FLUORESCENCE BASED OPTICAL SENSOR FOR PROTEIN DETECTION

by

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A THESIS

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This work mainly involves detection of protein by fluorescence based optical sensors. One is the profile design of optical fiber serving as a type of biosensors, the other is the study of an improved fluorescence polarization immunoassay (FPI) method using antibody coated magnetic nanoparticles.

A theoretical model using ray tracing method is developed to design the probe. The model could explain the observed fact that the maximum signal for a given realistic tapered length is at a probe radius smaller than that expected from V-number matching condition. It is shown that to obtain maximum fluorescence signal a realistic optimum taper length needs to be chosen. We found that, in air environment, this requirement is easy to achieve than that in aqueous condition. These facts were confirmed experimentally.

A novel idea is proposed with regard to FPI, that to introduce antibody coated magnetic nanoparticles into this immunoassay can greatly enhance the detecting range of biomolecules with various molecular weights. Detailed scheme is described in this work including related optical setup construction method, suitable dye and magnetic nanoparticle selection, magnetic field construction strategy, method of data processing,
etc. Experimental setup was built up and preliminary experiments have been carried on towards the development of this method. A homogeneous immunoassay was performed, and enhanced fluorescence anisotropy was observed with increasing amount of antibodies, which validated the usability of the optical setup. The developed FPI method using magnetic nanoparticles can be used for sensitive protein detection in future.
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CHAPTER 1
INTRODUCTION

As for the detection of bio-molecules such as proteins, the traditional methods are radioimmunoassay (Wide 1966), Enzyme-Linked ImmunoSorbent Assay (ELISA) (Engvall 1971) and western blot (Burnette 1981). However researchers wish to develop new approaches to enhance the detection capability, or enable researchers to study the characteristics of biomaterials of interest. The developments of fluorescence materials that have sound photophysical properties provide convenience to the study of biological principles using optical devices. Some research work has been conducted on the fluorescence based immunoanalytical systems (Marquette, 2006). The emphases of this thesis will be focused on the further development of fluorescence based fiber optical sensing (Wolfbeis 2006, Campbell 2006, Ko 2006) and fluorescence polarization immunoassay (FPI) (Shim 2004, Tsuruoka, 1991).

In recent years fiber-optic biosensors attract considerable research effort because of their potential sensitivity, detection speed and adaptability to a wide variety of assay conditions. In a fiber optic sensor, a fiber probe acts as a transduction element. It can generate absorption, fluorescence or scattering signal that proportions to analyte. There are various kinds of fiber optic sensors (Ahmad 2005, Anderson 1994, Golden 1994), one
of the most sensitive fiber-optic biosensor is based on excitation and detection of fluorescence using evanescent wave associated with the propagating modes in an optical fiber. Lots of work has been done with the aim of enhancing the acquisition efficiency of fluorescence signal (Kapoor 2004, Prasad 2003)

The design of fiber probe is a crucial factor for enhancing the signal-to-noise ratio in a fiber-optic sensor. The most popular fiber probes currently under study are long tapered probe (Anderson 1994, Geng 2004) and fiber with a thin waist as the probe part (Guo 2003, Villatoro 2004). The first kind of probe shape can be easily achieved by molding with polystyrene material, and the second combination shape is usually made by torching and stretching glass fibers. By comparative study of the signal acquisition from straight fiber and tapered optical fibers theoretically, we have shown that combination tapered fiber, which has a shape of combination of tapered part and straight part, have the highest sensitivity than fibers with other kinds of shape. In this thesis, we propose a combination probe shape using glass fiber. This will have the advantage that it will have very low noise signal which is typically generated by carbon constituent in plastic fiber. With the help of acid etching method, very long probe part can be obtained which is relatively difficult to be achieved by torching and stretching method. Although this kind of probe shape has already been studied in some theoretical work (Nath 1998), a more detailed mathematical model is needed which describes the relationship between fluorescence intensity and parameters like probe radius and refractive index of the environment. Some studies show that the taper angle of the taper part will affect the factors like transmission.
and penetration depth, and finally the fluorescence intensity obtained (Guo 2003, Angela 2006). However, a thorough study of the effect of the taper part on the collected fluorescence is still lacking. A criterion needs to be established for the optimum parameters of the taper zone for optimum signal generation and collection.

Another promising area is fluorescence polarization immunoassay which is presently in widespread commercial use in several instruments and still has a vast area to be explored (Shim 2004, Tsuruoka 1991, Li 2007). Briefly, the fluorescence polarization of a labeled macromolecule depends on the fluorescence lifetime and the rotational correlation time. For the use in analytical biochemistry, the antigen-antibody interactions and hormone-receptor interactions will change the rotational correlation time of the labeled molecules, the change of polarization anisotropy ensuing (Dandliker 1973). This method has an essential limitation that when it comes to the detection of large weight molecules, the anisotropy changes little which limits the dynamic range of the FPI method. Some studies make use of luminescent complex which has advanced characteristics like long lifetimes, high quantum yields and high emission polarization etc. to reduce the limitation of FPI (Guo 1998). Another approach which is proposed in this thesis could be to increase the weight of unlabeled molecules by attaching nanoparticle. Due to the high weight of nanoparticle used, a great dragging effect will happen to the targeted protein after immunoreaction. This will result in a change in fluorescence polarization. Based on this, an ensuing idea is proposed which in principle can further enhance the capacity of this method, that is to use antibody coated magnetic
particles in the FPI, so that a switching magnetic field can be applied to modulate the FP change.

Thesis Outline

This thesis is organized in the following way: It contains two parts-Part I including Chapter 2 will describe the design aspect of an optical fiber sensor, which is reprinted from published paper¹ and reprint permission has been granted by the publisher.

Part II including the following chapters will mainly focused on the development of FPI method using antibody coated magnetic nanoparticles.


CHAPTER 2

OPTIMUM PROBE DESIGN FOR MAXIMUM FLUORESCENCE SIGNAL FROM AN EVANESCENT WAVE FIBER OPTIC BIOSENSOR

Introduction

In recent years fiber-optic biosensors attracted considerable research effort (Love 1992, Axelrod 2001, Willer 2002, Amad 2005) because of their potential sensitivity, detection speed and adaptability to a wide variety of assay conditions. Their use as a probe or as a sensing element is increasing in clinical, pharmaceutical, industrial and military applications. Other main points in favor of the use of optical fibers in biosensors are excellent light delivery, long interaction length, low cost and ability not only to excite the target molecules but also to capture the emitted light from the targets. In a fiber optic sensor, a fiber probe acts as a transduction element. It can generate absorption, fluorescence or scattering signal that is proportional to the analyte concentration. There are various kinds of fiber optic sensors (Golden 1994, Kapoor 2004), one of the potentially sensitive fiber-optic biosensor is based on generation of fluorescence signal using evanescent waves associated with the propagating modes in an optical fiber. There are two main difficulties faced in fiber-based evanescent wave biosensors. Firstly, in comparison to distal end biosensors, only a small amount of power is available in the
evanescent wave sensors for generating a fluorescence signal (Snyder 1983). Secondly, in signal acquisition, there is only a low coupling efficiency of the fluorescence signal back to the fiber itself (Thompson 1995). Thus, there is a critical need for optimum design and fabrication of the fiber-based optical sensor leading to high excitations and a high level of fluorescence signal acquisition at the output end of the fiber.

In a fiber probe to generate an evanescent wave excited signal from the probe sensing region, cladding needs to be removed from the core along the distal end of a step-index optical fiber. An analyte recognition element needs to be immobilized on the decladded core region (Golden 1994). The decladding of core in the probe region leads to inefficient coupling back of fluorescence due to a mismatch between the V-number of probe and the cladded fiber part. It had been shown (Golden 1994) experimentally that Probes created with reduced sensing region radius exhibit improved response as reduced probe radius leads to reduction of V-number mismatch. It is further shown that tapering the radius of probe region can further improve the response. The most popular fiber probes currently under use are tapered fiber probes (Anderson 1994, Geng 2004). Detailed theoretical studies based on ray-tracing method have shown that the tapered optical fibers have higher sensitivity compared to straight fibers (Nath 1998) Theoretical work has also shown that among the tapered fibers, combination tapered fibers have higher sensitivity than continuous tapered fibers (Nath 1998). A combination tapered fiber has a shape of combination of tapered part and a straight part (Figure 2.1).
In this paper we are reporting the design conditions for an optimum fiber probe. A mathematical model based on ray tracing method is developed. The mathematical model is used to find variation of the total signal intensity with probe parameters, such as probe radius, taper length and refractive index of the environment. These theoretical results are compared with the experimental results obtained from in house fabricated combination taper fiber probes.

![Combination tapered fiber](image)

Figure 2.1. Combination tapered fiber. It has a linear tapered fiber sandwiched between a straight uncladded smaller diameter fiber (probe portion) and a cladded larger diameter fiber.

**Theory**

If we ignore the non-meridional rays, we can use a two-dimensional ray tracing model to simplify the problem. In an evanescent wave fiber optic based sensor, the evanescent wave generated on the fiber core is accessed by removing the cladding from a section of the fiber. Specific fluorophore labeled analytes are immobilized on the exposed
fiber core. These analytes are monitored by detecting the coupled back fluorescent signal.

The eventual fluorescent signal is the product of two processes. First is the excitation of fluorescence through the evanescent wave component of all the propagating rays inside the fiber. Second is the fraction of total emitted fluorescence coupling back into the detection end.

