of the polymer dissolved in an appropriate solvent, followed by air drying. Different thicknesses were attempted by using polymer solutions of different concentrations. The choice of solvent was governed by the need to have moderately slow evaporation rate at ambient temperature and pressure, while maintaining sufficient solubility for the polymer. For most of the polymers examined the optimum solvent was a 1:1 (v/v) mixture of tetrahydrofuran:toluene. It should be noted that this method does produce somewhat irregular coatings, with the polymer molecules having a number of orientations on the surface to produce different local thicknesses. However, the 2-mm diameter of the pump

Table II: Conditions for preparation of thin polymer films

Polymer	Solvent	Volume(μ L)	Film Diameter (mm)
Polyvinyl alcohol ^a	water	20	5
Polyvinyl alcohol ^b	water	20	5
Polyvinyl chloride ^c	THF:tol ^e	2	7
Polyvinyl chloride ^d	THF:tol	2	7
Polypropylene	THF:tol	2	9
Polystyrene	THF:tol	2	9

^a Low molecular weight (10 000 - 33 000),

^b High molecular weight (70 000 - 100 000),

^c High molecular weight (158 000),

^d Very high molecular weight (500 000),

^e Tetrahydrofuran:toluene (1:1 v/v mixture)

beam would sample a finite area of the film, generating an "effective" thickness which would represent a spatial average of the area sampled by the pump beam.

As a second stage of this work, a two-component composite polymer film was prepared in order to act as a model for the molecularly imprinted polymer films. Films were prepared in a similar manner as already described except that solutions of 10^{-6} g mL⁻¹ polyvinyl alcohol in water contained 1 ppm polystyrene latex microparticles. The result was the preparation of thin films in which LMP was embedded in a matrix of polyvinyl alcohol. The microparticles were selectively removed from the film by dissolution in 1:1 THF:toluene, which was a solvent for polystyrene but not polyvinyl alcohol. The SPR angular profile was evaluated before and after particle dissolution. Ideally it would be possible to distinguish between films which contained polystyrene LMP ($n=1.59$) and those which contained vacancies where the LMP had been removed, as a result of both the changes in effective thickness and refractive index of the composite film. SEM images of films with and without treatment were obtained to confirm complete removal of the microparticles.

The molecularly imprinted polymer, containing theophylline, was prepared according to the procedure outlined by Vlatakis *et al* [46]. To a 25 mL volume of chloroform was added 0.47 g theophylline, 0.9 g MAA, 9.4 g EDMA and 0.12 g 2,2'-azobis(2-methylpropionitrile) initiator (AIBN). The mixture was degassed in a sonicating bath under vacuum and flushed with nitrogen for 5 minutes. Polymerization began within 1 h and was continued for 24 h. The polymer was ground using mortar and pestle to produce a coarse powder. In order to produce anti-theophylline polymer the drug was

extracted from the polymer by Soxhlet extraction or supercritical fluid extraction (SFE). SFE was carried out using CO_2 at 400 atmospheres and 40°C (density 0.991 g mL^{-1}) with a flow rate of 0.480 mL min^{-1} . The extracted theophylline was collected by passing the CO_2 through two vials, each of which contained 4 mL of methanol, over 25 min.

Thin films of anti-theophylline polymer were prepared by evaporation from a 10^{-4} g mL^{-1} solution in 99:1 acetonitrile:acetic acid. The SPR angular profile was recorded for these films both before and after incubation at room temperature for 1 h with either water or a 4 mg mL^{-1} solution of theophylline in water and air-drying overnight.

RESULTS AND DISCUSSION

The SPR-PDS signal amplitude versus angle of incidence for a typical bare silver film is shown in Figure 13. Decay of non-radiative surface plasmons into heat generated refractive index gradients that were probed successfully by PDS. The signal profile obtained from the angular scan matches the theoretical response. In traditional detection of surface plasmons based on reflectance, a maximum loss in intensity of light reflected from the prism-silver interface coincides with resonance. In photothermal detection, it is the heat arising from the decay of non-radiative surface plasmons that is probed. The signal amplitude should then increase in the presence of resonant excitation of surface plasmons. Prior photoacoustic measurements using a microphone also yielded a positive-going peak in the place of the reflectance dip as did these PDS measurements [49]. While gas-microphone PAS is an excellent technique for many applications, it requires that the sample chamber be acoustically sealed. PDS is less susceptible to ambient noise and is ideally suited to open-air analysis. PDS is also generally more sensitive than gas-microphone PAS and has no background due to window absorption. A drawback with PDS is the somewhat more difficult optical alignment of two laser beams than PAS [3].

The photothermal system can be characterized by its thermal diffusion length, $\mu = (2\alpha/f)^{1/2}$, which for silver is of the order of 600 μm at a modulation frequency of $f=200$ Hz. As the thermal diffusion length is very much thicker than the metal film thickness (600 Å), which is in turn much thicker than the penetration depth of photons

Figure 13: SPR-PDS signal amplitude versus angle of incidence for a typical bare silver film.

Figure 14: Plot of SPR-PDS signal amplitude versus chopping frequency.

in silver (11 nm, calculated from the optical constants of silver), the system may be considered thermally very thin and optically opaque in this range of chopping frequencies. Such a system would generate a photothermal signal proportional to absorbance. This had been verified experimentally for a grating-coupled gas-microphone PAS system [49], and was confirmed in this work from reflectance measurements. The photothermal signal from a thermally thin system may be influenced by the backing or substrate material, which in our case, is the glass slide. However, since the glass is identical in any given batch of silver films and can be expected to vary little from batch to batch, its presence need not be taken into account. Photothermal theory predicts that for a thermally thin and optically thick system, the PDS signal amplitude should vary with f^{-1} [33]. It is interesting to note that the observed effect of modulation frequency on the SPR-PDS signal is actually closer to $f^{-1.3}$ as seen in the log-log plot of Figure 14.

The SPR-PDS signal amplitude for the bare silver film has a peak height of 20 μV for an incident power of 70 mW. The corresponding deflection angle of the probe beam could be estimated by first converting the SPR-PDS signal to a fractional change in beam position by using the slope at the inflection point of the one-dimensional spatial intensity profile for the probe beam shown in Figure 15. For small deflections, the angle is approximated by $\delta z/x$. Using a propagation distance of $x=53$ mm, which is the distance between the excitation beam spot and the photodiode, this calculation leads to a deflection angle of 7×10^{-5} rad.

Measurement of the reflected intensity at the resonance angle θ_0 and at an angle far from θ_0 gives an absolute calibration of the SPR system. The loss in reflected

Figure 15: One-dimensional, z , probe beam spatial intensity profile.

intensity of the excitation beam at resonance due to SPR yielded an absorbance value of approximately 0.8 for the bare silver films, indicating that some 85% of the incident photons were converted to surface plasmons. A peak SPR-PDS signal amplitude for bare silver of $40 \mu\text{V}$, with a typical signal-to-noise ratio of 1000:1 indicates that a change in the reflected intensity corresponding to an absorbance of 2×10^{-3} could be easily detected with a signal to noise ratio of 2:1. By using a point along the sharp linear portion of the SPR angular profile, it would be possible to discern a shift in resonance angle of 1×10^{-3} degrees, which would correspond to 2×10^9 molecules of IgG present on the silver surface, given that a full monolayer coverage of IgG gives rise to a total shift in resonance angle of 1.2° . In practice, this is not generally achievable, not due to a lack of sensitivity of the PDS detection but rather as a result of the level of reproducibility

Figure 16: Effect of exposure to ambient atmosphere on the resonance profile of a bare silver film. Individual data points have been omitted for clarity.

in the experimental system which typically showed variation of the order of 10% of the normalized SPR-PDS signal change (resulting from variation in silver films, variation in protein loading, position variation after replacing the prism in the mounting support).

The effect of exposure of the silver film to ambient atmosphere is shown in Figure 16 which tracks the shift in the SPR angular profile and resonance angle over a number of days. The rate of change in resonance angle for silver due to exposure to air is $\sim 0.1^\circ$ per day, which can be expected to vary from day to day depending on the relative humidity.

For bare silver films, the resonance angle θ_0 was determined experimentally for a variety of wavelengths. The resonance value for 633 nm was 43.4° and this value is comparable to that obtained by theoretical calculation and experimental determinations

at 632.8 nm made by other groups using other modes of detection [20],[29]. The variance among reported values for θ_0 can be attributed to slight geometrical variations, or to differences in the refractive index of the coupling prism used. The resonance position is also a function of wavelength [50]. The experimental resonance position scaled inversely with increasing wavelength, as represented graphically in Figure 17. The resonance angle θ was calculated by means of a simple Basic computer program for a series of wavelengths using the optical constants for silver from Johnson and Christy [51]. The variation of the dielectric constants of air and glass with wavelength was omitted since both are small over the visible range. As can be seen in Figure 18, there is reasonable agreement between the measured resonance angles and those predicted theoretically considering the approximations made. A more comprehensive program, written in Fortran (Appendix A), calculates the shift in resonance angle due to a thin coating on the silver surface of either a transparent or absorbing dielectric material. The program takes into account the effect of the silver film thickness on the resonance angle.

Figure 19 illustrates a comparison between two batches of silver films obtained from Interoptics. Batch C demonstrated a significantly broader SPR angular profile than did the previous batch B, though the resonance angle itself was unchanged. Presumably this was a result of a slower deposition rate which resulted in a rougher film, giving rise to the broader profile. It also explains the generally poorer adhesion of silver films to the glass substrates observed in this batch. Poorer adhesion makes sample preparation difficult and the broader bandwidth will ultimately limit the sensitivity of an assay. This

Figure 17: SPR angular profiles for a bare silver film at three different incident wavelengths.

Figure 18: Resonance angle θ_0 as a function of wavelength.

Figure 19: SPR angular profile for films from two batches obtained from Interoptics.

Figure 20: SPR angular profile for three blood proteins, BSA, IgG and IgA.

illustrates the need in SPR work to have good control over the metal film preparation in order to have the highest sensitivity and reproducibility.

Figure 20 shows the SPR angular profile for a variety of monolayers. The shift in resonance angle for species adsorbed to an SPR surface is related to the molecular weight of the species in so far as the molecular dimensions are related to molecular weight. Thinner films give rise to smaller absolute shifts in the resonance angle and has limited the applicability of SPR to larger molecular weight species [52]. Bovine serum albumin (BSA) (molecular weight of 66 000 daltons) exhibited a smaller shift in resonance angle, in keeping with its smaller molecular dimensions of the order of 4 nm, than IgG (146 000 daltons) which has molecular dimensions of the order of 5 nm [53]. The resonance angle was determined for a variety of monolayers as summarized in Table III.

Figure 21 confirmed that a 5 minute (300 s) exposure to a 20 ppm solution (*cf.* 200 ppm actually used in the assay) of IgG was sufficient to reach a full coverage. The protein layer was stable in air and could be analysed this way [14]; it certainly could still be measured reliably several days later although whether the protein layer would retain its immunological activity after a prolonged period of time has not been ascertained.

The reproducibility between IgG films was good as indicated by the excellent overlap observed in the curves for two different silver films each coated with a monolayer of IgG (Fig. 22). The shift from the resonance angle of bare silver was determined to be approximately 1.2° at 488 nm for the monolayer of IgG. Exposure of the IgG-coated film to a $50 \mu\text{g mL}^{-1}$ solution of anti-IgG results in a monolayer of the

antibody (Ab) bonded to the existing antigen (Ag=IgG) layer. The additional shift in

Table III: Measured Resonance Angles for a Variety of Substances

Substance	Resonance Angle (°)	Wavelength (nm)
Bare Silver Film	47.8	476.5
	45.8	488.0
	44.3	514.5
Monolayer of IgG	47.0	488.0
"monolayer" of α -IgG	47.5	488.0
"monolayer" of FITC-labelled α -IgG	48.0	488.0
monolayer of IgA	48.7	476.5
	47.3	488.0
monolayer of BSA	46.8	488.0
monolayer of human serum	48.7	476.5
	47.2	488.0
monolayer of rabbit IgG	47.2	488.0

resonance angle observed for a monolayer of adsorbed antibody was approximately 0.5° (Fig. 23). Since the Ab molecule is itself an IgG molecule, it might be expected to give rise to the same shift in resonance angle as did the original IgG monolayer by forming a film of the same thickness. However, since the antibody IgG molecule has two sites with which to bind to an antigen, it is not unreasonable to assume that one Ab can bridge between two Ag closely spaced on the surface. The resultant layer of antibody would be more sparsely populated, giving rise to a thinner apparent film. The exponential decay of the evanescent field strength might also be expected to contribute to the smaller

Figure 21: Effect of exposure time of the silver surface to IgG on the observed shift in resonance angle.

Figure 22: SPR angular profile for two silver films exposed to a solution of IgG. The curve on the right was generated after exposure of an IgG-loaded silver film to a solution of anti-IgG.

shift in θ_0 but since the thickness of the protein layer is very much smaller than the penetration depth of the evanescent wave it is generally treated as if the field was a constant [54].

In order to generate a calibration curve for antibody concentration, it is important to point out that it is the rate of Ag-Ab binding of which we are making use. Samples with higher concentrations of protein will adsorb faster (in terms of molecules s^{-1}) than those with lower concentrations. Since the exposure time in all cases was fixed, the change in the SPR-PDS signal gave the appropriate response with respect to concentration. The standard calibration curve in Figure 23 shows a linear dependence of the change in the SPR-PDS signal amplitude on anti-IgG concentration. The signal versus concentration plot is used here as an alternative to the signal versus log concentration plots more generally found in immunoassay studies; the r coefficients were found to be comparable in these series of experiments. Replicate data points on the calibration curve show the spread of replicate determinations. The detection limit for this study is 2×10^{-7} g, defined by an SPR-PDS signal change of three times the standard deviation of the replicate blank determinations. Using a molecular weight for the anti-IgG of 146 000 [55] and a sample volume of 100 μ L, this leads to a detection limit of 14 nM for this assay. There is more scatter visible in the curve than would be ideal and this hurts the initial detection limit. The observed scatter was most likely due to variations in the concentration of analyte due to loss by adsorption on vial walls. In an earlier immunosensing application of SPR, the antibody solutions themselves were analysed in a 2 mL chamber adjacent to the silver film [14]. This larger sample volume

Figure 23: Dependence of the relative change in SPR-PDS signal amplitude on concentration of anti-IgG.

Figure 24: New Calibration curve for anti-IgG in the presence of BSA.

precluded the need for an incubation time and with such large samples the amount of substance adsorbed was undoubtedly small compared to the total amount present in the sample. Smaller sample sizes are, however, desirable in clinical immunoassays and they can be applied simply by placing a drop of the sample solution on the silver surface and allowing it to air-dry. Although the use of capillary cells does avoid the larger sample volumes [16], analysis in air is generally preferable for a number of reasons. First, the SPR resonance peak in air is much sharper than that in water. Peaks generated for water solutions are broadened due to damping effects from the condensed phase [56]. Second, analysis in air is more convenient as it requires no sealed or leak-proof sample chamber, particularly when PDS detection with a probe beam propagating in air is employed.

