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Review

Electronic spectroscopic investigations of the stationary phase in reversed-phase liquid chromatography

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ABSTRACT

Electronic spectroscopy of probe molecules provides a powerful means of characterizing the stationary phase in reversed-phase liquid chromatography. In particular, both fluorescence and UV–visible absorption spectroscopies have been used to characterize these complex interfacial environments. This article reviews the progress made with these approaches for studying the structure of the stationary phase, the solute environment that it produces, and the dynamics of sorbed molecules in reversed-phase liquid chromatography. Fluorescence studies using either covalently attached probes, or physiosorbed probes are reviewed, along with total internal reflection fluorescence studies of flat, model interfaces. Dynamic effects due to excimer formation and quenching are shown to provide information about hydrocarbon ligand proximity, microviscosity, and contact of sorbed molecules with the mobile phase. UV–visible diffuse reflectance spectroscopy has also been used to characterize the dipolarity, polarizability and hydrogen bonding interactions of the reversed-phase surface environment. These electronic spectroscopic approaches lend insight into the organization, orientation, and polarity of the alkyl chains. In this article, the results of these studies are reviewed, and their impact on models for reversed-phase retention are discussed.

CONTENTS

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1. OVERVIEW AND INTRODUCTION

Over the past several years, many investigators have sought an improved understanding of the chemistry of stationary phases used in liquid chromatography. In particular, reversed-phase systems, where a relatively non-polar moiety is covalently attached to a silica gel surface, have been extremely popular in liquid chromatographic separations. Despite their popular use, the physical and chemical nature of these phases, and their interactions with solutes have not been well characterized. While a wide variety of techniques have been used to study these materials, the focus of this review is on the use of electronic spectroscopies of probe molecules, specifically UV–visible absorption and fluorescence spectroscopy, for the characterization of the stationary phases used in liquid chromatography. Although these techniques have been used to study modified silica in both the dry and solvated states, this article will emphasize studies of particulate surfaces in contact with typical mobile phase components, as it has been shown from chromatographic isotherm studies that C_{18} stationary phase materials sorb a considerable amount of mobile phase components under normal chromatographic conditions [1,2]. This can have a significant effect on the chemistry of the stationary phase, and complicates the interpretation of the experimental results. Results from both fluorescence and UV–visible absorption spectroscopy have advanced our understanding of this complicated interphase region. In particular, the results from the studies described in this review article have been able to address the following fundamental questions about the chemistry of alkyl-modified stationary phases:

Alkyl ligand structure: What is the distribution, organization and orientation of the alkyl ligands on the silica surface? How does sorption of mobile phase components affect these characteristics?

Stationary phase polarity: What is the polarity of the stationary phase environment? How do the specific interactions based on the dipolarity, polarizability, and hydrogen bonding characteristics of the phase contribute to the overall surface polarity? How does the sorption of mobile phase components affect the polarity of the stationary phase environment?

Dynamics, orientation and accessibility of sorbed solutes: What are the dynamic characteristics of chromatographic solutes within the stationary phase? What is their orientation with respect to the interface? How well do the alkyl chains protect chromatographic solutes from exposure to bulk mobile phase? What is the relative importance of adsorption *versus* partitioning retention mechanisms?

2. ENVIRONMENTAL EFFECTS ON ELECTRONIC STATES

The basis for all fluorescence and UV–visible absorption spectroscopic approaches for studying the stationary phase environment is the fact that the characteristics of an electronic state of a molecule depend highly on the molecular surroundings. The effect of the molecular environment on the ground electronic state and/or the first excited singlet state of an organic probe molecule is the most pertinent. Changes in the molecular environment can cause differences in state energies, transition probabilities, and excited state lifetimes. All of these effects have been utilized to probe the chemistry of the stationary phase environment. A brief summary of the basis for each of these effects is given here.

2.1. Transition energies

The energies of the ground and excited electronic states of molecules are strongly affected by the presence of solvent [3,4]. The ground state of an organic molecule will have an average solvation sphere that serves to stabilize the molecule, and lowers the energy of the state, as depicted in Fig. 1. In the case of a dipolar molecule, the energy of the ground state will be lower in a dipolar or polarizable solvent as compared to a non-polar solvent, since over time, the average orientation of the solvent dipoles or induced dipoles will align to stabilize the dipole of the probe molecule. The effect of the solvent on the energy of an excited state

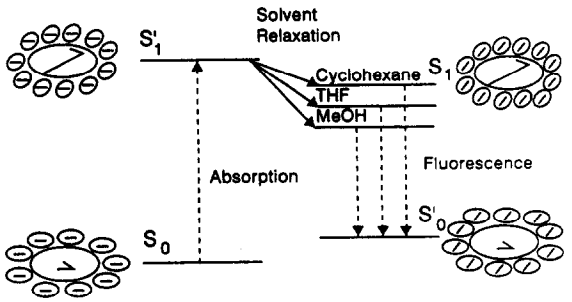

Fig. 1. Solvent effects on electronic state energies. S_0 = Ground state; S_1 = excited state; ——→ = radiative process; → = non-radiative processes; ⇀ = solute dipole; —— = solvent dipole; ○ = solvent electronic configuration. Adapted from ref. 4.

molecule depends upon the time scale of the measurement. Upon excitation of the molecule with UV–visible radiation (time scale *ca.* 10^{-15} s), the electronic distributions of the solvent molecules within the solvation sphere can adjust to stabilize the excited state, as shown in Fig. 1. If upon excitation, there is a substantial change in the dipole moment of the probe molecule, for example, an increase, then a dipolar and polarizable solvent will serve to stabilize the excited state more than the ground state, as compared to a non-polar solvent. In this case, the energy levels will be closer together, and the absorbance spectrum for this probe will be red-shifted in polar solvents. This effect is termed *positive solvatochromism*. The opposite effect, *i.e.*, a blue shift, is observed for molecules whose excited states have smaller dipole moments in their ground states; this is *negative solvatochromism* [5].

The time scale for fluorescence is much longer than that of absorption spectroscopy (*ca.* 10^{-8} s), and over this time period, the solvent molecules have a chance to rotationally reorient to stabilize an excited state dipole moment. The magnitude of this effect, called *solvent relaxation*, varies with the nature of the solvent, as shown in Fig. 1. This effect leads to a reduction in the energy of the excited state for a dipolar excited state in a dipolar solvent. Upon emission of radiation (fluorescence), the molecule returns to the ground state electronic configuration, but while the electronic configurations of the solvent molecules in the solvation sphere have a chance to reorient, the solvent molecules themselves do not have time to rotationally reorganize over the time scale of the fluorescence emission. The energy of this initial ground state is therefore usually larger than the equilibrium ground state configuration. If the ground state has a smaller dipole moment than the excited state, then this stabilization effect will not be as pronounced as that observed for the excited state, so that the primary effect in fluorescence emission spectra is to observe a red shift for polar solvents. An example of this kind of effect is seen with the dansyl probe of solvent polarity [6].

One comparison that can be made between the positive solvatochromism observed in absorption spectroscopy, and the solvent relaxation phenomenon manifested by red shifts in fluorescence spectroscopy, is that UV–visible absorption spectroscopic probes are normally much more sensitive to the polarizability, rather than the dipolarity of the solvent molecules. This is due to the fact that over the time scale of the absorption experiment there is only enough time for the electronic configurations of the solvent molecules to adjust to the presence of the new state. In the case of negative solvatochromism, the ground state is more dipolar than the excited state, so that the ground state equilibrium solvation conditions can more accurately reflect both the polarizability and dipolarity of the surrounding solvent. Note also, that if there is a significant difference in the propensity of the ground and excited states to form hydrogen bonds, this can also result in a red or blue shift in the presence of a hydrogen bonding solvent. Several probe dyes have been identified which respond in this way to either the hydrogen bond acidity or hydrogen bond basicity of the solvent [5].

Kamlet *et al.* [7] have investigated several solvatochromic dyes and have found them to be suitable probes of solvent dipolarity, polarizability, hydrogen bond acidity, and hydrogen bond basicity. The π^* scale of solvent dipolarity and polarizability is based on the solvent-dependent spectral shifts of the π to π^* transitions of a series of nitroanilines and related dyes, that have been shown to respond predominately to the

dipolarity and polarizability of the solvent, but not the hydrogen bonding characteristics [8]. The frequency of the absorbance maximum (ν_{max}) is determined, and normalized by the following equation

$$\pi^* = \frac{\nu_{max} - \nu_0}{s} \quad (1)$$

where ν_0 is the frequency of maximum absorbance for the dye in cyclohexane, and s is a normalization so that the π^* of dimethyl sulfoxide is one. Several dyes which give similar estimates for the π^* parameter are used to determine an average π^* value for each solvent, which is normally reproducible to within 0.02 to 0.05 π^* units.

