High-resolution fluorescence spectroscopy in immunoanalysis

by

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This is to certify that the doctoral dissertation of

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For the Major Program

To my wife Branka and my mother Duška - women that shouldered it all

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ABSTRACT

The work presented in this dissertation combines highly sensitive and selective fluorescence line-narrowing spectroscopy (FLNS) detection with various modes of immunoanalytical techniques. It has been shown that FLNS is capable of directly probing molecules immunocomplexed with antibodies, eliminating analytical ambiguities that may arise from interferences that accompany traditional immunochemical techniques. Moreover, the utilization of highly cross-reactive antibodies for highly specific analyte determination has been demonstrated. Finally, we demonstrate the first example of the spectral resolution of diastereomeric analytes based on their interaction with a crossreactive antibody.

CHAPTER 1

GENERAL INTRODUCTION

I. General Overview

Immunochemical techniques are widely used for the analysis of biological and environmental samples [1-5]. All immunoanalytical techniques, regardless of their format and design, rely on an antibody's capability to specifically recognize and (reversibly) bind an analyte of interest from a given sample [6]. Numerous assay formats arise from this basic concept depending on the nature of the analyte, the separation of bound and unbound compounds, and whether a labeled antigen (competitive assay) or a labeled secondary antibody (sandwich assay) is used [7].

Traditional detection schemes in immunoassays utilize radioactive, fluorescent, or enzyme labels (i.e. ELISA) [7, 8]. In immunosensors, various transducer systems (electrochemical, optical, microgravimetric) are used [9]. Nevertheless, both reporter labels and mainstream biosensor readout systems report solely on a binding event between an antibody and an antigen, and not on the intrinsic characteristics of a captured analyte. These methodologies will only yield valid analytical results when an analysis is free of analytical interferences, i.e., non-specific interactions. Although antibody-antigen complexation is believed to be one of the most specific molecular recognition events in nature, many antibodies crossreact with structurally similar molecules (cross-reactants) to the molecule for which the antibody was elicited [10-17]. Consequently, the validity of an

immunochemical method that utilizes antibodies as the only mean of selectivity may be seriously compromised.

Another challenging aspect of immunoanalysis is its use for multiple analyte detection. According to the “one antibody – one antigen” dogma, different analytes can only be distinguished by different antibodies, which requires the development of multi-channel assays or array sensors. In most of the published work multianalyte immunoassays include incubation of the sample with a mixture of all relevant antibodies together with the corresponding labels (tracers) [7]. For each analyte different labels have to be used to allow for independent analysis. This approach is, however, limited by the number of tracers which can be distinguished within a single assay under identical conditions. An alternative method relies on the spatial separation of different affinity reactions during the analytical procedure, which then allows for the use of only one antibody label for all analytes [3, 6, 7, 18-21]. The detection of different analytes is achieved by a corresponding spatially resolved signal transduction. As a consequence, the number of analytes that can be detected simultaneously, is limited by the degree of spatial resolution achievable by the signal transduction device. Nevertheless, in all multianalyte formats of immunoanalysis, the issue of possible crossreactive interactions remains unsolved.

Multidimensional detection in immunoanalysis has been rather neglected in recent years, while the focus has moved toward the architectural arrangement of elements (spatial resolution) and the integration of simple, monodimensional detection schemes. While information-rich detection methods such as mass spectrometry, NMR, wavelength-resolved UV-VIS spectroscopy, high resolution

fluorescence and Raman spectroscopies have proven valuable when coupled with various modes of chromatography and electrophoresis [22], immunoanalytical formats still rely on one-dimensional, often diagnostically inconclusive signals.

Fluorescence Line-Narrowing Spectroscopy (FLNS) has proven to be an extraordinarily powerful technique for the characterization and differentiation of structurally similar fluorescent molecules. The underlying principles and features of FLNS are briefly addressed in the following section. For a more comprehensive theoretical background and a summary of its applications the reader is referred to several excellent books [23-28] and recent reviews [29, 30].

The motivation for the work presented in this dissertation arises from the following question: is it possible to utilize the crossreactivity of an existing repertoire of promiscuous antibodies to eliminate analytical ambiguity by coupling immunoanalytical techniques with information-rich detection methods? As an alternative to laborious and expensive targeted antibody production, the screening of an assortment of existing antibodies may prove to be valuable not only for the selection of antibodies with desired properties, but also for their new applications. In this sense, FLNS detection that is capable of differentiating between structurally related species in their free and immunocomplexed forms, could be of great value in achieving the ultimate discriminatory quality of otherwise nonspecific analysis.

The main intention of this dissertation is to explore the implementation of FLNS as a readout method for various modes of immunochemical analysis. The focus will be in the development of highly specific immunoanalytical methodologies that overcome nonspecific determinations arising from antibody crossreactivity.

Moreover, we will explore the utilization of crossreactive antibodies in combination with FLNS detection for highly specific analyses.

II. Theoretical aspects of FLNS

FLNS is a low temperature (typically less than 10 K), site selective spectroscopic technique that eliminates or significantly reduces the contribution of thermal and site inhomogeneous line broadening to molecular spectral profiles. Molecular spectra contain a great deal of information regarding the emitting or absorbing molecular species. Electronic spectra observed in UV and visible energy regions are of the greatest interest, because they reflect transitions that include all degrees of freedom of the molecular chromophores. However, under conventional conditions of spectral analysis (room temperature, nonselective excitation, etc.) spectra of polyatomic species are broad and exhibit little structure, thus providing limited information about internal molecular structure and its immediate environment. In solid systems, however, spectral broadening can be classified as either homogeneous (Γ_{hom}) or inhomogeneous (Γ_{inh}), as depicted in Figure 1, frames A and B, respectively. Homogeneous broadening is identical for all molecules of a given compound where each isolated molecule contributes the same way to the transitions. In an absolutely perfect crystal all three transitions would have the same frequency, $\omega_1 = \omega_2 = \omega_3$, and the resulting narrow absorption band would be only homogeneously broadened [23]. Γ_{hom} electronic transition profile has a Lorentzian shape and mostly originates from interactions between guest molecules and lattice

Figure 1. Schematic representation of the optical absorbance lines for three identical chromophores in a perfect crystal lattice (A) and in a an amorphous host (B).

vibrations (phonons) of the matrix [24]. During the electronic excitation of the guest molecule, periodic movements of matrix molecules can perturb the phase relationship (guest-host equilibrium geometry) between the ground and excited state as defined by the total optical dephasing time (T_2):

$$\frac{1}{T_2} = \frac{1}{2T_1} + \frac{1}{T_2'} \quad (\text{II.1})$$

where T_1 is the excited state lifetime and T_2' is the pure dephasing time. T_2' is due to the modulation of the single site transition frequency, which results from the interaction of the excited state with bath phonons and is temperature dependent [23]. Since the contribution of T_2' to Γ_{hom} is significantly greater than that of T_1 , low temperature is necessary to approach theoretical spectral resolution. The temperature dependent Γ_{hom} can be expressed as:

$$\Gamma_{\text{hom}} = (\pi T_2 c)^{-1}, \quad (\text{II.2})$$

where c is the speed of light in cm s^{-1} and T_2 is the total dephasing time [31]. The typical value of the homogeneous width is $< 1\text{cm}^{-1}$ (at 4.2 K).

Inhomogeneous broadening arises from the fact that an analyte residing in a disordered host (glasses, polymers, proteins, DNA) can occupy a large number of energetically inequivalent sites, as shown on the left of Frame B of Figure 1. Consequently, optical bands of chromophores embedded in amorphous matrixes have large inhomogeneous broadening. Different mechanisms lead to broadening:

strains, random electric fields, field gradients of charged defects, interactions with collective excitations (such as phonons), and interactions between centers/chromophore [23]. The immediate individual micro-environments of the identical guest molecules in the disordered matrix are different, resulting in the different absorption energies ω_1 , ω_2 , and ω_3 (solid lines). This is illustrated by the three narrow spectra shown in Frame B of Figure 1. For inhomogeneous broadening, the statistical distribution of the inequivalent sites leads to a broad Gaussian absorption profile (dashed line), with a typical width of several hundred wavenumbers. Assuming very weak coupling with phonons (see below), the full width at half maximum of the Gaussian distribution refers to the inhomogeneous broadening, Γ_{inh} . The strength of the broadening can be expressed by the ratio of $\Gamma_{inh}/\Gamma_{hom}$, which can range from approximately 1 to about 10^5 , depending on the host matrix [23, 24]. Therefore, narrow laser excitation of chromophores, either directly within the inhomogeneously broadened absorption profile (referred to as (0,0) excitation) or into a vibronic region (i.e. vibronic excitation), is the essential feature of FLNS.

An inhomogeneously broadened fluorescence origin band ((0,0) band) has no structure at low temperature (as shown by the solid line in Figure 2A), but consists of many sharp bands which correspond to the fluorescence zero-phonon lines (ZPL) of the "guest" molecules occupying inequivalent sites. A zero-phonon transition is one for which no net change in the number of phonons accompanies the electronic transition. Building to lower energy on each ZPL is a broad phonon (lattice vibrational) wing, referred to below as the phonon sideband (PSB). As mentioned

Figure 2. A: Schematic of the inhomogeneously broadened fluorescence origin band; FWHM is approximately given by $\Gamma_{inh} + S\omega_m$. ω_L is the laser excitation frequency. B: Schematic view of the resulting fluorescence zero-phonon line (ZPL) and its phonon sideband (or phonon wing, PW).

above, each single site ZPL carries a homogeneous linewidth, Γ_{hom} , which is determined by the total dephasing time of the optical transition. Since the PSBs contribute to the absorption and/or fluorescence origin bands, the width of the Gaussian shown in Figure 2A (solid line) is approximately given by $\Gamma_{\text{inh}} + S\omega_m$, where ω_m is the mean phonon frequency (for organic molecules typically about 20-30 cm^{-1}) and S is the Huang-Rhys factor [23]. If excitation occurs at ω_L using a spectrally narrow laser excitation, as schematically shown in Figure 2A, only those chromophores can be excited whose transition energies coincide with the frequency of the laser. If the concentration of chromophores is sufficiently low, no energy transfers to other chromophores occurs. Subsequently, resonant fluorescence occurs, providing a fluorescence line-narrowed (FLN) spectrum, as shown in frame B of Figure 2.

The band shape of the single site fluorescence profile, as shown in Figure 2B, consists of two portions:

$$F(\omega, T) = \phi_{ZPL}(\omega, T) + \phi_{PSB}(\omega, T) , \quad (1)$$

where the first term describes the ZPL and the second describes the phonon side band (phonon wing).

It has been established that the S_1 state vibrational dynamics, frequencies, and intensities, are the most sensitive to subtle changes in the structure of the chromophore and its environment [29, 30]. Thus, this mode of excitation has great

potential in analytical applications. To probe the S_1 -state vibrational structure, vibronically excited FLN experiment is employed, as illustrated in Figure 3. For simplicity, ω_L excites only two overlapping vibronic transitions $(1_\alpha, 0)$ and $(1_\beta, 0)$ which will not be resolved in the absorption spectrum. Fluorescence occurs from these two energetically distinct isochromats and produces a "doubling" of every line in the FLN spectrum. As a result, two strong ZPLs $[(0,0)_B$ and $(0,0)_A]$ are observed. The displacements between ω_L and the doublet components of the origin transition, $[(0,0)_B$ and $(0,0)_A]$, yield the excited-state vibrational frequencies, i.e. ω_α' and ω_β' . The weaker vibronic bands that build on $[(0,0)_B$ and $(0,0)_A]$ are not shown for simplicity. The structure of the ZPLs is referred to as *multiplet origin structure*. Vibronically excited FLN is now used exclusively for identification and conformational analysis of DNA adducts because of its vastly superior selectivity. A key point is that by tuning ω_L across the $S_1 \rightarrow S_0$ absorption spectrum one can determine the frequencies and of all active excited-state vibrations [24]. Furthermore, each ω_L yields a distinct ZPL "fingerprint".

It should be noted that all the high-resolution fluorescence spectra presented in this thesis are obtained under the vibronic excitation mode in FLNS experiments. Some of the most important information regarding the analytical implications provided by low-temperature, high-resolution spectroscopy include; Inhomogeneous broadening (a distribution of $(0,0)$ transition energies) and homogeneous linewidth, Γ_{hom} , which contain information regarding molecular dynamics; Electron-phonon coupling, which provides a sensitive probe of the

Figure 3. Principles of vibronic excitation in FLNS. Frame A: selective laser excitation (ω_L) of two subsets of molecules (A and B) within the vibronic region. Resulting fluorescence spectrum with two $(0,0)_A$ and $(0,0)_B$ transitions is shown at the bottom of the frame A. ω_A and ω_B are the vibrational frequencies in the excited state. Frame B: an example of vibronically excited FLN spectrum of the *syn*-DB[a,h]PDE-14-N7Gua adduct; $\lambda_{\text{exc}}=376.0$ nm, $T=4.2$ K. The sharp ZPLs at 749 and 772 cm^{-1} correspond to excited state vibrational frequencies.

interaction of molecule with the local matrix environment; Vibrational frequencies in the ground and excited states can be measured, with latter being used as an analytical tool for providing a "fingerprint" of a molecule and/or to identify compounds in complex mixture [23].

The applicability of FLNS spans from small organic and inorganic molecules [24, 30, 32], to a range biomolecules including antenna protein-pigment complexes [33], photosynthetic pigments [34], and hemoglobin [35], to name a few. Another attractive application of FLNS is in the characterization of nucleoside/nucleotide-, oligonucleotide-, DNA-, and protein-adducts of polycyclic aromatic hydrocarbons (PAHs) [36-41]. Moreover, FLNS is capable of differentiating between external, base-stacked, and intercalated conformations of PAH-DNA adducts and stereoisomeric PAH-DNA adducts [42-47]. Due to its high sensitivity, FLNS showed remarkable success in the identification of various PAH-DNA adducts formed in *in vitro* [30, 48] and *in vivo* [42, 43, 49-51] experiments. Finally, FLNS offers insight into the nature of antibody/ligand interactions and mechanisms of immunocomplex formation and geometry [52].

III. Dissertation Organization

Chapter 2 describes the design of a novel immunosensor for simultaneous detection of PAH-derived DNA adducts using low-temperature fluorescence detection. In Chapter 3, FLNS study of the interactions of DNA adducts and metabolites of polycyclic hydrocarbons with their specific monoclonal antibodies is described. In Chapter 4 we report the results of the spectroscopic characterization

of mechanisms of immunocomplex formation of the highly crossreactive anti-PAH monoclonal antibody with pyrene and benzo(a)pyrene. Chapter 5 presents the first example of a high-resolution spectroscopic differentiation of the two enantiomeric couples based on their differential geometrical interactions with a highly cross-reactive antibody. Chapter 6 explores the utility of highly cross-reactive anti-PAH MAb as a medium for affinity electrophoretic chiral separation of diastereomeric benzo(a)pyrene tetrols. General conclusions are addressed in Chapter 7.

Chapters 2 and 3 are papers that have been published in peer-refereed journals. Chapters 4 and 5 are papers submitted for publication in peer-refereed journals. The results presented in Chapter 6 will be part of a future publication.

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CHAPTER 2

**NOVEL BIOSENSOR CHIP FOR SIMULTANEOUS DETECTION OF
DNA-CARCINOGEN ADDUCTS WITH LOW-TEMPERATURE
FLUORESCENCE**

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Abstract

A monoclonal antibody (MAb)–gold biosensor chip with low temperature laser-induced fluorescence detection for analysis of DNA-carcinogen adducts is described. Optimization of the detection limit, dynamic range, and biosensing applicability of the MAb–gold biosensor chip was achieved by: 1) using dithiobis (succinimidyl propionate) (DSP), as a protein linker and 2) employing recombinant protein A to provide oriented immobilization of the MAbs. The use of DSP, which has short methylene chain length led to faster protein binding kinetics and higher protein surface density than a chip with a longer dithiobis (succinimidyl

undecanoate) (DSU) linker. The incorporation of recombinant protein A increased the distance between MAb-bound analytes and the gold surface. The increased distance minimized fluorescence quenching, resulting in about a ten-fold increase in the fluorescence signal in comparison with chip without protein A. The improved chip architecture was used to demonstrate that biosensing of two structurally similar benzo[a]pyrene (BP)-derived DNA adducts, BP-6-N7Gua and BP-diolepoxide-10-N²dG, bound to two specific MAbs immobilized from a mixture at the same address on the chip, is feasible. These mutagenic adducts are formed by one-electron oxidation and monooxygenation pathways, and are depurinating and stable DNA adducts, respectively. It is shown that the DNA adducts can be easily identified at the same address using time-resolved, low temperature, laser-based fluorescence spectroscopy. The current limit of detection is in the low femtomole range. These results indicate that a single biosensor chip consisting of a Au/DSP/protein A/MAb nanoassembly, with analyte-specific MAbs and low-temperature fluorescence detection should be suitable for simultaneous detection and quantitation of the above adducts, as well as the luminescent antigens for which selective MAbs exist.

Keywords: biosensor, fluorescence detection, fluorescence line-narrowing spectroscopy, DNA adducts

¹Abbreviations

BP, benzo[a]pyrene; BPDE, benzo[a]pyrene diolepoxide; dG, deoxyguanosine, BP-6-N7Gua, (benzo[a]pyren-6-yl)guanine; *anti-trans*-BPDEdG, 7R,8S,9S-Trihydroxy-

10S-(N²deoxyguanosyl-3'-phosphate)-7,8,9,10-tetrahydrobenzo[a]pyrene; DSP, dithiobis(succinimidyl propionate); DSU, dithiobis(succinimidyl undecanoate); FLNS, fluorescence line-narrowing spectroscopy; Gua, guanine, IA, immunoassay; IRS, infrared reflection spectroscopy; MAbs, monoclonal antibodies; ODT, octadecanethiol; SAM, spontaneously-adsorbed monolayer.

I. Introduction

In recent years the development of biosensors capable of distinguishing multiple analytes in a single sample has become an actively pursued research goal. Although a number of biosensors have been successfully demonstrated for single analyte detection [1], simultaneous multi-analyte biosensing remains a challenge. Nevertheless, many recently developed biosensors, designed for simplicity and ease of use, exhibit clear advantages over traditional analytical methodologies. In particular, low material consumption and lower cost are important requirements in the development of biosensors, spanning a wide range of applications (e.g., medicinal analysis, environmental screening, and quality control). Reduction or elimination of sample pretreatment (extraction, preconcentration, and separation), which lead to reduced analysis time, is another important goal in biosensor research.