Performance was subsequently improved by preparing the antibody standards in the presence of a supporting protein, BSA in great excess. When exposed to the vial prior to the introduction of the antibody, BSA served to coat the vial walls thereby limiting the adsorption of the antibody protein. The presence of BSA in the assay does not represent an interference to the assay as anti-IgG has been shown to exhibit no cross-reactivity with BSA. This is also indicated by the excellent overlap between the curves observed for BSA before and after exposure to a 50 ppm solution of anti-IgG (see later Fig. 26). The new analytical calibration curve is shown in Figure 24. The detection limit was lowered to 75 ppb or 0.8 IU per mL. It should be pointed out that this corresponds to 1×10^{10} molecules of anti-IgG, using a sample volume of 30 μ L. Another calibration curve, Figure 25, was generated for a different system of IgA and

Figure 25: Calibration curve for anti-IgA.

Figure 26: Angular profile of a monolayer of BSA before and after exposure to a solution of anti-IgG.

anti-IgA, as expected.

Cullen and Lowe pointed out that one problem to be addressed is the inherent susceptibility of an immunosensor interface to non-specific adsorption of other components found in a complex sample [6]. Towards this end, the effect of undesirable interactions between the analyte antibodies and other biological components was examined. Since the antibody-antigen binding is known to be highly specific [57], the aim of this investigation is to explore any other interactions such as non-specific adsorption which may inadvertently contribute to a shift in SPR resonance angle thereby interfering with the analysis. To test for non-specific interactions, a variety of protein monolayers were prepared and then tested with either anti-IgG or anti-IgA solutions. The angular scan was obtained for the monolayer before and after exposure to the test solution. Typical results are shown in Figure 26 and results are summarized in Table IV. No interference was experienced as a result of non-specific adsorption of other serum components when the silver film substrate was prepared first with a layer of antigen (typically IgG or IgA). With such a well-prepared surface, the specificity of the Ab-Ag interaction led to good selectivity for the assay. Certainly development of this technique into a commercially available system would need to incorporate this first layer of either antibody or antigen (depending on the analyte) to achieve the necessary selectivity as well as limiting the amount of manipulation required of the ultimate user.

If the bare silver film substrate were exposed to a sample containing a variety of serum components, all the protein components would be expected to compete for adsorption sites on the silver. Exposure of that surface would then exhibit reactivity to

the antibodies in question. This type of mixed-antigen surface preparation may be useful in some applications where a qualitative yes or no to whether any of a class of antibodies was present in a sample such as in allergy testing. Certainly, the use of such a method would allow faster screening of a large number of samples and should single out ones that warrant fuller quantitative analysis.

Table IV: Summary of Observed Interactions Between Various Biological Substances

Protein Monolayer	Specific Antibody	
	anti- human IgG	anti- human IgA
human IgG	✓	×
rabbit IgG	×	×
human IgA	×	✓
human serum without IgG (removed by manufacturer, Sigma)	×	✓
bovine serum albumin	×	×

Saturation Studies

In an attempt to improve the detection limit of SPR-PDS, the pump laser power was increased to excite more of the surface plasmons in the Ag film. At moderate incident laser intensities the PDS response was linear with intensity as expected. However, at higher laser intensities, above 1200 mW (for 2 mm diameter pump beam this corresponds to 40 W cm^{-2}), the PDS signal appeared to saturate as shown in Figure 27. In order to obtain the highest intensities it was necessary to use the all-lines output

Figure 27: SPR-PDS signal amplitude versus incident laser power.

Figure 28: Intensity of reflected light and light scattered normal from the silver surface as a function of incident intensity.

from the argon ion laser. Since the SPR resonance condition is a function of wavelength, the use of all lines did increase the bandwidth of the resonance peak but this effect was deemed small since the SPR-PDS signal from the individual lines at 488 and 514.5 nm began to saturate at approximately the same intensity.

At the same time, the reflected beam intensity remained a linear function of the incident intensity. This indicates that the excitation of surface plasmons was unchanged although the non-radiative decay of those plasmons was either not taking place to the same degree or not being detected by PDS. If the amount of energy entering the system was linear with incident intensity, as suggested by the reflectance data, but the non-radiative decay, as probed by PDS, was not linear, then the logical assumption is that the radiative decay of surface plasmons was taking place at the expense of the non-radiative decay of plasmons. This was indeed confirmed by the simultaneous light scattering measurements made normal to the silver film. The light, scattered directly by coupling through roughness present on the film surface, was collected by a fiber optic bundle and detected by a photomultiplier tube. The scattered intensity indeed began to rise disproportionately with increasing incident intensity (Fig. 28).

A possibility for the saturation effect may be the result of Joule heating. At higher intensities of CW pump laser radiation, the temperature of the surface can be expected to increase. Such a rise in temperature was in fact measured by a thermocouple placed on the surface at a discrete distance from the pump laser spot. The total difference in temperature at the maximum attainable intensity was 15°C relative to ambient temperature. The film thickness is not expected to increase appreciably; at any

rate film expansion would tend to decrease the field strength of the evanescent wave and would tend to minimize the radiative mode (the opposite to what was observed) since the coupling of surface plasmons to a light wave decreases with increasing metal film thickness, disappearing only for $d \rightarrow \infty$ [31]. Alternatively, a temperature increase will cause small changes in the dielectric function of the silver [23] and it is possible that these changes may induce the saturation phenomenon. It has been discussed in the literature that the relaxation dynamics of surface plasmons strongly depends on the electron temperature [58]. Surface plasmons decay as a result of the finite conductivity of the metal and an increase in temperature is known to decrease the conductivity of silver [59]. If this increase in temperature and decrease in conductivity led to an increase in the effective lifetime of surface plasmons, which is typically on the femtosecond timescale, it should result in a longer decay length. It is possible to speculate that the longer propagation distance of surface plasmons along a surface of finite roughness might increase the overall probability of radiative decay through coupling to surface roughness.

This direction of research was not carried further in the interests of time. The effect of temperature on the SPR-PDS system should be investigated in future work. It would be interesting to see whether it is possible to induce changes in the SPR behaviour by using different ambient temperatures and/or atmospheres.

Fluorescent Tag and Particle Enhanced Immunoassay

In a recent application, SPR was incorporated into a traditional fluorescence-based

assay to form a hybrid technique [52]. Through the focusing of the electromagnetic field in the region of the immobilized labelled analyte, which is a result of the SPR mode of excitation, fluorescence emission was increased.

Fluorescent tags could be used in a reverse fashion for enhancing the SPR angular shift, as shown by the results presented in Figure 29. In this scheme the analyte solution was first exposed to the silver surface, generating the first curve. For simplicity the analyte was taken to be IgG and not anti-IgG as has been done up to this point. In comparison, exposure of an IgG-loaded silver film to a solution of anti-IgG led to the next curve shifted from the first by 0.5° . Exposure of another IgG-loaded film to a solution of FITC-labelled anti-IgG instead led to the third curve which was shifted from the IgG-loaded silver film by 1.2° . Clearly, the use of tagged antibody generated a larger total angular shift and thus leads to higher sensitivity for the assay. The availability of labelled antibodies has become great with the increased use of fluorescence-based techniques. The third curve did show a larger bandwidth as expected since light absorption in a thin film, while it does not affect resonance position, does increase damping and hence bandwidth. The increased bandwidth resulting from the tagged antibody would not represent much of a problem, however, since the leading edge of the SPR angular profile that is used for assay purposes remains reasonably steep.

The use of a second antibody in a sandwich method to enhance the analytical signal in an immunoassay is well accepted in the literature [15]. For small proteins, drugs and hormones, the low molecular weights of such substances would generate thin monolayers, insofar as the molecular weight determines the molecular dimensions, and

Figure 29: Fluorescent label enhancement of SPR-PDS immunoassay.

Figure 30: Sandwich method SPR-PDS immunoassay.

thus small overall shifts in the resonance angle which are difficult to determine accurately. This was illustrated in our lab by the smaller overall shift in resonance angle obtained for a monolayer of BSA (MW 66 000 daltons) compared to that for a similar monolayer of IgG (MW 146 000 daltons) (see Fig. 20). The results obtained for the sandwich assay method employing an FITC-labelled second antibody is shown in Figure 30. The first curve was obtained after exposure of the silver surface to the analyte antibody solution. Subsequent treatment with a concentrated solution of IgG (200 ppm) generated the second curve, exhibiting a shift of 1.2° . Treatment of that layer to a concentrated solution of FITC-labelled anti-IgG (50 ppm) led to the third curve which exhibited an additional shift of 1.3° . While more time consuming because of the extra analytical step, it is clear from the figure that there is the potential for greatly enhanced sensitivity since the total shift observed has increased to 3° from the previous 0.5° obtained for the standard procedure already discussed. Note that the use of FITC-labelled antibody as the second antibody is doubly advantageous in light of the results presented in Figure 29. Standard antibody used in a sandwich assay would still enhance the total shift, albeit somewhat less.

Kooyman *et al* suggested in 1988 the use of polystyrene as an intermediate layer between the metal and the protein layer as a means of minimizing the effect of changes in the bulk sample medium on the resonance position [13]. In that work, a 20 nm thick film of polystyrene was employed to limit the evanescent field strength in the sample medium, which could potentially be blood or serum, thereby improving the performance of the sensor. The effectiveness of the polystyrene film was limited by the increased

bandwidth of the SPR angular profile [13]. In this work, since the samples are analyzed in air the SPR results are far less prone to changes in the bulk medium. Polystyrene microparticles were then incorporated into this work as a means of increasing the effective dielectric constant of the analyte layer and thus the total shift in resonance angle. It was possible to generate a shift in the resonance angle on exposure of the anti-IgG coated silver film to a suspension of microparticles coated with IgG (CL) ($0.204\ \mu\text{m}$ in diameter). The total shift in resonance angle obtained when a full monolayer of anti-IgG was exposed to the CL was 0.68° while that obtained for a partial monolayer of anti-IgG (prepared from a 50 ppm solution of anti-IgG containing 100 ppm BSA) was a smaller shift of 0.25° . This was approximately the same as that exhibited by the normal methodology (see p. 46) and as such represents no enhancement. While the larger dielectric constant of the CL relative to the protein IgG alone would be expected to generate a larger overall shift in resonance angle, the efficiency of CL adhesion to the anti-IgG layer was low as evidenced by subsequent SEM examination. It was also apparent that the larger CL particles ($0.944\ \mu\text{m}$ in diameter) did not adhere as well as smaller ones ($0.204\ \mu\text{m}$ in diameter). Antibody layers treated to CL suspensions showed significantly fewer $0.944\ \mu\text{m}$ particles adhering to the surface. Presumably this is a result of rinsing which sheared the larger particles from the antibody layer. Moreover, the full-width at half maximum (FWHM) bandwidth of the SPR angular profile for anti-IgG monolayers exposed to CL was also greatly increased (to $>5^\circ$) to the point where it is no longer useful. Future use of significantly smaller particles, of the order of 20 nm (*cf.* 200 nm currently used) would in all likelihood improve the efficiency of sticking

on the surface but might not go a long way to improving the bandwidth.

SPR-PDS Evaluation of Thin Polymer Films

Figure 31 shows the resonance curves obtained for a range of polymer thicknesses (which is proportional to the mass of polymer deposited on the bare silver film) for PVC. The resonance angle shifts to higher values and the half-width of the SPR angular profile gets larger with thickness. The shift in the resonance angle resulting from progressively thicker PVC layers shows a linear response to film thickness (which is related to the concentration of the polymer solution used to deposit the film) (Fig. 32), as would be expected from theoretical calculations in this thickness region (see Appendix A). The scatter observed in the plot is an indication of the variations in film thickness resulting from our method of film formation. Polystyrene gave the most reproducible response presumably as a result of the ability to better control the spot size with water as the solvent. It was possible to detect a film prepared using a 20 μL volume of 10^{-7} g mL^{-1} polystyrene. This corresponded to 2 ng of polystyrene on the silver surface. Assuming a spot area of 20 mm^2 , and using the density of 1.4 g cm^{-3} for bulk polystyrene, 2 ng will translate to a thickness of 2 nm. The actual film thickness would be expected to be thicker than this since the density of polymer in a thin film is significantly lower than the bulk value. The sensitivity of SPR for quantification of film thickness can be improved by the appropriate choice of wavelength used to excite the plasmons. As illustrated in Appendix A, the slope of the linear region depicted in Figure B increases for shorter wavelengths. The enhanced sensitivity is bought at the price of decreased linear dynamic

Figure 31: SPR angular profiles for a variety of PVC thin films.

Figure 32: Shift of SPR resonance angle versus quantity of polymer deposited for a variety of polymer films. The large degree of scatter is a reflection of the simple method used to prepare the thin films.

range as is evidenced by the curve shown for 397 nm which begins to rise rapidly above 10 nm. The measurements in this work were carried out at 476.5 nm. This wavelength represents a good compromise between high sensitivity and extended range.

The technique may be used to discriminate between polymers of different molecular weights. If one plots the slope obtained in Fig. 32 versus molecular weight for the variety of polymers tested, one is rewarded with a jumble of points which indicate no progression based on molecular weight. If we limit ourselves to similar polymers, those with similar values of dielectric constant (or refractive index), it appears possible to distinguish between polymers of different molecular weights. Figure 33 shows the resonance shift versus thickness plots for high molecular weight (HMW) and very high molecular weight (VHMW) PVC. The change in resonance angle for a given mass of VHMW PVC on the surface was greater than that for a like amount of HMW PVC which is surprising since a fixed mass of polymer is present in both cases. This is attributed to the fact that the longer polymer chains of higher molecular weight may result in a thicker film possibly due to twisting and tangling of the longer polymer chains. This phenomenon was evaluated with another polymer pair of high and low molecular weight PVA. Although a similar increase in slope for the higher molecular weight species was observed, the effect was very much smaller and well within the scatter of the data. Possibly the large range of molecular weights present in the PVA (10 000 to 33 000 versus 70 000 to 100 000) tended to camouflage the effect but it is more likely that this phenomenon is not generally observable with this type of crude film preparation. It should be pointed out that although Langmuir-Blodgett assemblies were

Figure 33: Plot of SPR angular shift versus mass for HMW and VHMW PVC films.

not used in this study, the technique of SPR may be used very effectively to study molecular weight of monolayer polymer films. In such well-ordered films where the molecular orientation on the surface is regular, thickness is a direct reflection of the length of the chain and hence molecular weight.

SEM images of PVA films with LMP imbedded, before and after removal of the microparticles by dissolution in 1:1 THF:toluene, are presented in Figure 34. In order to make the effect more visible, the images obtained were for a much thicker PVA film than would normally be used in SPR-PDS applications. In the first image, the latex microparticles can be seen embedded just under the film surface as lighter small spots. In the second image, the thin skin of PVA has been dissolved and the microparticles have been removed to leave pores. In the thinner PVA films used for SPR-PDS work, SEM images showed that the particles essentially sat on the surface, the polymer film being too thin to be directly observed with SEM. After flushing the surface with the solvent, no particles could be seen on the surface. It was, of course, impossible to visually ascertain any changes in the polymer film itself. Ideally it would be possible to determine a shift in the resonance angle before and after the removal of LMP. However, no distinct resonance peaks for these films could be observed, presumably as a consequence of the great deal of scattering and radiative decay found with these rough film surfaces after the removal of LMP. Hence SPR-PDS evaluation of thin films of PVA embedded with LMP was inconclusive. As such it was not possible to use them as a model for our initial work on molecularly imprinted polymer films.