Using a similar procedure to that described above, other dyes have been identified which give rise to the α scale of hydrogen bond acidity and the β scale of hydrogen bond basicity [9]. These dyes normally give spectral shifts which reflect both the hydrogen bonding ability and the dipolarity/polarizability characteristics of the solvent. The β scale is based on comparisons of the shifts for several dyes with differences in the hydrogen bond donor (HBD) ability in the ground and excited states, relative to dyes with similar structures with no differences in HBD characteristics between states. A plot of ν_{max} for the HBA sensitive dye *vs.* the ν_{max} for the dye that is insensitive to hydrogen bonding interactions is made, and those solvents incapable of HBA interactions should fall on a straight line. The remaining solvents (those capable of HBA interactions with the HBD dye) will be displaced from the line by an amount proportional to their HBA basicity, and in a direction consistent with the excited state chemistry of the solvatochromic dye. These displacements are directly proportional to the solvent β, where the scale is normalized by setting the β value for hexamethylphosphortriamide (HMPT) to 1.00. Other dyes can be analyzed in an analogous way, and the values are pooled to give a scale of solvent HBA basicity. These β values may be supplemented with values obtained from IR and NMR measurements. A similar approach is used to generate the α scale of solvent HBD acidity.

2.2. Transition probabilities

One of the most widely used solvent polarity probes is pyrene [10,11]. The basis for the pyrene scale (Py) of solvent polarity is the change in intensity of the highest energy, 0–0 transition in the emission spectrum (also called the I band). In non-polar solvents, emission from the vibronic origin of this Lb (short axis polarized) band is symmetry forbidden. In polar solvents, the intensity of this band is increased due to symmetry-lowering perturbations from the solvent environment that allow mixing with the much stronger La (long axis polarized)

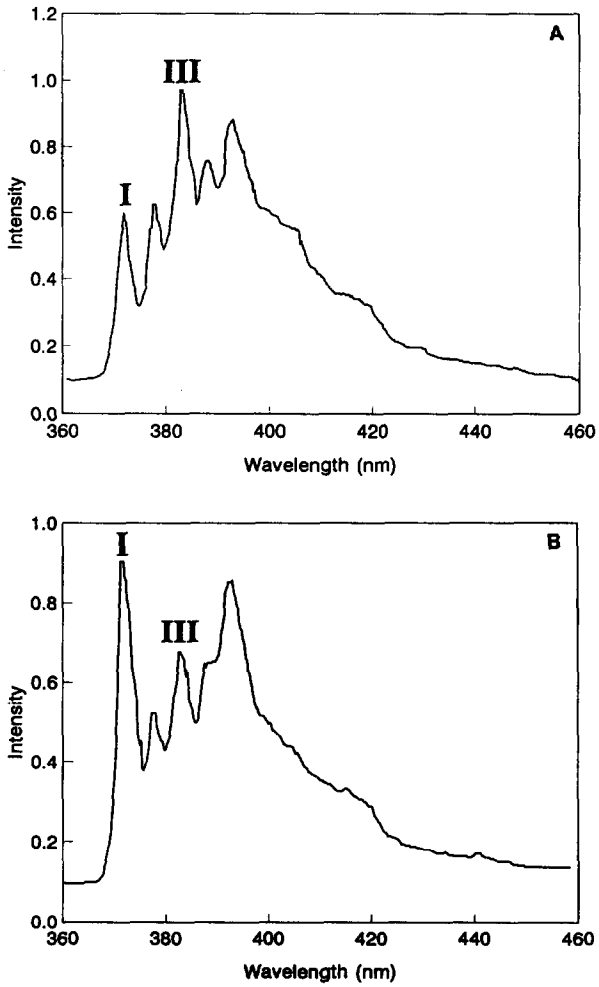

Fig. 2. Solvent effects on the spectrum of pyrene. (A) Pyrene in isooctane; (B) pyrene in methanol. Adapted from ref. 11.

transition [12]. By monitoring changes in the intensity of this band, the polarity of pyrene surroundings can be determined. Normally, the III/I band ratio is used, since the intensity of the third vibronic band is relatively invariant with solvent polarity. The spectra of pyrene obtained in isooctane and methanol are shown in Fig. 2, where the decreased intensity of the I band in the non-polar solvent is clearly observed. Stahlberg and Almgren [13] have shown that the III/I band ratio is strongly correlated with a function of the dipole moment (D) and the molar volume (V), at least for hydroxylic solvents, as shown in Fig. 3. Other polyaromatic hydrocarbons (PAHs) besides pyrene show similar behavior; a summary of solvent dependent effects in PAHs has recently been given by Acree *et al.* [14].

2.3. Excited state decay kinetics

Fluorescence offers additional means for characterizing molecular motions and encounters within the fluorophor environment. The lifetime of an excited singlet state reflects the rates of competing processes for de-excitation of the excited state. The excess energy may be lost through collisions, by energy transfer to other species (quenching), or via fluorescence. If a fluorophor is in a environment that is protected from collisions with quenchers, a longer lifetime will result. A fluorophor in multiple environments may exhibit multiexponential decay kinetics, representing different degrees of deactivation in each environment. Quenching studies thus offer a good means for evaluating the accessibility of the fluorophor to molecular encounters. Normally, an increase in the concentration of a quencher causes a decrease in the fluorescence lifetime, according to the Stern–Volmer equation [3]

$$1/\tau = 1/\tau_0 + k_Q[Q] \quad (2)$$

where τ is the observed lifetime for the fluorophor, τ_0 is the lifetime in the absence of quencher, [Q] is the concentration of quencher, and k_Q is the quenching rate constant. Data which follow the above equation are said to represent dynamic quenching, where the quencher and fluorophor diffuse together and interact during the excited state lifetime. When the magnitude of k_Q is limited only by diffusion, measurement of k_Q permits the estimation of the viscosity of the medium through the use of the Stokes–Einstein equation [3].

A related phenomenon is the formation of *excimers*. Upon excitation of the fluorophor to the first excited singlet state (M*), a second, ground state fluorophor molecule (M) may collide and interact with the first species to form an excited state dimer (D*) or excimer [15]. This process is summarized in Fig. 4. The excited state dimer has its own characteristic emission response, distinct from the emission of the monomer and shifted to longer wavelengths, as shown in Fig. 5. Several features of excimer fluorescence allow this phenomenon to be distinguished from the emission observed from excitation of ground state dimers (dimers that are preassociated before excitation). First, the excitation spectra observed for both the monomer and excimer emission bands should be virtually the same. In addition, the requirement that excimers form by diffusional encounters of excited states with ground state fluorophores means that the emission from an excimer band should have a finite rise time, as shown in Fig. 6.

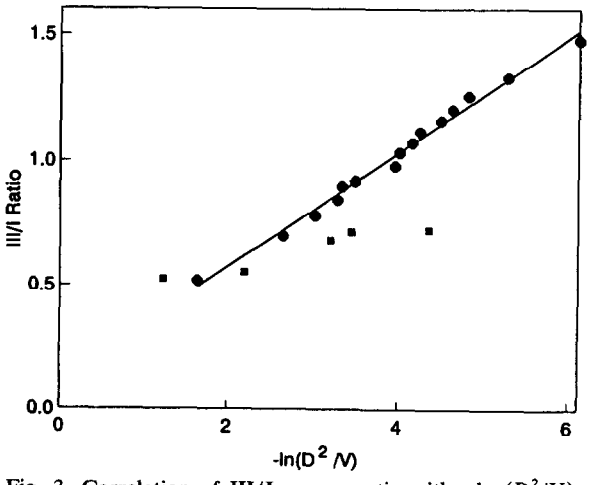

Fig. 3. Correlation of III/I pyrene ratio with $-\ln(D^2/V)$. ● = hydroxylic solvents and solvent mixtures; ■ = non-hydroxylic solvents. Adapted from ref. 13.

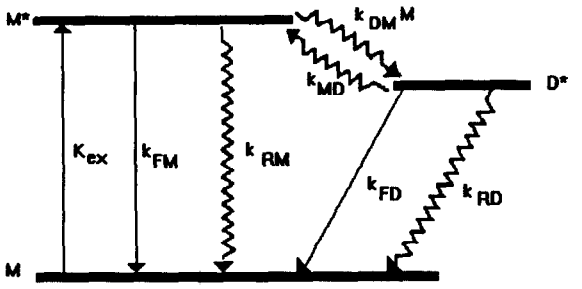

Fig. 4. Excited state diagram for excimer formation. M = Ground state monomer; M* = excited state monomer; D* = excited state dimer; ex = excitation; FM = monomer fluorescence; RM = monomer relaxation (non-radiative); DM = D* formation from M*; MD = M* formation from D*; FD = excimer fluorescence; RD = excimer relaxation (non-radiative). Adapted from ref. 15.

Finally, the excimer fluorescence intensity should be stable over long times to rule out the formation of photoproducts. Excimer fluorescence becomes more prominent at higher concentrations of fluorophor (see Fig. 5), since the ground and excited state species must be within a short enough distance to be able to diffuse together during the excited state lifetime. The ground and excited state precursors can also be different species; in this case the complex is called an

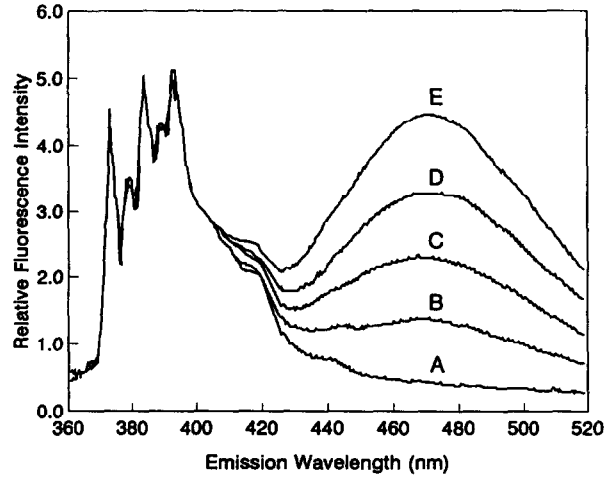

Fig. 5. Pyrene fluorescence spectrum as a function of added pyrene. M_0 is the concentration of pyrene in the C_{18} layer, assuming an 18 Å thickness and 350 m^2/g surface area. (A) $M_0 = 4.4 \cdot 10^{-4}$ M; (B) $M_0 = 4.0 \cdot 10^{-3}$ M; (C) $M_0 = 7.9 \cdot 10^{-3}$ M; (D) $M_0 = 1.2 \cdot 10^{-2}$ M; (E) $M_0 = 1.6 \cdot 10^{-2}$ M. Adapted from ref. 15.