Removal of cladding, however, results in critical angle mismatch between the clad portion and the sensing portion of the fiber. Critical angle becomes larger for the cladded region than that of the sensing region, where an aqueous medium or air replaces the cladding. Critical angle $\alpha_c$ is defined in terms of the core refractive index $n_{co}$ and cladding refractive index $n_{cl}$ as

$$\alpha_c = \sin^{-1}\left(\frac{n_{cl}}{n_{co}}\right)$$

On the basis of geometric optics we can make an argument that out of all the fluorescence rays generated on the probe surface only those can get coupled back into the fiber, which make an angle $\alpha$ with the normal to the interface such that $\alpha_{c2} \leq \alpha \leq \pi / 2$, where $\alpha_{c2}$ is the critical angle in the uncladded probe part. When the fluorescence rays reach back in the cladded fiber zone, only rays satisfying the condition $\alpha_c \leq \alpha \leq \pi / 2$ can enter that zone and rest will be lost. Refractive index of cladding in the probe zone is taken as the refractive index $n_{aq}$ of the aqueous solution or air. Since $n_{aq} < n_{cl}$, therefore $\alpha_c > \alpha_{c2}$, where $\alpha_c$ is the critical angle in cladded zone. Now out of all the coupled back fluorescence rays in the probe zone, the rays making angle in the range
\( \alpha_{c_2} \leq \alpha \leq \alpha_c \) will be lost after reaching the cladded fiber zone. This observation indicates that the fluorescence coupling back efficiency can be improved if somehow the ray angle \( \alpha \) can be continuously increased as the ray propagate from the probe portion of fiber to the cladded or detection portion of fiber.

Another important factor responsible for generation of fluorescence signal in a fiber probe is the number of reflections (total internal) an excitation ray makes in the probe portion. Each such reflection generates evanescent field on the probe surface. Since signal is always proportional to the total strength of evanescent field per unit area, therefore larger the number of these reflections in probe zone better it is. If a ray makes an angle \( \theta \) with the fiber axis, the number of reflections per unit length \( f \), in a straight fiber with radius \( r \) can be given as:

\[
f = \frac{1}{r \cot \theta}
\]

This equation indicates that smaller the probe radius \( r \), better will be the signal.

Figure 2.2. Equivalent geometrical path of optical ray in a linear tapered part of combination
Ray Trajectory in a Tapered Fiber

In a linear tapered fiber with $\Gamma$ taper angle, propagation of a guided ray is shown in Figure 2.1. As a ray propagates from larger radius side to the smaller radius side, the angle $\theta$, between an incident ray and the fiber axis, increases by angle $\Gamma$ with each reflection. This property of a tapered fiber can be used to design a combination tapered fiber that has a linear tapered fiber sandwiched between a straight uncladded smaller diameter fiber (probe portion) and a cladded larger diameter fiber (Figure 2.1). Such a design has two advantages, first enhancement of the fluorescence coupling back efficiency and generation of more fluorescence due to increase in the number of total internal reflections.

For optimum signal the taper angle should be chosen in such a way that neither the excitation power nor the collected fluorescence power is lost during transmission. This condition can be satisfied if the value of $\Gamma$ is such that an excitation ray making a maximum angle $\theta = \theta_a$ at the entrance of the linear tapered fiber (larger radius side) could make an angle $\theta_c + \Gamma$ at the exit of the linear tapered fiber (smaller radius side). Where $\theta_a$ is the maximum acceptance angle in cladded part and $\theta_c$ is the complementary of the critical angle $\theta_c$ on the probe side. This condition is equivalent to saying that a fluorescence ray making an angle $\theta_c$ in the probe portion should make an angle $\theta_a$ in the cladded portion after transmitting through the linear taper part. The
optimum value of taper angle $\Gamma$ satisfying this condition can be obtained by drawing (Figure 2.2) an equivalent geometrical path (Snyder 1983) of optical ray between P and Q in Figure 2.1. The length of path between successive reflections and the angles it makes with the taper interface at P, R, S and Q in Figure 2.1 are identical to the corresponding values for the straight length PRSQ in Figure 2.2. Thus PQ makes angle $\theta_c + \Gamma$ with OQ, and geometry $OQ = ON = r_i / \tan(\Gamma)$, $OP = ON + l$. On applying the sine rule to triangle OQM, we can get following equation to compute the optimum value of taper angle $\Gamma$.

$$\frac{r_i \sin(\theta_c + \Gamma)}{r_o \sin(\theta_a)} = 1$$

(3)

where $r_0$ is the cladded fiber core radius and $r_i$ is the probe radius. The maximum acceptance angle $\theta_a$ of a fiber is given as

$$\theta_a = \sin^{-1} \left( \sqrt{\frac{n_{co}^2 - n_{cl}^2}{n_{co}^2}} \right)$$

(4)

The Eqn. 3 is slightly different than the one derived by Snyder et. al., (Snyder 1983) as we have assumed that the rays making maximum acceptance angle $\theta_a$, enters at the middle (black ray in Figure 2.1) of the linear taper face instead of entering near the perimeter of the tapered fiber (gray ray in Figure 2.1).

As per mode analysis argument (Marcuse 1988, Golden 1994, Nath 1998) the removal of cladding results in $V$ number mismatch between the clad portion and the sensing portion of the fiber. $V$ number is defined as

$$V = \frac{2\pi r_0}{\lambda} \sqrt{n_{co}^2 - n_{cl}^2}$$

(5)

where $\lambda$ is the wavelength of the propagating ray. Because of mismatch in the $V$
number a fraction of signal coupling into higher order modes in the sensing region is lost on entering the cladded fiber. This signal loss becomes substantial because fluorescent emission is predominantly coupled in the higher order modes ((Marcuse 1988). To avoid this loss an optimum ratio of the uncladded probe radius $r_i$ and cladded portion core radius $r_0$ will provide the V number matching. V-number match ratio is given as

$$\frac{r_i}{r_0} = \sqrt{\frac{n_{co}^2 - n_{el}^2}{n_{co}^2 - n_{aq}^2}} = \frac{\sin(\theta_o)}{\sin(\theta_c)}$$

(6)

If we compare Eqn. 6 to Eqn. 3, we can see that V-number matching condition can be satisfied when $\Gamma \ll \theta_c$. In air this condition is easier to satisfy than in any liquid as the complementary critical angle $\theta_c$ is much larger in air than in liquids. Eqn. 3 can be used to calculate optimum taper length $l_o$ for a given set of fiber radii $r_0$ and taper angle $\Gamma$

$$l_o = \frac{r_0 - r_{opt}}{\tan(\Gamma)}$$

(7)

where $r_{opt}$ is the optimum probe radius obtained with the help of Eqn. 3. It's relationship with taper angle is illustrated in Figure 2.3 (a) for silica fiber with core radius $r_0 = 300 \mu m$. Optimum taper lengths, were also computed for various values of taper angle in three different probe environments as shown in Figure 2.3 (b).

**Characteristics of Evanescent Wave**

When a ray of light undergoes total internal reflection at the interface of media with
different refractive indices, the transmitted beam ceases to exist and a standing wave is generated at the interface (Feynman 1997). The electric field of the standing wave decays exponentially in the media with lower refractive index as

$$ E = E_0 \exp(-\delta / d_p) $$

where $\delta$ is the distance from the interface and $d_p$ is the penetration depth which is given as,

$$ d_p = \frac{\lambda}{2\pi \sqrt{n_{co}^2 \sin^2 \alpha - n_{sl}^2}} $$

where $\alpha$ is the angle between incident ray and normal to the interface, $\lambda$ is the excitation laser wavelength, $n_{co}$ and $n_{sl}$ are the refractive indices of the fiber core and sample medium respectively.

Figure 2.3. Optimum probe radius and taper length in terms of taper angle.

**Absorption of Evanescent Power.** In an absorbing medium of thickness $dx$ the change in
absorbed power per unit area $dI$ is proportional to the absorption constant $\gamma$ and incident power per unit area $I$.

$$dI = -\gamma I dx$$ \hspace{1cm} (10)

Although Eqn. 10 is used for a propagating waves but it is also valid for evanescent waves and can be rewritten as

$$dI = -\gamma I \exp(-x/d_p) dx$$ \hspace{1cm} (11)

Total absorbed power per unit area $\Delta I$, of an evanescent wave in a uniform absorbing sample of thickness $\delta$ on the interface of two mediums can be obtained by solving Eqn. 11

$$\Delta I = I_o[1 - \exp(-2\gamma d_p[1 - \exp(-2\delta/d_p)])]$$ \hspace{1cm} (12)

where $I_o$ is the incident power per unit area, at the interface. For weak absorption, $\gamma d_p \ll 1$ and above equation can be written as

$$\Delta I \approx 2I_o\gamma d_p[1 - \exp(-2\delta/d_p)]$$ \hspace{1cm} (13)

Fluorescence Signal from Combination Fiber

In a fiber probe total detected fluorescence signal $S$ is proportional to the product of total absorbed evanescent wave power $P_{abs}$ and total fluorescence coupling back efficiency $\eta_f$.

$$S \propto \eta_f \times P_{abs}$$ \hspace{1cm} (14)

Evanescent Absorption. If a laser beam is focussed on the input face of a fiber, the
power \( P_{\text{ray}}(\theta) \) carried by rays between angle \( \theta \) and \( \theta + d\theta \) is given as
\[
P_{\text{ray}}(\theta) = \frac{2P_0 \tan(\theta) \sec^2(\theta)d\theta}{\tan^2(\theta_a)}
\]
(15)

where \( P_0 \) is the total incident laser power on the fiber surface and \( \theta_a \) is the maximum acceptance angle of the cladded fiber portion. Love et al.\(^1\) have shown that during single reflection, the absorbed evanescent power \( dP_a \) of all the rays, making an angle between \( \theta \) and \( \theta + d\theta \) with the central axis of a fiber, in a uniform absorbing medium of thickness \( \delta \) on the probe surface is given by
\[
dP_a \propto \Delta P_{\text{ray}}(\theta) \frac{n_{\text{rel}}}{n_{\text{rel}}^2 - 1} \left[ 1 + \frac{2n_{\text{rel}} \cos^2(\theta) - 1}{(n_{\text{rel}}^2 + 1) \cos^2(\theta) - 1} \right]
\]
(16)

where \( n_{\text{rel}} \) is equal to \( n_{\text{co}} / n_{\text{aq}} \), \( \Delta P_{\text{ray}}(\theta) \) is power lost to absorbing medium of thickness \( \delta \), by all the rays making an angle between \( \theta \) and \( \theta + d\theta \) with the central axis. As per Eqn. 13 and Eqn. 15 \( \Delta P_{\text{ray}}(\theta) \) can be given as
\[
\Delta P_{\text{ray}}(\theta) = P_{\text{ray}}(\theta)n_{\text{co}} \sin^2(\theta)\gamma d_p[1 - \exp(-2\delta / d_p)]
\]
(17)

If \( f \) is the number of reflections per unit length by a ray making angle \( \theta \) with the fiber axis and \( L \) is the probe length, the total absorbed evanescent power on the probe surface can be given as
\[
\frac{P_{\text{abs}}}{P_0} \propto \frac{P_0}{\tan^2(\theta_a)}
\]
\[
\int_{\theta_{\text{max}}}^{\theta_{\text{max}}} \int_0^L f \tan(\theta) \sec^2(\theta)n_{\text{co}} \sin^2(\theta)\gamma d_p[1 - \exp(-2\delta / d_p)] \frac{n_{\text{rel}}}{n_{\text{rel}}^2 - 1} \left[ 1 + \frac{2n_{\text{rel}} \cos^2(\theta) - 1}{(n_{\text{rel}}^2 + 1) \cos^2(\theta) - 1} \right] d\theta dL
\]
(18)
where $\theta_{\text{max}}$ the maximum possible angle an excitation ray can make in the probe fiber.