Regardless, characterization by SPR-PDS of molecularly imprinted polymers was

a)

b)

Figure 34: SEM images of a) thin PVA film impregnated with LMP b) same film after removal of LMP.

proven successful. The angular profiles of molecularly imprinted PMAA-EDMA is illustrated in Figure 35. The first figure displays the overlapping angular profiles obtained before and after a 1 hour exposure of the anti-theophylline polymer (prepared by SFE of the theophylline) to a blank solution containing no theophylline. There was good overlap between the curves, indicating no shift of the resonance angle. The second figure shows the effect of a 1 hour exposure to a 4 mg mL⁻¹ aqueous solution of theophylline. A shift of the resonance angle of 0.25° is apparent in the profiles. Clearly, the uptake of theophylline molecules into the active sites of PMAA-EDMA caused sufficient change in the dielectric constant of the polymer layer to be monitored via a measurable shift in the SPR resonance angle. There may exist some question as to whether the theophylline is actually being taken up in the molecular pores and not just being caught in defects and cracks of the polymer film. However, that these results were obtained after substantial rinsing with distilled water, does seem to suggest that the binding is reasonably strong. This work supercedes the work of Nylander *et al* who employed a thin layer of silicon oil spin-coated onto the silver surface to quantify the anesthetic gas halothane in a gas sample [60] as molecular recognition was not there. As such the initial feasibility study of the use of SPR-PDS to characterise MI materials has been completed.

Future work will undoubtedly need to ascertain the detection sensitivity of the MI polymer for the drug theophylline. This will require optimization of the PMAA-EDMA film thickness and will be a compromise between thicker films which provide more sites for theophylline sorption into the matrix and thin films which generate the sharpest and

Figure 35: SPR angular profile for molecularly imprinted PMAA-EDMA films exposed to a) blank solution and b) 4 mg mL^{-1} theophylline solution

therefore most sensitive resonance peak. It will also be necessary to improve the extraction efficiency of the theophylline from the polymer prior to SPR film formation. The initial SFE extraction is estimated to have removed only about 25% of the total theophylline present in the bulk polymer, although Soxhlet extraction using methanol and acetic acid (9:1 v/v) over 24 hours was able to extract nearly all the available theophylline. Improved SFE extraction of theophylline may be realized by employing methanol as a modifier to the CO₂ to increase the polarity and hence solvent power of CO₂.

It will also be desirable to optimize the solution used to introduce the theophylline sample to the MI polymer. Water was used for the preliminary work; however Vlatakis *et al* pointed out that the theophylline substrate will bind more strongly to the polymer in an apolar solvent with a small amount of acetic acid added to suppress non-specific binding [46]. The use of a less polar solvent may improve either the total amount of theophylline binding to the MI polymer or it may improve the dynamics of binding such that shorter incubation times become possible.

Another area that will need to be investigated is the reversibility of the theophylline binding to the polymer by examining the leaching of sorbed theophylline by a blank solution. Vlatakis addressed the issue of dissociation constants and determined that the polymer, like naturally occurring polyclonal antibodies, contained sites which exhibited a variety of affinities for the print molecule. The degree of reversibility of the polymer will determine whether or not the polymer films will be reusable as the sensing element of an optical sensor for continuous monitoring applications.

CONCLUSIONS

SPR has been coupled successfully to PDS detection to yield a simple and sensitive immunoassay method that has a detection limit of 0.8 IU per mL of sample (10^{10} molecules of anti-IgG). Sensitivity can be enhanced by a variety of ways including the use of sandwich assay methodology, FITC labelled immunoglobulins or CL. Sensitivity may not be improved indefinitely by the use of higher pump beam powers as the non-radiative decay of surface plasmons, which generates the SPR-PDS signal, tends to saturate in favour of radiative decay at very high intensities. Current sensitivity is limited by the reproducibility of mechanical factors and silver film preparation and does not yet compete with the sensitivity exhibited by RIA or FIA. The technique exhibits good selectivity which results from the specificity of the Ab-Ag interaction. The method is reasonably insensitive to interferences (ie. non-specific adsorption) from serum proteins provided that the first stage of the antibody assay involves formation of a complete monolayer of the appropriate antigen (or antibody if it is the antigen that is being quantified). SPR may also be used in a homogeneous assay manner in the case of liquid samples although it has been used in a heterogeneous manner in this work to make use of the sharper resonances observed in air.

SPR may be used to yield semi-quantitative information of film thickness and molecular weight. The SPR resonance angle increases linearly with film thickness generally for films thinner than 20 nm. Employing shorter excitation wavelengths

enhances the sensitivity to film thickness, albeit with a narrower linear dynamic range for thickness.

SPR-PDS has been used successfully to characterize MIA materials as a first stage toward the creation of synthetic substitutes for naturally occurring antibodies. A thin film of polymer, which had been previously molecularly imprinted with theophylline, gave rise to a 0.25° shift in resonance angle on exposure to an aqueous solution of theophylline. SPR will be a suitable technique to characterize MI materials for other drugs peptides and proteins.

CHAPTER 2: Latex Microparticle (LMP)

INTRODUCTION

In the previous chapter, an optical immunosensor was developed in which a thin film of immunoglobulin was immobilized onto a flat metal surface and subsequently exposed to a sample containing antibody. While sensitive the method as developed does represent a heterogeneous assay method, or one that requires an additional step to separate bound from unbound antibody. Radioimmunoassay (RIA) and fluorescence immunoassay (FIA) are both standard, sensitive techniques in the field of immunoassay but share with SPRIA a heterogeneous methodology. Both RIA and FIA require some means of separating the bound from the unbound label. There is a need for homogeneous immunoassay techniques that require no separation step and in which all reagents are contained in one tube and analysed as such [61], although the issue of matrix interferences plaguing many homogeneous assays will be a difficult obstacle. In spite of the belief held by some researchers that it is unlikely that any homogeneous assay will ever find global applicability while maintaining the highest sensitivity [1], the desire for higher throughput in busy hospitals attainable through automation and the lure of simplicity will continue to make the search for homogeneous assay methods a very valid undertaking for years to come.

Particle assays, where small particles are used as labels, were developed as an alternative to heterogeneous approaches. Such techniques offer simplicity yet sufficient sensitivity for many applications. In particle immunoassays, the reaction between antigen

and antibody, when present in the right concentration and proportions, is made visible through precipitation. Most concentrations in the bodily fluids, however, are much too low to produce directly visible results [62]. Erythrocytes were originally used in the area of particle-based assays but as they are naturally produced, they displayed variable characteristics that tended to lower the ultimate reproducibility of the technique [62]. Polystyrene latex microparticles (LMP) display much more constant properties and as such have almost completely removed the use of erythrocytes. A few specialized LMP-based assays have been developed for home pregnancy tests, such as the product from Abbott Labs which is capable of detecting 0.25 IU HCG per liter of urine [63]. One of the first and simplest general tests using LMP's, however, was the latex slide test (LST) which can detect 250-500 IU of human chorionic gonadotropin (HCG) L^{-1} of urine [62]. The LST is a very simple test to perform but for higher sensitivity it is necessary to probe earlier stages of agglutination, before they have grown large enough to be visible.

One such method is the use of quasi-elastic light scattering (QELS) which reflects the average diffusion constant by examining the change in frequency when light is scattered by particles, which is a function of size. The method is sensitive, quantitative, accurate and has a detection limit of 10-25 $\mu g L^{-1}$ [62]. Use of angular anisotropy improves this further with 2 IU HCG L^{-1} of urine [62]. The drawback of such techniques is that the interpretation of data is not straightforward and the instrumentation remains quite sophisticated.

Particle counting immunoassay (PACIA) represents another popular method for

Figure 36: Representation of the IgG molecule.

particle immunoassay. Using a blood cell counter, it probes the number of residual unagglutinated particles and can detect $10 \mu\text{g L}^{-1}$ of ferritin in buffer [64]. Immunoglobulin G (IgG) of goat raised against human placental lactogen (HPL) or α -fetoprotein (AFP) can be coated onto LMP. The presense of either HPL or AFP causes agglutination and a decrease in the number of single particles. Serum interferences can be suppressed with dithiothreitol to exhibit a detection limit of $100 \mu\text{g L}^{-1}$ of serum. The use of F(ab')_2 fragments (two covalently linked Fab fragments) and high ionic strength in the reaction medium further reduces the interference. The combination of F(ab')_2 fragments, slightly dissociating conditions by use of the chaotropic agent ammonium thiocyanate, and chelation of bivalent cations with EDTA allows the detection of $13 \mu\text{g}$

of ferritin L^{-1} [62]. PACIA is considered a good technique because it requires no separation step, its incubation times are short and the process can be fully automated. PACIA system is the most sensitive and reliable of the various instrumental techniques for reading agglutination [64], [65]. The disadvantage is that it requires large and costly equipment and the sensitivity does not completely match that of RIA.

Application of colloidal gold particles might alleviate some of the problems. The gold sol, which is comprised of 50 nm gold particles coated with antibody, displays considerably improved sensitivity and represents an entirely new way for reading the endpoint of a homogeneous sol particle immunoassay (SPIA) [62]. The specific absorbance at 540 nm is monitored and decreases with agglutination. It is a simple test to perform since all reagents are contained in one vessel. The method exhibits a sensitivity of 100 IU L^{-1} of urine HCG. Sample interference and matrix effects remain problems as in other methods of homogeneous assays [62],[66].

The desire in our undertaking was to develop a homogeneous technique which held some of the desirable features of PACIA (high sensitivity, use of readily available LMP) while maintaining the simplicity of the gold SPIA. In a recent work by Kitamori *et al*, a homogeneous assay was developed in which agglutinations of a given size were selectively probed using photoacoustic spectrometry [67]. There was a significant enhancement of the PAS signal, resulting from resonant scattering, when the particle or agglutination size matched that of the excitation wavelength. This was in direct contradiction to the findings of Oda *et al*, who had shown PAS signals to be independent of particle size since PAS signals are proportional to the absorbed energy and are

unaffected by scattered radiation [68]. The phenomenon enabled the detection of specifically-sized agglutinations to be quantified without the need for separation.

The above mentioned method would represent an attractive methodology since it is a homogeneous assay, is amenable to automation, was claimed to be highly sensitive and has the potential to be simpler than PACIA. The technique has the attractive feature of being a direct assay method, probing the number of agglutinates rather than the number of particles left unagglutinated as is the case in PACIA and as such would be straight-forward and more sensitive. In this work, the resonant enhancement phenomenon is more closely examined. The work will be carried out along three lines: (i) to verify the resonance phenomenon, (ii) to develop some of the theoretical aspects of the phenomenon and (iii) to make modifications and improvements in its application to immunoassay.

THEORY

Light Absorption and Scattering

Scattering is related to the inherent inhomogeneity of the given system. In one sense or another, all things except absolute vacuum is heterogeneous, and as such will scatter light. It is appropriate to make the distinction between scattering by fluctuations (ie. concentration) and scattering by particles. Generally, though the mathematics is similar, the underlying thermodynamic arguments are not. The scattering by fluctuations is ignored as it is much less than scattering by particles. Discrete electric charges are present in any given system. Illumination by an electromagnetic wave causes the charges to oscillate due to the electric field. Accelerated charges radiate electromagnetic energy in all directions (this secondary radiation is known as the radiation scattered by the obstacle).

Some assumptions should be made regarding the system. The first is that the particle is embedded in a homogeneous medium in which the atomic and molecular heterogeneity is small compared to the wavelength. Secondly it is assumed that the particle is composed of matter that is at all points describable in macroscopic terms (ie the optical properties). The study is also limited to elastic scattering where the scattered radiation is of the same frequency as the incident radiation and we do not look at Mandel'stam-Brillouin or Raman scattering. It is assumed that the particles are isolated such that multiple scattering does not take place; in practice the sample is diluted until the scattering is proportional to particle concentration [69].

When the particle size (or diameter, $d=2a$) is much smaller than the wavelength of incident radiation, the scattering obeys Rayleigh ($d \ll \lambda$) or Rayleigh-Gans ($d < \lambda$) scattering [69]. In this region, scattering is dominated by volume effects and will exhibit a d^3 dependence. When the particle diameter is larger than the excitation wavelength, Mie scattering predominates. In this size region, light can rarely penetrate into the interior of the particle so the majority of the scattering takes place at the surface, exhibiting a d^2 dependence. When the particle has a diameter comparable to the exciting wavelength, easy predictions of scattering behaviour are not possible [70],[71]

In brief, the scattering process may be described in the following manner:

An arbitrary particle is composed of a number of small regions. An applied oscillating field (namely the electromagnetic wave) induces a dipole moment in each region. The dipoles oscillate at the frequency of the applied field and will scatter secondary radiation in all directions. At some distant point P, the total scattered field is obtained by superimposing scattered wavelets (after taking into account the phase difference).

Phase relations (which also depend on geometrical factors such as size and shape) change with scattering direction. Therefore, the scattered field may vary with the scattering direction. If a particle is small with respect to the wavelength, then all the wavelets are essentially in phase and will display very little variation of scattering with respect to direction. Larger particles have a higher probability of mutual enhancement or cancellation of scattered wavelets. The result is more peaks and valleys observed in the scattering pattern.

2 OF 2

PM-1 3 1/2" x 4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

Collections of particles can complicate the issue as the particles are electromagnetically coupled; each is excited by the external field as well as the resultant field scattered by all others. The scattered field depends on the total field that a given particle experiences. The problem is simplified when separation between particles is large and when it is possible to assume single scattering as opposed to multiple scattering. The total scattered field would then be the simple sum of the fields scattered by the individual particles and each particle can be assumed to be acted upon in isolation by the external field. The particles are further assumed to be random in separations and be many in number so that there is no systematic relation among phases of scattered waves and thus the total irradiance scattered is the sum of the irradiances scattered by individuals (incoherent scattering). Scattering remains coherent under these conditions in the forward direction and as such forms the basis for the method of coherent forward scattering (CFS).

Very small particles are called Rayleigh scatterers. The scattered field is proportional to the polarizability (ie. the number of electrons) and therefore to the particle volume. The scattered light intensity is proportional to the [particle volume]². This region extends up approximately $1/20 \lambda$. Light scattering methods can be used for particle sizing when the particle is of the same order as the incident wavelength.

The extinction coefficient Q_{ext} is a function of size and approaches the limit of 2 as the size parameter increases and this value is twice that for the limit of geometrical optics (where particle size is $\gg \lambda$). It is often referred to as the extinction paradox since it implies that the microsphere returns twice as much light as is incident upon it. Figure

37 displays the plot of extinction coefficient versus particle size as calculated by the Fortran program in Appendix B.