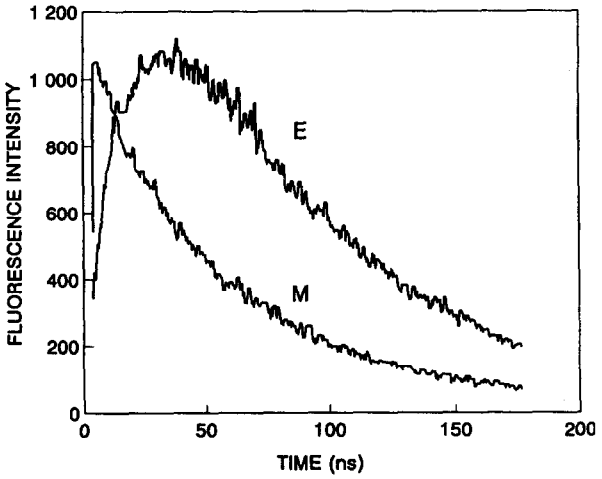

Fig. 6. Fluorescence decay curves for pyrene. $M_0 = 5.3 \cdot 10^{-3}$ M; M = monomer; E = excimer. Adapted from ref. 15.

exciplex. Changes in the charge-transfer character of exciplexes with electron donors can be a sensitive probe of local dielectric constant [16]. Pyrene and other PAHs show a tendency to form excimers and exciplexes, due to the interaction of the aromatic rings through excitation resonance and charge transfer interactions, respectively.

While excited-state quenching, and excimer and exciplex formation are used to probe bimolecular encounters, fluorescence depolarization can be used to study the unimolecular dynamics of fluorescent species. These studies allow rotational relaxation times to be measured, and can give an indication of the orientation and mobility of the fluorophor. To control the angle and polarization of the incident and detected radiation with respect to the interface, these studies are carried out using total internal reflection fluorescence spectroscopy and optically flat silica plates which serve as models for silica gel surfaces.

3. EXPERIMENTAL APPROACHES

Three classes of fluorescence techniques for studying chromatographic stationary phases have been developed. The first involves the covalent coupling of a fluorescent moiety to the silica surface, at a low surface coverage, followed by derivatization with an appropriate alkyl silane

reagent [17]. This approach has the advantage that the location of the fluorophor is known, and no interference from mobile phase species is possible. A second approach is based on the use of a suitable probe molecule that partitions into the stationary phase. These probes serve as models for actual chromatographic solutes, however, problems may occur due to interference from probe species that remain in the mobile phase. In either of these approaches, it is advantageous to measure the spectrum using a cell that allows the surface of the support material to be solvated with the selected mobile phase. An example of such a cell is shown in Fig. 7. A third approach that has been utilized is based on total internal reflection fluorescence (TIRF) spectroscopy, where the appropriate alkyl silane reagent is reacted with a flat silica plate, and the fluorescence from a probe molecule that has partitioned into the alkyl surface layer is monitored. An example of an experimental set-up for TIRF spectroscopy is shown in Fig. 8. This approach

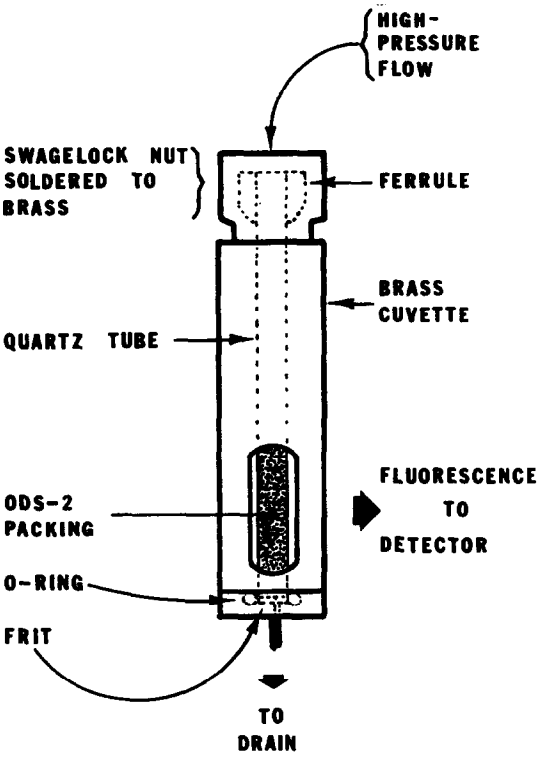

Fig. 7. Cell for measurement of fluorescence from silica interfaces. Adapted from ref. 17.

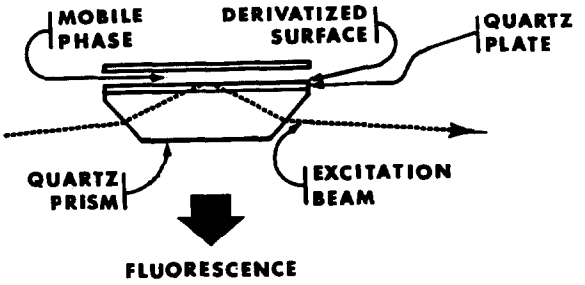

Fig. 8. Experimental arrangement for measurement of TIRF spectra.

has the advantage that the fluorescence is excited by an evanescent wave, which propagates only a short distance past the silica surface, giving a high degree of surface selectivity. However, with this technique, a flat interface is used, so no information can be obtained about the influence of the pore structure on the chemistry of the stationary phase. Results obtained from all three fluorescence techniques have given considerable insight into the chemistry of typical reversed-phase liquid chromatographic stationary phases.

Absorption spectroscopy has also provided information about the chemistry of stationary phases through the use of solvatochromic dyes, as discussed previously. In this case, diffuse reflectance spectroscopy has been used to measure the absorption bands of these dyes sorbed to the surface of the stationary phase. An example of a diffuse reflectance detector is shown in Fig. 9 [18]. In this case, a sample cell containing a suspension of the mobile phase/stationary phase combination to be studied is placed in the position for the reflection sample. The interior of the sphere is covered with a uniform coating of $BaSO_4$, and the photomultiplier detector mounted in the base of the sphere detects the diffuse reflectance signal. The use of UV–visible spectroscopy for characterizing solvated stationary phase environments does present some problems, however. First, the sensitivity of the absorption experiment is generally lower than that of a fluorescence experiment. In addition, the use of a scattering medium presents special problems for absorption studies. The presence of scattered light can change the shape of the spectral response, or in some cases, may even shift the spectrum. And, as for the case for

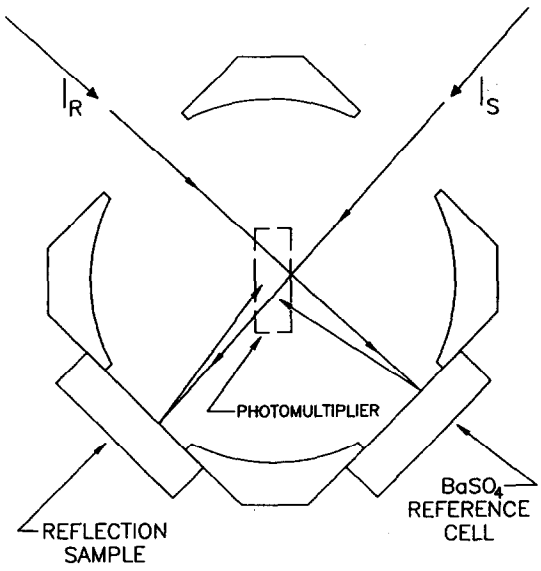

Fig. 9. Diffuse reflectance detector. I_S = Sample beam; I_R = reference beam. The reflection sample and reference cell are used for opaque samples. Adapted from ref. 18.

fluorescence probes, consideration of potential interferences from species remaining in the mobile phase is necessary. Due to the above factors, substantially fewer studies on the UV–visible absorption characterization of chromatographic stationary phases have appeared in the literature as compared to the number of fluorescence studies. However, since methodology has been developed to resolve hydrogen bonding effects from dipolarity/polarizability effects, the use of absorption probes holds considerable promise for further characterization of the stationary phase environment.

4. STATIONARY PHASE CHARACTERIZATION

4.1. Alkyl ligand structure

Lochmuller *et al.* [17] described the use of [3-(3-pyrenyl)propyl]dimethylchlorosilane (3PPS) covalently bound to silica substrates for characterization of the organization of these groups on a silica surface. The spectra were measured in the presence of a methanol overlayer, and the relative intensities of the excimer and monomer emissions were characterized. A shift in the excitation spectra for the excimer in high-coverage samples was attributed to inner filter effects, rather than the presence of ground state dimers. This was confirmed by dilution studies. These investigators found that even at low coverages the 3PPS groups were not isolated on the silica surface, but tended to aggregate in clusters [17]. These investigations indicated that clusters of stationary phase ligands form on the silica surface, and that an evenly distributed "brush" or "picket fence" model may not be appropriate.