Immunosensors involve antibodies or antibody fragments (as the capture elements), which provide highly specific binding interaction with antigens (or haptens). In general, preparation of an immunosensor requires immobilization of antibody or antibody-derived molecules on a solid support. Because antibodies are high-molecular weight biomolecules with several reactive groups and a well-defined

three-dimensional structure, control of the coupling reaction and the resulting orientation of the antibody binding sites are crucial for efficient sensing [2,3].

Recently, fluorescence line-narrowing spectroscopy (FLNS) has been employed as a direct readout methodology for detection and quantitation of fluorescent BP-6-N7Gua adducts via their selective binding to a monoclonal antibody (MAb) immobilized on a biosensor chip [4]. The FLN spectra allow for information rich detection that minimizes identification ambiguity [5]. Additionally, FLNS has been interfaced with capillary electrophoresis and high performance liquid chromatography for on-line detection and characterization [5,6].

For multi-analyte sensing in complex mixtures, array-based sensors are usually employed, in particular when indirect detection with signal generating labels (e.g. enzyme, electroactive compounds, fluorescent labels) is used. Array fabrication is achieved using various techniques for controlled surface modification to provide spatial separation of different addresses [7]. However, these techniques are generally complex, labor intensive and are often non-reproducible [8]. For example, a derivatized secondary antibody, specific for the primary antibody or different epitope of the same antigen, can be detected by Raman- or fluorescence-based spectra [9]. While such indirect methodology is applicable for the identification of large antigens, it cannot be used for the detection of relatively small DNA-carcinogen adducts. Besides, indirect detection can also be compromised by nonspecific detection arising from possible interferences (i.e. antibody cross-reactivity, nonspecific adsorption, contamination) associated with the system of interest. Therefore, the development of direct (i.e., label-free), information-rich

detection modes for biosensors (e.g., FLNS) that are capable of distinguishing between multiple analytes immobilized at the same address is of considerable importance since it would simplify biosensor production and minimize uncertainties in analyte(s) identification.

We report here on improvements of our previously designed biosensor chip that was based on a silicon wafer/Au/dithiobis(succinimidyl undecanoate) (DSU) /MAb nanoassembly for detection of a single analyte [4]. In this work, two linkers, DSU (which has to be synthesized) and the commercially available dithiobis(succinimidylpropionate) (DSP), are tested and compared for the rate of their deposition onto a gold surface, reactivity toward base catalyzed hydrolysis, and protein immobilization kinetics. The above two linkers differ only in length of their aliphatic chains (see below). It is shown that the shorter DSP linker is more efficient in protein immobilization and that additional incorporation of recombinant protein A as a mediator for oriented immobilization of MAbs minimizes fluorescence quenching by the gold substrate, which results in a lower limit of detection. Finally, the improved chip architecture is used to demonstrate that a biosensor chip with two specific MAbs immobilized at the same address can be used for the detection and identification of the depurinating (BP-6-N7Gua) and stable (BP-diolepoxide-10-N²dG) DNA adduct types formed by one-electron oxidation and monooxygenation pathways, respectively. We believe that this approach can be expanded to allow for simultaneous detection of multiple analytes (e.g., DNA adducts) without patterning and/or addressing.

II. Materials and Methods

Caution: *Benzo[a]pyrene is a hazardous chemical and should be handled carefully in accordance with NIH guidelines. Piranha solution is highly oxidizing and should be handled with extreme care.*

2.1. Preparation of gold substrates. Silicon wafer (100) single crystals, (Montco Silicon) cut into 10 x 20 mm sections or glass slides (Fisher) were used as solid supports for metal deposition in sensor preparation. Prior to metal deposition, the silicon substrates were sonicated in water followed by methanol for 20 min each and then dried with a stream of argon. The glass slides used for infrared reflection spectroscopy measurements were sonicated in Contrad 70 detergent (Fisher) and in methanol for 20 min each, and then dried with a stream of argon. These substrates were then coated with a 15 nm adhesive layer of chromium at a rate of 0.1 nm/s followed by deposition of 300 nm of gold (99.9% purity) at a rate of 0.3 nm/s. The depositions were carried out in an Edwards 306A cryo-pumped evaporator at a pressure of 2×10^{-6} Torr. Prior to linker deposition, the gold-coated surfaces were exposed to Piranha solution (3:1 H_2SO_4 and H_2O_2), rinsed with deionized water, and dried under a stream of argon. All other reagents were used as received. DSU was synthesized using a combination of procedures [10,11]. DSP was obtained from Pierce.

2.2. Linker - monolayer formation and MAb immobilization. Infrared reflection spectroscopy (IRS) performed on gold-coated glass slide supports was

used for the determination of monolayer formation kinetics and reactivity, as well as characterization of protein immobilization. The formation of linker monolayers was achieved by spontaneous deposition on the gold surface exposed to 0.1 mM ethanol solutions of DSU or DSP. These linkers have identical functional groups, but different methylene chain lengths; ten CH₂ groups for DSU and two CH₂ groups for DSP. Following linker deposition, the slides were rinsed with ethanol and dried under the stream of argon. MAb and recombinant (streptococcal) protein A (Pierce) were deposited from borate buffer solutions (100 mM H₃BO₃, 100 mM KCl, pH 8.5, 1% Tween 80; pH 8.5) onto a freshly prepared linker monolayer. The protein concentration was 50 µg/mL, and the total volume used was ten-fold the level required for surface saturation.

To study the protein binding efficiency of the DSP and DSU linkers mouse IgG (Pierce, 31204; BD 636550) was used. The CB53 MAb, specific for BP-6-N7Gua adduct, was obtained from Dr. G. Casale (Eppley Institute for Research in Cancer; Omaha, NE), while the 8E11 MAb, specific for the BP-diolepoxide-10-N²dG adduct was purchased from Trevigen, (Gaithersburg, MD). We note that the MAbs used on the biosensor chips were CB53 and/or 8E11; protein binding efficiency was comparable for IgG and the MAbs used on the biosensor chip. The samples were rinsed with buffer and deionized water and dried under a stream of argon. IR spectra of the linker monolayers and immobilized proteins were acquired with a Nicolet 750 FT-IR spectrometer purged with boil-off from liquid N₂. Spectra were obtained in an external reflection mode using *p*-polarized light incident at 82° with respect to the surface normal. A liquid-N₂-cooled HgCdTe detector was used for all

measurements. The spectra were recorded as $-\log(R/R_0)$ where R is the reflectance of the sample and R_0 is the reflectance of an octadecanethiolate- d_{37} monolayer-coated Au(111) reference sample. The spectra are an average of 512 scans and were taken at a resolution of 2 cm^{-1} with Happ-Genzel apodization.

2.3. Chip preparation. Immunosensor chips were fabricated on $10 \times 20\text{ mm}$ gold-coated silicon substrates. A poly(dimethylsiloxane) (PDMS) stamp [12] which had 2 mm diameter holes, was used to define the active areas on the chip. The stamp was soaked in octadecanethiol (ODT) and pressed against the gold surface for $\sim 30\text{ s}$. After rinsing with ethanol, the surface was dried under a stream of argon and immediately immersed in a 0.1 mM ethanol solution of DSU or DSP. After the deposition of the linker, substrates were rinsed with ethanol and dried under a stream of argon. This process provides reproducible, well-defined linker-modified areas surrounded by a hydrophobic ODT-coated region. Multiple on-chip addresses allow for testing of several, similarly prepared samples under comparable experimental conditions, with only small changes in chip positioning during the spectroscopic measurements. This results in improved reliability of the calibration measurements and enables better comparison of different samples.

For protein immobilization linker-activated areas of the chip were exposed to $10\text{ }\mu\text{L}$ solution containing the desired protein (proteins A and MAbs) or a mixture of MAbs at 0.5 mg/mL in borate buffer (pH 8.5) for 5 h . Laser-excited fluorescence detection of BP-derived DNA adducts bound by the corresponding MAbs was used to evaluate the performance of the chip. For that purpose, MAb-coated active

regions of a chip were incubated for 10 h with solutions of BP-6-N7Gua and anti-trans-BPDE-dG, or mixtures of the two (1×10^{-6} – 1×10^{-9} M) in 50 mM Tris-(hydroxymethyl)-aminomethane (TRIS) buffer (150 mM NaCl, pH 7.5) with added 1% dimethyl sulfoxide (DMSO). After incubation, the chips were rinsed with TRIS buffer and Nanopure (18 M Ω) water and dried under argon or helium.

2.4. Fluorescence measurements. The FLNS instrumentation is described in detail in Ref. [4]. Briefly, a Lambda Physik FL 2002 Scanmate pulsed dye laser system (10 Hz) pumped by Lambda Physik Lextra 100 XeCl excimer laser was used as the excitation source. A 1-m McPherson Monochromator (model 2601) and a Princeton Instruments photodiode array were used for dispersion and detection of fluorescence. A Princeton Instruments FG-100 pulse generator was used for time-resolved spectroscopy with detector delay times from 0 to 80 ns and a gate width of 200 ns. For spectroscopic measurements the chips were immersed in a glass cryostat (with quartz optical windows) filled with liquid helium or nitrogen. Prior to immersion, the active area(s) of the chip was covered with a thin layer (~ 300 μ m) of spectroscopic-grade glycerol (Aldrich). To minimize the amount of excitation light reaching the detection system, a grazing angle of $\sim 30^\circ$ of the laser beam relative to the sample surface was used. All fluorescence spectra are the average of 10 one-second acquisitions. The resolution for FLN and fluorescence non-line narrowed (FNLN) spectra was 0.1 and 0.8 nm, respectively.

III. Results and Discussion

In our previous work [4] a DSU linker was used to bind MABs to the chip surface. In this work we explore the efficiency of protein binding by the shorter DSP linker, and compare its performance in protein immobilization with that of DSU. A choice of a suitable linker may increase the MAB density and, thus, the number of binding sites.

3.1. Characterization of linkers by infra-red reflection spectroscopy.

Representative IR spectra of DSP- and DSU-derived monolayers chemisorbed on a gold surface are shown in Figure 1, spectra a and b, respectively. The three bands at 1817, 1788, and 1751 cm^{-1} are assigned to the C=O stretch of the ester, and the in-phase and out-of-phase C=O stretches of the succinimidyl endgroup, respectively [13]. The bands at 1218 cm^{-1} (C-N-C stretch) and 1077 cm^{-1} (N-C-O stretch) also arise from the succinimidyl endgroup. The DSU and DSP linkers, whose structures are shown in Figure 1, were chosen because they form spontaneously adsorbed monolayers (SAMs) on gold [11,14,15]. The Au-S bond ensures efficient chemisorption of the linker to the gold surface, while the succinimidyl ester group provides an efficient immobilization of proteins via covalent binding to the primary amines.

Spectra a and b of Figure 1 (60 min immersion time) are nearly indistinguishable, except for some differences in the symmetric and asymmetric C-H stretching modes for the $-\text{CH}_2$ groups at $\sim 2850 - 2925 \text{ cm}^{-1}$. As expected, the intensities of these modes in curve a are much weaker since the DSP linker

possesses a much shorter aliphatic chain. The weak band at 2950 cm^{-1} most likely corresponds to the CH_2 stretching mode of the succinimidyl group of the DSU and DSP molecules.

The results in Table 1 show that the frequency of the symmetric and asymmetric C-H stretching modes depends slightly on immersion time, with the DSU C-H modes at $2857/2925\text{ cm}^{-1}$ and $2850/2921$ for 5sec and 5 min of immersion time, respectively. Within the experimental uncertainty there is no further shifting after one minute. The 7 and 4 cm^{-1} red shifts for the longer immersion time indicate that the chemisorbed linker molecules subtly rearrange into a more ordered chair structure. In contrast, the C-H stretching modes of the DSP linker have somewhat higher frequencies, 2865 and 2925 cm^{-1} , respectively, and do not change with immersion time (spectra not shown). The larger $\nu_{\text{C-H}}$ values observed for the DSP linker and the absence of structural rearrangements with immersion time suggest that DSP is less ordered with possible gauche defects [16]. Thus, we conclude that:

1. the C-H modes in the $2850\text{-}2925\text{ cm}^{-1}$ region are conformationally sensitive and depend on the packing conformation of chains assembled on the surface; and
2. the packing of DSP linkers on the surface appears to be less constrained (less ordered) than that of DSU, predominantly due to DSP shorter length and, as shown below, also to its lower density of coverage.

Immersion Time (min)	DSU ν_{C-H} (cm ⁻¹)		DSP (ν_{C-H} (cm ⁻¹))	
	asym	sym	asym	sym
0.083	2925	2857	2925 ^a	2865 ^a
0.25	2922	2854		
0.5	2921	2851		
1	2921	2851		
5	2921	2850		
30	2921	2850		
150	2921	2850		
600	2920	2849		
1440	2920	2849		

^a Within the experimental uncertainty (± 2 cm⁻¹) these modes did not depend on the immersion time.

Table 1. Immersion time dependence of the symmetric and asymmetric C-H stretches (CH₂ groups) during formation of DSU and DSP monolayers.

3.2. Kinetics of the spontaneously adsorbed monolayer formation.

Figure 2 shows the time dependence of SAM formation for DSP (curve a) and DSU (curve b) obtained by monitoring the intensity of the 1751 cm^{-1} band as a function of immersion time in ethanolic solution. These reproducible results ($\pm 5\%$) show that surface saturation is reached within about 5 minutes for both DSP and DSU. Although no significant difference in the deposition rate of the DSU and DSP linkers is observed, these data indicate that the intensity of the 1751 cm^{-1} band in curve a (DSP) is lower than that in curve b (DSU). This difference, assuming minimal differences in end-group orientation, suggests that the surface density of the DSU monolayer is larger than that of DSP monolayer. This conclusion is supported by X-ray photoelectron spectroscopy (XPS) measurements which showed that DSU monolayer contains about 50% more nitrogen atoms (data not shown). The higher surface density of the DSU monolayer is also consistent with the lower C – H stretching frequencies for the DSU linker molecules, *vide supra*. Based on the IR and XPS data we conclude that the packing density of the DSU linker is about 40-50% higher than that of DSP, reflecting the stronger intermolecular hydrophobic interactions of DSU.

3.3. Reactivity and protein binding. The relative reactivities of the DSU and DSP linkers can be compared by measuring the hydrolysis rate of their succinimidyl endgroup. Such comparison is important because hydrolysis of the ester competes with protein binding. The rate of hydrolysis was determined by monitoring the succinimidyl C=O band intensity at 1751 cm^{-1} for DSU and DSP

derivatized surfaces as a function of exposure time to borate buffer solution (pH 8.5). The results for DSP and DSU linkers are shown in Figure 3, curves a and b, respectively. The intensity decrease of the 1751 cm^{-1} band is the result of hydrolysis of the succinimidyl endgroup, as schematically illustrated in the figure. The data show that hydrolysis rate for DSP is much faster than that for DSU. Complete hydrolysis of DSP is attained after about five hours. These results are consistent with those in [17], which showed that the rate of base-catalyzed ester monolayer hydrolysis is strongly influenced by monolayer packing density and linker orientation. Moreover, the hydrolysis rate for the DSU monolayer is about 1000 times slower than in solution [18]. The slow hydrolysis rate of the DSU monolayer was attributed to the more rigid and denser packing of the DSU molecules, which results in sp^2 hybridized carbon atoms being sterically blocked towards nucleophilic attack [19]. Such hindrance is much less severe in DSP because of its lower packing density and greater structural disorder.

Protein binding was studied next by monitoring the IR spectra of MAb bound to the DSP and DSU linkers. Data obtained following exposure of the linkers to MAb for 3 h are shown in Figure 4A. Spectra a and b correspond to the Au/DSP/MAb and Au/DSU/MAb chips, respectively. Protein binding efficiency can be determined by monitoring the intensity of the protein amide band near 1665 cm^{-1} . The width of this band (see solid arrow) indicates a broad distribution of the amide C=O stretch frequencies within the protein molecules and the newly formed amide linkages. Comparison of the intensity of the 1665 cm^{-1} band in spectra a and b suggests that the DSP linker binds more protein (see below). This observation is consistent with

the much faster decrease of the 1751 cm^{-1} band of the Au/DSP/MAB chip (spectrum a). The kinetics of disappearance of the 1751 cm^{-1} band in the presence of protein binding and competing hydrolysis of the succinimidyl endgroup is plotted in frame B of Figure 4. Curves a and b are for Au/DSP/MAB and Au/DSU/MAB, respectively. A cartoon of the reaction scheme is shown as an inset. Comparison of these results with the hydrolysis data shown in Figure 3 reveals that protein binding is more efficient than hydrolysis of the succinimidyl endgroup, especially for DSP. For DSP, MAB binding and hydrolysis are completed after $\sim 3\text{ h}$ (see arrow).

The protein binding kinetics, as determined by the increase in the intensity of the amide band at 1665 cm^{-1} , is plotted in Figure 4C. Comparison of curves a (DSP) and b (DSU) in frame C indicates that although the rates of protein binding are similar for the two linkers, the DSP monolayer at $t = 3\text{ h}$ binds $\sim 40\%$ more protein than the DSU monolayer. We suggest that the more densely packed protein on the DSP monolayer is a consequence of a lower packing density for DSP, which leads to enhanced flexibility in binding to the protein. Protein binding to DSP is completed in $\sim 3\text{ h}$ (see arrow). Although for $t > 3\text{ h}$ the results might suggest that binding of the protein to DSU is still occurring, comparison of curve b in Figure 3 and curves b in frames B and C of Figure 4 indicates that at times longer than $\sim 5\text{ h}$ mostly hydrolysis of unreacted DSU molecules is taking place. In fact, hydrolysis of DSU was not completed even after 20 h of reaction (data not shown). In contrast, the protein binding and succinimidyl group hydrolysis for DSP was completed in about 3 h . The finding that DSP is completely reacted by the time protein binding is

completed is important because there is no need to block the unreacted endgroups against non-specific binding as was the case for DSU mediated MAb immobilization.