Figure 37: Extinction coefficient versus size for non-absorbing spheres.

Photoacoustic Spectroscopy (PAS) of Particles and Scattering Media

Many researchers in the area have assumed that photoacoustic and photothermal spectroscopies (PTS) are essentially independent of scattering, the signal being a direct reflection of the absorption cross-section [68]. Yasa *et al.* pointed out in a seminal paper, however, that the total amount of energy absorbed by a system is also a function of the light energy distribution in the sample and that the thermal signal, S , so generated should be affected by scattering [72].

The scattering coefficient α_s is a decreasing function of λ for $\lambda/d > 1$. Thus for a fixed particle diameter and absorption coefficient, S may increase as λ is lowered to $\lambda = d$ if $0.1 \leq \alpha_s l \leq 10$. This implies a monotonic increase in S , for sub-micron particles, as λ is decreased towards UV if $\alpha_s l \approx 1$. When particle diameters are comparable to the wavelength, the scattering is peaked in the forward direction. It is possible that this generates a larger PAS signal due to the fact that photons are scattered in a direction where they can interact with other particles in the cylindrical sample volume. With other particles, where the light is not scattered in the forward direction, light can be expected to be scattered out of the cell through the side walls thereby avoiding secondary interaction with other particles.

Helander *et al* studied the effects of internal scattering in a sample medium. Aqueous solutions of dyes were used with latex particles added to change the scattering behaviour of the samples [73]. They found that the photoacoustic signal was increased by scattering. Their work was developed for gas-microphone PAS and stated that the scattering of light concentrated the photons towards the surface (in a gas-

microphone arrangement) and resulted in an increased temperature for a given absorption. Two ways in which scattering can affect the photoacoustic signal are 1) by scattering of the light onto the cell walls and absorption there and 2) by internal scattering in the sample which changes the photon distribution and thus the temperature distribution in the sample, resulting in enhancement of the acoustic signal. For the first type they used a highly reflecting cell design that had as little as possible area exposed to scattered light as possible. In our situation, although there is a significant area exposed to the scattered radiation, it is transparent and absorption in the glass should be minimal. In addition, the presence of the latex alters the thermal properties (specifically heat capacity C_p) which in turn could affect the acoustic signal. This effect should, however, be very exceedingly small since the heat capacity is changed by 2 % for 4 % solids content. We are using particle concentrations many orders of magnitude lower than this and thus we can safely ignore the effect on the heat capacity of the sample. For large scattering coefficient, the PAS signal varied slowly with scattering coefficient while for small scattering coefficient the PAS signal varied strongly with scattering coefficient. This means that the effect of scattering on the PAS signal (the ratio of PAS signals with and without scattering) is more significant at the smaller scattering coefficients [73].

EXPERIMENTAL

Setup

The photoacoustic signal was detected by means of a piezoelectric transducer (PZT). The transducer consisted of a cylindrical tube of polycrystalline lead zirconate titanate (American Piezo Ceramics, Mackeyville, PA, type APC850). The piezoelectric element had silver electrodes fired onto the inner and outer surfaces to facilitate the connection of electrical leads. Its dimensions were 2 cm in length and 1 cm in inner diameter with a wall thickness of 1 mm. The resonant frequency was found experimentally to be 100 kHz.

The liquid sample to be analyzed by PAS was contained in a cylindrical cell. The photoacoustic cell itself, after going through a number of developments to improve sensitivity, is depicted in Figure 38. The path length of the cell was 10 cm and had an internal cell volume of 3.8 mL. At the ends of the cell were optical windows through which the excitation beam entered and exited the cell. The PZT was shielded from light scattered directly from the sample by the use of an aluminum sleeve glued in place between the ceramic and the cell itself. The raw piezoelectric signal was then fed directly into a voltage follower preamplifier. The preamplifier signal was then fed into a lock-in amplifier in the case of CW experiments and into either a digital storage oscilloscope or a boxcar averager in the case of pulsed experiments.

Figure 38: Photoacoustic sample cell.

Latex Microparticles and Solutions

Latex microparticles (LMP) were obtained from Seradyn Inc. (Particle Technologies Division, Indianapolis, IN) in the size range from 0.2 μm to 1 μm diameter (see Table V). Stock suspensions of 0.2% (w/v) were prepared for each size from 10% (w/v) solids by dilution with 18 M Ω water.

Several stock solutions were prepared that are standard for immunoassays:

- 1) Glycine buffered saline (GBS): One litre of this solution contains 0.1 mol NaCl, 0.17 mol glycine and 0.04 g NaN₃. The pH of this solution is adjusted to 9 with concentrated NaOH.
- 2) Normal saline : This solution contains 9 g (0.15 mol) per litre reagent grade NaCl.
- 3) Bovine serum albumin (BSA) : Lyophilized BSA (Sigma Chemical #7638) was dissolved in normal saline to yield a 5 mg mL⁻¹ solution.

A variety of immunochemical stock solutions were also prepared:

- 1) Human immunoglobulin (IgG) : Lyophilized IgG (Sigma Chemical #4506) was made up to 5 mg mL⁻¹ in normal saline.
- 2) Goat-raised anti-human immunoglobulin (anti-IgG) : Lyophilized anti-human IgG (Sigma Chemical #3382) was made up to 50 μg mL⁻¹ solution in normal saline.

All solutions and dilutions were prepared using 18 M Ω water. All chemicals were reagent grade or better. In general, the biological solutions were stored frozen in

Table V: Latex Microparticles

Particle Diameter (μm)	Standard Deviation ^a ($\pm\mu\text{m}$)
0.204	0.002
0.305	0.0084
0.305 ^b	0.0084 ^c
0.460	0.0048
0.640	n/a
0.805	0.005
0.944	0.013

^abatch-specific values supplied by Seradyn from SEM examination

^bdye black with Sudan Black

^cSEM examination (this work) revealed a small number of very large particles ($>1\mu\text{m}$ in diameter) present in this batch

working-sized aliquots. The need for this was due to the fact that storage at room temperature is not very effective over the long-term as the solutions were prone to bacterial growth. The use of working aliquots prevented the need for repeated freezing and thawing which can damage the proteins, especially if the temperature is lowered slowly. This is the result of the formation of large crystals of ice which leave pockets of solution of drastically higher salt concentrations; proteins in these regions can easily be denatured. It is also generally better to store proteins at high concentration ($>1\text{ mg/mL}$) since diluted samples are known to lose activity much more rapidly than neat or more concentrated stock solutions. Convenience dictated, however, that the antibody stock solutions were stored at 50 ppm. To guard against activity losses, the addition of a carrier protein, sufficient to make up to 1 mg mL^{-1} in BSA, was used to prevent non-specific adsorption losses on surfaces of glassware and vessels.

Pulsed Laser Experiments

The schematic diagram of the pulsed laser system is presented in Figure 39. PAS measurements of latex suspensions were carried out using the frequency-doubled line at 532 nm from a Nd-YAG pulsed laser (Lumonics, Rugby, U.K., model HY400). Typical average powers were in the 50 mW range, corresponding to pulse energies in the 5 mJ range. The signals from the piezoelectric transducer were fed into a gated boxcar averager and subsequently into a computer interface (Stanford Research Systems, Sunnyvale, CA, models SR250 and SR245). The gate delay was scanned and set at the optimum value of 2 μ s and the gate width was typically 900 ns. A small portion of the laser pulse was reflected to a photodiode which was connected to the boxcar trigger input. One hundred samples were averaged and the repetition rate of the laser was set at 10 Hz. Alternatively, with a 200 MS/s digital storage oscilloscope (Gould, Valleyview, OH, model 465), it was then possible to collect and average whole waveforms from the transducer and peak height could be read directly from the waveform.

Standards were prepared by simple dilution of the stock suspensions of LMP. Calibration curves of each of the six available sizes of LMP were obtained.

CW Laser Experiments

CW laser experiments were carried out using the system shown in Figure 40 with an argon ion-dye laser (Spectra-Physics, California, U.S.A., models 164 and 375B). The dye laser was filled with DCM dye which gave a usable wavelength range of 605-660

Figure 39: Schematic diagram of the pulsed laser system.

Figure 40: Schematic diagram of CW experimental system.

nm. This would allow convenient study of the 0.640 μm particles that were available. The excitation wavelength was varied by means of a tuning wedge fitted with an ultra fine etalon which had a nominal bandwidth of 9 GHz (0.01 nm).

A number of experiments were carried out to examine the claim made by Kitamori *et al*. The first was to use a fixed wavelength of 640 nm and to generate calibration curves for particles of different sizes as well as for 0.640 μm . The next set of experiments involved the measurement of a given suspension at a variety of wavelengths in the vicinity of the particle diameter.

Immunoassay by LMP-PAS

The procedure used to coat the latex was modified from Masson *et al* to include the increased surface area of our particles (0.2 μm) over those of Masson *et al* (0.8 μm) [74]. To 80 μL GBS was added 150 μL of 10 mg mL^{-1} BSA (used to prevent overloading of IgG on the LMP) and 750 μL of 0.2 mg mL^{-1} IgG. The mixture was equilibrated at 37 °C for 10 minutes. In order to lessen the losses of IgG adsorption on the vessel walls, care was taken to ensure that the BSA was added first. A 10 μL volume of 10 % solid LMP (0.204 μm in diameter) was added and the suspension was incubated for 15 minutes at 37°C with occasional stirring. After incubation, the suspension was collected on a 0.1 μm filter and washed with several milliliters of saline solution in order to remove any residual IgG. The LMP was then re-suspended with sonication into 10 mL saline and stored unfrozen at 2°C in 500 μL aliquots. The approximate solid content in this final suspension was 100 ppm. The exact concentration

of coated LMP in suspension would necessarily vary from preparation to preparation since some particles will become permanently trapped in the filter. Any variation in signal due to variation in coated latex (CL) concentration would, however, be reflected in the blank and as such eliminated by normalization of the blank-subtracted signals.

In order to test for antibodies, 65 μL of CL, was sonicated in an ultrasonic bath for 5 minutes in order to separate any spontaneous agglutinations which are not uncommon with whole IgG [65]. Analyte solution, 1.0 mL in GBS, made up to 1 mg mL^{-1} in BSA (which has been shown in Chapter 1 not to cross-react with anti-IgG), was then added. Incubation was carried out at 37°C for 30 minutes with occasional shaking. After incubation, the sample was diluted to 10.0 mL in order to reach an appropriate concentration (~ 500 ppb) for PAS analysis. This concentration was chosen as it is the highest concentration that will not exhibit any significant multiple scattering as evidenced by a straight line plot of scattered intensity versus particle concentration. A calibration curve of PAS signal versus anti-IgG concentration in the test solution was thus generated.

Scanning Electron Microscopic (SEM) Analysis

As an absolute check on the agglutination procedure itself, samples were collected to be examined and counted by SEM. A 10 μL sample of the agglutination sample was suspended in a 1 mL of distilled water which was then filtered through a 0.1 μm membrane filter. Two factors were taken into account in choosing the volume to be filtered: first, the filter coverage had to be sufficiently low that the aggregates collected on the filter were in fact a true representation of aggregates rather than a result of build-

up of layers and second, there would be statistical numbers present in the field of view to generate meaningful histograms. Once on the filter, the aggregates were washed with 1 mL of distilled water to remove the salt residue which tended to interfere with the SEM images. The sample was then air dried at 37 °C overnight to ensure that water would not interfere with the vacuum of the microscope. Prior to SEM examination, all samples were plasma-coated with 15 nm of palladium/gold to enhance the resolution of the images. Since the samples were dried it was not necessary to use a cold-stage. Images were obtained using a JEOL 6400 Scanning Electron Microscope (Japan Electron Optical Ltd, Japan) with a eXL LZ 4 X-ray analyzer (Link Systems). Operating voltages were typically 10 kV.

For the images used to generate histograms, a representative low magnification image was obtained (1000 - 2000 x) before three higher magnification (5000 - 7000 x) images from different parts of the filter were collected. Regions of the filter surface exhibiting exceptionally large agglutinations were avoided as much as possible since it was assumed that large aggregates would display a greater tendency to trap smaller agglutinations as they were carried past with the filtrate, thus skewing the results in that region of the filter.

Heterogeneous Immunoassay by LMP-PAS

The development of a direct heterogeneous method was carried out as well as the homogenous assay. It is desirable to have a direct method that is fast, simple and can be carried out at the bed side with a reasonably compact apparatus. The assay should

also require very little skill on the part of the operator. This work is based on the selective separation and collection of agglutinates on commercially available membrane filters followed by quantification by PAS. This would represent a compromise for those assays that aim for bed side analysis and for those facilities that cannot afford to have the newest technologies. Moreover, the samples, once collected on filters, could be transported elsewhere for analysis, the timing no longer being critical.

The coating procedure was identical to that developed in the preceeding section, except that dyed LMP (0.305 μm in diameter) were used. The LMP has the dye located in the interior and it has been shown not to affect the surface chemistry of the particles [75]. Prior to its use in a given assay, the CL was sonicated for 5 minutes to break apart spontaneous agglutinations that form on storage of the CL. It was essential that prolonged sonication be avoided since this tended to greatly reduce the sensitivity and the reproducibility of the assay presumably as a result of the significant temperature rise observed during longer sonication times.

The best amount of CL to add was ascertained by replicate trials using 100, 150, 250 and 500 μL of CL suspension (containing 100 ppm CL by mass). The incubation time at 37°C was varied to achieve the highest LMP-PAS sensitivity. Identical samples were prepared simultaneously and allowed to incubate for 5, 15, 30 or 60 minutes. It was also necessary to determine the optimal volume of agglutination sample to be filtered.

Quantification of agglutinates collected on a filter was carried out using the PAS system depicted in Figure 41. Excitation was provided by a HeNe laser (Melles-Griot,

Irvine, CA, model 05 LHR 981, wavelength = 632.8 nm) which had a nominal output power of 16 mW. The excitation beam was mechanically chopped before entering the photoacoustic cell containing the sample. The PAS cell was coupled to a sensitive microphone and sound meter (Brüel & Kjær, Nærum, Denmark, models 4144 and 2109). The output signal from the sound meter was sent to a lock-in amplifier (Stanford Research, Sunnyvale, CA, model SR 510) and recorded on a personal computer.

Figure 41: Schematic diagram of the gas microphone PAS system.

RESULTS AND DISCUSSION

In the work by Kitamori *et al* it was stated that the enhancement of the PAS signal was due to a resonant effect in which the absorption coefficient of the particles was greatly enhanced when the diameter closely matched the excitation wavelength. Intuitively, the statement is plausible since it is possible to imagine a standing wave type of excitation in which the effective cross-section would be greatly enhanced through multiple passes through any given particle.