These investigators followed up this study by characterizing 3PPS at surface loadings ranging from 0.13 to 1.10 μmol/m^2 and used hexane,

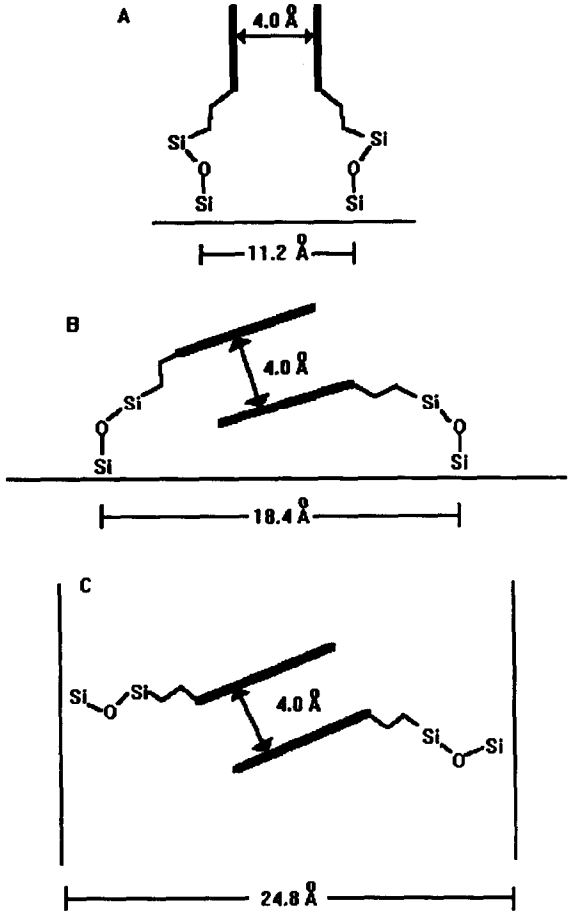

Fig. 10. Possible structural conformations for 3PPS bound to a silica surface. (A) Fully extended; (B) collapsed; (C) pore or interparticle conformation. Adapted from ref. 19.

tetrahydrofuran, acetonitrile, and methanol as the overlying solvents. Time-resolved fluorescence spectroscopy was used and three populations of probe molecules could be identified on the surface. One isolated or monomer population was relatively invariant with surface coverage, and was assumed to correspond to 3PPS bonded to isolated silanol groups. A second monomer population was found to be available for excimer formation, since the fractional contribution from this species decreased as the 3PPS surface coverage increased. Fig. 10 depicts several conformations that could lead to excimer formation. The conversion of this latter population from monomer to excimer (isolated to clustered) appeared to be random, following a Poisson statistical model for encounter probability. This shift in population is shown by the squares in Fig. 11. Even though the aggregation was statistical, the probability of encounter was high especially in hostile solvents. In addition, significant differences in the probe organization were found for the different overlying solvents. For hexane, there was evidence of substantially improved solvation of the 3PPS groups and a decreased tendency to cluster, relative to acetonitrile and methanol, as shown by the data plotted in Fig. 12 [19].

This study was extended by also characterizing

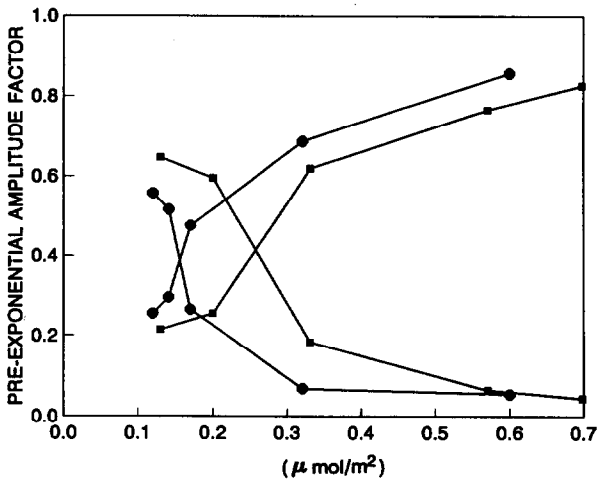

Fig. 11. Monomer population distribution as a function of alkyl chain length and surface concentration. Solvent, methanol. ● = 3PDS; ■ = 3PPS. Adapted from ref. 20.

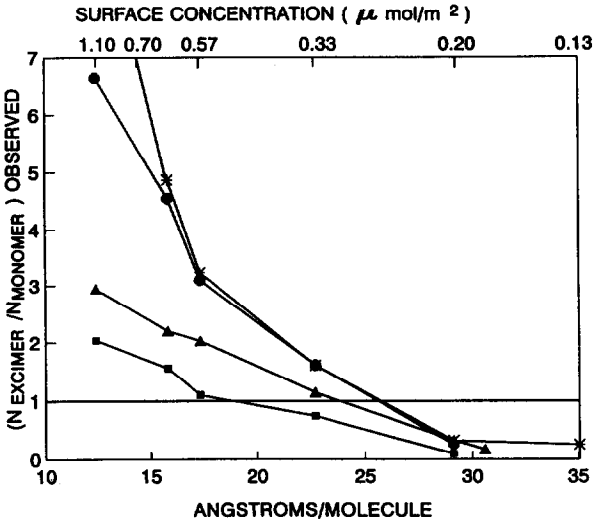

Fig. 12. Observed excimer/monomer population ratio as a function of surface concentration and average distance between bonding sites for different solvents. * = Acetonitrile; ● = methanol; ▲ = tetrahydrofuran; ■ = hexane. Adapted from ref. 19.

the behavior of [10-(3-pyrenyl)decyl]dimethylchlorosilane (3PDS) derivatized surfaces at various surface coverages, to compare the earlier results obtained for the 3PPS derivatized surfaces. The shift from monomer to excimer for the 3PDS probe, shown by the circles in Fig. 11, occurs at a lower surface coverage as compared to the data for 3PPS. This is due to the fact that the longer spacer group in the 3PDS molecule allows excimers to form at lower surface coverages. The rise times for excimer emission for the longer chain 3PDS derivative were shorter than those for the 3PPS derivatized surfaces. This behavior was interpreted to indicate that the long chain groups are more ordered. In these more ordered phases, it is more likely that two pyrene groups are in the "sandwich" configuration required for excimer formation. The excimer rise times tended to be longer for "good" solvents for the 3PDS groups (*i.e.*, hexane and tetrahydrofuran). This trend was also observed for low-coverage 3PDS silicas that were subsequently reacted with octadecyldimethylchlorosilane. These results were interpreted to indicate that the alkyl chains tend to be more fully extended in good solvents, but are collapsed onto the surface when the silica particles are in

contact with a "hostile" solvent, in this case, methanol [20].

4.2. Stationary phase polarity

The term polarity has been used to indicate an overall measure of a solvent's ability to interact with a solute. Solvent polarity is comprised of contributions from dispersion, induction, orientation, and hydrogen bonding interactions. The studies that will be discussed in this section cover experiments done with general polarity probes, as well as with probe molecules that characterize more specifically the interactions between the solute and solvent.

Lochmuller *et al.* [21] performed some of the earliest investigations on the use of fluorescence spectroscopy to probe the polarity of the mobile phase–stationary phase interface in liquid chromatography. Silica gel was derivatized with propyl amine functionalities, which were then reacted with dansyl chloride to produce a covalently bound probe of the silica surface environment. The wavelength of maximum emission intensity for the dansyl moiety ranges from 441 nm in hexane to 538 nm in water. The emission maximum for dansyl groups on otherwise bare silica surfaces was approximately 500 nm, when the overlying solvent was methanol or acetonitrile, and the maxima were similar to those observed for an analogous solution phase probe. This was not observed for either very polar solvents (*i.e.*, water) or non-polar solvents (*i.e.*, hexane). The silica surface in contact with water was less polar than bulk water, and the silica surface in contact with hexane was significantly more polar than bulk hexane. The dry materials exhibited absorption maxima at around 480 nm, which is similar to the value observed for a bulk solvent like ethyl acetate. Methyl, octyl and octadecyl derivatized silicas were also studied; the C_{18} derivatized surface in the dry state was slightly less polar than the analogous silica surface. In the presence of methanol and acetonitrile, the position of the emission maximum for the C_{18} surface was increased, indicating a more polar environment was being experienced by the dansyl groups. This polarity was slightly less than the analogous system with no C_{18} groups present. In the presence of tetrahydrofuran, the polarity of the phase was lower, and in the presence of water, the polarity of the C_{18} surface was unchanged from the dry state. These surfaces were also examined with an overlayer of a 50% mixture of acetonitrile, methanol or tetrahydrofuran with water. These C_{18} surfaces gave emission maxima that were similar to those obtained for the C_{18} surfaces exposed to the pure organic solvents. These studies have provided some of the first spectroscopic evidence that C_{18} derivatized surfaces are significantly more polar than a solution of the analogous alkane. In addition, these studies have given evidence that the nature of the mobile phase can affect the polarity of the stationary phase [21].