By substituting the value of $f$ from Eqn. 2 we get

$$p_{\text{abs}} \propto \frac{P_0 L}{\tan^2 \theta_a r_1}$$

$$\int_0^{\theta_{\text{max}}} \tan \theta \tan \theta \sec^2 \phi \eta_{\text{ex}} \sin^2(\theta) \gamma d\rho [1 - \exp(-2\delta / d_{\rho})] \frac{n_{\text{rel}}}{n_{\text{rel}}^2 - 1} \left[ 1 + \frac{2n_{\text{rel}} \cos^2 \theta - 1}{(n_{\text{rel}}^2 + 1) \cos^2 \theta - 1} \right] d\theta$$

(19)

where $r_1$ is the probe radius. In a combination fiber total excitation power $P_0$ delivered to the probe part, can be divided in two parts. One fraction $\eta_1$ is delivered by the linear taper part and other fraction $\eta_2$ is the one directly reaching the probe entrance without going through any reflections in the linear taper part. If $l$ is the taper length, we can assume that $\eta_1$ and $\eta_2$ are proportional to the effective fractional area of taper part and probe fiber at the entrance of the probe fiber and are given as

$$\eta_1 = \frac{(l \tan(\theta_a) + r_0)^2 - r_1^2}{(l \tan(\theta_a) + r_0)^2}$$

(20)

$$\eta_2 = \frac{r_1^2}{(l \tan(\theta_a) + r_0)^2}$$

(21)

The value of $\theta_{\text{max}}$ is not same for both parts of delivered power therefore Eqn. 19 can be written as a sum of two integrals. For probe part of integral, $\theta_{\text{max}} = \theta_a$. For linear taper part the value $\theta_{\text{max}}$ can be computed with the help of linear taper Eqn. 3. Consider a pump ray making maximum acceptance angle $\theta_a$ in cladded part, as long as the taper geometry and probe radius are fixed, the corresponding output angle $\theta_{\text{out}}$ at the lower radius end of taper can be computed from rewritten Eqn. 3 by substituting the given value.
of \( \Gamma \), and replacing \( \theta_c \) with \( \theta_{\text{out}} \).

\[
\theta_{\text{out}} = \sin^{-1}\left[ \frac{r_0 \sin(\theta_c)}{r_i} \right] - \Gamma 
\]  

(22)

Once the value of \( \theta_{\text{out}} \) is known we can set the value of \( \theta_{\text{max}} \) as

\[
\theta_{\text{max}} = \begin{cases} 
0 & \text{if } \theta_{\text{out}} < 0 \\
\theta_{\text{out}} & \text{if } 0 \leq \theta_{\text{out}} \leq \theta_c \\
\theta_c & \text{if } \theta_{\text{out}} \geq \theta_c 
\end{cases}
\]  

(23)

Eqn. 19 can be rewritten as

\[
P_{\text{abs}} \propto \frac{P_0 L}{r_1}
\]

\[
\left( \frac{\eta_1}{\tan^2 \theta_{\text{out}}} \right) \int_0^{\theta_{\text{max}}} \tan^2 \theta \sec^2 \theta n_{\text{co}} \sin^2(\theta) \gamma d_p [1 - \exp(-2\delta / d_p)] \frac{n_{\text{rel}}}{n_{\text{rel}}^2 - 1} \left[ 1 + \frac{2n_{\text{rel}} \cos^2 \theta - 1}{(n_{\text{rel}}^2 + 1) \cos^2 \theta - 1} \right] d\theta 
\]

\[
+ \frac{\eta_2}{\tan^2 \theta_{\text{out}}} \int_0^{\theta_c} \tan^2 \theta \sec^2 \theta n_{\text{co}} \sin^2(\theta) \gamma d_p [1 - \exp(-2\delta / d_p)] \frac{n_{\text{rel}}}{n_{\text{rel}}^2 - 1} \left[ 1 + \frac{2n_{\text{rel}} \cos^2 \theta - 1}{(n_{\text{rel}}^2 + 1) \cos^2 \theta - 1} \right] d\theta
\]  

(24)

Normalization factor \( 1 / \tan^2(\theta_{\text{out}}) \) in the first integral of Eqn. 24 is replaced with \( 1 / \tan^2(\theta_{\text{max}}) \) as the total integral range is taken to be \( \theta_{\text{max}} \).

**Fluorescence Coupling Back Efficiency.** Love et al. (Love 1992) have shown that fluorescence coupling back efficiency follows the law of reciprocity, therefore \( \eta_f \) is given as

\[
\eta_f = \int_0^{\theta_{\text{max}}} n_{\text{aq}} \gamma d_p [1 - \exp(-2\delta / d_p)] \frac{\sin^2(\theta)n_{\text{rel}}^3}{n_{\text{rel}}^2 - 1} \left[ 1 + \frac{2n_{\text{rel}} \cos^2 \theta - 1}{(n_{\text{rel}}^2 + 1) \cos^2 \theta - 1} \right] d\theta
\]  

(25)

where the value of \( \theta_{\text{max}} \) can be computed again with the help of linear taper Eqn. 3.
Consider a fluorescence ray making maximum complimentary critical angle \( \theta_c \) in probe fiber, as long as the taper geometry and probe radius are fixed, the corresponding input angle \( \theta_{in} \) at the larger radius end of the linear taper can be computed from rewritten Eqn. 3 by substituting the given value of \( \Gamma \), and replacing \( \theta_a \) with \( \theta_{in} \).

Once the value of \( \theta_{in} \) is known we can set the value of \( \theta_{max} \) as

\[
\theta_{max} = \begin{cases} 
\theta_c & \text{if } \theta_{in} \leq \theta_{out} \\
\theta_{out} & \text{if } \theta_{in} > \theta_{out}
\end{cases}
\]  

(26)

Experiments

**Probe Preparation**

Each probe was an 8 cm long 600 \( \mu \)m core multimode optical fibers (Ocean Optics Inc.). Approximately 1.5 cm of protective polyimide buffer surrounding the fiber was removed from one end by burning it with bunsen burner. The fiber was then decontaminated by sonicating it in a soap solution. This was followed by sonicating the fiber in a solution of de-ionized water to get rid of any carbon soot on the surface of the fiber. The cladding of probe part was removed by immersing the 1.5 cm uncoated part into 5% hydrofluoric acid solution. Probes of various diameters were obtained by adjusting the time duration of immersion. Tapered part between the etched probe and cladded fiber was obtained by capillary action. Some acid capillarily ascend into the space between fiber probe and polyimide buffer. Tapered angle obtained by this method was found to be nearly constant for all the probes. After taking out of the hydrofluoric
acid, the probes were sonicated for four minutes each in deionized water and then in acetone. The taper angle for each probe was measured using a microscope. The average taper angle was found to be 0.056 ± 0.004 rad. A photograph of a typical combination fiber prepared in our lab is shown in Figure 2.4. The taper lengths varied from 1.2 mm to 4.6 mm as the probe radius changed from 226 μm to 50 μm. The etched part of each probe was sensitized by immersing it in 2% APTS solution (Dry acetone as solvent) for 1 minute. The sanitized probes were then washed in acetone. To coat a fluorescence dye layer on probe surface, the sanitized probes were kept in 10 nM Alex Fluor 488 solution (DMSO as solvent) for 1 hour. Then the probes were rinsed with DMSO and distilled water. After drying, the probes were ready to record evanescent wave induced fluorescence signal.

Figure 2.4. A photograph of a typical combination fiber.

*Experimental Setup*
A schematic of our experimental setup is shown in Figure 2.5. A 476 nm laser diode (Nichia, Japan) was used for excitation. A dichroic band pass filter (476 nm, band width 10 nm) was placed in front of ethanol diode laser to block any red tail emission. The diode light passes through a dichroic beam combiner/splitter. The beam combiner/splitter is a short pass filter with high transmission (90%) for the excitation wavelength at 476 nm and high reflectivity (95%) at wavelengths longer than 488 nm. The peak emission wavelength for Alexa 488 dye is around 530 nm. Laser output is a collimated beam and a short focal length lens is used to focus the laser beam into a 600 μm core probe fiber. The evanescent wave induced fluorescence from the probe surface couples back into the probe fiber and gets transmitted into the collection fiber of a miniature charged coupled device (CCD) based fiber-optic spectrometer (Ocean Optics Inc., model HR2000). To further improve the signal to noise ratio, unwanted scattered light from diode laser was blocked by placing a razor edge 488 nm cut off long pass filter (Edmund Optics Inc) in front of the collection fiber. This filter has about 95% transmission for all the wavelengths longer than 500 nm but extremely small transmission (10–6%) for wavelengths shorter than 500 nm. The signal from the spectrometer is coupled to a computer (DELL). All the spectra were collected with the help of this computer.