Optical resonance of small particles does exist as outlined in van de Hulst [71]. However, the resonance region is usually discussed in terms of small size parameter, x ($=2\pi a/\lambda$), and large relative refractive index, $m = \eta_{\text{particle}}/\eta_{\text{medium}}$, in which magnetic and electrical vibrations are nearly self-sustaining. Since the m value for polystyrene in water is only of the order of 1.2, it does not seem to fit the criteria. Plots of extinction coefficient versus x show sharp features superimposed on the normal curve for large m values ($\sim m=9$) that decrease in sharpness down to $m=1.33$. For the smaller m values (<2), however, the resonance peaks become very broad and shallow, and are almost indistinguishable from the normal extinction curves. This does not agree with the behaviour of Kitamori's system which showed a very sharp enhancement peak with a bandwidth of the order of 3 size units.

In the work by Kitamori *et al*, there was a significant dependence of the PAS signal on the particle size in the region close to the excitation wavelength. One point

they claimed is not strictly correct and was supported only by the slope of a line between two points: below $0.2 \mu\text{m}$ they observed Rayleigh behaviour. This is not correct as the assumptions for the Rayleigh approximation hold only up to roughly $1/20 \lambda$. The size region below $0.2 \mu\text{m}$ may be more properly described as Rayleigh-Gans scattering. In this size region it is assumed that m is small ($|m-1| \ll 1$) and that the phase shift is small ($2ka|m-1| \ll 1$, where $k=2\pi/\lambda$). The restriction is then not that the size be much smaller than the wavelength but rather that the size should be $\ll \lambda |m-1|$. This is indeed the region in which we are working as $a=0.3 \pm 0.2 \mu\text{m}$ and this is very much smaller than $\lambda/|m-1| = 2.66 \mu\text{m}$. [69]

Resonance effects have been discussed by Sell [76], in which particles with a circumference equal to an integral number of wavelengths exhibited periodic peaks at each multiple. This does not seem to be the case since only one feature was observed in the work by Kitamori over a size range that might be expected to exhibit several peaks. There is also the difficulty in their assumption that the resonance effect would also be maintained in a polydisperse mixture of various-sized agglutinates. There enhancement was some 50 x and presumably this could be expected to lead to only a very small background resulting from other particle/agglutinate sizes. The third difficulty is that in an immunoassay, the aggregates are not in fact spherical particles but rather agglomerates which may possess a number of interfaces across the diameter of the aggregate. The scattering theory developed in the theory section for particle size dependence of the PAS signal is modified somewhat by the fact that the aggregates are not symmetrical homogeneous spheres [70]. For the theoretical treatment to be of use,

the aggregates are required to be as spherical and ordered as possible. The linear chain type of aggregates, which were found by SEM analysis to be quite common in neutral or acidic media and are to be discouraged as it is unlikely that they would exhibit behaviour similar to spheres and may also display orientation effects. Also, large clusters can be expected to display d^2 dependence and would tend to swamp the signal generation. It was apparent that long chains as well as very large aggregates were much less common in basic media pH 9. As such, agglutinations were carried out under basic conditions. Basic conditions were easily achievable by carrying out the agglutination in GBS solution which was already buffered to pH 9. Their empirical results would, however, seem to support their claim of resonant enhancement in spite of the obvious difficulties.

Our experiments have confirmed that the phenomenon does seem to be real, showing a small enhancement, of the order of 3 times, for particle diameters close to the excitation wavelength. This was observed for both pulsed and CW experiments using one wavelength and different particle diameters (Fig. 42). It was also observed using a fixed particle diameter and using the dye laser to obtain a range of wavelengths (Fig. 43). An enhanced PAS signal was even observed for a mixture of 0.204 μm and 0.460 μm LMP. A 5% mixture of 0.460 μm LMP in 0.204 μm LMP was distinguishable from a suspension of the same total concentration composed entirely of 0.204 μm LMP.

The enhancement (Fig. 44), however, is much smaller than that exhibited by Kitamori *et al* and this is a source of confusion. Examination of the histograms generated by SEM analysis of the agglutination samples after filtration (Fig. 45) did

Figure 42: PAS signal amplitude versus particle concentration for a variety of particle diameters.

Figure 43: Plot of PAS signal amplitude versus wavelength for a suspension of 0.640 μm microparticles.

indicate that the immunoreaction was taking place as it should in the presence of anti-IgG. The histogram for the blank sample showed that a large majority of single particles were present on the filter surface. The 1.0 ppm and 10.0 ppm anti-IgG samples showed progressively larger amounts of aggregates as well as a concomitant decrease in the number of single particles.

It is possible that our observed enhancement was low due to a poor match in particle diameter to the exciting wavelength. If so an improved enhancement factor should be obtained if the experiments were repeated using the newly-installed 476.5 nm mirror of the argon ion laser. This wavelength differs from the 0.460 μm particle diameter by only 16 nm which is close to the 12 nm difference used in Kitamori's experiments and is much closer than the ~ 100 nm for the pulsed laser experiments and the ~ 30 nm of the CW experiments. When this was tested the enhancement factor of 0.460 μm particles over 0.204 μm particles was not significantly altered from that previously obtained (of the order of three-fold enhancement).

This dependence of the PAS signal on particle diameter is in direct contradiction to the findings of Oda *et al*, who had shown the PAS signal to be independent of particle size since PAS signals are proportional to the absorbed energy and is unaffected by scattered radiation [68]. It is not in direct contradiction to Yasa *et al* [72] and Helander *et al* [73] who both concluded that scattering affected PAS measurements by affecting the distribution of photons in the sample. It is conceivable that the enhancement of the PAS signal had more to do with the photoacoustic mode of detection than a fundamental behaviour of the particles with respect to absorption and scattering effects. The cell used

Figure 44: Observed sensitivity as a function of particle diameter.

Figure 45: Histogram of size of agglutination for a variety of antibody concentrations.

was almost identical to that used by Kitamori's group, lacking only a flow apparatus which is more for convenience than for any improvement in analytical signal. The cell and PAS detection was presumably working correctly since the output signal exhibited $1/f$ frequency dependence on modulation frequency for LMP samples as would be expected for a constrained liquid (Fig. 46) [77]. Similar behaviour was obtained for an I_2 in CCl_4 test sample. Their detection limits for particle concentration have not been matched by us and there is a question as to how much this may affect our resonant enhancement results. They determined LMP in the range of 0.1 to 1 ppb which for $0.2\ \mu\text{m}$ particles represents some $2\text{ to }20 \times 10^3$ particles per mL and this represents extraordinary sensitivity especially considering that the particles are not highly coloured. Certainly there have been reports in the literature of spectroscopy carried out on single particles but generally these are limited to particles sizes on average two orders of magnitude larger [78]. Attempts at contact with Kitamori's group to illuminate any discrepancies in procedure were ignored by them. Multiple scattering effects may be ignored for the suspensions were diluted to the point where the scattered intensity was proportional to particle concentration [69]. This was experimentally verified for the particle concentrations in the range of 500 ppb. A linear plot was seen to extend at least to 1 ppm.

When the phenomenon was used to generate a standard calibration curve for anti-IgG, the results were less than satisfactory (Fig. 47). The best assay yielded a detection limit of ~ 5 ppm which is barely adequate to meet the needs of clinical immunoassay. More often than not, the PAS signal showed little response whatsoever to the presence

Figure 46: PAS signal with modulation frequency for dyed and plain LMP as well as for I_2 in CCl_4 .

Figure 47: Analytical calibration curve for anti-IgG concentration employing resonant enhancement of PAS.

of anti-IgG. Given that our observed enhancement is much smaller than that observed by Kitamori's group, it is impossible to develop the phenomenon as the basis for any realistic method. If an assay based on resonant enhancement of PAS is ever going to become a reality, it will be necessary to obtain deeper theoretical insight into the process itself and to clarify the procedural facets of Kitamori's original work. To the author's knowledge, no other immunoassay work since the original study by Kitamori *et al* has incorporated this phenomenon. This may suggest that an important aspect of the work has been overlooked by them in the publication.

Heterogeneous Immunoassay by LMP-PAS

The effect of amount of CL on the sensitivity of the heterogeneous assay by LMP-PAS is illustrated in Figure 48. Results indicated that the greater the amount of CL added, the greater the sensitivity. Arbitrarily, the upper limit of CL to be used was 500 μ L of 100 ppm solid suspension since use of too large a quantity of CL would tend to clog the membrane filters. A secondary consideration would be the blank signal which also increased with the amount of CL added in the immunoassay.

The use of additives such as polyethylene glycol (PEG) had been found to be useful in some antibody-antigen systems to accelerate the agglutination reaction [79]. Masson *et al* used PEG routinely as an additive in their PACIA work even though it was not effective for all the systems they tested [74]. The use of additives such as PEG was not employed in this work because it represented an additional reagent and more importantly because the actual role played by PEG in enhancing the Ab-Ag interaction

Figure 48: PAS signal amplitude versus antibody concentration for a variety of quantities of coated latex.

had not been clearly defined.

The incubation time was also optimized. Incubation of 5 min gave sporadic results while 15 and 30 min gave progressively larger signals. An incubation time of 30 minutes was used throughout the rest of the study. Incubation times greater than 30 minutes can be expected to generate larger overall signals; however, excessively long incubations times would also be considered a drawback to the method development.

The volume of agglutinated sample to be filtered was governed by both time and background signal. A few particles or aggregates normally small enough to pass the pores can be expected to be trapped by the filter, either because they may land on a portion of the filter lacking a pore or because a larger aggregate has clogged a pore in the vicinity. Ideally, filtering the largest possible volumes would give the largest absolute PAS signal; however, a compromise was made to limit the background signal arising from the above-mentioned sources. The volume filtered was determined as the maximum volume which gave rise to a PAS signal amplitude for the blank that was essentially indistinguishable from the small PAS signal obtained for the bare filter.

Reduction of the blank signal (Fig. 49) was limited by the fact that the dyed LMP obtained from Seradyn was contaminated by a small proportion of single particles of 1-3 μm in diameter. It was assumed that these large particles were coloured and would generate a noticeable PAS signal. Since the original LMP stock suspensions were highly concentrated (10% w/v), selectively filtering the large interfering particles was problematic (due to clogging of filters) and could not be done prior to the immunoassay. Ultimately 500 μL of the agglutination sample was filtered as this volume was convenient

Figure 49: PAS signal amplitude versus amount of blank agglutination sample filtered.

Figure 50: PAS signal versus modulation frequency for a blank anti-IgG sample.

and gave good results in terms of signal and reproducibility.

Preliminary work was carried out using mixed esters of cellulose filters (Millipore, Bedford, MA) with a nominal pore diameter of $0.45\ \mu\text{m}$. As the blank signal obtained from the cellulose ester filters was high, they were changed to polycarbonate membrane filters (Millipore) with a nominal pore diameter of $0.8\ \mu\text{m}$. In the polycarbonate filters, the pores were ion etched channels instead of the tortuous path type available from the cellulose-based filters. This ensured a cleaner separation between those aggregates that were retained on and unused CL that was allowed to pass through the filter. With the larger pore size of $0.8\ \mu\text{m}$, small agglutinations of up to three particles in number were also allowed to pass through the filter.

The microphone output of the PAS cell versus modulation frequency is shown in Figure 50. The cell has a reasonably low resonant frequency that can be seen centred at 550 Hz. PAS signals acquired at this resonant frequency would be larger in magnitude and the immunoassay was carried out at both 550 Hz and 200 Hz to determine whether the use of the resonant frequency would enhance performance in terms sensitivity (Fig. 51). While the sensitivity, in terms of the slope of the calibration curve (mV/ppm anti-IgG), did appear somewhat greater when cellulose mixed esters filters were used (52 mV/ppm versus 30 mV/ppm), it was even less noticeable when this was repeated using the polycarbonate filters (9.4 mV/ppm versus 7.9 mV/ppm). Presumably the slightly higher slope obtained with the cellulose based filters is a result of a reduction in the thermal diffusion length accompanied by higher modulation frequencies. The cellulose filters ($\sim 100\ \mu\text{m}$) were much thicker than the polycarbonate filters ($\sim 10\ \mu\text{m}$) and

Figure 51: PAS signal versus anti-IgG concentration for two modulation frequencies for heterogeneous LMP-PAS employing 0.45- μm cellulose ester filters.

Figure 52: Analytical calibration curve for anti-IgG using heterogeneous LMP-PAS immunoassay using 0.8- μm pore filters. Modulation frequency = 200 Hz.

generated a larger background PAS signal. Reducing the thermal diffusion length would serve to increase the fraction of the total signal resulting from the agglutinates, thus generating a slightly larger background or net PAS signal. Thus the use of the resonant frequency did not significantly enhance the sensitivity of the heterogeneous LMP-PAS assay.

The analytical calibration curve for anti-IgG employing 0.8 μm -pore size polycarbonate filters is shown in Figure 52. The assay gave a linear response with antibody concentration up to about 10 ppm. Higher concentrations, greater than 10 ppm, exhibited drastically higher PAS signals, undoubtedly due to clogging of the membrane pores which resulted in trapping of unagglutinated particles. Filtered samples thus prepared were highly coloured, visible to the eye. The detection limit of the assay was estimated to be 500 ppb. This detection limit was not hindered by the sensitivity of the PAS detection mode but rather on the reproducibility of sample preparation. Currently, the samples are filtered individually by hand and it is difficult to ensure the pressure, and thus the shearing forces, is constant from sample to sample. Better detection limits may result if this matter was addressed by automation or at the very least standardized mechanical sample filtration.

While there does seem to be a better limit of detection using the filters with smaller pores, the filters themselves tend to clog at very low concentrations thus limiting its utility in immunoassay.

CONCLUSIONS

The enhancement of PAS sensitivity for particles whose diameters closely match the excitation wavelength was confirmed, exhibiting as much as three times the sensitivity of smaller or larger particles. This is however a significantly smaller enhancement than the 50 times enhancement reported by Kitamori *et al.* Consequently, the LMP-PAS immunoassay only yielded a detection limit of 5 ppm (50 IU mL⁻¹) which is unsuitable for most purposes. More theoretical investigation into the enhancement phenomenon is needed before any significant improvement can be made.

The heterogeneous LMP-PAS assay based on collection of coloured agglutinates on small-pore filters was found to be fast (less than 5 min of analysis time per sample) and simple. The method displayed only a moderate detection limit of 500 ppb (or 5 IU mL⁻¹) which may be improved with better reproducibility in sample preparation.

**CHAPTER 3: Determination of Mercury(II) by Complexation
into Dithizone-impregnated Latex Microparticles**

INTRODUCTION

The determination of trace levels of toxic heavy metals continues to represent an important area of analytical chemistry. Mercury has been determined over the years in trace levels by reaction with the ligand phenylazothioformic acid 2-phenylhydrazide, more commonly known as dithizone [80]. Dithizone forms complexes with many heavy metals of which a number are photochromic. Photochromism refers to the reversible colour change that the compounds undergo on exposure to visible light. Analysis of these photochromic species by photoacoustic spectroscopy (PAS) has been quite successful. The method is sensitive and is quite specific for mercury if samples are prepared in solid films; no other heavy metal complexes with dithizone exhibit photochromism in the solid state [81]. Frequently however, the preparation of solid samples can be somewhat time-consuming. In this work, a novel technique is presented in which aqueous Hg(II) is sequestered into small uniform polystyrene LMP which are previously impregnated with dithizone. The particles can then be easily turned into a solid sample suitable for photochromism-induced photoacoustic spectroscopy (PCPAS) analysis by simple filtration onto commercially available small-pore membrane filters. The method is fast, simple and has the potential for simultaneous quantification and clean-up of Hg(II) in environmental waters.