Lochmuller *et al.* [6] proposed that examination of excitation wavelength dependent emission maxima of the covalently attached dansyl probe could lend insight regarding the different microenvironments experienced by the dansyl moiety, and allow the heterogeneity of the probe environment to be assessed. Potassium iodide was used as a quencher to ascertain the exposure of the different populations of dansyl probes to bulk solvent. Comparisons were made between the dry form of C_{18} derivatized silica and the same material in an acetonitrile suspension, and substantially different results were obtained, again confirming through spectroscopic means that solvation of the alkyl surface layer by the mobile phase is an important process to consider [6]. This approach was also used to characterize the behavior of different end-capping reagents used to block access to residual silanols. Two reagents, trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) were compared. It was found that HMDS was more effective in blocking acidic sites on the surface, and gave a surface with a lower polarity, as evidenced by the blue shift of the dansyl probe emission spectrum, but that TMCS treatment of the surface provided a more homogeneous environment. Chromatographic data were obtained that were in agreement with the results from the spectroscopic studies [22].

Lochmuller *et al.* [16] used exciplex emission from surface bound pyrene or biphenyl groups

and N,N-dimethylaniline to characterize silica surfaces reacted with either TMCS or HMDS. The exciplex emission from the pyrene–aniline and biphenyl–aniline excited state complexes indicated that, in contrast to the previous work, the HMDS-treated surface was more polar than the TMCS-treated surface. In addition, the nature of the solution overlayer had a significant effect on the emission wavelength of the complexes. A dielectric constant of approximately 4.4 for silica in contact with hexane was estimated from the spectroscopic data [16].

Stahlberg and Almgren [13] were some of the first investigators to examine the behavior of physiosorbed pyrene on C_{18} surfaces with solvent overlayers of typical reversed-phase mobile phases. These investigators studied both C_{18} and C_2 phases, in contact with water and organic solvent water mixtures containing up to 30% methanol or acetonitrile in water. Under these conditions, the pyrene is strongly sorbed to the stationary phase. Based on the examination of the III/I band ratio, these workers concluded that the polarity of the C_{18} surface in contact with water was similar to bulk 1-octanol. Upon addition of methanol, the polarity decreased slightly and then leveled off. This was attributed to an initial blocking of the available silanol groups by hydrogen bonding with the methanol, with little subsequent sorption of methanol by the C_{18} chains. In the case of acetonitrile, the polarity initially decreased, but began increasing as the percentage of acetonitrile increased past 10%. This difference in behavior between methanol and acetonitrile was attributed to the fact that the amount of methanol sorbed reaches a constant value, while the amount of acetonitrile sorbed by the C_{18} chains continues to increase over the solvent composition range measured [13]. These studies, along with the studies described above using covalently attached dansyl groups, were the first to indicate that typical C_{18} reversed phases in contact with the mobile phase were considerably more polar than the analogous bulk alkane solvent.

Carr and Harris [23,24] also used the III/I band ratios of physiosorbed pyrene to characterize the polarity of monomeric and polymeric C_{18} phases in the presence of acetonitrile–, methanol– and tetrahydrofuran–water mixtures. Care was taken, through measurements of capacity factors, to ensure that the fluorescence signal was predominantly due to species associated with the stationary phase. Mobile phase compositions were 50 to 80% methanol in water, 20 to 70% acetonitrile in water and 25 to 45% tetrahydrofuran in water. The effective polarity experienced by the pyrene decreased as the amount of water in the mobile phase increased, however, the polarity was significantly greater than either a dry C_{18} phase or a bulk alkane. The polarity was always lower than the surrounding bulk solution, indicating that the C_{18} chains protected the pyrene from exposure to bulk solvent. The main determinant of stationary phase polarity was shown to be the sorption of mobile phase components, rather than residual silanol effects. The monomeric phase showed a more highly polar environment for a low concentration of organic modifier; for higher concentrations of modifier, there was no significant difference between the results for the monomeric and polymeric phases [23]. This study was expanded by developing a protocol for studying a broader range of mobile phase compositions in a single experiment. Methanol–water mobile phases, ranging from 2 to 80% methanol were characterized. The results obtained are shown in Fig. 13. These data confirmed the previously observed behavior that indicated that the polarity of the stationary phase, as reported by the pyrene probe, reached a minimum at 50% methanol in water in the mobile phase [24]. These results again demonstrate that the sorption of mobile phase components can significantly affect the stationary phase chemistry, and show that the chemistry of the stationary phase depends on the composition of the mobile phase. This helps to strengthen the view (also supported by studies discussed in other articles in this issue) that the stationary phase *cannot* be viewed as a passive receptor for chromatographic solutes that are excluded from the mobile phase via solvophobic interactions.

Hartner *et al.* [25] demonstrated that TIRF spectroscopy could be used to characterize flat silica surfaces that have been derivatized with octadecyl silane. The exciting evanescent wave

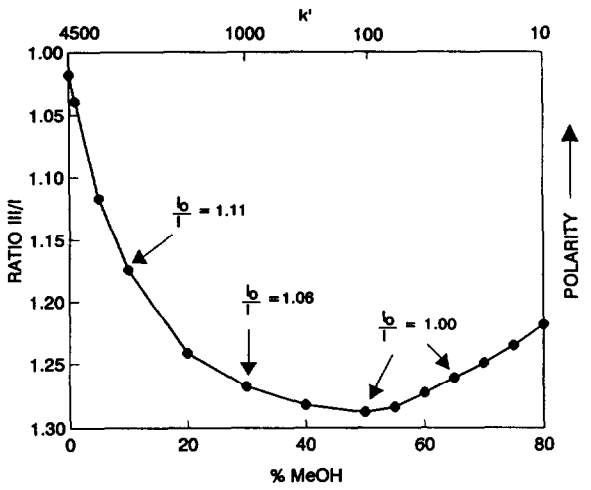

Fig. 13. Microenvironmental polarity of pyrene in a polymeric C_{18} stationary phase as a function of solvent composition. The pyrene III/I band ratio is plotted as a function of the percent volume of methanol. The I_0/I values indicate the quenching effect of 0.10 *M* KI, where I_0 is the original fluorescence intensity, and I is the fluorescence intensity after exposure to quencher. The capacity factors for pyrene retention on the C_{18} phase are shown on the scale at the top. Adapted from ref. 24.

propagates only a short distance past the interface, so that TIRF spectroscopy is a highly surface-selective technique. The polarity of this C_{18} interface in contact with solutions ranging from 40 to 55% methanol in water was probed using the III/I band ratios of physiosorbed pyrene. These results showed a decrease in surface polarity with increasing water content in the overlying solvent, which was consistent with previous studies on porous silica, although the polarity of the flat silica surfaces was much lower that the analogous C_{18} derivatized porous silica surface. The degree of interference from solution phase pyrene species was calculated, and ranged from 12% for the methanol–water (55:45) system to 3% for the methanol–water (40:60) system. Experiments where the solution overlayer was replaced with solvent-saturated vapor confirmed a small interference from solution phase species for the methanol–water (55:45) solvent. A slight offset in the polarity values was observed and was attributed to scatter-excited fluorescence of solution phase pyrene. A major conclusion of this work was that the C_{18} derivatized flat silica surfaces showed a much higher degree of chain ordering, leading to less absorption of mobile phase solvent components and a lower surface polarity, as compared to the previously studied porous silica materials [25]. This is an important conclusion which indicates that while TIRF spectroscopic studies may give useful information about C_{18} stationary phases, caution should be used in extrapolating conclusions drawn from TIRF studies to porous particles.

Rangnekar and Oldham [26] also used TIRF spectroscopy of pyrene sorbed to C_{18} layers and used different incidence angles which allowed the III/I ratios of the pyrene spectra to be characterized as a function of distance from the surface. The incidence angles studied gave penetration depths ranging from 90 to 200 nm. Polymeric and monomeric phases were compared, and similar results were obtained for these two phases [26].

In contrast to the studies described above, Men and Marshall [27] have studied the behavior of two more polar probes, *n*-propanedansylamide and *n*-decanedansylamide. In studying mobile phase compositions ranging from 50 to 100% methanol, these investigators found that the shifts in the wavelength of maximum emission for the dansyl moiety indicated that the fluorescent probes were residing in an environment with a polarity similar to a mixture of 10% water in methanol. The responses were verified to arise from probe molecules associated with the stationary phase through chromatographic retention measurements and quenching studies with KI. These authors concluded that this more polar probe was residing in a different stationary phase environment than the pyrene in the probe studies described above. They also postulated that the main effect of the increasing sorption of methanol by the stationary phase as the methanol concentration in the mobile phase increased might lead to mainly volume changes in the stationary phase, rather than actual polarity changes [27].

Handreck and Smith [28] studied the π^* dye, 4-nitroanisole, as an indicator of the surface polarity of a zeolite catalyst in the dry state, and found a dependence of the π^* value on the amount of aluminum in the catalyst [28]. Lindley

et al. [29] used several of the π^* dyes to characterize silica surfaces, and found that dry silica showed much higher values for the dipolarity/polarizability than bulk solvents, and showed a greater dispersion in the results from different dyes. They proposed that the interactions of the dye with the surface were much more specific than the analogous solution phase interactions, and concluded that the dispersion in the results could be due to the presence of different energy sites [29].