Results and Discussion

Probes of 10 different radii from 50μm to 160μm were prepared. Signal for each
probe was recorded in air, water and ethyl alcohol with refractive index 1.0, 1.333 and 1.36 respectively. To further improve the signal to noise ratio, the signal for each probe was obtained by computing the area under the recorded spectrum curve. It was found that the observed signal in air reduce by 20-40% when the probe end surface just touch the water or ethyl alcohol surface, although the full probe length of 1.5 cm was still in air. This observation was valid for probes of all radii and one plausible explanation for such observation could be that when the launched light reaches the probe end, it is reflected by the medium interface. The reflected excitation light will further increase the fluorescence signal. When air is replaced by water or ethyl alcohol, the Fresnel reflection from the interface will reduce thus the observed reduction in the signal. But the computed change in signal by using Fresnel equations show that the contribution of this reflection from a plane interface will be quite smaller than the observed change. Since our probe end surface was not a polished surface therefore the back reflection from the rough probe surface is almost two to three times more. For comparison of results obtained in air with those in water and ethyl alcohol, the signal recorded in air was corrected for the experimentally measured scattering factor.
Figure 2.5 Experimental setup. LD- Laser Diode, CL- Collimation Lens, BF- Band-pass Filter, ND- Neutral Density filter, LF -Long-pass Filter and SF- Short-pass Filter.

Figure 2.6. Variation of fluorescence signal with probe radius. A comparison of simulation and experimental result.
Variation of experimentally recorded fluorescence signal in air, water and ethyl alcohol as a function of probe radii is shown in Figure 2.6 (scattered points). Simulated fluorescence signal for different probe radii were also generated. The other parameters used in this simulation are fiber core radius \( r_0 = 301\mu\text{m} \), fiber core refractive index 1.46, numerical aperture of fiber 0.22, thickness of absorbing medium on probe surface 0.1 \( \lambda \) (\( \lambda \) is the excitation laser wavelength), the length of straight probe part was 8 mm (sensitized region), and the value of taper angle was 0.056 rad. All these probe parameters were the actual parameters of the probes used for experimental signal recording. The variation of normalized simulated fluorescence signal in air, water and ethyl alcohol as a function of probe radii is also shown in Figure 2.6 (solid lines). It can be seen that the experimentally recorded signals are quite comparable to the simulated signal.

Conclusions

The results obtained by the simulation are in agreement with experimentally obtained results. For a given realistic taper length, the maximum signals are obtained at probe radius smaller than V-number matching radius. In practical conditions it seems total utilization of excitation light from cladding part to probe zone of a fiber sensor is difficult to achieve in aqueous environment. Theoretical studies show that taper angle or taper length, and probing environment play important roles in signal acquisition besides
probe radius. Agreement of simulated results with experimental results indicate that the developed model can provide optimum probe parameters for designing sensor probes in a given detection environment.


CHAPTER 3
INTRODUCTION

Backgorund of Magnetic Nanoparticles Usage

Magnetic microspheres and nanoparticles have been used for a variety of applications and have even been incorporated into medical diagnostic techniques (Haukanes 1993, Olsvik 1994, Pankhurst 2003, Gijs 2004). Utilizing magnetic particles coated with antibody to separate target molecules is a widely used separation method (huang-hao yang and Wang 2004). Besides this, there are a few novel methods proposed recently aiming at biomolecule detection with magnetic particles.

Weitschies etc. (Weitschies 1997) proposed a novel magnetic relaxation/remanence immunoassay (MARIA) using a superconducting quantum interference device (SQUID) (Koelle 1999) as a magnetic field sensor. In the technique, an immobilized target is immersed in a suspension of superparamagnetic nanoparticles bound to antibodies specific to that target. A pulsed external magnetic field is applied to align the dipole moments of the particles. The SQUID detects the magnetic field from the particles bound to the target. In the presence of this aligning field the nanoparticles develop a net magnetization, which relaxes when the field is tuned off. Unbound nanoparticles relax rapidly by Brownian rotation and contribute no measurable signal. Nanoparticles that are
bound to the target on the film are immobilized and undergo Neel relaxation\textsuperscript{2}, producing a slowly decaying magnetic flux, which is detected by the SQUID. (Y. R. Chemla 2000)

McNaughton etc. (Brandon H. McNaughtona 2007; McNaughtona 2007) make use of shifts in the nonlinear rotational frequency of magnetic microspheres, driven by an external magnetic field, developed an approach for the detection of single bacterial cells. At low frequency of the rotating magnetic field, magnetic particles rotates continuously and synchronously (linear response regime) with the external field. At sufficiently high external driving frequencies, the particle becomes asynchronous (nonlinear) with the driving field. When a bacterium attaches to a nonlinearly rotating magnetic microsphere, the volume and shape of the rotating system are drastically changed, increasing the drag and thus slowing the rotation rate. The rotational process can be observe and recorded by standard microscopy equipments.

An interesting method to track the rotation of nano- or microparticles worth mentioning is the ‘MOONs’ method introduced by Behrend etc (CalebJ. Behrend1 2005). Coating nanospheres with a metal hemisphere shell breaks the particles’s optical symmetry, allowing its orientation to be tracked using fluorescence and reflection.

\textsuperscript{2} The anisotropy energy barrier of the particle, $E$, which is proportional to its volume, inhibits the dipole moment from rotating but may be overcome with sufficient thermal energy $k_B T$ ($T$ is the temperature and $k_B$ is Boltzmann’s constant). Thus, Neel relaxation occurs on a time scale $\tau_N = \tau_0 e^{E/k_BT}$, which depends exponentially on the particle volume. In addition to Neel relaxation, nanoparticles in suspension undergo Brownian rotation, which randomizes the orientation of the dipole moments. Typically, for an ideal single-domain, 20-nm magnetic particle $\tau_N \sim 1s$ and $\tau_B \sim 1\mu s$. 
Fluorescence Polarization Theory

Fluorescence polarization (FP) was first theoretically described by Perrin in 1926 (Perrin 1926); this description was subsequently developed both in theory and experiment. FP was developed for use in analytical biochemistry, including antigen (Ag)-antibody (Ab) interactions (Dandliker 1973) and hormone-receptor interactions (Levison 1976). Quantitative and qualitative measurement of various types of molecules and bioconjugates has been reported (Wei 1993, Sipior, 1997). In fact, FPI technology has been applied to several commercially available instruments.

\[
\phi = 50\text{ns} \quad \text{IgG} \quad \phi = 150\text{ns}
\]

Figure 3.1. Intuitive illustration of a Fluorescence polarization immunoassay. \(\phi\) is the rotation correlation time.

A FPI requires that emission from the unbound labeled antigen be depolarized, so that an increase in polarization may be observed upon antigen binding to antibody. For
depolarization to occur, the antigen must display a rotational correlation time much shorter than the lifetime of the probe. The fluorescence life times of various types of fluorophore ranges from a few nanoseconds to a few microseconds. Among various types of dyes, long-lifetime fluorophores have an advantage in monitoring binding interaction of high-molecular-weight antigens, because long-lifetime fluorophores can make the fluorescence of the labeled high-molecular-weight antigens more depolarized compared to short lifetime fluorophores. Therefore they are more suitable to be used in FPI except some fluorophores like Eu$^{3+}$ and Tb$^{3+}$ that do not show polarized emission. An intuitive graph is depicted in Figure 3.1 which illustrates this process.

The fluorescence polarization ($P$) of a labeled macromolecule depends on the fluorescence lifetime ($\tau$) and the rotational correlation time ($\phi$):

$$\frac{1}{P} - \frac{1}{3} = \frac{1}{P_0} - \frac{1}{3} (1 + \frac{\tau}{\phi}),$$  \hspace{1cm} (27)

where $P_0$ is the polarization observed in the absence of rotational diffusion. The rotational correlation time is related to molecular weight ($M_r$) and the molecular volume of the protein by

$$\phi = \frac{\eta M_r}{RT} (\bar{v} + h),$$ \hspace{1cm} (28)

where $R$ is the ideal gas constant, $\bar{v}$ is the specific volume of the protein, and $h$ is the hydration. In aqueous solution at 20 °C ($\eta = 1$ cP)$^3$, one can expect a protein such as

---

$^3$ The SI physical unit of dynamic viscosity is the pascal-second (Pa·s), which is identical to 1 kg·m$^{-1}$·s$^{-1}$. The cgs physical unit for dynamic viscosity is the poise (P), named after Jean Louis Marie Poiseuille. It is
HSA ($M_r \approx 65000$, with $\nu + h = 1.9$) to display a rotational correlation time near 50 ns.

Polarization is related to the transitional dipole direction of molecules, so reflects the spatial orientation and rotation dynamics of fluorescent molecules. Another concept fluorescence anisotropy (FA) is defined as (Lakowicz, 1999)

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}, \quad (29)$$

where $I_\parallel$ and $I_\perp$ are fluorescence intensities with polarization parallel and perpendicular to the excitation polarization, respectively. $I_\parallel + 2I_\perp$ is the total fluorescence intensity.

Since $P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp}$, the anisotropy and polarization are related by $r = \frac{2P}{3-P}$, $P = \frac{3r}{2+r}$.

The values of $P$ are more often used in FPI because they are entrenched by tradition and are slightly larger than the anisotropy values. The parameter $r$ is preferred on the basis of theory.

FA can be used to study the rotation diffusion of molecules, which is related to the size of the molecules and their interactions with the surrounding. In biological studies, FA measurements have been used to study conformational changes and torsional motion of the biomolecules and molecular binding interactions (Tramier 2000, Canet 2001).