Much work has been carried out to make use of the very regular surfaces of LMP [?]. The particles have recently been used as a support on which silver needles were grown for surface enhanced raman spectroscopy [82]. Many substances have

been adsorbed onto the surfaces of LMP, not the least of which being immunoglobulins for the now well established agglutination immunoassays such as LST and PACIA already described [74]. The technique for dyeing microparticles has been well established and particles are available in a variety of colours, usually to aid in visual determination of agglutination assays. Fluorescent dyes have also been used for a number of spectroscopic techniques. In these cases the dye has been shown to be deep in the interior of the particle and does not alter the surface chemistry [75]. A number of iron(III) complexes have been recently incorporated into sodium dodecyl sulphate micelles and studied spectroscopically [83], but these were limited to the case of the continuous phase being aqueous. This work is the first reported in the literature, to the author's knowledge, of an analytical reagent being put inside LMP. The reagent-impregnated LMP is stable in aqueous suspension and can be removed from the aqueous phase along with the Hg(II) for analysis under ambient conditions. Additionally, the potential for a variety of organic complexing agents being incorporated in LMP to create tailor-made reagents for toxic clean-up is great.

THEORY OF PHOTOCHROMISM (PC)

Photochromism, or simply the coloration by light, refers to the reversible change of certain chemical species between states that exhibit distinct absorption spectra, induced in at least one direction by light [84]. At least nine primary metal dithizonates exhibit photochromism in solution while mercuric dithizonate, $\text{Hg}(\text{HDz})_2$, is the only one to do so in the solid phase as well [85]. In the case of $\text{Hg}(\text{HDz})_2$ the colour change is from orange to blue and the absorption spectra of both ground and excited state species are presented in Figure 53.

Figure 53: Visible absorption spectra for ground and excited state $\text{Hg}(\text{HDz})_2$.

EXPERIMENTAL SECTION

Dithizone Impregnation and Hg(II) Extraction.

Dithizone (H_2Dz) was purified by Irving's method and the purified dithizone solution in spectroscopic grade CCl_4 was stored under a layer of 1 N H_2SO_4 in the dark. Solutions stored this way keep very well [86].

In a small vial, 50 μL of 0.01 % (w/v) H_2Dz solution in CCl_4 was added to 900 μL of deionized water. The H_2Dz was extracted into the aqueous phase by addition of 10 μL of 25 % (v/v) NH_4OH followed by gentle shaking. The CCl_4 layer was carefully removed after all traces of the green H_2Dz were gone. A 100 μL aliquot of 0.2 % (w/w) LMP suspension was added and the suspension, a soft orange-yellow colour, was acidified with 10 μL of 3 % (v/v) H_2SO_4 as the pH needed to drive H_2Dz into the LMP is below 6. The resulting green suspension of H_2Dz -impregnated LMP (see Figure 54) was added to 1.0 mL Hg(II) samples in 100 μL aliquots. This volume contained sufficient H_2Dz to completely complex the most concentrated standard plus a modest excess. The samples were filtered through 0.1 μm pore size filters (Nuclepore, Toronto, ON, Canada) which were then removed from the filter housing and allowed to air dry before analysis.

PCPAS measurement.

The schematic diagram of the PCPAS system is illustrated in Figure 54. The

Figure 54: Schematic showing the impregnation and extraction processes.

Figure 55: Schematic diagram of the PCPAS apparatus.

excitation source was an argon ion-pumped dye laser (Spectra-Physics, Mountainview, CA, models 164 and 375b). The output beam from the dye laser, 605 nm in wavelength and typically 90 mW in power, was directed through a window into the photoacoustic cell after being mechanically chopped at 200 Hz. The samples (membrane filters supporting LMP) were placed directly into the gas-tight cell which was fitted to a condenser microphone and a sound meter which contained a preamplifier and an octave filter set (Brüel and Kjær, Pte. Claire, PQ, models 4144 and 2209). The baseline PAS signal from the sample (see Figure 56) was first recorded using a lock-in amplifier (Stanford Research, Sunnyvale, CA, model SR510). The sample was then illuminated by the light of a projector lamp (80 W) focused into an optical fibre bundle. The optical fibre bundle was brought close to the photoacoustic cell and directed onto the sample through the back window. The PAS signal was again recorded for sufficient time to achieve a steady-state signal. The absolute difference in the signal voltages before and after projector light illumination, the PCPAS signal amplitude, was taken as an indication of the amount of Hg(II) present in the sample.

Interference from other heavy metals was examined by preparation of mixed metal standards. The dithizone present in the 100 μ L aliquot remained in excess of the total amount of extractable metals. These samples were analyzed in the same way as described above.

Figure 56: Typical PCPAS signal for Hg(II) dithizonate in latex microparticles. The signal rises abruptly when exposed to a bright light source due to higher absorptivity of the excited state complex.

RESULTS AND DISCUSSION

The chemistry of the H_2Dz -impregnated LMP preparation and $Hg(II)$ extraction is very interesting. It is not clear yet how H_2Dz , and then $Hg(II)$, is transported into the LMP centre. These solid particles of polystyrene, with a rigid structure allowing them to be filtered while retaining their shape, possess a negatively charged surface due to residual surfactant from their production. It is unlikely, then, that H_2Dz adsorbs onto the surface at any time to be followed by seepage into the particle interior. It may be that the presence of residual CCl_4 facilitates the transfer of H_2Dz in a manner similar to the procedure involved with dyeing the particles. In dyeing particles, the particles are initially swollen with a dye solution prepared in a solvent which also dissolves polystyrene, such as CCl_4 . The solvent is then carefully boiled off, trapping the dye inside the latex [75]. Although very little CCl_4 ($50 \mu L$) was involved in the H_2Dz impregnation step, the transport process might be analogous since the latex volume itself was quite small. The particles were probably slightly swollen by the organic solvent, allowing enough fluidity or flexibility to the structure to allow transfer of H_2Dz across the interface.

The pH of the extraction is generally important to achieve quantitative results. As illustrated in Eq. 12, a large H_2Dz concentration will tend to drive the reaction

towards completion. Sigmoid curves of the percentage extraction of primary dithizonates

into a solution of 10^{-5} M H_2Dz in a suitable organic solvent reach 100% above pH 4 while the extraction drops to zero below pH 3. Lower concentrations of H_2Dz in the organic phase exaggerate the curves and push them to higher pH regions; a 10^{-8} M solution will require a pH of 8 to achieve quantitative extraction [87]. In this work, the concentration of H_2Dz was essentially constant at approximately 2% (w/w) in the latex. This represented a larger concentration of H_2Dz in the latex phase compared to solution-phase extractions which are typically carried out in the 10^{-7} to 10^{-5} M range. Hence, the control of pH was less critical and a pH of ca. 6 was maintained in the extraction vessel to ensure quantification. The extraction was assumed to be complete after 15 minutes of occasional shaking, although the reaction was seen to be much faster in those samples concentrated enough to observe the colour change visually. The quantification was ascertained by reanalyzing the filtrate from aqueous samples after the primary reaction and filtration. The data verified that extraction efficiency was greater than 99% for 50 and 100 ng Hg(II) in aqueous samples under the given experimental conditions.

Photoacoustic analysis of Hg(II) dithizonate-impregnated LMP was straightforward. The presence of essentially non-absorbing latex polystyrene surrounding the analyte species did not complicate the issue of signal generation appreciably at a chopping frequency of 200 Hz. Calibration curves of LMP dyed dark blue with Sudan Black (Seradyn, Inc.) showed a linear response with quantity as expected. The calibration curve for the determination of Hg(II) using 0.204- μ m LMP is shown in the upper trace of Figure 57. The current detection limit, determined at twice the standard

deviation of the blank, is 500 pg (in a 1.0 mL aqueous sample) or 3 pmole of mercury. It should be noted that the actual amount of Hg(II) being sampled by the excitation beam, and thus generating the signal, is about 1 %, or 5 pg, of the total amount of Hg(II) in the microparticles trapped on the filter surface. A ten-fold improvement in the detection limit, from 500 to 50 pg, was conveniently achievable simply by reducing the filtration area from the current commercially available size of 130 mm² to 13 mm². Samples prepared in this manner did generate measurable signals for 50 pg Hg(II) standards, with a signal-to-noise ratio of 2.

Given that H₂Dz does not exhibit any appreciable photochromism at the excitation wavelength of 605 nm, excess H₂Dz in the microparticles did not add to the observed PCPAS signal amplitude. The amount of residual H₂Dz in each sample varied roughly inversely with the concentration of the standard. This meant that, since H₂Dz absorbed at this wavelength, the baseline signal amplitude did necessarily vary from sample to sample but since it was only the photochromism-induced signal change that was taken as a measure of mercury content, it did not represent a problem to the analysis. Nevertheless, the excess H₂Dz produced a background PAS signal which, if large enough, could limit the sensitivity range used on the sound meter. It appeared possible to remove some of the excess dithizone from the microparticles by addition of NH₄OH to bring the pH to 9 after complexation with Hg(II) was complete. In most cases, however, the excess of dithizone was modest and did not affect the analysis to any significant extent.

The lower trace of Figure 57 shows the resulting calibration curve when 0.544-

Figure 57: Analytical calibration curve for Hg(II) dithizonate in latex microparticles. The error bar shows the typical standard deviation of replicate samples.

Figure 58: Calibration curve for Hg(II) (▲) and for mixed metal standards containing equal quantities of Hg(II) and Ag(I) (●) and Zn(II) (+).

μm microparticles were used for the extraction. There did not appear to be any difference in the amount of H_2Dz that could be sequestered into equal volumes of particles of different sizes. Since the surface area to volume ratio did not seem to affect the impregnation, it was confirmed that H_2Dz , and hence Hg(II) , was being transferred into the interior of the latex and not being adsorbed onto the surface. The calibration results did show enhanced detection sensitivity when smaller particles ($0.204\ \mu\text{m}$) were used to collect Hg(II) . This was undoubtedly due to a combination of factors, including optical shielding of the interior of the larger particles ($0.944\ \mu\text{m}$) which inhibited the photochromic activity and higher surface area of the smaller particles which lead to more efficient transfer of heat to the gas microphone in the photoacoustic cell.

In the present experimental setup, the projector illumination entered the PAS cell through the back window. While this simple design avoided interference with the incident laser beam, it had a tendency to limit the intensity of projector light reaching the sample as the original filters were a mixed esters/cellulose acetate type and were strongly scattering. The useful dynamic range of PCPAS analysis was drastically improved by changing to Nuclepore filters, which were a polycarbonate type and were much more transparent to visible light (average absorbance in the visible range = 0.75). The new filters allowed more than enough light to reach the samples to ensure that small variations in projector intensity did not affect the generation of excited state Hg(II) dithizonate (ie. the PAS signal versus light intensity is flat). Additionally, the switch served to improve the ease of filtration. The polycarbonate pores are formed by nuclear bombardment followed by etching leaving a clear channel; the cellulose acetate filters are of the

tortuous path type. The result was less back pressure allowing larger volumes of dilute samples to be extracted for preconcentration of Hg(II). Many tens of millilitres of dilute solutions can be incorporated into very small quantities of latex; a 50 mL volume of 50 ppb Hg(II) will only require ca. 300 μg of dithizone-impregnated LMP.

The selectivity of the PCPAS determination for Hg(II) was quite good using the LMP as it is in other solid sample preparations of $\text{Hg}(\text{HDz})_2$. Samples of Ag(I) and Zn(II) dithizonates showed only small PCPAS signals at the excitation wavelength of 605 nm even at very high concentrations. Comparable quantities of either Ag(I) or Zn(II) in a standard of Hg(II) did not add appreciably to the measured PCPAS signal amplitude for Hg(II) as shown in Figure 58. Note that the slopes of these calibration curves were essentially the same; the variation from curve to curve was due to slight variations in the preparation of dithizone-impregnated LMP. This variation usually will not affect a calibration series from the same preparation.

The presence of interfering heavy metals becomes a problem, however, if the total amount of extractable metals is in excess of the amount of H_2Dz introduced to the sample. There will be competition between the metals for extraction into the LMP under this circumstance. A smaller overall PCPAS signal for $\text{Hg}(\text{HDz})_2$ will be produced since some Hg(II) ions will remain in the aqueous phase and be washed away with the filtrate. If large amounts of other heavy metals are suspected, a larger amount of H_2Dz -impregnated LMP will be needed. This may generate smaller PCPAS signals for Hg(II) as a result of the inner filter effect of the non-photochromic but absorbing, interfering metal dithizonates. Again, the use of stronger projector illumination to induce

photochromism should surmount the difficulty. Besides, intentionally swamping the LMP with heavy metals will help remove any excess H_2Dz after extraction of $Hg(II)$ from a sample solution, resulting in a lower background signal, providing that the choice of added metal does not interfere spectrally.

CONCLUSIONS

A novel technique has been demonstrated for the determination of Hg(II) at picomole levels in aqueous samples. The extraction of Hg(II) into H₂Dz-impregnated LMP is both rapid and quantitative. PCPAS detection for Hg(HDz)₂ in these particles remains selective over other toxic heavy metals, and the sensitivity of the technique is good for environmental analysis.

Since there is a noticeable colour change of the H₂Dz-impregnated LMP upon reaction with heavy metals (regardless of PCPAS detection ability), a packed column of the microparticles can be applied to water streams as a visual indicator for heavy metals contamination. Additionally, the latex microparticles are a potential tool for water clean-up as they can be collected easily on a membrane filter for safe disposal after quantitative removal of the heavy metals. It remains to be seen, however, whether it will be economically possible to successfully scale the production of H₂Dz-impregnated LMP up to meet environmental needs.

CLAIMS TO ORIGINAL RESEARCH

1. Development of novel chemical sensor incorporating optical phenomenon of surface plasmon resonance and photothermal detection spectrometry detection.
2. The SPR-PDS technique has been used for the heterogeneous immunoassay of immunoglobulins IgG and IgA.
3. Development and evaluation of techniques to enhance performance of the SPR-PDS technique with respect to small proteins. Use of fluorescently-tagged antibodies and antibodies coated onto polystyrene latex microparticles was investigated.
4. The investigation of high intensity domain indicated that the SPR-PDS signal saturated. Investigation suggested that the radiative decay of surface plasmons increases at the expense of non-radiative decay.
5. The feasibility of SPR-PDS as a means of characterizing molecularly-imprinted materials as a first step in preparing synthetic alternatives to natural antibodies has been ascertained.
6. Development of heterogeneous assay based on selective collection of agglutinations of dyed latex microparticles quantified via photoacoustic spectrometry (PAS).
7. First reported incorporation of an analytical reagent (dithizone) into polystyrene latex microparticles.
8. Development of technique for the extraction and determination of Hg(II) in aqueous samples based on the extraction of Hg(II) into dithizone-containing particles for the subsequent quantification by photochromism-induced PAS.