3.4. Protein A mediated antibody immobilization on a DSP monolayer.

To increase the distance between the antigens (DNA adducts in this case) and the gold surface protein A was incorporated between DSP (or DSU) linker and the MAb. This modification leads to space-directed coupling of MAbs [2]. A confirmation of protein A binding to the linker, as well as subsequent MAb (CB53) binding to protein A was obtained by IRS. Typical IR spectra of protein A and protein A/MAb covalently bound to the DSP linker based chip are shown in spectra a and b of Figure 5, respectively. The intense bands near 1665 and 1542 cm^{-1} are due to the amide I and II modes, respectively. The broad band at about 3300 cm^{-1} is the N-H stretch vibration [20]. The presence of these bands proves that protein A and MAb are immobilized on the biosensor surface. The amide modes and the N-H stretch in spectrum b are five times more intense than those in spectrum a, proving that the MAb efficiently binds to the DSP/protein A nanoassembly. Somewhat less MAb (CB53) binding was observed for the DSU/protein A based chip (data not shown), in agreement with the results in Figure 4.

Based on the results presented previously [4] the average distance (d) between the MAb binding site and the gold surface in the Au/DSP/MAb based biosensor (hereafter chip 1) is about 9 nm, after correction for the length of the DSP linker. For the chip with bound protein A (Au/DSP/protein A/MAb), hereafter chip 2, d is estimated to be 18-19 nm. The significant increase of d is due to incorporation

of protein A (an additional 3-4 nm in height [21] and directed MAb immobilization that leads to upright binding of the MAb. This binding further increases the value of d by about 5-6 nm since the height of the MAb "Y" structure is ~14 nm [22,23]. The distances estimated above could be somewhat smaller since the depth of the binding pocket is unknown. This uncertainty, however, does not affect our comparative studies of fluorescence intensity measured for chips 1 and 2 since only relative enhancements are considered in the next section

3.5. Detection of BP-6-N7Gua adducts using the Au/DSP/protein A/MAb chip 2 architecture. Since fluorescence quenching of an analyte depends critically on the distance between the analyte and the gold surface [24] one expects that fluorescence quenching for chip 2 should be significantly weaker than for chip 1. Quenching can be due to energy transfer from the fluorophore to the metal surface [25]. The low temperature laser-induced fluorescence results that follow for chip 2 with the BP-6-N7Gua adduct bound to the CB53 MAb are consistent with the expectation discussed above.

Figure 6 shows non-line-narrowed (NLN) 77 K fluorescence spectra for BP-6-N7Gua adducts immobilized on chip 1 and 2, spectra a and b, respectively. The two spectra were obtained under identical conditions. The surface of each chip was covered with a thin layer of glycerol (300-500 μm) prior to cooling. The glycerol layer prevents evaporation of buffer and keeps the protein hydrated, which prevents denaturation. Other effects of glycerol will be discussed below.

The fluorescence spectra shown in Figure 6 with an origin band at 407 nm (for 20 ns delay) are in agreement with those in [4]. Comparison of spectra a and b shows that the fluorescence intensity of BP-6-N7Gua adducts measured on chip 2 is a factor of twelve times higher than that on chip 1. This is illustrated by the bar plot in Figure 6. Experiments with several chips gave an intensity increase factor of 10-12. Note that the only difference between the chips is that in chip 2 the adduct-gold surface separation is approximately twice that of chip 1, as illustrated in the lower part in Figure 6. The increase could be expected since fluorescence quenching due to energy-transfer process is proportional to βd^{-3} [25]. However, a doubling of d would only account for an 8-fold increase in fluorescence intensity for chip 2, while the observed increase is a factor of 10-12. The difference may be due to: *i*) change in β , which is proportional to the orientational parameter θ that is 1.5, 0.75, and 1 for perpendicular, parallel, and isotropic dipole configurations, respectively [24,25] and/or *ii*) an increase in the number of available binding sites per number of immobilized MAbs [26,27]. Moreover, an additional deactivation pathway that involves electron-transfer between excited DNA adducts and gold substrate could also contribute to the fluorescence quenching [28]. Nevertheless, it appears that the increase of d by a factor of 2 is largely responsible for the observed fluorescence enhancement.

3.6. Simultaneous biosensing of the two adducts. Figure 7 shows 77 K non-line narrowed fluorescence spectra of the BP-6-N7Gua and *anti-trans*-BPDE-N²dG adducts bound by MAbs immobilized on chip 2 obtained with 20 (a), 40 (b) and 80 ns (c) delay times of the observation window relative to excitation,

respectively. Spectra a-c were normalized at the 377.4 nm band that is the origin band of the *anti-trans*-BPDE-N²dG adduct (labeled in Figure 7 as (0,0)_{II}) [29]. However, spectrum a contains additional (and somewhat broader) bands at 407.0 nm (labeled as (0,0)_{II}) and near 430 nm. Based on the results in [4], they correspond to the origin and vibronic band of the BP-6-N7Gua adduct. Comparison of spectra a, b, and c indicates that BP-6-N7Gua adduct has a shorter fluorescence lifetime (τ_{FL}) than that of *anti-trans*-BPDE-N²dG. This difference is consistent with our estimates of τ_{FL} for BPDE-N²dG and BP-6-N7Gua adducts, based on integrated time-resolved 77 K spectra (obtained at several delay times), which are 50±10 and 20±10 ns, respectively. This explains why the relative contribution of BP-6-N7Gua decreases at 40 ns delay (spectrum b), and disappears at 80 ns delay of the observation window (spectrum c).

The maximum of the (0,0)_{II} band is similar to that of BP-6-N7Gua in a standard glycerol glass, except for a small (~1.0 nm) matrix-induced red shift. Since at longer delay times (80 ns) BP-6-N7Gua does not contribute to the fluorescence, spectrum c is assigned solely to the *anti-trans*-BPDE-N²dG with a (0,0)_I band at 377.4 nm. The pure spectral contribution from BP-6-N7Gua adduct is obtained by the difference between spectrum a and spectrum b or spectrum b and spectrum c. The latter is spectrum d. The (0,0)_{II} in spectrum d is blue-shifted by 1.5 nm relative to its position in spectrum a. This shift is in agreement with the results in [30] which showed that the position of the origin band for the BP-6-N7Gua adduct shifts to higher energies with longer delay time of the observation window. This change was interpreted in terms of a distribution of BP-6-N7Gua conformers and a matrix-

induced frequency dependent transition dipole moment change across the inhomogeneously broadened absorption profile. This effect was not observed for the *anti-trans*-BPDE-N²dG adduct. We note that the BP-6-N7Gua adduct can be selectively excited at 386 nm, and thus differentiated from the BPDE-type adduct (data not shown). We conclude that: 1) BP-6-N7Gua and BPDE-10-N²dG bind efficiently to the CB53 and 8E11 antibodies, respectively, immobilized on chip 2; and 2) the above adducts bound at the same active area on chip 2 can be easily distinguished either by time resolved spectroscopy or selective laser excitation.

Considered next are the vibronically excited FLN spectra in Figure 8 obtained with chip 2, 4.2 K. Frames A and B are for BP-6-N7Gua and BPDE-N²dG adducts, respectively. Spectra a and b in frame A were obtained with an excitation wavelength (λ_{ex}) of 385.0 and 387.0 nm, respectively, and are characteristic for BP-6-N7Gua [4,31]. The vibrational frequencies of the S₁ excited state at 1134, 1169,

IV. Concluding Remarks

The results presented in this paper show a higher density of MAb on the DSP/protein A modified surface than on the DSU/protein A assembly. In addition, the modified chip architecture, i.e., Au/DSP/protein A/MAb (chip 2), provides an oriented immobilization of MAb and increases the distance (by a factor of two) from the fluorescent analytes to the gold surface. The larger distance decreased fluorescence quenching due to the decreased energy- and/or electron-transfer processes to the gold surface. The reduced quenching in this novel chip design led to significantly stronger (by a factor of 10-12) fluorescence of the laser-excited DNA

adducts, and as a result, to a better limit of detection (LOD) that is currently in the low femtomole range. The concentration LOD for the BP-6-N7Gua adduct is $\sim 10^{-9}$ M. A slightly better LOD was observed for the stable *anti-trans*-BPDE-10-N²dG adduct that possesses a longer fluorescence lifetime. We emphasize that the modifications in chip 2 not only increased the distance between the analytes and the gold surface, but also increased the number of available binding sites per active area due to the upright MAb binding.

It was also shown that a biosensor chip with two MAbs: 1) CB53 specific for BP-derived DNA adducts formed by one-electron oxidation and 2) 8E11 specific for monooxygenation type adducts can be used to identify these adducts at the same address using time-resolved, laser-based, low temperature fluorescence spectroscopy. To our knowledge this is the first report of a sensor that can simultaneously measure the two major DNA adduct types derived from BP, which is known to be one of the most potent carcinogens [35]. We conclude that with a proper calibration curve quantitation of the BP-6-N7Gua and BP-diolepoxide-10-N²dG adducts should be possible using a single chip with CB53 and 8E11 MAbs immobilized at the same address. This concept could be accomplished by exposing the biosensor first to DNA supernatant to collect depurinating adducts, and then to the corresponding DNA digest to bind the BPDE-type adducts on the same chip. We anticipate that the new chip methodology can be scaled up to accommodate detection of multiple luminescent antigens for which MAbs can be generated. For example, biosensors with specific MAbs could be used for identification of DNA

adducts derived from the same carcinogen activated by different metabolic pathways.

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Figure 1. Spectra a and b are the infrared reflection spectra for DSP- and DSU-derived monolayers deposited on a gold surface. The intense bands at 1077, 1218, and 1751 cm^{-1} correspond to the succinimidyl endgroup of the linker molecules. The insets show the molecular structures of the immobilized DSP and DSU.

Figure 2. Kinetics of DSP (a) and DSU (b) monolayers formation measured by monitoring band intensity at 1751 cm^{-1} .

Figure 3. Hydrolysis rate of DSP (a) and DSU (b) derivatized surfaces recorded at the 1751 cm^{-1} vibrational mode, as a function of the immersion time in a borate buffer solution (pH 8.5).

Figure 4. Frame A: IR spectra obtained after 3h of protein deposition on DSP (a) and DSU (b) monolayers. The bands at 1751 and 1665 cm^{-1} are the out-of-phase C = O stretch of the succinimidyl group and the amide mode I, respectively.

Figure 4. *Frame B:* Curves a and b describe the decrease in time of the 1751 cm^{-1} band intensity during the incubation of the DSP and DSU monolayers with a protein (pH 8.5), respectively. *Frame C:* The rate of protein immobilization on the DSP (a) and DSU (b) monolayers monitored by the amide I band 1665 cm^{-1} .

Figure 5. Infrared reflection spectra of protein A (a) and protein A/MAb assembly (b) obtained on a biosensor chip surface with the DSP linker. The more intense bands at 1542, ~1665, and ~3300 cm^{-1} , observed in spectrum b, show that MAb is efficiently deposited on protein A (curve a).

Figure 6. Spectra a and b are the 77 K NLN fluorescence spectra of BP-6-N7Gua adducts obtained with 20 ns delay time of the observation window on chip 1 and chip 2, respectively. ($\lambda_{\text{ex}} = 381 \text{ nm}$). The vertical bars in the inset correspond to the integrated fluorescence based on spectra a and b, chip 1 and 2, respectively. The lower part of the inset shows the basic features of chip design.

Figure 7. NLN fluorescence spectra obtained with chip 2 exposed to a mixture of BP-6-N7Gua and *anti-trans*-BPDE-10-N²dG. The concentration of each adduct was 10⁻⁶ M. Spectra a, b, and c were obtained with 20, 40 and 80 ns delay times of the observation window (T = 77 K). $\lambda_{\text{ex}} = 343$ nm. Spectrum d is the difference between spectra b and c. The solid arrows in spectrum a indicate the positions of the fluorescence origin bands. Spectra c and d reveal pure contribution from the *anti-trans*-BPDE-10-N²dG and BP-6-N7Gua adducts, respectively; the insets show the corresponding adducts structures.

Figure 8. FLN spectra of BP-6-N7Gua and BPDE-10-N²dG adducts on chip 2, frames A and B, respectively. Spectra a/b in frames A and B were obtained with λ_{ex} of 385.0/387.0 and 367.0/363.0 nm, respectively. Zero phonon lines are labeled with their excited state vibrational frequencies (in cm⁻¹). T = 4.2 K.

CHAPTER 3

**PROBING THE INTERACTION OF BENZO[A]PYRENE ADDUCTS
AND METABOLITES WITH MONOCLONAL ANTIBODIES USING
FLUORESCENCE LINE-NARROWING SPECTROSCOPY**

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Abstract

A new approach for studying antibody/antigen interactions of DNA adducts and metabolites of polycyclic hydrocarbons (PAHs) is demonstrated in which fluorescence line-narrowing spectroscopy (FLNS) is used. It is based on the fact that the relative intensities of the line-narrowed bands that correspond to the excited vibrations in an FLN spectrum are, in general, strongly dependent on the local environment of the fluorophore. Information on the nature of the interactions can be

obtained by comparing the FLN spectra of the antigen/antibody complexes to the spectra of the antigen in solvents of different types (H-bonding, aprotic, π -electron containing) recorded under the same conditions. The antigens used were the DNA adduct 7-(benzo[a]pyren-6-yl)guanine (BP-6-N7Gua) and the metabolite (+)-*trans-anti*-7,8,9,10-benzo[a]pyrene-tetrol (BP-tetrol) of benzo[a]pyrene; two monoclonal antibodies (MAb) have been developed to selectively bind these compounds. It is shown that for BP-tetrol, H-bonding solvents have a pronounced effect on the FLN spectra. The presence of π -electrons in the solvent results in relatively small but still significant changes in the spectra. When BP-tetrol is bound to its MAb, however, neither of these effects is observed; its spectrum is very similar to the one obtained with aprotic solvent methylcyclohexane. Therefore, we can conclude that the MAb has an internal binding site in which the interaction with BP-tetrol is of a hydrophobic character. For BP-6-N7Gua, however, there is a strong effect of the presence of π -electrons in the solvent. The FLN spectrum of this antigen bound to its MAb is very similar to its spectrum in acetone and dimethylsulfoxide, indicating that π - π interactions play an important role in the binding.

Keywords: Monoclonal antibodies; antigen/antibody binding interaction; benzo[a]pyrene (B[a]P) adducts; benzo[a]pyrene metabolites; solvent effect; fluorescence line-narrowing spectroscopy.

I. Introduction

In recent years monoclonal antibodies (MAbs) have become increasingly popular in bioanalytical chemistry [1]. When immobilized on a chip or optical fiber, they can be used as biosensors [2,3]. Because MAbs can be developed that selectively bind the compound(s) of interest, sample pre-treatment and separation requirements can be less stringent in the total analytical scheme. Even for the analysis in complex biological matrices like blood, urine and plasma they can often be used directly [1]. Several papers have addressed the mechanisms of ligand-MAb binding [4-7]. Nonetheless, not in all cases are MAbs specific enough; they show cross-reactivity [8]. Thus, there is a need for simple methodologies that provide insights on the nature of antibody-antigen interactions.

One application of MAbs is the monitoring of DNA adducts and metabolites of polycyclic aromatic hydrocarbons (PAHs) in urine [9]. PAHs are formed after incomplete combustion of organic material and many PAHs are potent carcinogens. Benzo[a]pyrene (B[a]P), known as one of the most carcinogenic PAHs, is present in relatively high concentrations in traffic exhaust, cigarette smoke, and grilled or smoked food products [10,11]. In vertebrates, PAHs are oxidized in order to form more polar and, therefore, more easily excretable metabolites. However, some metabolic processes lead to reactive-electrophilic intermediates that can initiate cancer. B[a]P can be metabolically activated via three main pathways [12-14]: one-electron oxidation to yield radical cations, monooxygenation to produce bay-region diol epoxides; and, thirdly, the formation of reactive and redox active o-quinones by dihydrodiol dehydrogenases. The active intermediates can bind to DNA to form

stable and/or depurinating adducts [12-14]. The depurinating adducts are released from DNA by destabilization of the N-glycosidic bond and excreted in urine. Determination of depurinating adducts or metabolites in urine can be used for cancer risk assessment studies. For that purpose antibodies have been developed that selectively bind such compounds [15].

Since most PAHs are highly fluorescent, fluorescence spectroscopy can be used for the detection of PAH adducts bound to MAbs. However, most MAbs lack the specificity required to bind only one adduct or metabolite, so that conventional fluorescence is not always adequate for distinguishing between different antigens possessing the same parent fluorophore. Under such conditions a more specific, high-resolution technique like fluorescence line-narrowing spectroscopy (FLNS) can be used for additional selectivity [8,15]. FLNS has proven to be useful for the detection of PAH adducts and metabolites, since it can distinguish between (stereo-)isomers and between metabolites bound to different nucleophilic sites [16]. FLN spectra of PAH-derived metabolites and/or PAH-DNA adducts bound to a MAb have also been reported [17]. In this paper we will focus on one particular BaP adduct and one particular B[a]P metabolite for which MAbs have been developed. The adduct, 7-(benzo[a]pyren-6-yl)guanine (BP-6-N7Gua), is formed upon one-electron oxidation, whereas the metabolite (+)-*trans-anti*-7,8,9,10-benzo[a]pyrenetetrol (BP-tetrol), is formed upon monooxygenation and hydrolysis (see Scheme 1). Both the metabolite and adduct are biomarkers and can be found in urine; BP-tetrol is a biomarker of exposure and metabolism and BP-6-N7Gua is a biomarker of actual DNA damage [18,19].