After being excited by linearly polarized light, polarization of freely rotating

---

more commonly expressed, particularly in ASTM standards, as centipoise (cP). The centipoise is commonly used because water has a viscosity of 1.0020 cP (at 20 °C; the closeness to one is a convenient coincidence). $1 \text{P} = 1 \text{g \cdot cm}^{-1} \cdot \text{s}^{-1}$. The relation between poise and pascal-seconds is: $10 \text{P} = 1 \text{kg \cdot m}^{-1} \cdot \text{s}^{-1} = 1 \text{Pa \cdot s}$; $1 \text{cP} = 0.001 \text{Pa \cdot s} = 1 \text{mPa \cdot s}$
fluorescent molecules decays exponentially during the lifetime of the excited state. When the polarization of fluorescence can be characterized by fluorescence anisotropy (FA) as defined in Equ. 29, the temporal average of the time dependent FA function, $r$, is related to the fluorescence lifetime of the molecules $\tau$. (Dandliker 1973, Terpetschnig 1995)

$$r = \frac{r_0}{1 + \frac{\tau}{\phi}}, \quad (30)$$

where $r_0$ is the maximum FA without rotational diffusion and $\phi$ is the rotational correlation time of molecules. If the correlation time is much larger than the lifetime ($\phi \gg \tau$), then the measured anisotropy ($r$) is equal to the fundamental anisotropy ($r_0$). If the correlation time is much shorter than the lifetime ($\phi \ll \tau$), then the anisotropy is zero. $r_0$ is typically near 0.3 for most fluorophores, although the theoretical limit given collinear transition dipoles for absorption and emission is 0.4.

The expression of $r$ is a form of Perrin equation. It can be derived from first principle based on diffusion steps (Weber 1966). The derivation of this equation begins with the time-resolved decay of anisotropy $r(t)$ for a spherical molecule:

$$r(t) = r_0 e^{-t/\phi} = r_0 e^{-6D/t}, \quad (31)$$

the rotational correlation time is related to the rotational diffusion coefficient $\phi = (6D)^{-1}$. Only spherical molecules display single exponential anisotropy decay. Steady-anisotropy can be calculated from an average of the anisotropy decay by doing integration over time, which yields the Perrin equation.

According to Stokes-Einstein-Debye equation
\[ \phi = \frac{\eta V}{kT}, \tag{32} \]

where \( \eta \) is the viscosity of the solution; \( V \) is the volume of the molecule; \( T \) is the temperature; \( k \) is the Boltzmann constant. \( \phi \) is a parameter that reflects the rotational mobility of molecules.

The polarized excitation can also be realized using multi-photons excitation (Gryczynski 1995), but it is not the issue that will be studied in this thesis.

Some Explanations of Anisotropy Theory

Calculation of Rotational Correlation Time of Proteins

For globular proteins the rotational correlation time is approximately related to the molecular weight (\( M \)) of the protein by

\[ \phi = \frac{\eta V}{RT} = \frac{\eta M}{RT} (\bar{v} + h) \tag{33} \]

where \( V \) is the molar volume after hydration, \( \bar{v} \) is the specific volume of the protein, and \( h \) is the hydration, \( T \) is the temperature in \( \degree K \), \( R = 8.31 \times 10^7 \) erg/mol\( \degree K \), and the viscosity \( \eta \) is in poise (P). Values of \( h \) for proteins are typically near 0.73 ml/g, and the hydration is near 0.23 g \( \text{H}_2\text{O} \) per gram of protein. This expression predicts that the correlation time of a hydrated protein is about 30% larger than that expected for an anhydrous sphere. Generally, the observed values of rotational correlation time are about twice that expected for an anhydrous sphere (Yguerabide, 1970).

For example, for anhydrous protein sphere with a 10 kD molecular weight, with
\( h = 0 \text{ml/g} \) and \( \eta = 1 \text{cP} = 0.01 \text{P}, \ \bar{v} = 0.75 \text{ml/g}, \) then the value of rotational correlation time can be calculated as

\[
\phi \approx \frac{0.01 \times 10 \times 10^3 \text{g/mol} \times 0.75 \text{ml/g}}{8.31 \times 10^7 \text{erg/(mol} \circ \text{K}) \times 293 \circ \text{K}} \approx 3.1 \text{ns}
\]

The expected anisotropy values for a range of photoluminescence lifetimes are simulated in Figure 3.2. The calculations were based on the assumption that the limiting anisotropy \( r_0 = 0.3 \) in the absence of rotational diffusion, the solution viscosity was 1 cP, and \( \bar{v} + h = 1.9 \text{ml/g} \) for the protein.

![Figure 3.2](image_url)

**Figure 3.2.** Molecular-weight-dependent anisotropy for a protein-bound luminophore with luminescence lifetimes of 5, 50, 1000, and 3000 ns. The curves are based on assumption that the aqueous solution is at 20 \( ^\circ \text{C} \) with a viscosity of 1 cP and \( \bar{v} + h = 1.9 \text{ml/g} \).
Why Is the Total Intensity Equal to $I_\parallel + 2I_\perp$?

For any fluorophore the total intensity is equal to the sum of the intensities along the three axes: $I_T = I_x + I_y + I_z$. Each intensity is given by the square of the transition moment projection on each axis. According to the definition of anisotropy, it is equal to the ratio of the polarized component to the total intensity ($I_T$):

$$r = \frac{I_z - I_y}{I_x + I_y + I_z} = \frac{I_z - I_y}{I_T}$$

When the excitation is polarized along the z-axis, emission from the fluorophores is symmetric around the z-axis (Figure 3.3). Hence $I_x = I_y$. Recalling $I_y = I_\perp$ and $I_z = I_\parallel$, thus $I_T = I_\parallel + 2I_\perp$. Notice that a dipole oriented along z-axis does not radiate along the axis, and cannot be observed with a detector located on this axis (Figure 3.4).

Figure 3.3. Polarization of Fluorescence.
CHAPTER 4

NOVEL IDEA INTRODUCED TO FPI

Before Terpetschniq etc. discovered a Ruthenium complex dye that displays polarized emission and has a lifetime of about 400 ns, a limitation exists in the immunoassay using FPI. The lifetimes of Fluorophores used were usually very short. For example, fluorescein, displays a lifetime near 4 ns. A FPI requires that emission from the unbound labeled antigen be depolarized, so that an increase in polarization may be observed upon antigen binding to antibody. For depolarization to occur, the antigen must display a rotational correlation time much shorter than the lifetime of the probe. Short fluorophore life time will limit the dynamic range of FPI for antigens with low molecular weights (Figure 3.2)

Guo etc. (Guo, Castellano et al. 1998) developed a FPI based on luminescence from
a Re(I) metal-ligand complex which has a lifetime of 2.7 μs. This long-lifetime dye enhanced the potential of FPI to detect antigens with molecular weight above $10^7$ Daltons in theory.

While long-lifetime dye provides a possibility to FPI measurement for large molecular weight antigens, for very large molecular weight antigens, above $10^6$ Daltons for example, the small dynamic range problem still exist, and the same problem with the detection of very small molecular weight proteins. After antibody binding of small molecular weight protein, the total weight of antigen and antibody usually do not exceed $10^6$ Daltons, and the eventual anisotropy will not be greater than 0.1 with fluorophore lifetime of 3 μs according to Figure 3.2., which is a theoretical simulation and the detailed procedures are described later.

To solve the above problems, a novel method is proposed in this study that uses antibody coated magnetic nanoparticles instead of antibody to do the FPI measurements. Since the nanoparticles usually consist of iron oxide, each particle has a much greater weight than a single antibody. When they are coated with antibody, and the immunoreaction take place, the binding will generate a dragging effect and significantly change the rotation correlation time of antigens. By FPI measurement, the utilization of magnetic nanoparticles can result in a more appreciable anisotropy change. Further more, by applying a DC magnetic field on the sample, the magnetic dipole moments of nanoparticles are aligned in the same direction, inhibiting thermal agitation and rotation, thus can further enhance the anisotropy change. Even when the dynamic range of
anisotropy change has already been small for the antigens with molecular weight greater than a certain level, the anisotropy change can be modulated using a step-down magnetic field with an appropriate frequency. The modulated anisotropy change, though small and may not be appreciable, can be picked out by lock-in amplifier.

The advantages of this FPI method using antibody-coated magnetic nanoparticles are that it becomes feasible to perform FPI on a wide range of antigens with different molecular weights using a single type of fluorophore, and enhance the dynamic range of anisotropy change, which results in more precise immunoassay.

The magnetic particles used should be superparamagnetic particles. Compare to paramagnetic particles, superparamagnetic particles usually have smaller dimensions. Because of the small scale of superparamagnetic particles, the ambient thermal energy is sufficient to change the direction of magnetic moment of a particle. The random rotation of particles will cause a certain extent of depolarization of fluorescence, which is an essential condition in order to observe the anisotropy change after applying the magnetic field.
CHAPTER 5

KNOWLEDGE OF MAGNETISM FOR DETERMINING SUITABLE NANOPARTICLES TO BE USED

It would be useful to clarify some concepts in magnetism before getting into the part of type selection of magnetic particles.

Definitions and Units

There are three magnetic vectors: H, Magnetic field; M, Magnetization; B, Magnetic induction.

A magnetic field H will be produced at the center of a current loop given by

\[ H = \frac{i}{2r} \, \text{[Amperes/meter, A/m]} \]

The current loop has a magnetic moment, m, associated with it

\[ m = i \times \text{Area} \, \text{[Am}^2\text{]} \]

The intensity of magnetization, M, is magnetic moment per unit volume

\[ M = m / V \, \text{[A/m]} \]

Susceptibility is the ratio of magnetization to magnetic field, \( k = M / H \) [dimensionless].

In SI system, the relationship between B, H and M is given by \( B = \mu_0 (H + M) \) [Tesla, T]. The constant \( \mu_0 \) is called the permeability of free space. In SI it is equal to \( 4\pi \times 10^{-7} \) Henry/m.
In CGS, $\mu_0$ is set equal to unity, which makes the relation among the three vectors to be $B = H + 4\pi M$, but each have a different unit name, i.e. Gauss for $B$, Oersted for $H$, and emu/cm$^3$ for $M$.

Some units conversions between SI and CGS are listed in the Table 5-1. It should be noted that when talking about a magnetic “field” of 100 milliTesla (mT) in some cases that are not very rigorous, it really means $\mu_0 H = 100\text{mT}$.