Appendices

APPENDIX A

The presence of a thin dielectric coating affects the properties of the surface plasmons propagating at the silver surface. Consider the system shown below. Medium 0, the non-absorbing glass prism, is

assumed to have a real dielectric constant,

ϵ_0 . The silver film adjacent to the glass

has thickness d_1 and has a complex

dielectric constant, represented by

$\epsilon_1 = \epsilon_1' + i\epsilon_1''$. The dielectric coating, of

thickness d_2 , is represented by a real

dielectric constant if it is transparent and

by the complex expression $\epsilon_2 = \epsilon_2' + i\epsilon_2''$

if it is absorbing. The complex dielectric

constant is related to the complex

refractive index $\eta = n + ik$ through the optical constants n and k , where $\epsilon_1' = n^2 - k^2$ and

$\epsilon_1'' = 2nk$. The absorption coefficient, α , is related through $\alpha = 4\pi k/\lambda$. The non-

absorbing medium 3 outside the prism coupler, normally either air or water, is

represented by ϵ_3 . Surface plasmons propagate at the interface between medium 1 and

three for a bare silver surface. The presence of a dielectric material at this interface

alters the resonance condition.

Figure A: Schematic diagram of a four-component system composed of prism, metal film, dielectric layer and air.

It is possible to obtain an exact, though cumbersome, expression for the dispersion relation from Maxwell's equations. By developing the exact expression and retaining expressions only up to second order with respect to dielectric film thickness, d_2 , and ϵ''/ϵ' , Pockrand was able to develop a simplified explicit expression which agreed very well with the exact expression [56]. The expression is limited to thin films and for metals with small ϵ_1'' (silver is such a metal, where $\epsilon_1'' \ll \epsilon_1'$). The approximate expression for the surface plasmon dispersion is given by:

$$K_x^{sp} = K_x^0 + K_x^{1C} + K_x^{2C} + K_x^{1M} + K_x^{2M} + K_x^{2CR}$$

The first term, K_x^0 , is the usual dispersion expression, presented already in the theory section, for surface plasmons at a silver surface. The terms, K_x^{1C} and K_x^{2C} are first and second order expressions governing the effect of the dielectric coating. The terms K_x^{1M} and K_x^{2M} represent the effect of the silver film thickness on the dispersion relation. The effect of metal film thickness on the resonance position is small for thicknesses normally used in SPR work (ca. 600 Å). The final term, K_x^{2CR} , is an expression for the effect of the coating on the radiation damping. The expressions for the individual terms will not be reproduced here.

Following Pockrand, this program was written to calculate the changes in resonance position to be expected for a variety of coating thicknesses. The full surface plasmon dispersion relation, K_x^{sp} , is first calculated and then equated to the evanescent wave $K_x^{ev} = K_g \sin \theta$, the condition for resonance. The arcsin of this value leads to the resonance angle, reported in degrees. A DO loop is used increment the dielectric film thickness.

The program, written in Fortran 77, can accommodate coloured or transparent dielectric coatings. Note that absorption in the coating does not affect the resonance position, but increases the half-width and decreases the magnitude of the resonance peak through increased damping. The program prompts the user for complex dielectric constants of the metal and the coating (in the case of a non-absorber the imaginary portion is 0), the wavelength and the nominal thickness of the metal film. The current program assumes that the system is in contact with air ($\epsilon_3 = 1.0$) but can easily be altered to a water system ($\epsilon_3 = 1.76$).

Testing of the program's performance was accomplished by comparison of our results with those presented by Pockrand. Results are presented for our experimental conditions: dielectric constant of glass prism, ϵ_0 , is 2.2953, dielectric constant of protein, ϵ_2 , is 2.16, silver film thickness is 600 Å and optical constants for silver were obtained from the literature [39]


```

1:C      PROGRAM FILM 6
2:C      =====
3:C      THIS PROGRAM CALCULATES THE EFFECT OF A DIELECTRIC
4:C      COATING ON THE SP RESONANCE ANGLE.
5:C      IT INCLUDES THE EFFECT OF METAL FILM THICKNESS AND
6:C      THE EFFECT OF THE COATING ON RADIATION DAMPING
7:C      =====
8:      COMPLEX KSP, KX0, KX1C, KX2C, KX1M, KX2M, KX2CR
9:      COMPLEX E1, E2, J, KX1A, KX1B, KX2A, KX2B
10:     COMPLEX K0Z, K1Z, R01, KX1N, KX2
11:     REAL L, A, D2, THETA, S, E1R, E1IM, E2R, E2IM, D1
12:     J=CMPLX(0,1)
13:     D2=0.0
14:     E0=2.21217
15:     E3=1.0
16:     WRITE(*,2000) 'ENTER COMPLEX DIELECTRIC FUNCTION OF METAL'
17:     READ (*, *) E1R, E1IM
18:     WRITE(*,2000) 'ENTER COMPLEX DIELECTRIC FUNCTION OF COATING'
19:     READ(*, *) E2R, E2IM
20:     WRITE(*,2000) 'ENTER METAL FILM THICKNESS (IN ANGSTROMS)'
21:     READ(*, *) D1
22:     WRITE(*,2000) 'ENTER WAVELENGTH (IN ANGSTROMS)'
23:     READ(*, *) L
24:     A=6.2832/L
25:     E1=CMPLX(E1R, E1IM)
26:     E2=CMPLX(E2R, E2IM)
27:     WRITE(*,2000) 'EFFECT OF COATING THICKNESS ON RESONANCE ANGLE'
28:     WRITE(*,2000) '===== '
29:     WRITE(*,3000) 'E0=', E0, ' E1=', E1R, ' +J', E1IM
30:     WRITE(*,3000) 'E2=', E2R, ' +J', E2IM, ' E3=', E3
31:     WRITE(*,2000) '===== '
32:     WRITE(*,4000) 'WAVELENGTH IS', L, ' ANGSTROMS'
33:     WRITE(*,2000) '===== '
34:     WRITE(*,4000) 'METAL FILM THICKNESS IS', D1, ' ANGSTROMS'
35:     WRITE(*,2000) '===== '
36:     WRITE(*,2000) 'FILM THICKNESS      RESONANCE ANGLE'
37:     WRITE(*,2000) ' (ANGSTROMS)      (DEGREES)'
38:     KX0=(A*(CSQRT((E1*E3)/(E1+E3))))
39:     DO 10 N = 1, 21
40:         KX1A=A*((E2R-E3)/E2R)*(((E1R)/(E1R+E3))*(E1R/(E1R+E3)))
41:         KX1B=((E2R-E1R)/(E3-E1R))*((-E1R)**(-0.5))
42:         KX1C=KX1A*KX1B*(A*D2)
43:         KX2A=(2*(2*(E3*E3)-(E2*E2))/(E3*(E3-E2)))+((E1R+E3)/(-E3))
44:         KX2B=0.5*(KX1C/(REAL(KX0)))*(KX2A)-(0.5*J*(E1IM/E1R))
45:         KX2C=KX1C*KX2B
46:         K0Z=CSQRT(E0*(A*A)-(KX0*KX0))
47:         K1Z=CSQRT(E1*(A*A)-(KX0*KX0))
48:         R01=((K1Z/E1)-(K0Z/E0))/((K1Z/E1)+(K0Z/E0))
49:         KX1N=A*R01*(2/(E3-E1R))*(((E1R*E3)/(E1R+E3))**1.5)
50:         KX1M=KX1N*EXP((-12.566*D1/L)*(-E1R/(SQRT(-E1R-E3))))
51:         KX2N=(0.5*KX1M/REAL(KX0))*((2*E1R+E3)/(-E3))

```

```

52:      KX2M=KX1M*(KX2N-(J*((E1IM)/(E1R-E3))))
53:      KX2=((-E1R/E3)+((2*E3)/(E3-E2))+(E2/(2*E3)))
54:      KX2CR=((KX1M*KX1C)/REAL(KX0))*KX2
55:      KSP=KX0+KX1C+KX2C+KX1M+KX2M+KX2CR
56:      S=REAL(KSP/((SQRT(E0))*A))
57:      THETA=ASIN(S)
58:      THETA=THETA*(57.2958)
59:      WRITE(*,1000) D2, '      ', THETA
60:1000    FORMAT(8X,F9.4,A,F9.4)
61:      D2=D2+50.0
62: 10      CONTINUE
63:2000    FORMAT(5X,A,5X)
64:3000    FORMAT(5X,A,F8.3,A,F8.3,A,F8.3,5X)
65:4000    FORMAT(5X,A,F6.0,A,5X)
66:      STOP
67:      END

```

EFFECT OF COATING THICKNESS ON RESONANCE ANGLE

```

=====
E0=  2.295  E1= -20.095  +J  .448
E2=  2.161  +J  .000  E3=  1.000
=====

```

```

=====
WAVELENGTH IS 6596.  ANGSTROMS
=====

```

```

=====
METAL FILM THICKNESS IS 600.  ANGSTROMS
=====

```

FILM THICKNESS (ANGSTROMS)	RESONANCE ANGLE (DEGREES)
.0000	42.6024
50.0000	42.9704
100.0000	43.3940
150.0000	43.8745
200.0000	44.4133
250.0000	45.0120
300.0000	45.6726
350.0000	46.3975
400.0000	47.1892
450.0000	48.0510
500.0000	48.9867
550.0000	50.0006
600.0000	51.0983
650.0000	52.2862
700.0000	53.5721
750.0000	54.9661
800.0000	56.4805
850.0000	58.1315
900.0000	59.9402
950.0000	61.9361
1000.0000	64.1609

Figure B: SPR resonance angle as a function of dielectric film thickness.

APPENDIX B

This program is taken directly from Bohren and Huffman's review book which included this useful program for calculation of extinction, scattering and absorption coefficients [70].

```

PROGRAM CALLBH (INPUT=TTY,OUTPUT=TTY,TAPES=TTY)
C =====
C C/  ?H CALCULATES THE SIZE PARAMETER (X) AND RELATIVE
C REFRACTIVE INDEX (REFREL) FOR A GIVEN SPHERE REFRACTIVE
C INDEX, MEDIUM REFRACTIVE INDEX,RADIUS, AND FREE SPACE
C WAVELENGTH. IT THEN CALLS BHMIE, THE SUBROUTINE THAT COMPUTES
C AMPLITUDE SCATTERING MATRIX ELEMENTS AND EFFICIENCIES
C =====
C COMPLEX REFREL,S1(200),S2(200)
C WRITE (5,11)
C =====
C REFMED = (REAL) REFRACTIVE INDEX OF SURROUNDING MEDIUM
C =====
C REFMED=1.0
C =====
C REFRACTIVE INDEX OF SPHERE = REFRE + I*REFIM
C =====
C REFRE=1.55
C REFIM=0.0
C REFREL=CMPLX(REFRE,REFIM)/REFMED
C WRITE (5,12) REFMED,REFRE,REFIM
C =====
C RADIUS (RAD) AND WAVELENGTH (WAVEL) SAME UNITS
C =====
C RAD=.525
C WAVEL=.6328
C X=2.*3.14159265*RAD*REFMED/WAVEL
C WRITE (5,13) RAD,WAVEL
C WRITE (5,14) X
C =====
C NANG = NUMBER OF ANGLES BETWEEN 0 AND 90 DEGREES
C MATRIX ELEMENTS CALCULATED AT 2*NANG - 1 ANGLES
C INCLUDING 0,90 AND 180 DEGREES
C =====
C NANG=11
C DANG=1.570796327/FLOAT(NANG-1)
C CALL BHMIE(X,REFREL,NANG,S1,S2,QEXT,QSCA,QBACK)
C WRITE (5,65) QSCA,QEXT,QBACK
C WRITE (5,17)
C =====
C S33 AND S34 MATRIX ELEMENTS NORMALIZED BY S11.

```

```

C  S11 IS NORMALIZED TO 1.0 IN THE FORWARD DIRECTION
C  POL=DEGREE OF POLARIZATION (INCIDENT UNPOLARIZED LIGHT)
C  =====
S11NOR=0.5*(CABS(S2(1))**2 + CABS(S1(1))**2
NAN=2*NANG-1
DO 355 J=1,NAN
  AJ=J
  S11=0.5*CABS(S2(J))*CABS(S2(J))
  S11=S11+0.5*CABS(S1(J))*CABS(S1(J))
  S12=0.5*CABS(S2(J))*CABS(S2(J))
  S12=S12-0.5*CABS(S1(J))*CABS(S1(J))
  POL=-S12/S11
  S33=REAL(S2(J)*CONJG(S1(J)))
  S33=S33/S11
  S34=AIMAG(S2(J)*CONJG(S1(J)))
  S34=S34/S11
  S11=S11/S11NOR
  ANG=DANG*(AJ-1.)*57.2958
355 WRITE (5,75) ANG,S11,POL,S33,S34
65 FORMAT (//,1X,"QSCA = ",E13.6,3X,"QEXT = ",E13.6,3X,
  2"QBACK = ",E13.6)
75 FORMAT (1X,F6.2,2X,E13.6,2X,E13.6,2X,E13.6,2X,E13.6)
11 FORMAT (/"SPHERE SCATTERING PROGRAM"/)
12 FORMAT (5X,"REFMED = ",F8.4,3X,"REFRE = ",E14.6,3X,
  3"REFIM = ",E14.6)
13 FORMAT (5X,"SPHERE RADIUS = ",F7.3,3X,"WAVELENGTH = ",F7.4)
14 FORMAT (5X,"SIZE PARAMETER = ",F8.3/)
17 FORMAT (//,2X,"ANGLE",7X,"S11",13X,"POL",13X,"S33",13X,"S34"/)
  STOP
  END
C  =====
C  SUBROUTINE BHMIE CALCULATES AMPLITUDE SCATTERING MATRIX
C  ELEMENTS AND EFFICIENCIES FOR EXTINCTION, TOTAL SCATTERING
C  AND BACKSCATTERING FOR A GIVEN SIZE PARAMETER AND
C  RELATIVE REFRACTIVE INDEX
C  =====
SUBROUTINE BHMIE (X,REFREL,NANG,S1,S2,QEXT,QSCA,QBACK)
  DIMENSION AMU(100),THETA(100),PI(100),PI0(100),PI1(100)
  COMPLEX D(3000),Y,REFREL,XI,XI0,XI1,AN,BN,S1(200),S2(200)
  DOUBLE PRECISION PSI0,PSI1,PSI,DN,DX
  DX=X
  Y=X*REFREL
C  =====
C  SERIES TERMINATED AFTER NSTOP TERMS
C  =====
XSTOP=X+4.*X**.3333+2.0
NSTOP=XSTOP
YMOD=CABS(Y)
NMX=AMAX1(XSTOP,YMOD)+15
DANG=1.570796327/FLOAT(NANG-1)
DO 555 J=1,NANG