Jones and Rutan [30] characterized the behavior of two π^* dyes at the surface of silica and C_{18} derivatized silica thin-layer chromatographic plates, in the presence of methanol–water and acetonitrile–water mobile phase mixtures. Diffuse reflectance spectroscopy was used to measure the absorption spectra of the dyes for varying mobile phase compositions. Chromatographic capacity factors were used to determine the degree of interaction of the dyes with the stationary phase, and to assess interferences from mobile phase species. The results from these studies indicated that the C_{18} derivatized silica was considerably more polar than bulk alkane solvents, due to the large degree of solvation of the C_{18} chains with mobile phase components [30]. This study has been followed up in work by Hayashi *et al.* [31], where column chromatographic supports were investigated using acetonitrile– and methanol–water mobile phase mixtures. Both silica and C_{18} derivatized silica surfaces were characterized. In this work, a data analysis method, known as an adaptive Kalman filter, was used to correct for interferences from dye that remains in the mobile phase. Although this analysis results in slightly lower values for the π^* parameter for the solvated C_{18} surfaces, the polarity is still significantly higher than the values obtained for bulk alkanes. The π^* values for the solvated C_{18} phases were approximately constant over a range of solvent compositions between 50 and 100% organic modifier [31]. Some of these data for the dye N,N-diethyl-4-nitroaniline in methanol–water mixtures are shown in Fig. 14. These results are in agreement with those reported by Men and Marshall [27] for the sorbed dansyl probe of solvent polarity. These results are also consistent

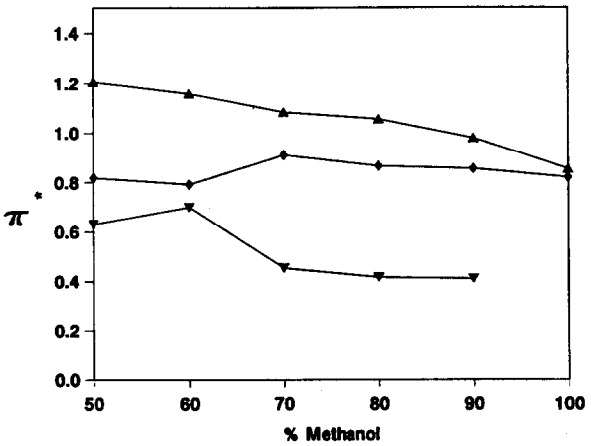

Fig. 14. Plot of π^* values *vs.* mobile phase composition for (▲) solution phase; (◆) C_{18} stationary phase (uncorrected data) and (▼) C_{18} stationary phase (corrected data). Adapted from ref. 31.

with fluorescence quenching studies that have indicated that the C_{18} chains are able to protect the solute from exposure to bulk mobile phase, and that the retention mechanism for this type of stationary phase–mobile phase combination is most likely partitioning.

A popular dye that has been used to help establish the α scale of HBD acidity is known as Reichardt's betaine, or ET-30 [5]. The spectroscopic shifts of this dye are quite pronounced, with a λ_{max} of 453 nm in aqueous solution, and a λ_{max} of 926 nm in hexane solution. Analysis of the spectroscopic behavior of this dye in a wide variety of solvents has led to the conclusions that these shifts are due to both dipolarity/polarizability effects and hydrogen bond acidity of the surroundings in approximately equal proportions. Michels and Dorsey [32] used this dye to characterize the surface of γ-alumina, as a function of thermal activation and water deactivation of the surface. These authors found that the surface polarity decreased as the amount of water added to the support was increased, and were able to correlate chromatographic retention data with the values for the $E_T(30)$ surface polarity parameter [32].

The ET-30 dye was also used by Jones and Rutan [30] to characterize silica and C_{18} derivatized silica thin-layer chromatographic plates in contact with the mobile phase solvent. In the

case of the silica surfaces, there was an abrupt decrease in the $E_T(30)$ parameter when the overlying solvent composition was changed from 100% acetonitrile to acetonitrile–water (90:10). This was attributed to a displacement of the ET-30 dye from the silanol groups by water. One additional difficulty with the use of this dye in these environments is that the dye tends to become protonated, and the solvent sensitive band disappears from the spectrum. A related dye that is a weaker base, ET-33, was used to alleviate this difficulty [33]. Approximate α values were calculated from the results from the ET-33 dye, and these data indicated that components within the solvated C_{18} phase were able to act as a hydrogen bond donor towards the ET-33 dye. A potential difficulty with these experiments is that the ET-33 dye and the reference dye sensitive only to the dipolarity/polarizability of the medium used to calculate the α parameter may not reside in the same environment, and the resulting α parameters may be in error. A similar analysis has been accomplished with a hydrogen bond donor dye which indicates the basicity of the dye surroundings, however, the same cautions must be used in interpreting the calculated β values. The β dyes investigated to date do not show a strong tendency to sorb to the stationary phase, but the preliminary β values that were obtained did indicate a significant propensity for the solvated C_{18} medium to accept hydrogen bonds as well [34].

Ionic fluorescent probes have also been used to characterize silica surfaces derivatized with alkyl chains. Dowling and Seitz [35] used the ammonium salts of 8-aniline-1-naphthalenesulfonic acid (ANS) to study C_{18}, C_8, C_1 and phenyl phases in contact with some quaternary ammonium cations commonly used for ion-pair chromatography with water or methanol overlayers. These investigators' results showed that the nature of the quaternary ammonium cation had a significant effect on the ability of the ANS to form ion pairs within the stationary phase. The tetrabutyl ammonium cation provided a stationary phase environment that was much less polar than the tetramethyl ammonium cation in the presence of water. In addition, when the cation concentration was decreased, the apparent polarity of the surfaces decreased. This was attributed to the decreased charge density at the surface under these conditions [35].

Carr and Harris [36] studied the temperature-dependent behavior of polymeric C_8 and C_9 stationary phases using physiosorbed pyrene as a probe of the interfacial polarity. The stationary phase was equilibrated first with a mobile phase of acetonitrile–water (50:50) containing the pyrene probe. The mobile phase was then replaced with pure water, and the system was allowed to come to equilibrium. Next the temperature was raised, then lowered, and a sigmoid transition in solvent polarity *vs.* $1/T$ was observed at 41 and 49°C for the C_8 and C_9 phases, respectively. Upon cooling, the dependence with $1/T$ was linear down to the original temperature. These results, in conjunction with chromatographic retention studies by Gilpin and Squires [37], indicated that some acetonitrile was "trapped" within the C_8 and C_9 chains, until the increased temperature caused the acetonitrile to be released. Carr and Harris [36] argued that this was an entropic, rather than an enthalpic effect, since the slopes of the ln k' *vs.* $1/T$ curves before and after the transition were indistinguishable, while the intercepts were significantly different. The results from both studies indicated that upon release of the acetonitrile the phase becomes less polar and less ordered. Once the acetonitrile is removed from the phase, the polarity still shows a significant temperature dependence, which was attributed to density changes analogous to those seen in bulk solution [36].

This study was followed up with a study on C_7 and C_8 monomeric phases, where the temperature of the transition (for both retention and polarity behavior) was dependent upon the equilibration time. This indicates that in the monomeric phase, loss of acetonitrile is a result of slow kinetics, rather than a distinct phase transition. Once the acetonitrile is removed from the stationary phase, the polarity of the phase is independent of temperature. These results indicate that the polymeric phases are considerably more ordered than monomeric phases, which is consistent with a multitude of retention studies [38].

4.3. Dynamics, orientation and accessibility of sorbed solutes

Fluorescence spectroscopic measurements have allowed a number of conclusions to be drawn about the dynamic behavior of chromatographic solutes. Several studies have characterized diffusion of solutes in the mobile phase within the pores, diffusion of solutes between the mobile and stationary phases, and motion within the stationary phase itself. Quenching studies, excimer experiments, and anisotropy measurements have been particularly useful in this regard, and the results from these studies will be described in this section.

Wong *et al.* [39] used covalently bound 3PPS to characterize the diffusion of the quencher, I_2, in the pores of bare silica, in contact with a series of straight-chain alcohols. Both 300 Å and 90 Å pore silicas were characterized. It was found that the diffusion could be modeled using a simple, semihemispherical model, for diffusion lengths less than the average pore diameter. For these average distances traveled by the quencher (80–280 Å), no evidence was found for differences in the viscosity of the solvent within the pores, relative to bulk solution [39]. These results indicated that the solvent within the pores of silica appears to retain many of its bulk properties, and that the local geometry of the porous silica surface does not influence encounter rates with the surface.

Harris and co-workers [40,41] followed up on the study described above, and also used the covalently bound 3PPS to characterize surface diffusion of I_2 on bare silica and on C_1 modified silica. In the case of the bare silica, the quenching of the pyrene moiety by I_2 could be explained solely by diffusion of the I_2 from bulk solution, whereas, for the C_1 surfaces, increased quenching rates indicated that adsorbed I_2 was able to diffuse across the C_1 derivatized surface. The rate of this surface diffusion was highest when the surface was in contact with an overlying solvent of 100% methanol; the estimated diffusion coefficients for surface diffusion were reduced by approximately 1/5 and 1/10 for methanol–water (75:25) and methanol–water (50:50), respectively. The behavior of an unretained polar quencher, $HgCl_2$, was substantially different from that observed for the I_2. In this case quenching from solution showed little dependence on either the nature of the surface (bare silica and C_1) or the amount of water in the surrounding solvent [40,41]. The results from this study indicated that surface diffusion may be an important contribution to the dynamics of retention for non-polar solutes.