The principles of FLN spectroscopy have been discussed in earlier work [20,21]. Briefly, in FLNS solute molecules are imbedded in an amorphous host matrix held at a temperature in the vicinity of 4 K. At such temperatures, thermal broadening of the origin and vibronic fluorescence bands is minimized. However, the structural disorder of the host results in severe static inhomogeneous broadening (typically $\sim 100\text{-}300\text{ cm}^{-1}$) of the origin and vibronic absorption bands of the ground state (S_0) to excited electronic state (S_1) transition of the solute molecule, with the excited state being fluorescent. FLN is a laser-based site excitation energy selective technique that eliminates the contribution from inhomogeneous broadening to the widths of the vibronic bands in the fluorescence spectrum. The basic idea is that with narrow line laser excitation, only those solute sites whose absorption transition frequency overlaps the laser profile are excited. At sufficiently low solute concentrations, only the sites excited fluoresce; thus, the line-narrowing phenomenon. Typically, such line-narrowing leads to about a two order of magnitude improvement in resolution as measured relative to inhomogeneous broadening. There are two modes of operation: excitation into the origin (0,0) absorption band of the $S_0 \rightarrow S_1$ transition and excitation into vibronic bands of the same transition. Spectra obtained in the former and latter modes are referred to as origin and vibronically excited. It has been shown that the latter mode provides the highest selectivity because it provides detailed information on the active vibrations of the S_1 state. It is this mode that was used in the present study. The sharpest bands in a FLN spectrum are referred to as zero-phonon-lines (ZPLs) because low frequency lattice vibrations (phonons) of the

solute/host system are not involved. However, electron-phonon coupling associated with the $S_0 \rightarrow S_1$ transition leads to a broad phonon sideband (PSB) that builds on the sharp ZPLs. Strong electron-phonon coupling leads to a significant loss in resolution since the intensity of the ZPL relative to that of the PSB decreases with increasing coupling strengths.

In the vibronically excited mode, laser excitation at the wavelength λ_{exc} typically excites several different vibrations. Once excited, these vibrations rapidly relax to the zero point vibrational level of the S_1 state after which fluorescence (relaxed) occurs. This results in the so-called multiplet origin structure in the FLN spectrum. The differences in energy between the ZPLs that contribute to the multiplet structure and λ_{exc} yield the excited state vibrational frequencies of the $S_0 \rightarrow S_1$ transition. A key point is that the relative intensities of the ZPLs are very sensitive to the interactions of the fluorophore and its local environment. Thus, changes in the local environment produce significant changes in the relative intensities of the ZPLs that contribute to the multiplet origin structure for a given value of λ_{exc} . By tuning λ_{exc} across the absorption spectrum one can determine the frequencies and Franck-Condon factors of the fundamental vibrations that contribute to the $S_0 \rightarrow S_1$ spectrum.

So far, few applications of FLNS for probing purposes have been reported. Kumke *et al*, used FLNS to probe pyrene - humic acid interactions [22], and Suh *et al* [23] showed that FLN spectra of intercalated B[a]P-DNA adducts are strongly influenced by π - π interactions with neighboring DNA bases. Here, it will be shown for the first time that the binding interaction between antibodies and antigens can be

studied using FLNS. The approach followed here is to compare the FLN spectra of an antibody/antigen complex with the FLN spectra of the antigen in various solvents of different chemical nature. Whereas other methodologies like surface plasmon resonance [5] and ELISA [6] give information about the affinity of antibody-antigen binding as such, our approach provides information on the nature of the intermolecular interactions that lead to binding. Furthermore, the crystal structure of the MAb is not needed, as in molecular modeling studies [4,7].

II. Materials and Methods

Caution: PAHs are hazardous chemicals and should be handled carefully in accordance with NIH guidelines.

The solvents used were dimethylsulfoxide, acetone, methylcyclohexane (Aldrich, Milwaukee, WI), ethanol (Fisher, Fair Lawn, NJ), glycerol (Spectrum Chemical Mfg. Corp., Gardena, CA) and an aqueous PBS buffer (0.1M sodium phosphate, 0.15 M sodium chloride). 10 μ M solutions of BP-tetrol and BP-6-N7Gua were used to record the spectra. The MAbs used in this study were monoclonal antibody CB53 (raised against BP-6-N7Gua, provided by Dr. G. Casale, University of Nebraska Medical Center, Omaha, NE) and *anti*-BPDE antibody 8E11 obtained from Trevigen (Gaithersburg, MD). The latter also has an affinity for BP-tetrol [24]. The antigen bound to the MAb was measured in solution: 2 mL of an aqueous solution containing 1 μ M of antigen and 5 μ g/ml MAb was incubated for 2 hours. The excess of antigen was removed using an ultrafree-4 centrifugal filter with Biomax-5 membrane (Millipore Corporation, Bedford, MA). Two additional washing steps were performed

and the samples were concentrated by reducing the volume to 100 μ L. These samples were diluted (1+1) with glycerol to obtain a glassy matrix under cryogenic conditions. 30 μ L samples were transferred to quartz tubes (2 mm i.d.) for FLN analysis, the irradiated volume was about 1 μ L.

The quartz tubes were immersed in liquid helium contained in a cryostat with quartz optical windows (H. S. Martin Inc., Vineland, NJ). The excitation source was a Lambda Physik Scanmate 2 pulsed dye laser, pumped by a Lambda Physik Lextra 100 XeCl excimer laser (Lambda Physik, Ft. Lauderdale, FL) at a repetition rate of 10-30 Hz. Fluorescence emission was collected with a 2-inch lens at a 90° angle to the excitation beam, focused onto the entrance slit of a 1-m monochromator (McPherson model 2061), and detected with an intensified linear photodiode array (model IRY-1024/GRB, Princeton instruments, Trenton, NJ) operated in the gated mode to reject scattered light. With the 2400 G/mm grating the spectral resolution was 0.05 nm (high-resolution conditions: FLN measurements of the ZPL origin multiplet structure. A 150 G/mm grating and a resolution of 0.8 nm was used for recording total fluorescence spectra at 77K (low-resolution conditions). Background subtraction was performed with Origin 5.0 (Microcal Software Inc, Northampton, MA).

III. Results and Discussion

In this study the FLN spectra of MAb-bound BP-tetrol and BP-6-N7Gua are compared to their spectra in various solvents of different chemical nature. For BP-tetrol, the stereochemistry of its four hydroxyl groups attached to the saturated ring

has a large effect on the FLN spectrum [18] and, thus, an effect of H-bonding solvents can be expected. In the case of BP-6-N7Gua, the only H-bonding atoms are located on the guanine moiety and any effect on the fluorophore from H-bonding is expected to be less pronounced. However, it has been shown that π - π interactions have a strong effect on the FLN spectra of B[a]P-DNA adducts, in particular they lead to strong electron-phonon coupling for base-stacked and intercalated configuration of the aromatic fluorophore. This is the result of interactions of the fluorophore with the DNA bases which results in the fluorophore's S_1 state possessing charge-transfer character. [23].

Apart from their chemical characteristics, the solvents were chosen such that under cryogenic conditions the solute molecules would be in a glass matrix. For this purpose, 50% glycerol is often added to aqueous solutions to ensure high quality of the glass. Therefore, also in this study the antigen/antibody complexes were dissolved in 50/50 glycerol/buffer solution. In order to study the effect of H-bonding two protic solvents were selected: ethanol (EtOH) and 50/50 glycerol/buffer. The latter was also used to dissolve the antibody/antigen complex, so a difference between the FLN spectrum of this complex and that of the antigen in the 50/50 glycerol/buffer glass would indicate that the antigen is not free in solution and therefore must be bound to the antibody. As an aprotic aliphatic solvent, methylcyclohexane (MCH) was chosen. For the study of the effect of π -electron containing solvents, acetone and dimethylsulfoxide (DMSO) were chosen.

BP-tetrol

The 77K fluorescence spectrum of BP-tetrol bound to MAb was recorded and compared to its spectra in the five abovementioned solvents, recorded under the same conditions, Figure 1. In the low-resolution spectra of Figure 1 no major differences in the positions and the relative intensities of the bands can be observed, only some minor shifts and changes in bandwidths. Although reproducible, these effects are relatively small compared to the spectral resolution used (0.8 nm) to record the spectra. Therefore, we concluded that high-resolution FLN spectra are required to obtain information on the nature of the antibody/antigen binding interactions.

Under FLN conditions the solvent effects for BP-tetrol in the (0,0) band region (375 to 380 nm), Figure 2 are much more pronounced. The FLN spectra show pronounced ZPLs superimposed on a relatively weak broad fluorescence background due to phonon sidebands (PBSs) and spectral congestion (dotted lines). The positions of the multiplet origin ZPLs are very similar in the different solvents, indicating that the intramolecular vibrational energies of the S_1 state of the parent fluorophore (pyrene) are not significantly affected by the environment. In Figure 3 for each spectrum a background (the dotted lines in Figure 2) is subtracted. This broad background fluorescence was subtracted in order to better compare the relative intensities of the ZPLs; these differences are more clearly observed in Figure 3 than in Figure 2. The numbers labeling the ZPLs in Figure 3 are the vibrational energies (cm^{-1}) of the S_1 state of the parent fluorophore pyrene. Although the vibrational energies of BP-tetrol bound to the MAb and in the various solvents are identical

within experimental uncertainty ($\pm 3 \text{ cm}^{-1}$), the ZPL intensity distributions for the aprotic solvents differ significantly from the distributions for the H-bonding solvents EtOH and Gly/buffer. This is most evident by comparing the intensities of the ZPLs at 958 cm^{-1} and 1111 cm^{-1} in the various spectra. For BP-tetrol in EtOH and 50/50 glycerol/buffer, the excited state mode at 958 cm^{-1} is much more intense than the mode at 1111 cm^{-1} . Conversely, in the aprotic solvents MCH, acetone and DMSO, the opposite is observed. This indicates that the spectrum is strongly influenced by H-bonding that leads to a blue-shift of the $S_0 \rightarrow S_1$ transition. (That $\pi\pi^*$ states are blue-shifted in H-bonding solvents is well established). Under close examination this shift is already noticeable in the 77K fluorescence spectra of Figure 1, but under FLN conditions the blue shift is much more apparent. For the two ZPLs at 1038 cm^{-1} and 1047 cm^{-1} there is a solvent effect as well, which is not related to H-bonding or blue shifting. In EtOH, 50/50 glycerol/buffer and MCH the two bands are of approximately equal intensity, whereas in acetone and in DMSO the intensity of the 1038 cm^{-1} band is considerably lower than that of the 1047 cm^{-1} band. These differences are presumably due to $\pi-\pi$ interactions between the pyrene moiety and acetone or DMSO; such interactions are absent for the other solvents..

The solvent dependencies discussed above can be used to describe the binding of BP-tetrol to the MAb. If H-bonding were important, the spectrum would resemble those recorded in EtOH or 50/50 glycerol/buffer. The intensity ratio between the 958 cm^{-1} and the 1111 cm^{-1} band observed for MAb indicates that this is not the case. Since the antibody is in 50/50 glycerol/buffer glass, the conclusion can be drawn that BP-tetrol is not located at the surface but well embedded inside the antibody. This also

confirms the affinity of the *anti*-BPDE MAb for BP-tetrol. Comparing the spectrum of BP-tetrol bound to MAb to its spectra in aprotic solvents, we note that the two peaks at 1038 and 1047 cm^{-1} are of approximately equal intensity, similar to the spectrum in MCH. Furthermore, the relative intensity of the 958 cm^{-1} and 1111 cm^{-1} bands in the spectrum of BP-tetrol bound to MAb is more similar to that in MCH than to those in acetone and DMSO. These observations indicate that the binding site in the MAb is of a nonpolar character, and that H-bonding and π - π interactions play no major role. Thus, it is most likely that the antigen/antibody binding is mainly due to hydrophobic interactions.

BP-6-N7Gua

Similar experiments were performed with BP-6-N7Gua. The 77K fluorescence spectra are shown in Figure 4. The origin bands are located near 405 nm and a major vibronic transition can be seen near 430 nm. Again, only small differences in the position of the origin band and in bandwidth could be observed under these conditions. Much more detailed information can be obtained from the 4K FLN spectra of the BP-6-N7Gua samples; these spectra differ considerably (see Figure 5). For BP-6-N7Gua bound to MAb and dissolved in acetone or DMSO, the ZPLs have a low intensity and are superpositioned on a broad background with a maximum around 408.5 nm. This red shifted background is due to increased electron-phonon coupling. In the spectra of BP-6-N7Gua dissolved in EtOH, 50/50 glycerol/buffer or MCH this broad background is much weaker. Therefore, the increased electron-phonon coupling is probably due to π - π interactions, similar to

intercalated B[a]P-DNA adducts [23]. Unlike the case of BP-tetrol, only a minor influence of H-bonding solvents was observed: the spectra in 50/50 glycerol/buffer and MCH are largely similar.

After background subtraction (the backgrounds used are shown in Figure 5 as the dotted lines) the relative intensities of the ZPLs can be more easily compared (see Figure 6). Although there is some noise at the red end of the spectra, the multiplet origin bands can be readily distinguished. It should be noted that their positions in the various solvents are virtually identical, indicating that the S_1 vibrational energies are not affected by the solvent. Some clear differences can be observed when comparing the relative intensities of the 1168 cm^{-1} and 1315 cm^{-1} bands. For EtOH and 50/50 glycerol/buffer these two bands have similar intensities. In MCH, however, the intensity of the 1168 cm^{-1} band is approximately two times higher. For BP-6-N7Gua bound to MAb and BP-6-N7Gua in acetone or DMSO, the opposite is observed: the 1315 cm^{-1} band is twice as intense as the 1168 cm^{-1} band. Another difference is the presence of the 1397 cm^{-1} band: in acetone and in the spectrum of BP-6-N7Gua bound to MAb it can be clearly observed, whereas in DMSO it is beyond detection.

These results show that, unlike the MAb developed for BP-tetrol, the binding site of the MAb for BP-6-N7Gua is not hydrophobic (see the difference with MCH spectrum). On the contrary, the similarity to the spectrum in acetone suggests that π - π interactions (for instance with carbonyl groups) are involved in the binding site of the MAb. Since only minor effects of H-bonding solvents were observed in the

spectra of BP-6-N7Gua, no conclusions can be drawn about the extent of hydrogen bonding (for instance to the guanine moiety) in the binding site.

IV. Concluding Remarks

It has been demonstrated that FLNS can provide qualitative information on the interaction between B[a]P derivatives and the monoclonal antibodies developed to selectively bind them. Based on our data BP-tetrol is located in the interior region of the protein with a binding site being of a hydrophobic nature. In contrast, the binding site of BP-6-N7Gua is not hydrophobic and appears to be dominated by the interaction between π -electrons of BP-6-N7Gua and those of the surrounding protein.

We note that the combination of FLNS and solvent dependent studies to investigate the nature of antibody/antigen binding interactions will only be useful for antigens (or ligands in general) that exhibit native fluorescence as well as fluorescence line-narrowing under cryogenic conditions. A second prerequisite is that the FLN spectra are solvent dependent. A phenomenon that is hardly described in the literature and needs further exploration.

It should be emphasized that in FLNS sample handling is simple – the only requirement is cooling to temperatures in the vicinity of 4 K – and furthermore that FLNS has a high inherent sensitivity. The information on the type of binding interactions that is obtained can be useful for the development of MAbs and the understanding of which compounds will show cross-reactivity. Currently the applicability of the FLNS approach is being extended by a study of binding sites of

enzyme receptors (for example the estrogen receptor) and cross-binding activity of various monoclonal antibodies.

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Scheme 1. Structures of BP-tetrol and BP-6-N7Gua.

Figure1. 77K low-resolution fluorescence spectra of BP-tetrol in various solvents. Excitation wavelength is 350 nm into $S_0 \rightarrow S_2$ transition of pyrene.

Figure 2. FLN spectra of the multiplet origin band of BP-tetrol in various solvents, excited at 363 nm. The dotted lines are the background fluorescence due to phonon sidebands and spectral congestion (see text).

Figure 3. Background subtracted FLN spectra of the multiplet ZPL origin region of BP-tetrol in various solvents, excited at 363 nm (background: see figure 2). The numbers labeling the ZPLs are the S_1 state vibrational energies in the units of cm^{-1} .

Figure 4. 77K low-resolution fluorescence spectra of BP-6-N7Gua, excited at $\lambda_{\text{ex}} = 360$ nm.

Figure 5. FLN spectra of the multiplet origin band of BP-6-N7Gua bound to MAb and in various solvents, excited at 386 nm. The dotted line is the background that is subtracted in figure 6.

Figure 6. Background subtracted FLN spectra of the multiplet origin band of BP-6-N7Gua in various solvents, excited at 386 nm (background: see figure 5). The given wavenumbers are the excited state vibrational energies.

CHAPTER 4

**CROSS-REACTIVITY AND CONFORMATIONAL MULTIPLICITY OF
AN anti-POLYCYCLIC AROMATIC HYDROCARBON MONOCLONAL
ANTIBODY**

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Abstract

Cross-reactivity and multispecific functionality of antibodies play a central role in the immune system. Antibody's promiscuity is attributed to structural flexibility and conformational multiplicity of its binding sites governed by the rearrangement of hydrogen bonding centers. However, antibodies whose recognition and binding rely on less directional hydrophobic interactions might follow different interaction pathways. We investigated interaction of anti-polycyclic aromatic hydrocarbon monoclonal antibody (anti-PAH MAb) with two biologically important cross-reactants - pyrene and benzo(a)pyrene. Complex formation was characterized by means of low temperature laser-induced fluorescence (LIF) spectroscopy in both low-

resolution, and high-resolution, fluorescence line-narrowing (FLN) modes. It is shown that the FLNS can identify various haptens cross-reacted with an antibody, as well as simultaneously differentiate between free and immunocomplexed haptens. In addition, our results suggest an interesting case of an antibody binding a particular hapten (cross-reactant) by adopting two distinct conformations of its binding sites. The existence of the multiple conformations for anti-PAH MAb that are trapped at low temperature can be rationalized through the existing models for antibody binding. Finally, as revealed by FLN spectra of immunocomplexed chromophores, π - π interactions, rather than hydrogen bonding, play the central role in complex formation.

Abbreviations: PAH, polycyclic aromatic hydrocarbons; Py, pyrene; BP, benzo(a)pyrene; MAb, monoclonal antibody; LIF, laser induced fluorescence; FLNS, fluorescence line-narrowing spectroscopy; NLN, non line-narrowing; ZPL, zero phonon line;

I. Introduction

The relationship between protein structure and function continues to be one of the most challenging of problems in molecular biology. Complicating the solution of this problem is the demise of the view that a given protein sequence has one structure and, therefore, one function. Indeed, examples of multifunctional proteins are numerous, as are examples in which a single binding site can bind a range of varied molecular shapes (1-3). This promiscuity has been explained by two different

models: molecular mimicry and preexisting equilibrium of structural isomers. The molecular mimicry assumes that the protein exists in a single conformation that can bind structurally similar ligands with low affinity and that subsequently the protein rearranges into a high affinity configuration (induced fit). Evidence for the induced fit model is that crystal structures of bound and unbound proteins show differences in the ligand binding site configuration (2, 4). The preexisting equilibrium model, on the other hand, assumes that the intact protein exists in multiple conformations, one of which has high affinity for the ligand; following binding, the equilibrium shifts in favor of this configuration (5). Recently, James et al. presented structural evidence for equilibrium between the two preexisting antibody isomers and demonstrated that a single antibody (SPE7, raised against 2,4-dinitrophenyl hapten) can bind structurally unrelated ligands through conformational selection (3). They have also investigated cross-reactivity of the same antibody involving a single conformational isomer (2). They found that while SPE7 can distinguish between closely related derivatives of the hapten it was elicited against, it can also bind a number of unrelated haptens. From partitioning experiments, it was shown that this multispecificity did not arise from "hydrophobic stickiness", but rather from hydrogen bonding that differed between the various haptens.