<table>
<thead>
<tr>
<th>Magnetic Term</th>
<th>Symbol</th>
<th>SI unit</th>
<th>CGS unit</th>
<th>conversion factor</th>
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</thead>
<tbody>
<tr>
<td>magnetic induction</td>
<td>B</td>
<td>Tesla (T)</td>
<td>Gauss (G)</td>
<td>$1 \text{T} = 10^4 \text{G}$</td>
</tr>
<tr>
<td>magnetic field</td>
<td>H</td>
<td>A/m</td>
<td>Oersted (Oe)</td>
<td>$1 \text{A/m} = 4\pi/10^3 \text{Oe}$</td>
</tr>
<tr>
<td>magnetization</td>
<td>M</td>
<td>A/m</td>
<td>emu/cm$^3$</td>
<td>$1 \text{A/m} = 10^3 \text{emu/cm}^3$</td>
</tr>
<tr>
<td>magnetic moment</td>
<td>m</td>
<td>Am$^2$</td>
<td>emu</td>
<td>$1 \text{Am}^2 = 10^3 \text{emu}$</td>
</tr>
<tr>
<td>permeability of free space</td>
<td>$\mu_0$</td>
<td>H/m</td>
<td>dimensionless</td>
<td>$4\pi\times10^{-7} \text{H/m} = 1 \text{(cgs)}$</td>
</tr>
</tbody>
</table>

Classes of Magnetic Materials

The origin of magnetism lies in the orbital and spin motions of electrons and how the electrons interact with one another. It can be classified by different ways materials respond to magnetic fields.

The five major groups are diamagnetism, paramagnetism, ferromagnetism, ferrimagnetism, and antiferromagnetism. Materials in the first two groups are those that exhibit no collective magnetic interactions and are not magnetically ordered. Materials in the last three groups exhibit long-range magnetic order below a certain critical
temperature. Besides the above five categories, a particular magnetism-superparamagnetism interests us most, because only magnetic particles in nano-scale have the possibility of showing superparamagnetic characteristics.

Diamagnetism

Diamagnetism is a fundamental property of all matter, although it is usually very weak. It is due to the non-cooperative behavior of orbiting electrons when exposed to an applied magnetic field. Diamagnetic substances are composed of atoms which have no net magnetic moments (i.e., all the orbital shells are filled and there are no unpaired electrons).

Paramagnetism

As for this class of materials, some of the atoms or ions in the material have a net magnetic moment due to unpaired electrons in partially filled orbitals. However, the individual magnetic moments do not interact magnetically, and like diamagnetism, the magnetization is zero when the field is removed. In the presence of a field, there is now a partial alignment of the atomic magnetic moments in the direction of the field, resulting in a net positive magnetization and positive susceptibility.

In addition, the efficiency of the field in aligning the moments is opposed by the randomizing effects of temperature. This results in a temperature dependent susceptibility,
known as the Curie Law.\footnote{Curie's law For low levels of magnetisation, the magnetisation of paramagnets follows Curie's law to good approximation: $M = \chi \cdot B = C \cdot \frac{B}{T}$, where $M$ is the resulting magnetization, $B$ is the magnetic flux density of the applied field, measured in teslas, $T$ is absolute temperature, measured in kelvins, $C$ is a material-specific Curie constant}

At normal temperatures and in moderate fields, the paramagnetic susceptibility is small (but larger than the diamagnetic contribution). Unless the temperature is very low (\(<\ll 100 \text{ K}\)) or the field is very high paramagnetic susceptibility is independent of the applied field. Under these conditions, paramagnetic susceptibility is proportional to the total iron content.

\textit{Ferromagnetism}

Unlike paramagnetic materials, the atomic moments in ferromagnetic materials exhibit very strong interactions. These interactions are produced by electronic exchange forces and result in a parallel or antiparallel alignment of atomic moments. Exchange forces are very large, equivalent to a field on the order of 1000 Tesla, or approximately a 100 million times the strength of the earth's field.

The exchange force is a quantum mechanical phenomenon due to the relative orientation of the spins of two electrons.

Ferromagnetic materials exhibit parallel alignment of moments resulting in large net magnetization even in the absence of a magnetic field. The elements Fe, Ni, and Co and many of their alloys are typical ferromagnetic materials.
There is a big difference between paramagnetic and ferromagnetic susceptibility. As compared to paramagnetic materials, the magnetization in ferromagnetic materials is saturated in moderate magnetic fields and at high (room-temperature) temperatures:

Table 5-2 Differences between paramagnetism and ferromagnetism.

<table>
<thead>
<tr>
<th></th>
<th>$H_{\text{sat}}$ (T)</th>
<th>$T$ range (K)</th>
<th>$\chi\ 10^{-6}$m$^3$/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>paramagnetism</td>
<td>&gt;10</td>
<td>&lt;&lt;100</td>
<td>~50</td>
</tr>
<tr>
<td>ferromagnets</td>
<td>~1</td>
<td>~300</td>
<td>1000-10000</td>
</tr>
</tbody>
</table>

**Ferrimagnetism**

In ionic compounds, such as oxides, more complex forms of magnetic ordering can occur as a result of the crystal structure. One type of magnetic ordering is called ferrimagnetism. The magnetic structure is composed of two magnetic sublattices (called A and B) separated by oxygens. The exchange interactions are mediated by the oxygen anions. When this happens, the interactions are called indirect or superexchange interactions. The strongest superexchange interactions result in an antiparallel alignment of spins between the A and B sublattice. In ferrimagnets, the magnetic moments of the A and B sublattices are not equal and result in a net magnetic moment. Ferrimagnetism is therefore similar to ferromagnetism.

**Antiferromagnetism**
If the A and B sublattice moments are exactly equal but opposite, the net moment is zero. This type of magnetic ordering is called antiferromagnetism.

Superparamagnetism.

There are materials that show induced magnetic behavior that follows a Curie type law but with exceptionally large values for the Curie constants. These materials are known as superparamagnets. They are characterized by a strong ferro- or ferrimagnetic type of coupling into domains of a limited size that behave independently from one another.

Superparamagnetism is a small length-scale phenomenon, where the energy required to change the direction of the magnetic moment of a particle is comparable to the ambient thermal energy. At this point, the rate at which the particles will randomly reverse direction becomes significant (Kryder 2005).

Normally, coupling forces in ferromagnetic materials cause the magnetic moments of neighboring atoms to align, resulting in very large internal magnetic field. This is what distinguishes ferromagnetic materials from paramagnetic materials. At temperatures above the Curie temperature (or the Neel temperature for antiferromagnetic materials), the thermal energy is sufficient to overcome the coupling forces, causing the atomic magnetic moments to fluctuate randomly. Because there is no longer any magnetic order, the internal magnetic field no longer exists and the material exhibits paramagnetic behavior. If the
material is non-homogeneous, one can observe a mixture of ferromagnetic and paramagnetic clusters of atoms at the same temperature, the superparamagnetic stage.

Superparamagnetism occurs when the material is composed of very small crystallites (1–10 nm). In this case even when the temperature is below the Curie or Neel temperature (and hence the thermal energy is not sufficient to overcome the coupling forces between neighboring atoms), the thermal energy is sufficient to change the direction of magnetization of the entire crystallite. The resulting fluctuations in the direction of magnetization cause the magnetic field to average to zero. Thus the material behaves in a manner similar to paramagnetism, except that instead of each individual atom being independently influenced by an external magnetic field, the magnetic moment of the entire crystallite tends to align with the magnetic field.

The energy required to change the direction of magnetization of a crystallite is called the crystalline anisotropy energy and depends both on the material properties and the crystallite size. As the crystallite size decreases, so does the crystalline anisotropy energy, resulting in a decrease in the temperature at which the material becomes superparamagnetic.

The differences among ferromagnetism, paramagnetism and superparamagnetism can be summarized as that: atoms of ferromagnetism have parallel aligned magnetic moments, atoms of paramagnetism have randomly oriented magnetic moments with the absence of magnetic field, while atoms of superparamagnetism only have aligned
magnetic moments in a small single domain, in a crystallite for example.

What Kind of Magnetic Particles Are Suitable for This Purpose?

According to the description from the last section, ferromagnetic nanoparticles and superparamagnetic nanoparticles can meet the requirement of FPI. Because these kinds of particles are apt to be agitated by ambient thermal energy so that they can provide the depolarization condition before applying a magnetic field to modulate the FP change. To determine the particle sizes that are suitable for FPI, let us start with the two magnetic relaxations of particles, that are characterized by the sized of particles.

After switching off the magnetic field that has aligned nanoparticles (MNPs), the magnetization of MNPs can relax either through Brownian or through a Neel relaxation mechanism. If the MNP is suspended in a liquid and if the magnetic anisotropy constant of the MNP is high enough to block the magnetization inside the MNP, then the MNP can undergo Brownian rotation which randomizes the orientation of the dipole moments. If the magnetization is unblocked, the internally-Neel relaxation occurs (Ludwig 2004).

The dominant relaxation process for MNPs suspended in a liquid depends on the particle size. The time constant of Brownian relaxation is given by \( \tau_B = 3V \eta / k_B T \), where \( V \) is the hydrodynamic volume of the MNP, \( \eta \) is the viscosity of the carrier liquid, \( k_B \) the Boltzmann constant and \( T \) the temperature. The time constant of Neel relaxation is given by \( \tau_N = \tau_0 \exp(KV / k_B T) \), where \( \tau_0 \) is usually taken as \( 10^{-9} \) s, \( K \) is the magnetic
anisotropy\(^5\) constant and \(V\) the volume of the MNP. The calculated relaxation times of Brownian and Neel relaxation are depicted in Figure 5.1 and 5.2.

Figure 5.1. Brownian relaxation time versus MNP diameter. \(T=300\) K.

Figure 5.2. Relaxation times of Brownian and Neel relaxation versus MNP core diameter. \(K=20\) kJ/m\(^3\) and \(T=300\) K were assumed.