Carr and Harris [24] characterized the accessibility of the physiosorbed pyrene probe using methanol–water mobile phases, ranging from 2 to 80% methanol. An ionic quencher (KI), and a polar, molecular quencher ($HgCl_2$) were used to assess the accessibility of the pyrene probe. The KI quencher, which has virtually no affinity for the stationary phase, was not able to quench the pyrene fluorescence until the organic modifier concentration in the mobile phase was reduced below 30%. This is indicated by $I_0/I > 1$ in Fig. 13, and corresponds to the region where the interfacial polarity (indicated by the III/I ratio) begins to increase as the organic modifier concentration is further reduced. These results were interpreted to indicate that the stationary phase chains were collapsed at higher water compositions, partially exposing the pyrene to bulk solvent. A dynamic quenching mechanism was inferred from the data; and quenching constants indicated that even at a mobile phase composition of 10% methanol, the pyrene is only partially quenched. The quenching data for the polar quencher, $HgCl_2$, could only be interpreted by invoking two populations of pyrene —one exposed to quencher, and one in a hydrophobic environment that was protected from quencher. The polarity of the exposed environment was found to depend much more heavily on the surrounding mobile phase composition, while the protected pyrene experienced a more consistent hydrophobic environment over a range of mobile phase compositions. These data also indicated that for high methanol concentrations, the stationary phase environment was on average, much more homogeneous [24].

Wong *et al.* [42] characterized bare silica, C_1, C_8 and C_{18} stationary phases in contact with methanol and methanol–water (75:25) mixtures using the covalently attached 3PPS probe. Life-

times and quenching rate constants (with HgI_2 as the quencher) were determined. These results indicated that for 75 to 100% methanol, the C_{18} chains were effective in protecting the 3PPS probe from exposure to bulk solvent, while the ability of the C_8 chains to protect the probe was reduced in solutions containing 25% water. The bare silica and C_1 surfaces yielded an environment similar in polarity to the corresponding bulk solution, as inferred from the lifetime data. Lifetimes were observed to be monoexponential in these instances, implying that over the excited state lifetime, the probe sees a single, homogeneous environment [42]. Since these results indicated that the C_{18} could protect the 3PPS probe from exposure to bulk solvent, they may also be able to shield chromatographic solutes from exposure to bulk mobile phase, which is consistent with a partitioning mechanism for retention.

Avnir *et al.* [43] studied the bifunctional probe, 1,3-di-1-pyrenylpropane adsorbed to silica and octadecyl silica in the dry state. Dynamic excimer formation was not observed on dry silica surfaces, however, when 1-octanol was coadsorbed to the silica surface, dynamic excimer formation was observed, indicating that the pyrene groups have increased their mobility. On C_{18} derivatized surfaces, dynamic excimers were observed at low concentrations of the pyrene probe, however, at high concentrations, the excitation spectrum was shifted, and there was no appreciable rise time for the excimer band, indicating that the emission at long wavelengths arises from ground state aggregates, rather than excimer formation [43]. Ware and co-workers [44,45] have also studied the emission of pyrene adsorbed to the surface of dry silica particles, and also concluded that much of the emission in the excimer region of the spectrum is due to the presence of ground state dimers.

Lochmuller and Wenzel [46] reexamined the results described by Ware and co-workers [44,45], and concluded that silica was not a strong sorbent for pyrene. In addition, concentration-dependent changes in the excimer excitation spectrum revealed that many of the previous experiments were done under optically dense spectroscopic conditions. With coadsorbed solvents, there was evidence that the pyrene existed in "pools" of solvent, possibly within the pores, and dynamic excimer formation was observed. It was also concluded that in many cases, the excimer emission from pyrene could be attributed to the formation of microcrystalline pyrene on the surface, rather than ground state dimers [46]. These conclusions indicated that many of the results from the earlier work were not pertinent to chromatographic stationary phases.

Stahlberg *et al.* [47] studied the fluorescence decay kinetics for the excimer band observed for pyrene sorbed to C_2, C_8 and C_{18} layers in the presence of a water layer. Dynamic excimer formation was observed for all experiments. The data were used to estimate the rate constants for the photophysical processes for excimer formation. The data were somewhat variable for the C_2 and C_8 phases, but the C_{18} phase showed temperature-dependent behavior that was used to estimate the viscosity of the C_{18} layer. The resulting value indicated more liquid-like properties, with a possible phase change occurring at approximately 20°C [47].

Bogar *et al.* [15] also used the lifetime information from pyrene excimer emission from C_{18} surfaces, in this case, in contact with a mixture consisting of methanol–water (75:25). No evidence for ground state dimer formation was observed, and excimer emission was seen only from the wetted C_{18} stationary phase. From the rate constant describing the formation of excimers from excited state monomers (k_Q), a diffusion coefficient was calculated that gave a value for the "microviscosity" of the stationary phase of 18 cP, which is similar to the value observed for bulk ethylene glycol. Although Stahlberg *et al.* [47] and Bogar *et al.* [15] used somewhat different assumptions in their analysis of the excimer lifetime data, and Stahlberg *et al.* used an overlayer of water, while Bogar *et al.* used an overlayer of methanol–water (75:25), the results obtained by the two groups are in reasonable agreement. These results indicate that solvated C_{18} layers covalently attached to silica gel can be viewed as a viscous liquid, which supports a partitioning rather than an adsorption type mechanism.

Rangnekar *et al.* [48] have also characterized

the stationary phase viscosity, in this case by using fluorescence anisotropy measurements in conjunction with TIRF methods. These authors were able to estimate a viscosity value using 1,6-diphenylhexatriene as a physiosorbed probe of the solvated C_{18} layer. Data for C_1 and C_3 layers yielded results indicative of adsorptive interactions, results for C_{18} layers indicated partitioning behavior, and results for C_8 layers gave intermediate results. The microviscosity of the C_{18} layer in contact with 100% methanol was estimated to be 18 cP, in agreement with the earlier results reported by Bogar *et al.* [15]. These authors cautioned, however, that this value should be considered a lower limit, due to potential mobile phase contributions under their experimental conditions.

Montgomery *et al.* [49] investigated the orientational dynamics of the hydrophobic probe, 1,4-bis(*o*-methylstyryl)benzene (bis-MSB), on silica plate surfaces derivatized with chlorodimethyloctadecyl silane in contact with water, methanol–water (20:80) and propanol–water (5:95). Although contact angle information indicated when the organic modifier was added to the water that the C_{18} surface was wetted, frequency-domain, fluorescence anisotropy decay data indicated that the orientation of the probe was still largely aligned along the surface, rather than normal to the surface, as would be expected if the chains were fully extended. These studies support the notion that the C_{18} chains assume a collapsed configuration when in contact with high water content phases [49]. Note that there is no analogous experiment that can be done using porous particles that yields information about the orientation of the solute within the stationary phase.

The bis-MSB probe was also used to characterize orientational behavior of C_{18} layers in contact with a sorbed layer of the surfactant, sodium dodecyl sulfate (SDS). The fluorescence anisotropy data indicated that the reorientation of the probe was less hindered in the presence of SDS, as compared to a pure water mobile phase. In addition, anomalous behavior of the amplitude ratios for the frequency domain data was observed near the critical micelle concentration (CMC) of the SDS molecule, indicating a change in the reorientation dynamics at this point [50]. Interestingly, the lowest barrier to reorientation was observed near the CMC, where probe reorientation was more facile than either above or below the CMC. This unique behavior occurs where the adsorption of surfactant to the C_{18} surface reaches a maximum and indicates that interesting changes in structure of the interface occur at this point. Given the practical interest in micellar chromatography, this observation clearly deserves further investigation.

Burbage and Wirth [51] used a similar approach with another probe molecule, acridine orange, to study octadecyl groups on silica surfaces using TIRF. Acridine orange is a charged, aromatic species, which is expected to reside at the interface between the polar solvent and the C_{18} chains, rather than partitioning into the chains, as was the case with the probe discussed above. The results indicated that the in-plane reorientation of the probe was facile, and this process was slowed by the addition of adsorbed alcohols, such as propanol and methanol. The orientation distribution indicated that the surface roughness increased with the addition of the adsorbed alcohols as the surface tension was lowered. There was also evidence that the out-of-plane reorientation (normal to the surface) was hindered, which is consistent with geometric considerations [51].

5. SUMMARY AND CONCLUSIONS

The studies described in this article have addressed a number of fundamental questions about the nature and role of the stationary phase in reversed-phase liquid chromatography. It is clear from these studies that the alkyl groups are not evenly distributed across the silica surface, but are aggregated to a degree that depends on the average bonding density and the nature of the overlying solvent. The mobile phase appears to affect the organization and orientation of the chains at the surface. For mobile phases with high organic content, there is evidence that the alkyl chains are organized and extended away from the surface. It is clear that the alkyl chains are solvated to a significant effect with the

organic component of the mobile phase, and that this has a profound effect on the dipolarity and polarizability of the stationary phase environment. For mobile phases with no water, the stationary phase has a very similar polarity to the mobile phase. Initial studies with UV–visible probes have indicated that hydrogen bonding also plays an important role in determining the overall polarity of the stationary phase environment.