```

```

      THETA(J)=(FLOAT(J)-1.)*DANG
555 AMU(J)=COS(THETA(J))
C  =====
C  LOGARITHMIC DERIVATIVE D(J) CALCULATED BY DOWNWARD
C  RECURRENCE BEGINNING WITH INITIAL VALUE 0.0 + I*0.0
C  AT J = NMX
C  =====
      D(NMX)=CMPLX(0.0,0.0)
      NN=NMX-1
      DO 120 N=1,NN
      RN=NMX+1
120 D(NMX-N)=(RN/Y)-(1./(D(NMX-N+1)+RN/Y))
      DO 666 J=1,NANG
      PI0(J)=0.0
666 PI1(J)=1.0
      NN=2*NANG-1
      DO 777 J=1,NN
      S1(J)=CMPLX(0.0,0.0)
777 S2(J)=CMPLX(0.0,0.0)
C  =====
C  RICCATI-BESSEL FUNCTIONS WITH REAL ARGUMENT X
C  CALCULATED BY UPWARD RECURRENCE
C  =====
      PSIO=DCOS(DX)
      PSI1=DSIN(DX)
      CHI0=-SIN(X)
      CHI1=COS(X)
      APSIO=PSIO
      APSI1=PSI1
      XI0=CMPLX(APSIO,-CHI0)
      XI1=CMPLX(APSI1,-CHI1)
      QSCA=0.0
      N=1
200 DN=N
      RN=N
      FN=(2.*RN+1.)/(RN*(RN+1.))
      PSI=(2.*DN-1.)*PSI1/DX-PSIO
      APSI=PSI
      CHI=(2.*RN-1.)*CHI1/X-CHI0
      XI=CMPLX(APSI,-CHI)
      AN=(D(N)/REFREL+RN/X)*APSI-APSI1
      AN=AN/((D(N)/REFREL+RN/X)*XI-XI1)
      BN=(REFREL*D(N)+RN/X)*APSI-APSI1
      BN=BN/((REFREL*D(N)+RN/X)*XI-XI1)
      QSCA=QSCA+(2.*RN+1.)*(CABS(AN)*CABS(AN)+CABS(BN)*CABS(BN))
      DO 789 J=1,NANG
      JJ=2*NANG-J
      PI(J)=PI1(J)
      TAU(J)=RN*AMU(J)*PI(J)-(RN+1.)*PI0(J)
      P=(-1.)**(N-1)
      S1(J)=S1(J)+FN*(AN*PI(J)+BN*TAU(J))

```

```

T=(-1.)**N
S2(J)=S2(J)+FN*(AN**TAU(J)+BN*PI(J))
IF(J.EQ.JJ) GO TO 789
S1(JJ)=S1(JJ) + FN*(AN*PI(J)*P=BN*TAU(J)*T)
S2(JJ)=S2(JJ)+FN*(AN*TAU(J)*T+BN*PI(J)*P)
789 CONTINUE
PSI0=PSI1
PSI1=PSI
APSI1=PSI1
CHI0=CHI1
CHI1=CHI
XI1=CMPLX(APSI1,-CHI1)
N=N+1
RN=N
DO 999 J=1,NANG
PI1(J)=((2.*RN-1.)/(RN-1.))*AMU(J)*PI(J)
PI1(J)=PI1(J)-RN*PI0(J)/(RN-1.)
999 PI0(J)=PI(J)
IF (N-1-NSTOP) 200,300,300
300 QSCA=(2./(X*X))QSCA
QEXT=(4./(X*X))*REAL(S1(1))
QBACK=(4./(X*X))*CABS(S1(2*NANG-1))*CABS(S1(2*NANG-1))
RETURN
END

```

REFERENCES

1. Ekins, R. P., in *Alternative Immunoassays*, W. P. Collins, ed., John Wiley and Sons, Chichester, 1985, Chapter 13.
2. Yeung, J.P., *personal communication*, September 16th, 1993.
3. Morris, M.D. and Peck, K., *Analytical Chemistry*, 1986, **58**, 811A-822A.
4. Herbert, W.J., Wilkinson, P.C. and Stott, D.I., eds., *Dictionary of Immunology*, 3rd edition, Blackwell Scientific, Oxford, 1985.
5. Hall, E.A., *Biosensors*, Open University Press, Milton Keynes, 1990, p.15.
6. Cullen, D. C. and Lowe, C. R., *Sensors and Actuators*, 1990, **B1**, 576-9.
7. Rothenhäusler, B. and Knoll, W., *Nature*, 1988, **332**, 615-7.
8. Hickel, W. and Knoll, W., *Acta Metallica*, 1989, **37**, 2141-4.
9. Schneider, F. W., *Non-linear Raman Spectroscopy and Its Applications*, Kiefer, W. and Long, D. A., eds., D. Reidel, Dordrecht, 1982, p. 461-70.
10. Cowan, J. J., *Optics Communications*, 1972, **5**, 69-72.
11. Villuendas, F. and Pelayo, J., *Sensors and Actuators*, 1990, **A21-A23**, 1142-5.
12. Lee, I., Callcott, T. A. and Arakawa, E. T., *Analytical Chemistry*, 1992, **64**, 476-8.
13. Kooyman, R. P. H., Kolkman, H., Van Gent, J. and Gerve, J., *Analytica Chimica Acta*, 1988, **213**, 35-45.
14. Liedberg, B., Nylander, C., and Lundström, I., *Sensors and Actuators*, 1983, **4**, 299-304.
15. Lukosz, W., *Biosensors and Bioelectronics*, 1991, **6**, 215-225.
16. Vadgama, P. and Crump, P.W., *Analyst*, 1992, **117**, 1657-70.
17. Arwin, H., Welin, S. and Lundstrom, I., in *Non-isotopic Immunoassay*, Ngo, T.T., ed., Plenum Press, New York, 1988.
18. Flanagan, L. T., and Pantell, R. H., 1984, *Electronics Letters*, **20**, 968-70.

19. Grygier, R.K., Knoll, W. and Coufal, H., *Canadian Journal of Physics*, 1985, **64**, 1067-9
20. Inagaki, T., Kagami, K. and Arakawa, E. T., *Physical Review B*, 1981, **24**, 3644-6.
21. Chard, T., *An Introduction to Radioimmunoassay and Related Techniques*, in *Laboratory Techniques in Biochemistry and Molecular Biology*, Work, T. S and Work, E., eds., Vol. 6, 1982, Elsevier Biomedical Press, Amsterdam.
22. Szentirmay, Z., *Spectrochimica Acta*, 1992, **48A**, 9-17.
23. Welford, K., *Optical and Quantum Electronics*, 1991, **23**, 1-27.
24. Raether, H., *Physics of Thin Films*, 1977, **9**, 145.
25. Ferrell, T. L., Callcott, T. A., and Warmack, K. J., 1985, *American Scientist*, **73**, 344-53.
26. Kretshmann, E., *Optics Communications*, 1972, **6**, 185.
27. Sun, X., Shiokawa, S., and Matsui, Y., *Journal of Applied Physics*, 1991, **69**, 3626.
28. Fuzi, Y., Bradberry, G. W., and Sambles, J. R., *Journal of Modern Optics*, 1989, **36**, 1405-10.
29. Hayashi, S., Yamada, T. and Kanamori, H., *Optics Communications*, 1981, **36**, 195-9.
30. Innes, R. A. and Sambles, J. R., *Solid State Communications*, 1985, **56**, 493.
31. Otto, A., *Zeitschrift fur Physik*, 1968, **216**, 398.
32. Inagaki, T., Nakagawa, Y., Arakawa, E.T. and Aas, D.J., *Physical Review B*, 1982, **26**, 6421-30.
33. Jackson, W. B., Amer, N. M., Boccara, A. C. and Fournier, D., *Applied Optics*, 1981, **20**, 1333-44.
34. Tam, A. C., *Reviews of Modern Physics*, 1986, **58**, 381-431.
35. Murphy, J.C. and Aamodt, L.C., *Journal of Applied Physics*, 1980, **51**, 4580-8.
36. Aamodt, L.C. and Murphy, J.C., *Journal of Applied Physics*, 1981, **52**, 4903-14.
37. VanderNoot, V. A. and Lai, E. P. C., *Spectroscopy*, 1991, **6**, 28-33.
38. Matsubara, K., Kawata, S., and Minami, S., *Applied Optics*, 1988, **27**, 1160-3.

39. Johnson, P. B. and Christy, R. W., *Physical Review B*, 1972, **6**, 4370-9.
40. Fotiou, K.F. and Morris, M.D., *Applied Spectroscopy*, 1986, **40**, 700-3.
41. Guo, R., *Ph. D Dissertation*, 1989, Carleton University, Ottawa, ON, Canada.
42. Murphy, J.C. and Aamodt, L.C, *Journal of Applied Physics*, 1980, **51**, 4580-8.
43. Giaver, I., *Journal of Immunological Methods*, 1978, **21**, 57-61.
44. Vanderlaan, M., Stanker, L. and Watkins, B., eds., *Immunoassays for Trace Chemical Analysis*, American Chemical Society, Washington, 1990.
45. Weiler, E.W., in *Pyrethroid Residues, Immunoassays for Low Molecular Weight Compounds*, Blab, W. and Weiler, E.W., Springer-Verlag, Berlin, 1990.
46. Vlatakis, G., Andersson, L.I., Müller, R. and Mosbach, K., *Nature*, 1993, **361**, 645-7.
47. Knoll, W., Hickel, W., Sawodny, M., Stumpe, J. and Knobloch, H., *Fresenius' Journal of Analytical Chemistry*, 1991, **341**, 272-8.
48. Ulman, A., *An Introduction to Ultrathin Organic Films*, Academic Press, Boston, 1991.
49. Inagaki, T., Motosuga, M., Yamamori, K. and Arakawa, E. T., *Physical Review B*, 1983, **28**, 1740-4.
50. Kou, E. F. and Tamir, T., *Applied Optics*, 1988, **27**, 4098-103.
51. Johnson, P. B. and Christy, R. W., 1972, *Physical Review B*, **6**, 4370-9.
52. Attridge, J.W., Daniels, P.B., Deacon, J.K., Robinson, G.A and Davidson, G.P, *Biosensors and Biotechnology*, 1991, **6**, 201-15.
53. Silin, V.I., Balcytic, G.A., Zhizhin, G.N. and Yakovlev, V.A., *Vibrational Spectroscopy*, 1993, **5**, 133-42.
54. Harrick, N.J., *Internal Reflection Spectroscopy*, 1987, Interscience, New York, p. 51.
55. Jolliff, C.L., *The Immunoglobulins, Beckman Technical Literature IG-1*, Beckman Instruments, Brea, 1989.
56. Pockrand, I., *Surface Science*, 1978, **72**, 577-88.

57. Cullen, C.R., *Biosensors*, 1985, 1, 3-16.
58. Groeneveld, R., Sprik, R., and Lagendijk, A., *Physical Review Letters*, 1990, 64, 784-7.
59. Lyman, T., ed., *Metals Handbook*, American Society for Metals, Cleveland, 1958.
60. Nylander, C., Liedberg, B., and Lind, T., 1982, *Sensors and Actuators*, 3, 79-88.
61. Campbell, A.K., Roberts, P.A and Patel, A. in *Alternative Immunoassays*, Collins, W.P., ed., John Wiley and Sons, 1985, Chichester, Chapter 10.
62. van Hell, H., Leuvering, J.H.W. and Gribnau, T.C.J., in *Alternative Immunoassays*, Collins, W.P., ed., John Wiley and Sons, Chichester, 1985, Chapter 4.
63. Tarcha, P.J., Misun, D., Finley, Wong, M. and Donovan, J.J., in *Polymer Latexes: Preparation, Characterization, and Applications (ACS Symposium Series No. 492)*, Daniels, E.S., Sudol, E.D. and El-Aasser, M.S., eds., 1992, American Chemical Society, Washington, Chapter 22.
64. Bernard, A.M. and Lauwerys, R.R., *Clinical Chemistry*, 1983, 29, 1007-11.
65. Limet, J.N., Moussebois, C.H., Cambiaso, C.L., Vaerman, J.P. and Masson, P.L., *Journal of Immunological Methods*, 1979, 28, 25-32.
66. Gray, K., *Clinical Diagnostics Today*, 1991, 11, 14.
67. Kitamori, T., Suzuki, K., Sawada, T., and Gohshi, Y., *Analytical Chemistry*, 1987, 59, 2519-22.
68. Oda, S., Sawada, T., Moriguchi, T. and Kamada, H., *Analytical Chemistry*, 1980, 52, 650-3.
69. Kerker, M., *Scattering of Light and Other Electromagnetic Radiation*, Academic Press, New York, 1969, p. 312.
70. Bohren, C. F. and Huffman, D. R., *Absorption and Scattering of Light by Small Particles*, John Wiley and Sons, New York, 1983.
71. van de Hulst, H. C., *Light Scattering by Small Particles*, Dover, New York, 1981.
72. Yasa, Z. A, Jackson, W. B., and Amer, N. M., *Applied Optics*, 1982, 21, 21-31.
73. Helander, P., Lundstrom, I. and McQueen, D., *Journal of Applied Physics*, 1980, 51, 3841-7.

74. Masson, P.L., Cambiaso, C. L., Collet-Cassart, D. and Magnusson, C.G.M, Richards, C.B. and Sindic, C.J.M., *Methods in Enzymology*, 1981, **74**, 106-139.
75. Bangs, L. B., *Uniform Latex Microparticles*, Seradyn Inc., Indianapolis, 1984, Chapter VII.
76. Campillo, A.J. and Lin, H.-B. in *Photothermal Investigations of Solids and Fluids*, Sell, J., ed., Academic Press, Boston, 1989.
77. Rosencwaig, A., *Photoacoustics and Photoacoustic Spectroscopy*, John Wiley and Sons, New York, 1980.
78. Wu, J., Kitamori, T., and Sawada, T., *Analytical Chemistry*, 1991, **63**, 217-9.
79. Harrington, J.C., Fenton, J.W., II and Pert, J.H., *Immunochemistry*, 1971, **8**, 413-21.
80. Sandell, E. B. and Onishi, H. *Colorimetric Determination of Traces of Metals*, 4th ed., Wiley-Interscience, New York, 1978, 391.
81. Chen, N., Guo, R. and Lai, E.P.C. *Analytical Chemistry*, 1988, **60**, 2435-39.
82. Wachter, E. A., Moore, A. K. and Haas, III, J. W. *Vibrational Spectroscopy*, 1992, **3**, 73-8.
83. Mazumdar, S. *Journal of the Chemical Society, Dalton Transactions*, 1991, 2091-6.
84. Brown, G.H., *Photochromism, Techniques in Chemistry*, Vol. III, Wiley-Interscience, New York, 1971.
85. Meriwether, L.S., Breitner, E.C. and Sloan, S.L., *Journal of the American Chemical Society*, 1965, **87**, 4441.
86. Irving, H. M. N. H. *Dithizone*; The Chemical Society: London, 1977; Chapter 8.
87. Irving, H. M. N. H. *Dithizone*; The Chemical Society: London, 1977; Chapter 5.

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