In this paper we address the question of antibody specificity for ligands structurally similar to that against which the antibody was raised. Our interest is cross-reactivity in the case where hydrogen bonding to the hapten is limited (or not possible) and the specificity is determined by less discriminating interactions (hydrophobicity and/or π - π interactions). The anti-PAH monoclonal antibody (MAb)

used here has been elicited against fluoranthene, a polycyclic aromatic hydrocarbon (PAH) that lacks any polar groups for which strong hydrogen bonding can occur. PAH are a class of compounds that consist of two or more condensed aromatic rings. Because PAH and their metabolic and mutagenic derivatives are relatively small, aromatic, nonpolar molecules they are highly susceptible to cross-reaction with antibodies developed for one particular molecule from the group (6, 7). From the perspective of chemical analysis and diagnostics this is vital, since the validity of immunoassays that are commonly used for determination of PAH and their derivatives can be seriously challenged. Therefore, revealing the nature of antibody-hapten interactions and patterns involved in antibody cross-reactivity, as well as the development of methodologies to discriminate between cross-reacted species is of substantial interest.

Being highly fluorescent, most PAH and their derivatives can be determined by fluorescence techniques. Particularly powerful is high-resolution, fluorescence line-narrowing spectroscopy (FLNS), attributed with fingerprinting capabilities and proven to provide unambiguous identification of structurally related fluorescent analytes, i.e. isomers (8, 9). Its physical principles and attributes are only briefly mentioned here, while for detailed description the reader is referred to recent reviews and book chapters (10, 11). FLNS is a low-temperature (typically $< 10\text{K}$) site-selective spectroscopic technique in which the contributions from thermal and inhomogeneous spectral broadening are largely reduced or eliminated. Upon selective laser excitation, spectral line-narrowed widths of vibronic transitions are

lifetime limited ($\sim 5 \text{ cm}^{-1}$) due to vibrational relaxation. Resulting fluorescence spectra are highly structured and characteristic for a given molecule.

Besides providing intrinsic information on the structure of a fluorescent molecule itself, FLNS offers an insight into the fluorophore's immediate environment. It is well known that relative intensities of vibronic bands, as well as the spectral shifts (solvatochromic effect) in fluorescence spectra of many luminescent molecules are heavily influenced by the properties (polarity, dielectric constant, electrostatic interactions) of the adjacent surrounding media (12). Moreover, careful examination of high-resolution spectra of fluorescent molecules in different matrices (solvents) or interacting with biomolecules may provide structural information about the host (13).

In this paper, anti-PAH MAb, elicited against fluoranthene was tested toward immunocomplex formation with two biologically significant cross-reactants, pyrene (Py) (inset in Figure 1) and benzo(a)pyrene (BP) (inset in Figure 2). Complex formation was characterized by means of laser-induced fluorescence (LIF) spectroscopy in both low-resolution, non-line narrowing (NLN) and high-resolution (FLNS) modes. In addition, to assess the structure of the antibody's binding site, FLN spectra of Py and BP in several different solvents, chosen to cover a range of polarities, hydrogen-bonding, and π - π interaction capabilities, were compared to representative spectra of immunocomplexed fluorophores.

It will be shown that: i) FLNS is capable of differentiating between various molecules cross-reactively complexed with a single promiscuous antibody as well as between the free and complexed antigen; ii) interestingly, two conformationally different subpopulations of antibody binding sites are responsible for binding a single

cross-reactant; and iii) antibody-hapten interactions are mostly of an aromatic π - π character, suggesting the presence of aromatic amino acids within the binding domain.

II. Materials and Methods

Caution: *PAH are hazardous chemicals and should be handled carefully in accordance with NIH guidelines.*

Purified Anti-PAH MAb was purchased from Strategic Biosolutions, Newark, DE, USA. Goat anti-horse IgG was purchased from Pierce, Rockford, IL and bovine serum albumin (BSA) was obtained from Sigma-Aldrich, USA. Py and BP standard solutions were purchased from Protocol Analytical Standards, Metuchen, NJ. Methylcyclohexane (Aldrich, Milwaukee, WI), ethanol, toluene, dimethylsulfoxide (Fisher, Fair Lawn, NJ) and glycerol (Spectrum Chemical MFG. Corp., Gardena, CA) were used as received. Phosphate-buffered saline (PBS) (0.1 M sodium phosphate and 0.15 M sodium chloride) was purchased from Pierce and prepared according to the procedure supplied by the manufacturer. All selected solvents form a glass under cryogenic conditions providing an amorphous matrix for solute molecules.

For low-resolution LIF measurements, high-energy laser excitation of 308nm was provided by a Lambda Physik Lextra 100 XeCl excimer laser. For FLNS measurements, the same laser was used as pump source for a Lambda Physik FL 2002 Scanmate tunable dye laser system (10 Hz). A 1-m McPherson monochromator (model 2601) and a Princeton Instruments photodiode array were used for dispersion and detection of fluorescence. A Princeton Instruments FG-100

pulse generator was used for time-resolved spectroscopy with detector delay times from 0 to 160 ns and a gate width of 200 ns. For spectroscopic measurements 30 μ l volumes of sample were placed in quartz tubes and immersed in a helium cryostat with quartz optical windows. All fluorescence spectra are the average of 10 one-second acquisitions unless stated differently. The resolution for FLN and NLN spectra was 0.1 and 0.8 nm, respectively. Corresponding accuracy in vibrational frequencies is ± 3 cm^{-1} . To ensure quantitative binding of haptens, immunocomplexes were formed by incubation of a 10-fold excess of antibodies relative to the concentration of haptens ($\sim 10^{-7}$ M).

III. Results

Figure 1 shows the normalized room temperature fluorescence spectra of free Py (traces a and b) and Py in the presence of a ten-fold excess of anti-PAH MAb (traces c and d). Spectra are shown for two different solvents: PBS, a commonly used buffer for immunoassays, and a 1:1 mixture of glycerol and PBS (G/PBS). G/PBS is more suited to low temperature spectroscopic applications and is thought to be compatible with proteins and protein-ligand interactions (14). Similar spectra for free BP and BP incubated with excess MAb are shown in Figure 2. For both PAH and in both solvents, the fluorescence markedly red shifts on binding to the MAb. Note that each spectrum in Figures 1 and 2 is normalized to the strongest band in the emission. The spectrum of free BP in PBS has relatively weaker fluorescence as is indicated by the poorer signal to noise of the trace a in Figure 2.

Spectra of the two PAH obtained at 4.2K are shown in Figures 3 and 4. In each of these figures, NLN spectra of the free PAH and the PAH in the presence of a ten-fold excess of MAb are shown (Frames A). The NLN spectra of free Py and free BP each show a blue shift relative to the room temperature spectra, as is commonly observed for low temperature spectra. In Figure 3A, spectrum b corresponds to the Py/MAb complex and shows a clear splitting of the fluorescence origin band. The two components of the split band are well fit as Gaussians shown by traces b^I and b^{II} with corresponding full widths at half maximum (FWHM) of 75 cm^{-1} and 130 cm^{-1} , respectively. In the case of BP, however, although some splitting is apparent on binding to MAb (Figure 4A, trace b), the components are less well resolved than in the Py case. For BP/MAb, however, time gated emission can more clearly detect the component with a peak at 405.5 nm, when a gate delayed by 160 ns relative to the excitation pulse is used (Figure 4A, spectrum c). For Py/MAb, time resolved spectra did not reveal any lifetime differences in the two spectral components within the observed delay time window.

In the bottom frames of Figures 3 and 4 are shown the FLN spectra of the free PAH and of the PAH complexed with the MAbs. The numbers labeling the sharp zero phonon lines (ZPLs) in the spectra are the difference, in wavenumbers, between the line position and the excitation energy. These correspond to excited state vibrational frequencies and serve as a fingerprint of a molecule. The free Py spectrum shows only the strong 577 cm^{-1} band, the band labeled ω_{ph} , and a few much weaker bands (Figure 3B, spectrum a). The band labeled ω_{ph} is a phonon sideband due to coupling of the electronic transition to low frequency vibrations of

the matrix. The Py/MAB spectra are more complex and vary with excitation wavelength. However, both spectra shown (b and c) show a strong 577 cm^{-1} band and corresponding phonon sidebands (ω_{ph}). For BP, the FLN spectra are somewhat more structured (Figure 4B). However, each of the spectra shown contains prominent vibronic bands at 512 , 578 , and 622 cm^{-1} revealing the identity of a BP molecule in each of the observed cases.

In order to obtain information regarding the nature of the MAB binding site, FLN spectra of Py and BP molecules in 5 different solvents were acquired and compared to the spectra of the two MAB-bound PAH. Protic solvents - ethanol, aqueous PBS buffer and G/PBS (1:1) matrix were used to study the H-bonding effect on the fluorescence spectra of the two haptens. Methylcyclohexane was used as an aprotic, aliphatic solvent, while dimethylsulfoxide and toluene were employed as π -electron-containing solvents. Figure 5 shows the effects of solvents and immunocomplexation on the FLN spectra of Py using 353 nm excitation. Spectra a-e show the origin band region of Py in G/PBS, methylcyclohexane, ethanol, dimethylsulfoxide and toluene respectively. Spectra f and g correspond to Py quantitatively complexed with anti-PAH MAB in PBS and G/PBS matrix respectively, while spectrum h corresponds to the Py/MAB complex in the same matrix with an excess of Py. Similar spectra for BP in the various solvents and bound to the MAB, excited with 398 nm selective excitation (data not shown).

IV. Discussion

Complexation of the Py and BP with the anti-PAH MAb is revealed by the red-shift of their fluorescence origin bands relative to those of the free fluorophores, as shown in Figures 1 and 2. Significant spectral shift toward lower energies is frequently observed for Py and BP molecules (and their derivatives) in other biological samples, as, for example, in intercalation of BP-derived adducts into a DNA strand, and is a result of π - π interactions (15). Moreover, as shown in Figures 1 and 2, while the free haptens exhibit somewhat different spectra (in origin band positions) depending on the matrix (PBS vs. G/PBS), immunocomplexed species show similar spectra regardless of solvent, indicating relative seclusion of the complexed fluorophores from the surrounding matrix. These findings can be rationalized by an abundance of aromatic amino acid residues in the variable regions of the antibodies that can form deep, solvent inaccessible binding pockets (16). As will be shown later, this is supported by findings deduced from FLN spectra of the fluorophores in π -electron-containing solvents. Tight binding and screening from solvent effects is also revealed by an increase in fluorescence intensity upon complexation with the antibody. The intensity of spectrum c in Figure 2, corresponding to the BP complex in PBS, is ~10 times higher than the unnormalized intensity of the spectrum of free BP, spectrum a. Such significant increase might arise from a reduced solvent dynamical or oxygen-induced fluorescence quenching of PAH and/or their increased solubility. Strong complexation and consequent shielding from solvent effects results in an increased signal from the bound fluorophore. The same trend, although to a lesser extent is observed for G/PBS

matrix, suggesting already reduced quenching and/or higher solubility in the more viscous, glycerol-containing matrix.

Complex formation was further proven by competitive assays with fluoranthene. Due to its low fluorescence quantum yield and a broad, relatively featureless fluorescence emission spectrum, it was not possible to spectroscopically characterize immunocomplexation of fluoranthene. Nevertheless, its slightly higher binding constant toward the antibody (~ 50% higher affinity according to manufacturer specifications) enabled us to use fluoranthene as an effective competitor and demonstrate the specific antibody binding of Py and BP and eliminate the possibility of nonspecific protein–chromophore interactions (data not shown). When competed against fluoranthene added in excess (10 fold relative to the concentration of a complex), immunocomplexed Py was completely excluded from the complex, as reflected in the blue shift of the origin band of the fluorescence spectrum to ~ 372.8 nm, which is characteristic, as shown in Figure 1, of free Py. Similar results were obtained for BP.

Lastly, from control experiments with bovine serum albumin and rabbit anti-horse monoclonal antibodies that produced no spectral changes upon incubation with Py or BP, it is concluded that the significant red shift arises from the fluorophores' specific interactions with unique binding pockets of the anti-PAH MAb (data not shown).

Anti-PAH MAb binds Py and BP by adopting two distinct conformations of its binding sites.

The observation of two distinct contributions in the low temperature fluorescence spectra of Py and BP involved in immunocomplexes was unexpected, yet highly intriguing. In the case of structurally and conformationally uniform protein binding sites, the origin bands in the fluorescence spectra of haptens occupying those sites are expected to be a single band, broadened by thermal effects and inhomogeneity, and shifted in frequency relative to the unbound molecule. However, our spectroscopic data suggest the existence of at least two conformationally inequivalent antibody binding sites that accommodate the haptens. The existence of two unique binding conformations for an antibody has previously been proven by crystal structures by James *et al.* In that case, the free antibody can form crystals which differ in the structure of the binding site. The two binding sites, labeled Ab₁ and Ab₂, bind different types of antigens. The Ab₂ structure binds small molecules similar to dinitrophenyl hapten, the hapten against which the antibody was raised. The Ab₁ structure binds a recombinant protein, Trx-Shear3. James *et al.* argue that the two unique free antibody structures are proof of the pre-existing equilibrium model. Small differences between the structures of the complexed and the free antibody argue for induced-fit effects also being operable in this example.

In the present case, the two spectroscopic forms observed for both Py/MAb and BP/MAb complexes indicate two distinct conformations of the antibody binding site accommodating *each* antigen. It is not possible (from our data) to determine if the two spectroscopic types of hapten/MAb complexes originate from a pre-existing

equilibrium or from a special case of molecular mimicry that allowed a single binding site to adopt two stable conformations through the induced fit rearrangement. Regardless of the mechanism involved, this is an interesting case of an antibody adopting two distinct conformations for binding a particular antigen. We believe that this flexibility may be a consequence of a "soft" hydrophobic binding pocket deficient in hydrogen bonding centers necessary for unique locking.

The absence of a doublet in the room temperature spectra of the complexes is primarily due to thermal broadening resulting in unresolved spectra, and, perhaps, the existence of a more complex equilibrium that involves multiple (more than 2) conformationally different complexes, whose spectral diversity cannot be resolved. Further discussion of spectral bandwidths is included below with the discussion of the observations from high-resolution measurements.

Turning to those spectra, multiplet origin structures, characteristic of FLN are revealed for both of the free haptens and their corresponding immunocomplexes as illustrated with the highly structured spectra shown in Frames B of Figures 3 and 4. Excited-state vibrational frequencies (in cm^{-1}) calculated as differences in energy between the ZPLs of the multiple origin structure and λ_{exc} are labeled. Spectrum a in Figure 3B is the FLN spectrum of free Py in G/PBS matrix excited at 363.0 nm. Several ZPLs are revealed with this excitation. For immunocomplexed Py, the two contributions observed under NLN conditions (I and II of Figure 3A) were successfully spectrally isolated under FLN conditions and can be assigned as Py on the basis of the vibronic structure, upon laser excitations of 364.3 (spectrum b^I) and 366.0 nm (spectrum b^{II}). The strongest, sharp line in all three spectra corresponds to

the characteristic 577 cm^{-1} vibrational frequency of Py. The higher frequency vibrational modes in spectrum b^I (721 and 824 cm^{-1}), located between 374 and 375 nm , belong to the red-shifted emission of Py in site II. Conversely, lower vibrational frequencies in the FLN spectrum b^{II} (453 and 495 cm^{-1}), positioned between 372 and 373 nm , correspond to the Py emission lines from the blue-shifted site I. As revealed by several more excitation wavelengths (data not shown), vibrational frequencies observed within the origin bands of components I and II were identical, proving the presence of the same fluorophore in different pockets. It is clear that the identification of Py cross-reacted with an antibody and its correlation with the standard spectrum of a free Py is easily attainable by FLN, even in the case of multiple contributions to the fluorescence origin band.

Similarly to Py, high resolution spectra of the free and immunocomplexed BP chromophore are shown in Figure 4B. Again, it was possible to select excitation wavelengths that demonstrate the characteristic multiplet structure from immunocomplexed fluorophore that can be correlated with the standard spectrum of BP. Spectrum a is a 394.0 nm -excited standard FLN spectrum of free BP in G/PBS matrix characterized with three major vibrational frequencies 512 , 578 and 622 cm^{-1} . In the case of the complexed fluorophore, 397.0 and 399.0 nm laser excitations (spectra b^I and b^{II} respectively), reveal the two contributions to the origin band, whose multiplet structures compare well with the standard FLN spectrum of free BP. As in the case of Py, identification and differentiation of unbound and bound forms of BP were easily accomplished. Further, FLNS allowed for complete resolution of the

two contributing subpopulations encompassed within the origin band of BP complex that were only partially resolved in the NLN spectra.

It is interesting to address the differences in relative widths of the spectral bands in NLN spectra of free and complexed haptens. Lowering the temperature from 300 K to 4.2 K substantially reduces the thermal broadening, i.e., full widths at half maximum (FWHM) of the origin bands in the fluorescent spectra of free Py are 235 cm^{-1} at room temperature and 145 cm^{-1} at 4.2 K. However, apart from thermal broadening, spectral widths in low-temperature measurements are also influenced by the degree of static structural disorder of guest molecules within the matrix (inhomogeneity) and the extent of electron-phonon coupling. As shown in Figure 3 A, spectral widths of bands I and II in the 4.2 K NLN spectrum of Py complex were resolved and estimated through the dual Gaussian fit of spectrum b (curves b^I and b^{II}). Their FWHMs were found to be 75 cm^{-1} and 130 cm^{-1} respectively. Since the degree of thermal broadening for all measurements performed at the same temperature (4.2 K) should be very similar, it is apparent that the differences in line-widths of the two contributions arise from differences in inhomogeneity and/or contributions from the phonons (less likely). Building on the lower energy side of ZPLs in Figure 3 B, noticeable in all of the three FLN spectra and marked as ω_{ph} , are broader phonon side bands (PSB), whose intensity is indicative of the strength of electron-phonon coupling. Relative intensities of PSB in spectra a, b^I and b^{II} seem to be in a good agreement with the trend values for FWHM in the corresponding NLN spectra in frame A. Moreover, the differences in the extent of electron-phonon

coupling and in inhomogeneity of two contributions further confirm the differences in the two binding sites.