\(^5\) Magnetic anisotropy is the direction dependence of a material’s magnetic properties. A magnetically isotropic material has no preferential direction for its magnetic moment in zero field, while a magnetically anisotropic material will align its moment to an easy axis.
Figure 5.2 indicates that there are different scenarios that can be used for the specific
detection of biomolecules according to characteristics of Brownian and Neel relaxation.
If the MNPs are immobilized by their binding to a specific biomolecules and if they have
a suitable size, they exhibit Neel relaxation whereas unbound ones relax via Brownian
rotation. In this way, bound and unbound MNPs can be clearly distinguished by their
different relaxation times. If the MNP binds to a biomolecule, both being in suspension
and consequently mobile, its hydrodynamic volume will be increased, resulting in an
increased Brownian time constant, dependent on whether it exceeds the corresponding
Neel time constant or not (Ludwig 2004).

In the study of FPI using magnetic nanoparticles, what we concern most is the
Brownian relaxation time. Because FPI is performed in liquid environment, and all the
particles are mobile and not immobilized to some sites. Moreover, in the expression of
fluorescence anisotropy, the rotational correlation time is actually Brownian relaxation
time according to their respective formula.

Since the dye lifetimes usually do not exceed a few microseconds level, according to
Figure 3.2 and 5.1, the nanoparticles can be used in FPI should be only a few tens of
nanometers in size. If the nanoparticles are too big, for one thing, the correlation time
becomes very long and may not be comparable with the fluorescence lifetime, and the
fluorescence anisotropy change would be very difficult to pick out by measuring
equipment. For another, large particles are apt to settle down in testing container, thus
cannot meet the requirement that the particles should be easy to be suspended in liquid for
some time to perform the FPI.

Possibility to Extract the Signal

After immuno-reaction, assume the nanoparticles attached with antigens have a
correlation time of 100 μs, and the dye used has a lifetime of 1 μs, then according to Equ.
30. the fluorescence lifetime is

\[ r = \frac{r_0}{1 + \tau / \phi} = \frac{0.3}{1 + 1/100} = 0.2970. \]

After applying magnetic field, particles are all aligned. The particles can be seen to be
immobilized and there is no Brownian relaxation happen. Thus the rotational correlation
time can be deemed as infinity, and the anisotropy has the assumed maximum value 0.3
here. The anisotropy change is 0.003. According to experimental measurement, the
anisotropy fluctuation is usually within 0.01, so the anisotropy change after applying
magnetic field cannot be observed directly. However, if a modulated magnetic field is
utilized, then the anisotropy change signal that is buried in noise can be pick out by a
lock-in amplifier.

Commercial Sources of Dye and Magnetic Nanoparticles

Long lifetime dye of around 1 μs or longer is required in this proposed method. A
commercial available ruthenium complex dye Fluka 71603 can be obtained from
Sigma-Aldrich Inc. Its synonym is Ru(bpy)$_2$(phen-5-isothiocyanate)(PF$_6$)$_2$, with
molecular formula $C_{33}H_{23}F_{12}N_{7}P_{2}RuS$. Its labeling process is quite easy and it can yield a high dye-to-protein labeling ratio (Szmacinski 1996).

Clemente Associates provides iron oxide magnetic nanoparticles with diameter around 50 nm. Those particles are pre-coated with antibodies or active chemicals. They also provide special order of particles with diameter around 35 nm.
To align the magnetic nanoparticles, a uniform and steady DC magnetic field is needed. A normal solenoid can generate this uniform magnetic field, but the analyte is required to be placed in the center of the solenoid, which is very difficult with the involvement of optical setup. In this case, a pair of helmholtz coils is a good choice since it will not block the optical paths in neither excitation nor detection besides excellent homogeneous characteristic of magnetic field it generates.

A helmholtz coil pair is made up of two, identical, circular coils aligned on the same axis and separated by a particular distance. This separation distance, which is equal to the radius of the coil, is the feature that makes the mixing process of immunoassay easy to achieve. A simple calculation gives the correct value of the field at the center point. If the radius is $R$, the number of turns in each coil is $N$ and the current flowing through the coils is $I$, then the magnetic flux density, $B$ at the midpoint between the coils will be given by,

$$B = 0.815 \frac{\mu_0 NI}{R},$$

where $\mu_0$ is the permeability constant $4\pi \times 10^{-7}$ T•m/A.

In the case of this research, the sample which is contained in a cuvette needs to be
place in the optical testing cube, which can prevent the material from the outer
background light. A rectangular pair of helmholtz coil design is introduced in this
magnetic field construction, because it have the following advantages:

(1) Ease of construction of the coil forms,

(2) Ease of alignment of the coils by making use of the optical testing cube, and

(3) Relative ease of field computations.

![Diagram of magnetic field calculation](image)

Figure 6.1. Magnetic field calculation of a segment of straight wire carrying current.

According to Biot-Savart Law (Preston 1991, Tipler 1999), if the differential element of
current is defined as $Id\vec{l}$, then the corresponding differential element of magnetic field is,

$$d\vec{B} = \frac{\mu_0}{4\pi} \frac{I d\vec{l} \times \hat{r}}{r^2},$$  \hspace{1cm} (34)

integrate this differential element along the straight wire segment as shown in Figure 6.1.
The magnitude of magnetic field at a point that has a distance $s$ away from the wire
segment can be easily obtained as,
\[ B = \frac{\mu_0 I}{4\pi s} (\cos \alpha - \cos \beta), \]

where \( \alpha \) and \( \beta \) are the angles shown in Figure 6.1.

The fields generated by a pair of rectangular coils can be easily derived by summing the contribution of each of the eight line segments of current. In this manner one can obtain an explicit expression for the field at any point without the use of elliptic integrals. Computer programs were developed to plot contours of the axial field deviation using MATLAB (Appendix B).

![Cartesian coordinate system for the rectangular Helmholz coils](image)

Figure 6.2. Cartesian coordinate system for the rectangular Helmholz coils.

The coordinate system used in the field plots, are Cartesian coordinate system \((x, y, z)\) as shown in Figure 6.2. The \(x\) and \(y\) axes are parallel to the coil sides and \(z\) axis is perpendicular to the coil planes and passes through their centers. The coils used in this computation are that with width of 6 cm, height of 6.5 cm and distance of 5 cm between
the two pairs. In Figure 6.3, and the contours of constant axial field deviation is defined as:

\[ \Delta = \frac{[B_z(x, y, z) - B_z(0,0,0)]}{B_z(0,0,0)} \]

Figure 6.3. Magnetic field contour of the designed helmholtz coils.
It can be seen from the contour graph that within the volume of 1cm³ cube located at the origin, the deviation of magnetic field is less than 2%. The field in gauss at the center of the pair of rectangular coils helmholtz coils, \( B_z(0,0,0) \), is given by,

\[
B_z(0,0,0) = 0.19199NI,
\]

where \( N \) is the number of turns on each coil, \( I \) the current in amperes.

A pair of plastic spools was manufactured to hold the rectangular helmholtz coils. They can be mounted on both sides of the optical testing cube as shown in Figure 6.4. Mounting spools on the cube provides an advantage that the coils don’t need stands to hold them and the alignment of the two coils are automatically achieved.

![Figure 6.0.4. Mounting spools onto testing cube (not to scale).](image)

The dimensioned drawings of the spools are shown in Figure 6.4.
DC Magnetic Field Construction and Calibration

The pair of spools was mounted on both sides of the testing tube, on each of which wounded 100 rounds of enameled copper magnet wire (WLS85135-40J, Sargent-Welch). The two spools of coil were connected in series and formed a close circuit by connecting them to anode and cathode of a power supply (CP32787-00, Sargent-Welch). A Gauss meter (GM1A, Applied Magnetics Laboratory Inc.) with transverse probe was used to detect the magnetic induction $B$ by placing the probe in the testing tube and facing the probe flake parallel to the magnetic field. When performing the calibration, a multi-meter was connected in the circuit in series to measure the circuit current. After increasing the current to a certain value, it is decreased to observe if there is big hysteresis. The
magnetic field in the center of testing tube versus magnetic current is listed in Table 6-1.

<table>
<thead>
<tr>
<th>Current (A)</th>
<th>Magnetic Field (Gauss)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/3</td>
<td>13.0</td>
</tr>
<tr>
<td>2/3</td>
<td>21.4</td>
</tr>
<tr>
<td>1</td>
<td>28.8</td>
</tr>
<tr>
<td>2/3</td>
<td>22.3</td>
</tr>
<tr>
<td>1/3</td>
<td>13.4</td>
</tr>
</tbody>
</table>

The measured data shows the magnetic field at current 2/3 A which was used in later designed Fluorescence Polarization experiment varies only about 0.04%, so it can provide an acceptably stable magnetic field.

Optical Setup

The whole experimental setup scheme is illustrated in Figure 6.6. A 405 nm laser diode (Nicha, Japan) controlled by a Laser diode controller (LDC 202C, Thorlabs) was used to generate excitation laser. After passing through a Glan-Thompson Polarizer to make the laser vertically polarized, the laser was reflected by a long pass filter in the
direction of where sample was placed. A short focal length lens mounted on a position tunable adapter was used to focus the laser in sample. Fluorescence generated by excitation of the laser was detected by the detection part of the system which mainly consisted of a Glan-Taylor polarizer and two CCDs (MiniSpectrometer, Hamamatsu; HR2000+, OceanOptics). The Glan-Taylor polarizer (GT10-A, Thorlabs) split the two polarization components of the fluorescence and directed each of them to a CCD by a lens (F220SMA-A, Thorlabs) and a piece of optical fiber. The spectrum were displayed and recorded in two software environments.

Figure 6.6. Experimental setup of Fluorescence Polarization.

*Glan-Thompson Polarizer*
The extinction ratio\(^6\) of Glan Thompson calcite polarizers (Figure 6.7) can reach up to 100,000:1. The polarizer consists of two cemented prisms made from the highest optical grade calcite. The operating spectral range is 350 nm to 2.3 μm.

Figure 6.7. Glan Thomson Polarizer. A Glan-Thompson prism deflects the \(p\)-polarized ordinary ray whilst transmitting the \(s\)-polarized extraordinary ray. The two halves of the prism are joined with optical cement, and the crystal axes are perpendicular to the plane of the diagram.