In the case of C_{18} derivatized surfaces, the alkyl chains are clearly capable of protecting the solute from exposure to bulk solvent, which is strong evidence for a partitioning mechanism. Stationary phases with shorter chains show more evidence for an adsorption type mechanism, with intermediate behavior seen for C_8 derivatized surfaces. Studies have also demonstrated that the stationary phase exhibits liquid-like behavior, with local viscosities similar to relatively viscous liquids.

Comparison of results obtained from alkyl derivatized flat silica surfaces to derivatized porous particles has demonstrated that there are some important differences in structure and chemistry in these two systems, and caution must be used in extending conclusions drawn from flat supports to porous chromatographic support materials.

While the above studies have demonstrated that dynamic information can be gained from time-resolved fluorescence about the transport of molecules both on and within the stationary phase and near its interface with the mobile phase, there are limitations to these experiments based on their nanosecond time window. UV–visible absorption experiments have an even more limited time-window that can probe only electronic relaxation dynamics. Many questions remain concerning kinetics of adsorption and desorption and the time evolution of stationary phase environments that are slower than these electronic spectroscopies can probe. Fluorescence detection can be coupled with relaxation kinetics measurements using pressure jump [52,53] or temperature jump [54] perturbations on a microsecond time scale. Longer time scales might also be addressed by investigations using excited triplet state probes. Future work along these lines should provide important information on band broadening mechanisms in liquid chromatography.

Another area with significant potential for future studies is the use of probes that are selectively sensitive to hydrogen bonding effects in the stationary phase. Most of the probe molecules used to date are primarily sensitive to the dipolarity and polarizability of their surroundings. Probe molecules that are more sensitive to hydrogen bonding effects should provide, with careful analysis, important information about the hydrogen bonding characteristics of stationary phases, that will supplement retention studies that have been used previously to characterize hydrogen bonding effects.

The use of electronic spectroscopies of probe molecules for characterizing reversed-phase stationary phases used in liquid chromatography has provided important information on the role of the stationary phase in reversed-phase retention, and should continue to be a useful tool in furthering our understanding of this complex interphase region.

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REFERENCES

1 R.M. McCormick and B.L. Karger, *Anal. Chem.*, 52 (1980) 2249–2257.
2 R.M. McCormick and B.L. Karger, *J. Chromatogr.*, 199 (1980) 259–273.
3 J.R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Plenum Press, New York, 1983.
4 T.L. Cecil and S.C. Rutan, *Anal. Chem.*, 62 (1990) 1998–2004.
5 C. Reichardt, *Solvent Effects in Organic Chemistry*, VCH, Weinheim, 2nd ed., 1988.
6 C.H. Lochmuller, D.B. Marshall and J.M. Harris, *Anal. Chim. Acta*, 131 (1981) 263–269.

7 M.J. Kamlet, J.-L.M. Abboud and R.W. Taft, *Prog. Phys. Org. Chem.*, 13 (1980) 485–594.
8 M.J. Kamlet, J.- L.M. Abboud and R.W. Taft, *J. Am. Chem. Soc.*, 99 (1977) 6027–6038.
9 M.J. Kamlet, J.-L.M. Abboud, M.H. Abraham and R.W. Taft, *J. Org. Chem.*, 48 (1983) 2877–2887.
10 D.C. Dong and M.A. Winnik, *Photochem. Photobiol.*, 35 (1984) 17.
11 D.C. Dong and M.A. Winnik, *Can.J. Chem.*, 62 (1984) 2560–2565.
12 F.W. Langkilde, E.W. Thulstup and J. Michl, *J. Chem. Phys.*, 78 (1983) 3372.
13 J. Stahlberg and M. Almgren, *Anal. Chem.*, 57 (1985) 817–821.
14 W.E. Acree, Jr., S.A. Tucker and J.C. Fetzer, *Polycyclic Aromat. Comp.*, 2 (1991) 75–105.
15 R.G. Bogar, J.C. Thomas and J.B. Callis, *Anal. Chem.*, 56 (1984) 1080–1084.
16 C.H. Lochmuller, M.T. Kersey and M.L. Hunnicutt, *Anal. Chim. Acta*, 175 (1985) 267–274.
17 C.H. Lochmuller, A.S. Colborn, M.L. Hunnicutt and J.M. Harris, *Anal. Chem.*, 55 (1983) 1344–1346.
18 *Instruction Manual for Integrating Sphere Assembly*, Shimadzu Corporation, Kyoto, 1988.
19 C.H. Lochmuller, A.S. Colborn, M.L. Hunnicutt and J.M. Harris, *J. Am. Chem. Soc.*, 106 (1984) 4077–4082.
20 C.H. Lochmuller and M.L. Hunnicutt, *J. Phys. Chem.*, 90 (1986) 4318–4323.
21 C.H. Lochmuller, D.B. Marshall and D.R. Wilder, *Anal. Chim. Acta*, 130 (1981) 31–43.
22 C.H. Lochmuller and D.B. Marshall, *Anal. Chim. Acta*, 142 (1982) 63–72.
23 J.W. Carr and J.M. Harris, *Anal. Chem.*, 58 (1986) 626–631.
24 J.W. Carr and J.M. Harris, *Anal. Chem.*, 59 (1987) 2546–2550.
25 K.C. Hartner, J.W. Carr and J.M. Harris, *Appl. Spectrosc.*, 43 (1989) 81–87.
26 V.M. Rangnekar and P.B. Oldham, *Anal. Chem.*, 62 (1990) 1144–1147.
27 Y.-D. Men and D.B. Marshall, *Anal. Chem.*, 62 (1990) 2606–2612.
28 G.P. Handreck and T.D. Smith, *J. Chem. Soc., Faraday Trans. I*, 84 (1988) 1847–1852.
29 S.M. Lindley, G.C. Flowers and J.E. Leffler, *J. Org. Chem.*, 50 (1985) 607–610.
30 J.L. Jones and S.C. Rutan, *Anal. Chem.*, 63 (1991) 1318–1322.
31 Y. Hayashi, R.S. Helburn and S.C. Rutan, in C.L. Wilkins (Editor), *Proceedings of the 4th Symposium on Computer-Enhanced Analytical Spectroscopy*, Plenum, New York, 1992, in press.
32 J.J. Michels and J.G. Dorsey, *Langmuir*, 6 (1990) 414–419.
33 M.A. Kessler and O.S. Wolfbeis, *Chem. Phys. Lipids*, 50 (1989) 51–56.
34 R.S. Helburn, Y. Hayashi, D. Mitchem, H. Lu and S.C. Rutan, presented at the *204th ACS National Meeting, Washington, DC*, 1992, Abstract No. 4.
35 S.D. Dowling and W.R. Seitz, *Anal. Chem.*, 57 (1985) 602–605.
36 J.W. Carr and J.M. Harris, *J. Chromatogr.*, 481 (1989) 135–146.
37 R.K. Gilpin and J.A. Squires, *J. Chromatogr. Sci.* 19 (1981) 195.
38 J.W. Carr, *Ph.D. Dissertation*, University of Utah, Salt Lake City, UT, 1987.
39 A.L. Wong, M.L. Hunnicutt and J.M. Harris, *J. Phys. Chem.*, 95 (1991) 4489–4495.
40 A.L. Wong and J.M. Harris, *J. Phys. Chem.*, 95 (1991) 5895–5901.
41 J.M. Harris, A.L. Wong and M.L. Hunnicutt, in H.A. Mottola and J.R. Steinmetz (Editors), *Chemically Modified Surfaces*, Elsevier, Amsterdam, 1992, pp. 39–56.
42 A.L. Wong, M.L. Hunnicutt and J.M. Harris, *Anal. Chem.*, 63 (1991) 1076–1081.
43 D. Avnir, R. Busse, M. Ottolenghi, E. Wellner and K.A. Zachariasse, *J. Phys. Chem.*, 89 (1985) 3521–3526.
44 K. Hara, P. DeMayo, W.R. Ware, A.C. Weedon, G.S.K. Wong and K.C. Wu, *Chem. Phys. Lett.*, 69 (1980) 105–108.
45 R.K. Bauer, R. Borenstein, P. deMayo, K. Okada, M. Rafalska, W.R. Ware and K.C. Wu, *J. Am. Chem. Soc.*, 104 (1982) 4635–4644.
46 C.H. Lochmuller and T.J. Wenzel, *J. Phys. Chem.*, 94 (1990) 4230–4235.
47 J. Stahlberg, M. Almgren and J. Alsins, *Anal. Chem.*, 60 (1988) 2487–2493.
48 V.M. Rangnekar, J.T. Foley and P.B. Oldham, *Appl. Spectrosc.*, 46 (1992) 827–831.
49 M.E. Montgomery, Jr., M.A. Green and M.J. Wirth, *Anal. Chem.*, 64 (1992) 1170–1175.
50 M.E. Montgomery, Jr. and M.J. Wirth, *Anal. Chem.*, 64 (1992) 2566–2569.
51 J.D. Burbage and M.J. Wirth, *J. Phys. Chem.*, 96 (1992) 5943–5948.
52 D.B. Marshall, J.W. Burns and D.E. Connolly, *J. Am. Chem. Soc.*, 108 (1986) 1087.
53 D.B. Marshall, J.W. Burns and D.E. Connolly, *J. Chromatogr.*, 360 (1986) 13.
54 S.W. Waite, J.M. Harris, E.H. Ellison and D.B. Marshall, *Anal. Chem.*, 63 (1991) 2365–2370.