π - π interactions are, rather than H-bonding, responsible for binding.

The spectra shown in Figure 5 are used to gain insight into the nature of the binding pocket in the MAb for the two PAH, as previously described by Bader et al. (13). Py emission spectra obtained with 353nm excitation show several excited state vibrational frequencies in the range from 1300 to 1618 cm^{-1} . Although the positions of ZPLs in the measured spectra of Py in different solvents and MAb correspond to the same excited state vibrational frequencies (most of them labeled in Figure 5), relative intensities of these lines show significant differences depending on the type of solvent used or MAbs involved in immunocomplexation. The absence of frequency differences is due to the weak solvent influence on the vibrations of Py and BP molecules. However, the energy difference between S_0 and S_1 is altered as a result of different solvent interaction with the luminescent molecules. Consequently, a shift to higher or lower energies is observed in the emission spectrum (solvatochromism), which in case of highly structured FLN spectrum results in the differences of the relative intensities of ZPLs. Polar solvents, capable of forming H-bonds, locate the origin band of the emission of Py at the higher energy side (blue shift) as seen in FLN spectra of Py in ethanol and G/PBS of Figure 5. For those spectra, the most intense lines belong to 1358, 1400 and 1448 cm^{-1} . Furthermore, relative intensities of ZPL in those spectra still differ significantly, emphasizing further differences between those solvents revealed with FLNS. As expected, spectra of Py in π -electron containing matrices – dimethylsulfoxide and

toluene, exhibited red shifted fluorescence. The strongest ZPL in the spectrum of Py in dimethylsulfoxide was 1448 cm^{-1} , accompanied with the appearance of higher frequency lines at 1525 and 1561 cm^{-1} . In the spectrum of Py in toluene, a highly developed multiplet of frequencies in the range of $372 - 374\text{ nm}$ was observed, with a characteristic strong 1561 cm^{-1} line. Spectra of immunocomplexed Py in PBS and G/PBS matrixes (f and g) are most similar to the Py spectrum in toluene, indicating strong π - π interactions most likely of an aromatic character. The absence of H-bonds within the binding pocket of the anti-PAH MAb is not surprising, since unsubstituted PAH are highly nonpolar molecules lacking functional groups that could form H-bonds. The aromatic character of these interactions is consistent with a high abundance of aromatic amino acid residues in the binding regions of antibodies, as opposed to other proteins.

Finally, spectrum h of Figure 5 is interesting as a demonstration of the capability of FLNS to unambiguously resolve free from immunocomplexed hapten. By comparing the spectrum h with the spectra of free Py in G/PBS (spectrum a) and immunocomplexed Py in the same matrix (spectrum g), it is clear that the spectrum h is composed of more components. While the portion of the spectrum h from $372 - 374.8\text{ nm}$ ($1448 - 1618\text{ cm}^{-1}$) clearly resembles the spectrum of Py complex (g), the multiplet of lines located from $370 - 371.5\text{ nm}$, with the most blue shifted one being 1300 cm^{-1} , belongs to a free Py chromophore in a polar solvent environment. This simple, yet useful observation may be utilized for very fast screening of the specificities and affinities of different antibodies. As expected, the effect of solvents or of MAb binding on the BP spectra (data not shown) lead to the same conclusion

as the corresponding Py spectra, confirming the presence of aromatic amino acid residues within a binding domain.

Unlike most antibodies whose crystal structures have been solved and specific antibody-antigen interactions attributed mostly to H-bonding, antibody recognition for the unsubstituted PAH relies mostly on π - π interactions.

V. Concluding Remarks

In this paper we present the first example of an antibody binding a particular hapten (cross-reactant) by adopting more than one distinct conformation of its variable binding regions. The existence of the two conformational substates for anti-PAH MAb that are trapped at low temperature can be rationalized through the existing models for antibody binding. As revealed by FLN spectra of immunocomplexed chromophores, π - π interactions, rather than hydrogen bonding, play the central role in complex formation. The nonselective π - π character and subsequent flexibility of binding regions can explain the immense promiscuity of antibodies elicited against molecules lacking H-bonding functionality. Finally, it is shown that FLNS can identify various haptens cross-reacted with an antibody, as well as simultaneously differentiate between free, immunocomplexed or non-specifically bound hapten.

The combination of the exceptional resolving power of FLN spectroscopy on one hand, and a high degree of promiscuity of antibodies developed for PAH and related compounds on the other hand, not only offer new insights into the

mechanisms involved in antibody recognition and cross-reactivity, but also open new perspectives in immunoanalysis.

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Figure legends

Figure 1. Normalized fluorescence emission spectra of free Py and Py/MAb complexes in PBS buffer (bottom) and G/PBS matrix (top). (a) free Py in PBS buffer, (b) free Py in G/PBS matrix, (c) Py/MAb complex in PBS buffer, (d) Py/MAb complex in G/PBS matrix. Spectra are recorded at 300K, $\lambda_{\text{exc}} = 308$ nm, CW mode of detection.

Figure 2. Normalized fluorescence spectra of free BP and BP/MAb complexes in PBS buffer (bottom) and G/PBS matrix (top). (a) free BP in PBS buffer, (b) free BP in G/PBS matrix, (c) BP/MAb complex in PBS buffer, (d) BP/MAb complex in G/PBS matrix. Spectra are recorded at 300K, $\lambda_{\text{exc}} = 308$ nm, CW mode of detection.

Figure 3. Normalized low resolution (Frame A) and corresponding FLN spectra (frame B) of free Py and Py/MAb complex in G/PBS matrix recorded at 4.2 K with 40 ns delay. Frame A, $\lambda_{\text{exc}} = 308$ nm: (a) free Py, (b) Py/MAb complex, (b') Gaussian fit

for peak I, (b^{II}) Gaussian fit for peak II. Frame B: FLN spectra of (a) free Py, $\lambda_{\text{exc}} = 363.0$ nm; (b^I) Py/MAb complex, $\lambda_{\text{exc}} = 364.3$ nm; (b^{II}) Py/MAb complex, $\lambda_{\text{exc}} = 366.0$ nm. Numbers in bold represent excited state vibrational energies in cm^{-1} . ω_{ph} label corresponding phonon side bands.

Figure 4. Normalized low resolution (Frame A) and corresponding FLN spectra (frame B) of free BP and BP/MAb complex in G/PBS matrix recorded at 4.2 K and CW mode. Frame A, $\lambda_{\text{exc}} = 308$ nm: (a) free BP, (b) BP/MAb complex, (c) BP/MAb complex recorded with 160 ns delay. Frame B: FLN spectra of (a) free BP, $\lambda_{\text{exc}} = 394.0$ nm, (b^I) BP/MAb complex, $\lambda_{\text{exc}} = 397$ nm; (b^{II}) BP/MAb complex, $\lambda_{\text{exc}} = 399.0$ nm. Numbers in bold represent excited state vibrational energies in cm^{-1} . Figure 5. FLN spectra of Py in various solvents and Py/MAb complex in two matrixes; (a) G/PBS, (b) Methylcyclohexane, (c) Ethanol, (d) Dimethylsulfoxide, (e) Toluene, (f) Py/MAb complex in PBS, (g) Py/MAb complex in G/PBS, (h) Py/MAb complex with an excess of Py in G/PBS. $\lambda_{\text{exc}} = 353$ nm and delay 40 ns. Numbers in bold represent excited state vibrational energies in cm^{-1} .

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Figure 1. Normalized fluorescence emission spectra of free Py and Py/MAB complexes in PBS buffer (bottom) and G/PBS matrix (top). (a) free Py in PBS buffer, (b) free Py in G/PBS matrix, (c) Py/MAB complex in PBS buffer, (d) Py/MAB complex in G/PBS matrix. Spectra are recorded at 300K, $\lambda_{\text{exc}} = 308 \text{ nm}$, CW mode of detection.

Figure 2. Normalized fluorescence spectra of free BP and BP/MAb complexes in PBS buffer (bottom) and G/PBS matrix (top). (a) free BP in PBS buffer, (b) free BP in G/PBS matrix, (c) BP/MAb complex in PBS buffer, (d) BP/MAb complex in G/PBS matrix. Spectra are recorded at 300K, $\lambda_{\text{exc}} = 308$ nm, CW mode of detection.

Figure 3. Normalized low resolution (Frame A) and corresponding FLN spectra (frame B) of free Py and Py/MAb complex in G/PBS matrix recorded at 4.2 K with 40 ns delay. Frame A, $\lambda_{exc} = 308$ nm: (a) free Py, (b) Py/MAb complex, (b^I) Gaussian fit for peak I, (b^{II}) Gaussian fit for peak II. Frame B: FLN spectra of (a) free Py, $\lambda_{exc} = 363.0$ nm; (b^I) Py/MAb complex, $\lambda_{exc} = 364.3$ nm; (b^{II}) Py/MAb complex, $\lambda_{exc} = 366.0$ nm. Numbers in bold represent excited state vibrational energies in cm^{-1} . ω_{ph} label corresponding phonon side bands.

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Frame B: FLN spectra of (a) free BP, $\lambda_{\text{exc}} = 394.0$ nm, (b^I) BP/MAb complex, $\lambda_{\text{exc}} = 397$ nm; (b^{II}) BP/MAb complex, $\lambda_{\text{exc}} = 399.0$ nm. Numbers in bold represent excited state vibrational energies in cm⁻¹.

Figure 5. FLN spectra of Py in various solvents and Py/MAB complex in two matrixes; (a) G/PBS, (b) Methylcyclohexane, (c) Ethanol, (d) Dimethylsulfoxide, (e) Toluene, (f) Py/MAB complex in PBS, (g) Py/MAB complex in G/PBS, (h) Py/MAB complex with an excess of Py in G/PBS. $\lambda_{\text{exc}} = 353 \text{ nm}$ and delay 40 ns. Numbers in bold represent excited state vibrational energies in cm^{-1} .

CHAPTER 5

**HIGH RESOLUTION SPECTRAL DIFFERENTIATION OF
ENANTIOMERS: BENZO(a)PYRENE TETROLS COMPLEXED WITH
A HIGHLY CROSS-REACTIVE ANTIBODY**

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Abstract

Interaction of the highly cross-reactive anti-PAH monoclonal antibody with four diastereomeric benzo(a)pyrene tetrols (BPT) is studied by means of high-resolution fluorescence line-narrowing spectroscopy (FLNS). It was shown that the interaction of enantiomers of cis-BPT and trans-BPT with the antibody involves different complex geometries. These spatially different ligand-protein interactions result in differential alterations of the excited-state vibrational frequency patterns in the FLN spectra of enantiomers, thus allowing for unambiguous spectroscopic resolution of all four enantiomeric isomers. This study represents the first example of a fluorescence spectroscopic method, capable of enantiospecific differentiation based

on the differential intermolecular interaction of chiral analogues with a highly cross-reactive monoclonal antibody molecule.

Abbreviations: PAH, polycyclic aromatic hydrocarbons; BP, benzo(a)pyrene; BPDE, benzo(a)pyrene dihydrodiol epoxide; cis-BPT, r-7,t-8,t-9,t-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene or cis-BP tetrol; trans-BPT, r-7,t-8,9,c-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene or trans-BP tetrol; MAb, monoclonal antibody; LIF, laser induced fluorescence; FLNS, fluorescence line-narrowing spectroscopy.

I. Introduction

Immunoanalytical methodologies (immunoassays and immunosensors) rely solely upon an antibody's discriminative properties as the fundamental element of their specificity. Although antibody-antigen complexation is believed to be one of the most specific biological events, examples of nonspecific (crossreactive) antibodies are more the rule than the exception. Constrained by the impotence of mainstream immunochemical detection techniques to differentiate between "desired" and "false" binding events, antibody crossreactivity may be detrimental to the validity of an immunoassay. Accordingly, most nonspecific antibodies are destined to either limited usage (group determination) or their complete elimination from analytical procedure.

Multidimensional detection in immunoanalysis has been rather neglected, while the focus has been directed toward architectural arrangement of elements

(spatial resolution) and integration of simple, monodimensional detection schemes. While information-rich detection methods (such as mass spectrometry, NMR, wavelength-resolved UV-VIS spectroscopy, high-resolution fluorescence and Raman spectroscopies) have found their place in integrated analytical schemes (through hyphenation with various modes of chromatography and electrophoresis [1]), immunoanalytical formats remain reliant on one-dimensional, non-informative signals. We recently demonstrated that high-resolution spectroscopy, i.e. fluorescence line-narrowing spectroscopy (FLNS), coupled with analytical separation techniques [1] and biosensors [2] successfully differentiates between structurally similar analytes, thus precluding potential qualitative analytical ambiguities of a procedure.

The question arises as to whether the combination of immunocomplexation (even with cross-reactive antibodies) and a highly selective multidimensional detection scheme can provide much higher selectivity and a more robust and accurate methodology. Further, such a detection scheme could effectively use existing antibody libraries that have been overlooked or thought to be ineffective for current diagnostic applications. In this sense, information-rich detection methodologies, that possess the capability to differentiate between structurally related molecules (crossreacting candidates) in both, their free form and in a complex with an antibody, could be of great value in achieving the ultimate indiscriminatory capability even with nonspecific immunoassays. In addition, it would provide an alternative to laborious and expensive targeted antibody production.

From the perspective of the specificity of an analytical method, chiral differentiation is probably the most challenging issue and requires involvement of another chiral element: chiral molecule or chiral electromagnetic wave. Molecular chirality and enantioselectivity are fundamental aspects of biological activity, and are reflected in remarkable discriminatory, antagonistic or side physiological effects of enantiomeric analogues [3-5]. Chemically induced carcinogenesis, for example, has been shown to evolve from enantioselective DNA damage initiated by prochiral mutagenic agents. The development of techniques that are capable of diastereomeric differentiation is crucial not only from a bioanalytical (diagnostic) standpoint, but also from the perspective of understanding the mechanism(s) that underlie enantioselective biological interactions. However, both, conventional low- and high-resolution spectroscopic techniques (based on randomly polarized excitation sources) are incapable of distinguishing between enantiomeric analogues.

In this study we present the first example of a fluorescence spectroscopic method, capable of enantiospecific differentiation based on the differential intermolecular interaction of chiral analogues with a highly cross-reactive monoclonal antibody molecule. The two pairs of chiral diastereoisomers of interest are metabolic derivatives of the mutagenic benzo(a)pyrene (BP): ($\pm$)r-7,t-8,t-9,t-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene (cis-BPT) and ($\pm$)r-7,t-8,9,c-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene or (trans-BPT) (Figure 1). It is well established that BP forms a variety of stable DNA adducts via monooxygenation metabolic pathway. Formation of stable BP-derived DNA adducts involves active diolepoxide mutagenic intermediar (BPDE). The DNA damage induced mutations to

the p53 tumor suppressor gene are believed to be critical for the induction of certain types of chemically induced lung cancers [6, 7]. BP tetrols are of interest as they are the final hydrolytic products of BPDE-derived DNA adducts [8]. Due to the stereoselective biological interaction of BPDE with DNA, resulting mixture of adducts (and metabolites) derived in *in vivo* and *in vitro* experiments consists of a combination of stereoisomers in various ratios [9] implying different enantiomer activities. While preserving the carbon backbone of the BP molecule, BP tetrols contain polyhydroxyl moiety residing on the flexible cyclohexenyl ring formed through the metabolic hydroxylation of the aromatic ring A of BP (Figure 1).

The highly cross-reactive anti-PAH MAb used here, and developed against fluoranthene, interacts with a number of polycyclic aromatic hydrocarbons (PAH), *i.e.*, pyrene and benzo(a)pyrene (my PNAS ref.). It is the intention of this study to examine the possibility of using the broad cross-reactivity of anti-PAH MAb for spectroscopic analysis of other molecules that possess polycyclic aromatic structure (*i.e.*, various PAH-derived DNA adducts and/or their dissociation derivatives).

II. Materials and Methods

Purified Anti-PAH MAb was purchased from Strategic Biosolutions, Newark, DE, USA. Methanol (Aldrich, Milwaukee, WI) and glycerol (Spectrum Chemical MFG. Corp., Gardena, CA) were used as received. Phosphate-buffered saline (PBS) (0.1 M sodium phosphate and 0.15 M sodium chloride) was purchased from Pierce and prepared according to the procedure supplied by the manufacturer.

Sodium phosphate, and phosphoric acid were purchased from Fisher Scientifics (Fair Lawn, NJ) and were used for preparation of a running buffer for capillary electrophoresis. The buffer was filtered through 0.22 μ m Non-pyrogenic filter (Costar, Corp.Corning, NY). All buffers and samples were prepared using water purified by a Milli-Q Water Purification System (Millipore).

Racemic *r*-7,*t*-8,*t*-9,*t*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene (cis-BPT) and ($\pm$)*r*-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene (trans-BPT) were purchased from Midwest Research Institute, NCI Chemical Carcinogen Reference Standard Repository, Kansas City, Missouri. Enantiomers were obtained by HPLC separation of racemic standards on the γ -cyclodextrin chiral stationary phase (Cyclobond II, 250 x 4.6 mm, Astec, Whippany, NJ, USA). The HPLC system consisted of two pumps (LC-10AD, Shimadzu), a UV detector (SPD-6A, Shimadzu), a Chromatopac integrator (CR601, Shimadzu), and a six-port injection valve equipped with a sample loop (10 μ L). Detection wavelength was 246 nm, which correspond to the absorption maxima of the two compounds. The preparative separation conditions used to obtain mg quantities of the pure enantiomers of cis-BPT and trans-BPT are as follows. Racemates of cis-BPT and trans-BPT were dissolved in methanol to a concentration of 32 mg/ml. 10 μ L of each sample were injected into a γ -cyclodextrin column and eluted with methanol/water, 20/80 (v/v). The enantiomers were collected manually and concentrated under vacuum at 60 °C. The mobile phase was degassed before use and the flow rate was 1.0 ml/min. The polarimetric detector (Chiralyser, Astec, Whippany, NJ, USA) was used to determine

the elution order. The wavelength is 430 nm. For both compounds, the (–) enantiomer was eluted first, followed by the (+) isomer.