A Glan-Thompson prism consists of two right-angled calcite prisms that are cemented together by their long faces. The optical axes of the calcite crystals are parallel and aligned perpendicular to the plane of reflection. Birefringence splits light entering the prism into two rays, experiencing different refractive indices; the \(p\)-polarized \(o\)-ray is totally internally reflected from the calcite-cement interface, leaving the \(s\)-polarized \(e\)-ray to be transmitted. The prism can therefore be used as a polarizing beam splitter. Traditionally Canada balsam was used as the cement in assembling these prisms.

The Glan-Thompson has a wide acceptance angle, but a much lower limit of maximum irradiance (due to optical damage limitations of the cement layer).

---

\(^6\) Extinction Ratio is a measure of the ability of a polarizer to attenuate plane polarized light. The extinction ratio can be calculated by measuring the ratio of the maximum transmission (\(T_{\text{max}}\)) when the polarizer is aligned to the polarization axis and the minimum transmission (\(T_{\text{min}}\)) when the polarizer is rotated 90 degrees. The ratio \(\text{ER} = T_{\text{max}}/T_{\text{min}}\) is often expressed in a form like 1000:1.
**Glan-Taylor Polarizer**

Glan-Taylor Polarizers offer extremely pure linear polarization with an extinction ratio greater than 100,000:1 for laser beams in the wavelength ranges of 0.25-2.3\textmu m (Fan 2003). The \(s\) polarization\(^7\) component is reflected through a 68° angle and exits the polarizer through one of the side ports. The \(p\) polarization\(^8\) passes straight through the polarizer.

![Diagram of a Glan-Taylor prism](image)

Figure 6.8. A Glan-Taylor prism reflects \(s\)-polarized light at an internal air-gap, transmitting only the \(p\)-polarized component. The optical axes are vertical in the plane of the diagram.

A Glan-Taylor prism is usually used as a polarizer or polarizing beam splitter. The prism is made of two right-angled prisms of calcite (or sometimes other birefringent materials) which are separated on their long faces with an air gap. The optical axes of the

---

\(^7\) S Polarization: A linear polarization state that is orthogonal to the Plane of Incidence and usually parallel with the optical table.

\(^8\) P Polarization: A linear polarization state that is parallel to the Plane of incidence and usually perpendicular with the optical table.
calcite crystals are aligned parallel to the plane of reflection. Total internal reflection of $s$-polarized light at the air-gap ensures that only $p$-polarized light is transmitted by the device. Because the angle of incidence at the gap can be reasonably close to Brewster's angle, unwanted reflection of $p$ polarized light is reduced, giving the Glan-Taylor prism very good transmission.
CHAPTER 7
FA Measurement and Data Processing

From the fluorescence components of parallel and perpendicular polarizations, FA can be calculated using the equation

\[ r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}, \]  

(36)

where \( I_\parallel \) and \( I_\perp \) are the fluorescence intensities with polarization parallel and perpendicular to the excitation polarization, respectively.

However, optical components may have different transmission efficiency for the two polarization components and the two detectors may also have different sensitivities. Even for a particular detector, it may have different sensitivities to different polarizations. This point can be illustrated by the following experiment: A 405nm vertically polarized laser was launched into the optical system, 99ul PBS and 1ul 1mg/ml Alex Fluor 430 labeled BSA were mixed together to serve as the testing sample. The fluorescence generated has a peak at 546.35nm. Hamamatsu spectrometer was used to detect the s (horizontal) polarized light while OceanOptics spectrometer was used to detect the p (vertical) polarized light. The intensity counts in the two spectrometers at wavelength 546.35nm were recorded for three times respectively. Then the fiber connectors at the two spectrometers were exchanged so that OceanOptics spectrometer was used to measure the s polarized light and Hamamatsu p polarized light. The results are shown in
Table 7-1.

Table 7-1 Comparison on sensitivity of two spectrometers to polarized light

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamamatsu_s</td>
<td>7339</td>
<td>7095</td>
<td>6935</td>
<td>7123</td>
</tr>
<tr>
<td>OceanOptics_p</td>
<td>11735</td>
<td>11067</td>
<td>10798</td>
<td>11200</td>
</tr>
<tr>
<td>Hamamatsu_p</td>
<td>12406</td>
<td>11482</td>
<td>11799</td>
<td>11895.67</td>
</tr>
<tr>
<td>OceanOptics_s</td>
<td>9872</td>
<td>9027</td>
<td>9254</td>
<td>9384.333</td>
</tr>
</tbody>
</table>

It can be seen that for s polarized light, OceanOptics Spectrometer has a higher sensitivity while for p polarized light, Hamamatsu gives greater intensity counts.

Effects of these differences are usually taken into account by a correction factor $G$ (Siegel 2003), which is the ratio of efficiencies for the parallel and perpendicular detection channels. $G$ can be determined experimentally once the optical path is set and well aligned. To determine the $G$, a method was developed as follows: For vertically polarized excitation, the paralleled fluorescence component is $p$ polarized and detected by CCD1 as $I_{vv}$, while the perpendicular fluorescence component detected as $I_{vh}$ is $s$ polarized. Using $S_v$ and $S_h$ to represent the combined transmission and detection efficiencies for $p$ and $s$ polarization detecting channels, relations can be obtained as

$$I_{vv} = S_v I_{\parallel},$$

$$I_{vh} = S_h I_{\perp},$$

If $G$ is the ratio between $S_v$ and $S_h$, then
For horizontally polarized excitation, the parallel fluorescence component $I_{\|}$ is $s$ polarized and detected by CCD2 as $I_{hh}$, while the $p$ polarized fluorescence component detected by CCD1 as $I_{hv}$ is vertically polarized. Similarly,

$$\frac{I_{\|}}{I_{\perp}} = \frac{S_h I_{vv}}{S_v I_{vh}} = G \frac{I_{hh}}{I_{hv}}$$

According to the above two equation,

$$G = \frac{I_{vv} I_{hv}}{I_{vh} I_{hh}}. \quad (37)$$

Since $\frac{I_{\|}}{I_{\perp}} = \frac{S_h I_{vv}}{S_v I_{vh}}$, then

$$r = \frac{S_h I_{vv} - S_v I_{vh}}{S_h I_{vv} + 2 S_v I_{vh}} \quad (38)$$

After dividing the nominator and denominator both by $S_h$, equation (1) can be modified to

$$r = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}}. \quad (39)$$

It can also be written in the horizontal polarized excitation form. Therefore, before the FA measurement, the $G$ factor needs to be determined by making horizontal and vertical polarized excitation measurement respectively. After the value of $G$ is determined by Equ. 37, it should be applied to each measurement to get the anisotropy value. And the ensuing measurement can be made only with vertical polarized excitation light which is suitable for the current optical setting, because the vertical polarized light
gives stronger signal due to different polarization reflectivity of the filter to the excitation light.
CHAPTER 8
RESULTS AND DISCUSSIONS

Determine the G Factor

1 μl Alex Fluor 430 labeled BSA was mixed into 99 ul PBS. The polarization components were recorded three times with vertically polarized excitation and horizontally polarized excitation respectively.

Table 8-1 Measurements for determining G factor.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vertically polarized excitation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I_{vv}$</td>
<td>12406</td>
<td>11482</td>
<td>11799</td>
<td>11895.67</td>
</tr>
<tr>
<td>$I_{vh}$</td>
<td>9872</td>
<td>9027</td>
<td>9254</td>
<td>9384.333</td>
</tr>
<tr>
<td>Horizontally polarized excitation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I_{hv}$</td>
<td>10666</td>
<td>10033</td>
<td>9790</td>
<td>10163</td>
</tr>
<tr>
<td>$I_{hh}$</td>
<td>7970</td>
<td>7488</td>
<td>7192</td>
<td>7550</td>
</tr>
</tbody>
</table>

Thus the value of G factor at wavelength 547 nm for this system is $G = \sqrt{(I_{vv} \cdot I_{hv}) / (I_{vh} \cdot I_{hh})} = 1.3063$. It was adopted in the following fluorescence anisotropy (FA) calculations.

Validation of the FA Measurement System
A homogeneous immunoassay for rabbit-anti-goat IgG was performed to examine the validation of the optical system. 100 μl Alex Fluor 430 labeled rabbit-anti-goat IgG was prepared. After recording the FA of the sample, increasing amount of goat-anti-rabbit IgG was added into the sample, ranging from 0 to 4 times that of rabbit-anti-goat IgG (Figure 8.1).

Increasing FA was observed, which proved the feasibility of the optical system.

Table 8-2 Calculated FA data of immunoassay for rabbit-anti-goat IgG

<table>
<thead>
<tr>
<th>After background subtraction</th>
<th>FA 1</th>
<th>FA 2</th>
<th>FA 3</th>
<th>Average FA</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 IgG</td>
<td>0.130481</td>
<td>0.131816913</td>
<td>0.139812</td>
<td>0.134037</td>
<td>0.005046</td>
</tr>
<tr>
<td>1 time amount of IgG</td>
<td>0.161503</td>
<td>0.165900235</td>
<td>0.162063</td>
<td>0.163156</td>
<td>0.002393</td>
</tr>
<tr>
<td>2 times amount of IgG</td>
<td>0.152344</td>
<td>0.163044999</td>
<td>0.167231</td>
<td>0.160873</td>
<td>0.007678</td>
</tr>
<tr>
<td>4 times amount of IgG</td>
<td>0.192725</td>
<td>0.167221948</td>
<td>0.186293</td>
<td>0.18208</td>
<td>0.013263</td>
</tr>
</tbody>
</table>
Figure 8.1. Steady-state fluorescence anisotropy of Alex Fluor 430 labeled rabbit-anti-goat IgG at various concentrations of goat-anti-rabbit IgG measured at 20°C. The excitation and observation wavelengths were 405 nm and 547 nm.
A novel idea of FPI using magnetic nanoparticles was proposed and detailed scheme was described in this study. This method has a promising potential in enhancing the accuracy and sensitivity of immunoassay. Experimental setup was constructed for this study, and preliminary experiments were carried on to evaluate the feasibility of the system.

After some necessary software support is developed to realize the time-resolved signal recording, the constructed setup can be used for sensitive protein detection in future using the proposed method.
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