For low-resolution (NLN) spectroscopic measurements, high-energy laser excitation of 308 nm was provided by a Lambda Physik Lextra 100 XeCl excimer laser. For FLNS measurements, the same laser was used as pump source for a Lambda Physik FL 2002 Scanmate tunable dye laser system (10 Hz). A 1-m McPherson monochromator (model 2601) and a Princeton Instruments photodiode array were used for dispersion and detection of fluorescence. A Princeton Instruments FG-100 pulse generator was used for time-resolved spectroscopy with detector delay times from 0 to 160 ns and a gate width of 200 ns. For spectroscopic measurements 30 μ l volumes of sample in PBS:glycerol (1:1) matrix were placed in quartz tubes and immersed in a helium cryostat with quartz optical windows. All fluorescence spectra are the average of 10 one-second acquisitions unless stated differently. The spectral resolution for FLN and NLN measurements was 0.1 and 0.8 nm, respectively. Corresponding accuracy in vibrational frequencies is ± 3 cm^{-1} . To ensure quantitative binding of haptens, immunocomplexes were formed by incubation of a 10-fold excess of antibodies relative to the concentration of haptens ($\sim 10^{-7}$ M).

CE running buffer used was 50 mM Na-phosphate buffer at pH 8.8. For the frontal analysis, samples were hydrodynamically injected with 20-mbar pressure for 3 s and electrokinetic separations were carried out at 21 kV (resulting current 20.0 μ A). UV-transparent capillaries (85 cm x 75 μ m i.d. and 365 μ m. o.d.) were obtained from Polymicro Technologies with a length of 70 cm (55 cm to the detection

window). Laser-induced fluorescence detection system consisted of CW excitation at 257nm provided by Lexel 95-SHG laser operated at intensity of 100 mW. Fluorescence was collected with a reflecting objective (N.A. 0.28), dispersed by a monochromator, and detected by an intensified CCD camera. Electropherograms were generated by on-line integration of the 320 - 450 nm spectral region in fluorescence emission window using WinSpec/32 software, Roper Scientific. Excitation used and spectral region observed enabled simultaneous observation of the fluorescence of BP tetrols, as well as the native antibody fluorescence using.

III. Results and discussion

Electronic spectra of molecules contain information on intrinsic molecular structure, and can be used for their identification [10, 11]. However, due to the thermal spectral broadening, classical room-temperature UV-VIS absorbance and luminescence spectroscopies are not capable of resolving the fine structure of molecular vibronic transitions [10]. As a result, spectroscopic differentiation between structurally related molecules (positional-, conformational-, and stereo-isomers) is often not possible [12]. Although low-temperature spectroscopy (<10K) significantly reduces the thermal spectral broadening (narrows the spectral bands and improves the resolution), the contribution from the inhomogeneous broadening precludes observation of the fine vibrational transitions [10, 11, 13].

Spectra A1 – D1 of Figure 2 represent the (0–0)-bands of low-temperature (4.2 K), non line-narrowing (NLN) spectra of four diastereomeric BP tetrols, obtained

with 308nm excitation source. It is evident that the low-resolution spectra of enantiomeric couples are identical, while small differences in the position of the (0-0)-band (0.3 nm) are only observed for cis-BPT relative to trans-BPT. Although additional minor differences between the spectra of cis-BPT and trans-BPT can be observed in the vibronic region (spectra not shown), differentiation based on low-resolution NLN spectra can be inconclusive and misleading primarily due to the high influence of the surrounding environment (matrix) on the molecular spectra. On the contrary, selective vibronic excitation in a FLNS experiment reveals a highly structured pattern of the excited-state vibrational frequencies that is characteristic for a given molecule.

As shown in Figure 2, comparison of FLNS spectra of enantiomeric ($\pm$) cis-BPT (spectra A2 and B2) and ($\pm$) trans-BPT (spectra C2 and D2), obtained with 363.0 nm excitation wavelength, reveals apparent differences. Several distinctive vibrational frequencies are identified for each isomer: 891 and 1027 cm^{-1} for cis-BPT, and 1017 and 1045 cm^{-1} for trans-BPT. Moreover, differences in the relative intensity of their common vibrational frequencies (*i.e.*, 953 (956) and 1110 cm^{-1}) can also serve as means of differentiation. Several other excitation wavelengths (ranging from 355 – 371nm) were used, each revealing a different portion of the characteristic S_1 excited-state vibrational frequencies, which are listed in Table 1. These numbers are consistent with those obtained previously for BPDE-derived DNA adducts and metabolites [14, 15] and can be used for positive identification of stereoisomeric BP tetrols. However, as expected, optical isomers do not reveal spectral differences even under high-resolution conditions, as evident from virtually

identical FLN spectra obtained for enantiomeric couples (spectra A2 and B2 for (–) cis-BPT and (+) cis-BPT, and spectra C2 and D2 for (–) trans-BPT and (+) trans-BPT, respectively).

Importantly, upon complexation of enantiomeric BP tetrols with anti-PAH MAb, significant differences in the position of the (0–0)-bands in low-resolution spectra of all four immunocomplexed ligands are observed. While (–) enantiomers did not reveal notable spectral changes upon complexation (spectra A3 and C3) relative to the spectra of uncomplexed ligands (spectra A1 and A3), spectra of immunocomplexed (+) enantiomers, compared to uncomplexed forms, exhibited significant red wavelength shifts (1.8 nm for (+) cis-BPT and 0.8 nm for (+) trans-BPT, spectra B3 and D3, respectively). Alterations of molecular electronic spectra arising from interactions of chromophores with the adjacent surrounding medium are commonly observed. Relative intensities of spectral vibronic bands, as well as the spectral band shifts (solvatochromic effect) in electronic spectra of many molecules are heavily influenced by the properties (polarity, dielectric constant, dipole moment, electrostatic interactions) of the interacting environment (12). Analogously, complexation of small chromophoric molecules with protein binding sites can induce similar spectral changes. Protein complexation relies on a variety of noncovalent binding interactions, most important being hydrogen bonds, polar, hydrophobic and π - π interactions. Depending on the molecular structure of its counterpart, antibody binding site accommodates amino acid sequence and geometry to favor specific intermolecular interactions and strong, reversible binding. In most reported studies of protein high affinity binding, hydrogen bonds are claimed to be responsible for

strong and specific complexation. Yet, there are cases where hydrogen bonding is limited or not possible, and the binding is restricted to other, less spatially directed interactions [16]. As demonstrated recently [17], π - π interactions within the antibody pocket are responsible for binding and broad crossreactivity of anti-PAH MAb and enable its strong binding to a number of different PAH (fluoranthene, pyrene, benzo(a)pyrene, etc). Hence, the expected interaction of BP tetrols with anti-PAH MAb is through the pyrene moiety that remained free from chemical modification and available for interaction with the antibody binding site. However, four hydroxyl groups residing on the cyclohexenyl ring of BP tetrols may involve additional steric interactions that can enable or hinder a molecule's favorable orientation and interaction with the antibody binding site.

Indeed, based on the red spectral shift observed in the low-resolution spectra of (+) enantiomers of cis-BPT/MAb and trans-BPT/MAb complexes, complexed pyrene moiety experiences significant π - π interactions, suggesting ligand inclusion into the binding pocket. As described previously [17], environment directing high π -electron density upon the π -electron cloud of pyrene chromophore shifts its origin band spectral envelope to higher wavelengths (red shift of the (0-0)-band). In FLN spectra this is reflected in the change of the relative intensities of the characteristic vibronic bands, as observed in spectra B4 and D4 of Figure 2 when compared to spectra of uncomplexed forms B2 and D2, respectively. Importantly, the changes in the energy difference between the S_1 and the ground electronic states induced by complexation did not qualitatively alter the excited state vibrational frequencies (no changes in excited-state vibrational frequencies). The preservation of vibrational

frequencies is important since it enables positive identification of a ligand regardless of its residence environment, while the alterations in the relative intensity of those frequencies enable differentiation between isomers that interact with the protein molecule through a different geometrical arrangement.

For (–) enantiomers no significant spectral changes are observed upon association with the antibody molecules (spectra A4 and C4) suggesting negligible π - π interactions directed toward the pyrenyl moiety of (–) cis-BPT and (–) trans-BPT, respectively. The lack of spectral alterations in this case, however, does not necessarily imply the complete absence of intermolecular interactions, and the possibility of alternative, possibly external surface interactions with a massive immunoglobulin molecule has to be accounted for.

To correlate the spectral behavior of complexed ligands with their binding strengths (and geometry), affinity constants for each ligand/antibody complex were determined using the frontal analysis (FA) method of capillary electrophoresis (CE) [18]. Figure 3 shows the plot of the fraction of ligand bound to the protein vs. free ligand concentration for the series of CE injections of four antibody-ligand mixtures. Numerical values for association constants (K_a) for (+) trans-BPT, (–) trans-BPT, (+) cis-BPT, and (–) cis-BPT, (calculated from the slopes) are: 6.0×10^6 , 6.1×10^5 , 2.4×10^6 , and $2.1 \times 10^5 \text{ M}^{-1}$, respectively. The (+) enantiomers of diastereomeric tetrols exhibit significantly higher values for the binding constants relative to their enantiomeric counterparts, which is in agreement with results obtained from spectroscopic studies described above. However, larger red spectral shift of the (0–0)-band in the case of (+) cis-BPT/MAb complex (1.8 nm) relative to the (+) trans-

BPT/MAb complex (0.8 nm) did not reveal a higher binding constant. The stronger binding of the (+) trans-BPT isomer can be rationalized through the additional stabilization of the immunocomplex due to favorable steric and electrostatic interactions of a flexible tetrahydroxy-cyclohexenyl moiety with the aminoacid assembly guarding the gate of the hydrophobic pocket.

Lower binding constants and the lack of spectral changes in the case of complexes of (–) enantiomers suggest weaker, most likely external interactions with the protein molecule, probably through the hydroxyl functionalities of tetrols. In this case, it is likely that pyrenyl moiety resides out of the hydrophobic binding pocket and is exposed to the solvent. Moreover, electrophoretic mobility shifts and band broadening in immunoaffinity CE experiment (data not shown) indicate lower mass transfer rates for complexes of (+) enantiomers relative to their (–) analogues. Lower mass transfer suggests different kinetic and geometry, and presents an additional supporting evidence for the inclusion nature of complexes of (+) enantiomers with anti-PAH MAb. However, to explicitly resolve the spatial arrangement in MAb/hapten complexes, *i.e.*, to reveal the cause for the observed spectral differences, crystal structures of antibody-ligand complexes are needed.

IV. Conclusions

It has been demonstrated that the interaction of anti-PAH MAb with the enantiomers of cis- and trans-BP tetrols involves different complex geometries. These spatially different ligand-protein interactions result in differential alterations of the excited-state vibrational frequency patterns in the FLN spectra of enantiomers,

thus allowing for unambiguous spectroscopic resolution of all four stereoisomeric BP tetrols. The ability of FLNS to directly probe and identify different fluorescent molecules complexed with promiscuous antibodies could be utilized for the development of highly versatile and specific immunoanalytical methods (immunosensors and immunoassays).

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Table 1. Most prominent excited state vibrational frequencies in FLN spectra of enantiomeric cis-BPT and trans-BPT. Excitation wavelength was scanned in the region from 355-371nm. Frequencies are given in wavenumbers with an accuracy of $\pm 3 \text{ cm}^{-1}$.

Figure Legends

Figure 1. Molecular structures of benzo(a)pyrene (BP), benzo(a)pyrene diolepoxide (BPDE), ($\pm$)r-7,t-8,t-9,t-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene (cis-BPT), and ($\pm$)r-7,t-8,9,c-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene (trans-BPT).

Figure 2. Non line-narrowed (NLN) ($\lambda_{\text{exc}} = 308 \text{ nm}$) and FLN spectra of free (spectra 1 and 2, respectively) and immunocomplexed diastereomeric BP tetrols (spectra 3 and 4, respectively). Frames A – D refer to (–) cis-BPT, (+) cis-BPT, (–) trans-BPT, and (+) trans-BPT, respectively. $T = 4.2 \text{ K}$ with a 40ns delayed detection.

Figure 3. Plot of $r/(1-r)$ against the concentration of free ligand, where r is the fraction of ligand bound per MAb. The slopes of linear fits to the experimental data points give the association constants (K_a) of BP tetrol/anti-PAH MAb complexes.

Figure 4. Non line-narrowed ($\lambda_{\text{exc}} = 308 \text{ nm}$) spectra of (a) uncomplexed racemic cis-BPT and (b) racemic cis-BPT complexed with anti-PAH MAb.

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(+)(-) c-BPT	(+)(-) t-BPT
453	449
479	—
495	503
538	546
578	578
617	—
646	646
740	715
770	760
830	830
891	—
953	956
—	1017
1027	—
—	1045
1110	1110
1384	1383
1445	1440
1562	1562

Table 1. Most prominent excited state vibrational frequencies in FLN spectra of enantiomeric cis-BPT and trans-BPT. Excitation wavelength was scanned in the region from 355-371nm. Frequencies are given in wavenumbers with an accuracy of $\pm 3 \text{ cm}^{-1}$.

Figure 1. Molecular structures of benzo(a)pyrene (BP), benzo(a)pyrene diol-epoxide (BPDE), ($\pm$)r-7,t-8,t-9,t-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene (cis-BPT), and ($\pm$)r-7,t-8,9,c-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene (trans-BPT).

Figure 2. Non line-narrowed (NLN) ($\lambda_{\text{exc}} = 308 \text{ nm}$) and FLN spectra of free (spectra 1 and 2, respectively) and immunocomplexed diastereomeric BP tetrols (spectra 3 and 4, respectively). Frames A – D refer to (–) cis-BPT, (+) cis-BPT, (–) trans-BPT, and (+) trans-BPT, respectively. $T = 4.2 \text{ K}$ with a 40ns delayed detection.

Figure 3. Plot of $r/(1-r)$ against the concentration of free ligand, where r is the fraction of ligand bound per MAb. The slopes of linear fits to the experimental data points give the association constants (K_a) of BP tetrol/anti-PAH MAb complexes.

CHAPTER 6.

**FLOW-THROUGH PARTIAL- FILLING AFFINITY CAPILLARY
ELECTROPHORESIS USING CROSS-REACTIVE ANTIBODY FOR
ENANTIOMERIC SEPARATIONS**

Manuscript in preparation

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I. Introduction

Functional proteins owe their physiological potency to selective recognition and reversible binding to various endogenous or exogenous molecules. Their discriminatory interaction goes beyond coarse architectural differentiation of molecules and, in fact, reaches extraordinary stereoisomeric specificity [1, 2]. Moreover, the production of some proteins, like antibodies launched through a cellular immune response, is exclusively aimed to achieve highly specific structural complementarity with the molecules that initiated their expression. The ability of the immune system to generate extraordinary pool of diverse binding molecules implies the most powerful combinatorial library in nature. Its strength is attributed to the versatility of antibody binding sites produced during an immunological reaction. In addition, the diversity of chemical functions present at the surface of antibodies

allows them to interact in variety of ways with a broad range of structurally unrelated substances. The most important interactions in antibody selective binding are hydrogen bonds, electrostatic and hydrophobic interactions. Because of the chiral nature of proteins, they may react differently with stereoisomers of chiral molecules. At specific binding sites it is possible for enantiomers to interact with an antibody in a qualitatively (i.e., with different binding sites) and/or quantitatively (i.e., with different binding strength) different fashion [3].

Although an antibody-antigen interaction is known to be one of the most specific biological events, examples of nonspecific (cross-reactive) antibodies are numerous. Structural and mechanistic studies have revealed two distinct mechanisms that are responsible for the crossreactive interaction of antibodies: 1. antibody conformational diversity – i.e. the pre-existing equilibrium of antibody conformers, each capable of binding structurally different antigens [4, 5], and 2. induced fit mechanism – i.e. the flexibility of an antibody binding pocket, usually operable for cross-reactivity in the case of shared ligand chemistry (molecular mimicry) [6, 7]. However, promiscuous antibodies can exhibit different degrees of heterospecificity [8], that is, broad-specificity within a family of similar molecules, or discrimination within a group of alike molecules, but significant interaction with unrelated antigen(s) [9].

Our recent work has been focused on developing applications of cross-reactive antibodies for highly specific analyses using multidimensional spectroscopic detection, primarily high-resolution, fluorescence line-narrowing spectroscopy (FLNS). We have demonstrated that a single, cross-reactive anti-PAH MAb can be

used for capturing structurally related molecules, such as fluoranthene (F), pyrene (P), benzo(a)pyrene (BP), and diastereomeric benzo(a)pyrene tetrols (BPT). FLNS detection is used to directly probe the molecule immunocomplexed with the antibody, giving unambiguous positive identification. Moreover, we have demonstrated that geometrically different interactions of diastereomeric cis- and trans-BP tetrols with anti-PAH MAb can be spectroscopically distinguished allowing chiral differentiation.

In this study, we explore the use of cross-reactive anti-PAH MAb as a chiral selector in affinity capillary electrophoresis (ACE) with laser-induced fluorescence (LIF) detection. More specifically, we utilize the flow-through partial-filling affinity electrophoresis (FTPFACE) approach to study the applicability of antibodies for resolution of four diastereomeric BP tetrols. We also study the binding parameters of antibody/ligand interactions. First, the capillary is partially filled with a plug of antibody followed by a plug of a sample containing ligands. Upon application of voltage gradient, and due to the high difference in the electrophoretic mobility, ligand and antibody zones temporarily overlap during the course of the electrophoretic run (Figure 2). Due to the binding interaction between the ligand(s) and the antibody, electrophoretic mobility shifts and/or changes in the peak shape will occur.

The experimental setup (Figure 3) assumed coupling of a deep UV laser (257nm) as an excitation source to the capillary observation window, which enabled us not only to observe strong fluorescence from the ligands, but also the native protein (antibody) fluorescence. In addition, the emission was dispersed with a monochromator and collected with an intensified CCD camera, which enabled

collection of a full emission spectrum at any given point in the electropherogram. This resulted in direct peak identification that was less dependent on migration times derived from standards. This method was used to resolve four stereomeric BP tetrols: (+) cis-BPT, (-) cis-BPT, (+) trans-BPT, and (-) trans-BPT, whose structures are depicted in Figure 1. In addition to the analytical outcomes of the chiral separation, the results are discussed in terms of the nature of the interaction between the ligands and the antibody, and correlated with spectroscopic data obtained for the same complexes presented in Chapter 5 of this dissertation.

II. Materials and Methods

Anti-PAH MAb was purchased from Strategic Biosolutions, Newark, DE, USA. Methanol (Aldrich, Milwaukee, WI) was used as received. Racemic r-7,t-8,t-9,t-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene (cis-BPT) and ($\pm$)r-7,t-8,9,c-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene (trans-BPT) were purchased from Midwest Research Institute, NCI Chemical Carcinogen Reference Standard Repository, Kansas City, Missouri. Enantiomers were obtained by HPLC separation of racemic standards on the γ -cyclodextrin chiral stationary phase (Cyclobond II, 250 x 4.6 mm, Astec, Whippany, NJ, USA). The HPLC system consisted of two pumps (LC-10AD, Shimadzu), a UV detector (SPD-6A, Shimadzu), a Chromatopac integrator (CR601, Shimadzu), and a six-port injection valve equipped with a sample loop (10 μ L). The detection wavelength was 246 nm, which correspond to the absorption maxima of the two compounds. The preparative separation conditions used to obtain pure enantiomers of cis-BPT and trans-BPT are as follows.

Racemates of cis-BPT and trans-BPT were dissolved in methanol to a concentration of 3.2 mg/ml. 10 μ L of each sample were injected onto a γ -cyclodextrin column and eluted with methanol/water, 20/80 (v/v). The enantiomers were collected manually and concentrated under vacuum at 60 °C. The mobile phase was degassed before use and the flow rate was 1.0 ml/min. A polarimetric detector (Chiralyser, Astec, Whippany, NJ, USA) was used to determine the elution order with a 430 nm wavelength. For both compounds, the (–) enantiomer eluted first, followed by the (+) isomer.

The CE running buffer used was 50 mM sodium-phosphate buffer at pH 8.8. Sodium phosphate, and phosphoric acid were purchased from Fisher Scientifics (Fair Lawn, NJ) and were used for the preparation of a running buffer. The buffer was filtered through 0.22 μ m Non-pyrogenic filter (Costar, Corp.Corning, NY). All buffers and samples were prepared using water purified by a Milli-Q Water Purification System (Millipore). For the flow-through ACE, samples were hydrodynamically injected with 20-mbar pressure for 6 s and electrokinetic separations were carried out at voltages ranging from 13 - 17 kV (resulting in currents ranging from 150-180 μ A). UV-transparent capillaries (85 cm x 75 μ m i.d. and 365 μ m. o.d.) were obtained from Polymicro Technologies and used with a length of 70 cm (55 cm to the detection window). The laser-induced fluorescence detection system consisted of CW excitation at 257nm provided by a Lexel 95-SHG laser operated at an intensity of 100 mW. Fluorescence was collected with a reflecting objective (N.A. 0.28), dispersed by a monochromator, and detected by an intensified CCD camera. Electropherograms were generated by on-line integration of

the 320 - 450 nm spectral region in the fluorescence emission window using WinSpec/32 software, Roper Scientific. The excitation used and the spectral region observed enabled simultaneous observation of the fluorescence of BP tetrols, as well as the native antibody fluorescence.

III. Results and discussion

Using the FTPFACE technique, we examined the interaction between anti-PAH MAb and neutral isomeric BP tetrols. The conditions are chosen to ensure that the antibodies are negatively charged (running buffer pH = 8.8, antibody pKa 7.5-8.3), while the ligands remained neutral. By injecting the antibody plug 0.5-1.0 min prior to the ligand plug, the antibody plug penetrates the ligand zone throughout the course of electrophoresis. (Figure 2). Continued electrophoresis permits either total penetration and final resolution of zones, or partial resolution of zones, depending on the antibody-ligand interaction strength and kinetics, as discussed below.

Figure 4, Frame A, line (a) shows the conventional CE electropherogram of a racemic mixture of (+) cis-BPT and (-) cis-BPT. The single peak represents the migration time of both enantiomers that are not resolved under achiral separation conditions. Electropherogram (b) represents an FTPFACE run in which an anti-PAH MAb injection plug was followed by an 1 min delay in the injection of a (-) cis-BPT plug, while electropherogram (c) represents the same CE run for (+) cis-BPT. Frame B of Figure 4 depicts a FTPFACE electropherogram of a racemic mixture of cis-BPT enantiomers with anti-PAH MAb.

Line (a) in Figure 4. gives the migration time of an electrophoretic run for the cis-BPT enantiomers not influenced by interactions with the antibody. Under such conditions, both enantiomers have the same migration time and cannot be resolved resulting in a single peak. Electropherogram (b) shows the migration time shift for the (–) cis-BPT isomer as a result of the flow-through interaction of the isomer with the antibody plug. The broad peak on the right, as confirmed by the on-line fluorescence spectrum collected for this ACE run (data not shown), to the free antibody band. Line (c) depicts the electropherogram in which a migration time shift and band broadening for (+) cis-BPT occurred as a consequence of the interaction with the antibody in the flow-through ACE experiment. Due to the obvious differences in the migration times and band shapes for two enantiomers (electropherograms (b) and (c)), the separation of two enantiomers should be feasible using anti-PAH MAb. Indeed, the Frame B of Figure 4 depicts the flow-through ACE separation of two enantiomeric cis-BP tetrols. According to the migration times and band shape peaks are identified as (–) cis-BPT, (+) cis-BPT, and free anti-PAH MAb in the elution order.

Figure 5, Frame A, line (a) represents the conventional CE electropherogram of a racemic mixture of (+) trans-BPT and (–) trans-BPT. Again, the single peak represents the migration time of both enantiomers that are not resolved under the achiral separation conditions. Electropherogram (b) represents an FTPFACE run in which an anti-PAH MAb injection plug was followed by 1 min delay in the injection of the (–) trans-BPT plug, while electropherogram (c) represents the same run for the (+) trans-BPT. Frame B of Figure 5 depicts a FRPFACE electropherogram of a

racemic mixture of the trans-BPT enantiomers with anti-PAH MAb. The peak in the electropherogram (a) shown in Figure 5. represents the migration of a free electrophoretic (no antibody plug) for the trans-BPT enantiomers. Similar to cis-BPT, both enantiomers have the same migration time and coelute as a single peak. Electropherogram (b) shows the migration time shift for the (–) trans-BPT isomer as a result of the flow-through interaction of the isomer with the antibody plug. Again, the broader peak (second in the elution order) belongs to the free antibody plug. In electropherogram (c), the single peak was found to be the co-elution of (+) trans-BPT and anti-PAH MAb. The on-line fluorescence spectrum of this peak shows a mixture of the (+) trans-BPT and the native protein (tryptophan) fluorescence, similar to the Frame C of Figure 7. Similar to cis-BPT, Frame B of Figure 5 represents the flow-through ACE separation of two enantiomeric trans-BP tetrols, where the first peak in elution order belongs to (–) trans-BPT and the second broad peak is identified as a mixture of (+) trans-BPT and anti-PAH MAb.

Finally, a multiple injection flow-through partial-filling ACE electropherogram of a mixture of racemic cis-BP tetrols and trans-BP tetrols with the anti-PAH MAb plug is presented in Figure 6. The separation was carried out in the following injection order: 1) racemic mixture of cis-BPT and trans-BPT; 2) 2 min delayed anti-PAH MAb; 3) 0.5 min delayed racemic mixture of cis-BPT and trans-BPT. The peak assignment was performed according to the data obtained from individual ACE runs, as well as from on-line LIF spectra obtained at the maxima of peaks (Figure 7). Within the given injection sequence, the first sample zone (mixture of ligands) does not undergo interaction (penetration) with the antibody plug, and passes the

detection window unaffected by antibody. This zone of mixed isomeric BP tetrols corresponds to the co-elution of all four ligands and is represented as the largest peak (first in the elution order) in the electropherogram. The following four peaks in elution order correspond to (–) trans-BPT, (–) cis-BPT, (+) trans-BPT, and (+) trans-BPT, respectively, as deduced from the standard migration time shifts and band shapes, as well as confirmed by the on-line fluorescence spectra obtained for each peak (Figure 7).

Slow on-and-off kinetics, i.e., the half time of the Ab-Ag complex is long compared with the time of the electrophoresis, lead to band broadening and results in far lower efficiency of the separation. Fast Ab-Ag interaction kinetics are characterized by a relatively short lifetime of the Ab-Ag complexes, and can yield to migration time shifts [3]. In our study, the migration times and band shapes reveal significant differences in the binding strength and kinetics of interaction between diastereomeric BP tetrols and anti-PAH MAb. Narrow peaks with small migration time shifts obtained for (–) enantiomers indicate a lower binding constant and faster kinetics of interaction of these enantiomers with the antibody relative to their (+) counterparts. These results are consistent with earlier results obtained in the spectroscopic study of the interactions of BP tetrol isomers with an antibody and their corresponding binding constants (Chapter 5). The electrophoretic mobility shifts and band broadening in the case of the (+) enantiomers indicate stronger binding and involvement of different interaction kinetics for these enantiomers with anti-PAH MAb. These results are in agreement with our spectroscopic data presented in Chapter 5 of this dissertation.

IV. Conclusions

This study demonstrates the applicability of cross-reactive anti-PAH MAb for the first enantiomeric separation of a mixture of four diastereomeric BP-tetrols by means of flow-through partial-filling affinity capillary electrophoresis. The CE runs are designed such that the antibody zone penetrates (interacts with) the plug consisting of different ligands. Due to the different interaction kinetics and binding strengths, the antibody changes the migration times and the band widths of all four ligands resulting in four distinct peaks in the FTPFACE electropherogram of a ligand mixture. In addition to the analytical outcomes in terms of a chiral separation of four isomeric ligands, mobility shifts and peak shape alterations are used to discuss the binding strengths and kinetics of the antibody-ligand interactions. It is shown that (–) enantiomers have lower binding constants and faster on-and-off kinetics with the antibody than (+) enantiomers of BP tetrols. These results were analyzed with respect to the spectroscopic data obtained for the same ligand-antibody complexes (Chapter 5) and were found to be in excellent agreement.

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Figure Legends

Figure 1. Molecular structures of diastereomeric cis-BPT and trans-BPT.

Figure 2. Schematic representation of the flow-through partial-filling technique. Compounds: (Y) antibody; (red circles) ($\pm$)cis-BPT; (blue circles) ($\pm$)trans-BPT.

Figure 3. Block diagram of CE-LIF instrumentation with CCD detection.

Figure 4. Frame A: The flow-through partial-filling ACE electropherograms of enantiomeric cis-benzo(a)pyrene tetrols (cis-BPT). (a) racemic mixture of (+) cis-BPT and (-) cis-BPT, (b) anti-PAH MAb plug followed by 1 min delay in the injection of (-) cis-BPT plug, (c) anti-PAH MAb plug followed by 1 min delay in the injection of (+) cis-BPT plug. Frame B: anti-PAH MAb plug followed by 1 min delay in the injection of a racemic mixture of (+) cis-BPT and (-) cis-BPT. 50 mM sodium-phosphate buffer at pH 8.8 was used as the CE running buffer. A 257 nm laser excitation source and a CCD camera were used for LIF detection.

Figure 5. Frame A: The flow-through partial-filling ACE electropherograms of enantiomeric cis-benzo(a)pyrene tetrols (cis-BPT). (a) racemic mixture of (+) trans-BPT and (-) trans-BPT, (b) anti-PAH MAb plug followed by 1 min delay in the injection of (-) trans-BPT plug, (c) anti-PAH MAb plug followed by 1 min delay in the injection of (+) trans-BPT plug. Frame B: anti-PAH MAb plug followed by 1 min delay in the injection of a racemic mixture of (+) trans-BPT and (-) trans-BPT. 50

mM sodium-phosphate buffer at pH 8.8 was used as the CE running buffer. 257 nm laser excitation source and CCD camera were used for LIF detection.

Figure 6. Multiple injection flow-through partial-filling ACE electropherogram of a mixture of racemic cis-BP tetrols and trans-BP tetrols with anti-PAH MAb.

Injection order: 1) racemic mixture of cis-BPT and trans-BPT; 2) 2 min delayed anti-PAH MAb; 3) 0.5 min delayed racemic mixture of cis-BPT and trans-BPT.

50 mM sodium-phosphate buffer at pH 8.8 was used as the CE running buffer. A 257 nm laser excitation source and a CCD camera were used for LIF detection.

Figure 7. On-line LIF spectra ($\lambda_{\text{exc}} = 257 \text{ nm}$) for FTPFACE separation of a mixture of four diastereomeric BP tetrols with anti-PAH MAb. Frame A: spectrum (a) corresponds to (–) trans-BPT obtained at the maximum of the electrophoretic peak labeled (–)t in Figure 6; spectrum (b) corresponds to (–) cis-BPT obtained at the maximum of the electrophoretic peak labeled (–)c in Figure 6. Frame B: spectrum of (+) cis-BPT corresponding to electrophoretic peak labeled (+)c in Figure 6). Frame C: mixed spectrum of (+) trans-BPT and anti-PAH MAb corresponding to the fronting part of the electrophoretic peak labeled (+)t in Figure 6). Frame D: Frame E: spectrum of anti-PAH MAb corresponding to the tailing part of the peak labeled Ab in Figure 6).

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Figure 5. Frame A: The flow-through partial-filling ACE electropherograms of enantiomeric cis-benzo(a)pyrene tetrols (cis-BPT). (a) racemic mixture of (+) trans-BPT and (–) trans-BPT, (b) anti-PAH MAb plug followed by 1 min delay in the injection of (–) trans-BPT plug, (c) anti-PAH MAb plug followed by 1 min delay in the injection of (+) trans-BPT plug. Frame B: anti-PAH MAb plug followed by 1 min delay in the injection of racemic mixture of (+) trans-BPT and (–) trans-BPT. 50 mM sodium-phosphate buffer at pH 8.8 was used as the CE running buffer. A 257 nm laser excitation source and CCD camera were used for LIF detection.

Figure 6. Multiple injection flow-through partial-filling ACE electropherogram of a mixture of racemic cis-BP tetrols and trans-BP tetrols with anti-PAH MAb. Injection order: 1) racemic mixture of cis-BPT and trans-BPT; 2) 2 min delayed anti-PAH MAb; 3) 0.5 min delayed racemic mixture of cis-BPT and trans-BPT. 50 mM sodium-phosphate buffer at pH 8.8 was used as the CE running buffer. A 257 nm laser excitation source and a CCD camera were used for LIF detection.

Figure 7. On-line LIF spectra ($\lambda_{\text{exc}} = 257 \text{ nm}$) for FTPFACE separation of a mixture of four diastereomeric BP tetrols with anti-PAH MAb. Frame A: spectrum (a) corresponds to (-) trans-BPT obtained at the maximum of the electrophoretic peak labeled (-)t in Figure 6; spectrum (b) corresponds to (-) cis-BPT obtained at the maximum of the electrophoretic peak labeled (-)c in Figure 6. Frame B: spectrum of (+) cis-BPT corresponds to electrophoretic peak labeled (+)c in Figure 6). Frame C: mixed spectrum of (+) trans-BPT and anti-PAH MAb corresponding to the fronting part of the electrophoretic peak labeled (+)t in Figure 6). Frame D: spectrum of anti-PAH MAb corresponding to the tailing part of the peak labeled Ab in Figure 6.

CHAPTER 7

GENERAL CONCLUSIONS

The work presented in this dissertation focuses on combining highly sensitive and selective fluorescence line-narrowing spectroscopy (FLNS) detection with various modes of immunoanalytical techniques. It has been shown that FLNS is capable of directly probing molecules immunocomplexed with antibodies, thus eliminating ambiguities that may arise from interferences (non-specific binding) that accompany traditional immunochemical techniques. Moreover, the utilization of highly cross-reactive antibodies for specific analyte determinations is demonstrated, including spectral and electrophoretic differentiation of diastereoisomers and enantiomers.

In Chapter 2 the simultaneous detection of two analytes on a mixed-antibody, single-channel immunosensor using time-resolved, laser-based, low temperature fluorescence spectroscopy was demonstrated. The single spot, mixed-antibody architecture combined with a multidimensional detection method enabled multianalyte detection without the need for spatial resolution of different antibodies. Chapter 3 discusses the spectroscopic (FLNS) method for studying antibody/antigen interactions of DNA adducts and metabolites of polycyclic hydrocarbons (PAHs). The method is based on the fact that the relative intensities of the excited-state vibrational frequencies in an FLN spectrum are strongly dependent on the local environment of the fluorophore. Information on the nature of the interactions can be

obtained by comparing the FLN spectra of the antigen/antibody complexes to the spectra of the antigen in solvents of different types (H-bonding, aprotic, π -electron containing).

Chapter 4 demonstrates that the FLNS can be used to identify various haptens cross-reacted with an antibody, as well as to simultaneously differentiate between free and immunocomplexed haptens. In addition, our results suggest an interesting case of an antibody binding a particular hapten (cross-reactant) in which the antibody adopts two distinct conformations of its binding sites. The existence of the two conformational substates for anti-PAH MAb can be rationalized through the existing models for antibody binding. As revealed by FLN spectra of immunocomplexed chromophores, π - π interactions, rather than hydrogen bonding, play the central role in complex formation.

In Chapter 5, utilization of the highly cross-reactive anti-PAH MAb for spectral resolution of four stereomeric analytes has been demonstrated. This study represents the first example of a fluorescence spectroscopic method, capable of enantiospecific differentiation based on the geometrically different intermolecular interactions of chiral analogues with a single, highly cross-reactive antibody.

Finally, the results presented in Chapter 6 demonstrate the utility of a cross-reactive antibody as a chiral selector in flow-through partial-filling affinity capillary electrophoresis (FTPFACE) with laser-induced fluorescence (LIF) detection.