ARTICLE

Mixed Self-Assembled Monolayers in Chemical Separations

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Chemical separations of many biomolecules and pharmaceuticals are limited by their electrostatic interaction with the surfaces of the separation medium. Mixed self-assembled monolayers of octadecyl and methyl chains organize into a dense, two-dimensionally cross-linked network over the chromatographic silica surface to reduce acid dissociation of the surface silanols. Molecular models predict that two-dimensional cross-linking is sterically possible for pure methylsiloxane monolayers, silicon-29 nuclear magnetic resonance measurements show that cross-linking predominates for mixed monolayers of primarily methylsiloxane, and chromatographic measurements confirm that electrostatic interactions are reduced when the monolayer is primarily methylsiloxane. Chromatographic separation of genetic variants of a highly charged protein, cytochrome c, demonstrates the promise of self-assembled monolayers in separations of biomolecules.

The chemical analysis of complex mixtures typically requires spatial separation of the chemical components, which is often accomplished by means of chromatography. In this technique, the mixture is injected as a narrow zone into a mobile phase and then passed through a column packed with a stationary phase, which is typically silica gel with a monolayer coating. A detector senses each component as it elutes. Ideally, the surface of the silica gel interacts differently with each component as the mixture flows through the column, resulting in a different rate of elution to give a spatial separation. Silica has desirable properties as a material in separations: low compressibility, controllable pore structure and particle size, and surface silanol (SiOH) groups that allow covalent attachment of a variety of functional groups to comprise a monolayer. The most commonly used monolayer for organic and pharmaceutical analyses is the monomeric C_{18} monolayer (Fig. 1), consisting of dimethyloctadecylsiloxane chains covalently bonded to the silica surface. Steric hindrance limits the coverage of alkyl chains, leaving the silica substrate between the chains exposed to the mobile phase. About half of the original silanols remain unreacted, and these groups are acidic, creating negatively charged sites on the surface. Problems arising from these charged sites are widespread in chromatography.

The principal problem in separating and purifying biomolecules on silica supports is their unwanted electrostatic interaction with the negatively charged silica surface (*1*). Pharmaceuticals and proteins are frequently positively charged because of the pervasive amino functionality. Electrostatic interaction slows desorption kinetics (*2*), resulting in the widely observed tail on the eluting zone. For example, when the pH of the mobile phase is 2, protonation of the silanols on a monomeric C_{18} surface is maintained, keeping it nearly neutral, whereas when the pH is 8, deprotonation of the silanols is allowed, giving a strongly charged surface. The arrival of $Ru(bpy)_3^{2+}$ (bpy, 2,2′-bipyridyl) at the detector is delayed at pH 8 because of its stronger electrostatic interaction with the surface at that pH (Fig. 2); the asymmetry of the zone is obvious. Highly positively charged proteins can have asymmetric zones even at pH 2, where the silica surface is only slightly charged.

Steps to reduce electrostatic interactions have been devised from an understanding of the chemical control of surface charge. A charged surface has a nonzero electrostatic potential ϕ_0, which gives rise to an electrostatic interaction energy

$$E_{es} = ze\phi_0 \quad (1)$$

between the silica surface and a cation of charge z (e is the fundamental unit of charge). The proportionality between E_{es} and z underscores why proteins are difficult to elute without tailing. Gouy-Chapman theory describes the relation between the electrostatic potential at the surface and the charge density σ at the surface (*3*)

$$\sigma = \sqrt{8kT\varepsilon\varepsilon_0 I}\,\sinh\frac{ze\phi_0}{2kT} \quad (2)$$

This relation accounts for a mobile phase of dielectric constant ε and ionic strength I (ε_0 is the permittivity of free space and kT is the thermal energy). Because $\sinh x \approx x$ under typical chromatographic conditions, the electrostatic interaction increases nearly linearly with surface charge density.

The tactics used to reduce zone tailing can be understood from these equations. First, one can minimize the surface charge density by using silica gel that has minimal acidity. The presence of metal impurities within the silica increases surface acidity considerably (*4*); therefore, pure silica is the most desirable material. The acidity of the surface of pure silica has a complicated relation to the concentration of silanol groups, exhibiting a maximum in acidity at a silanol concentration of 3 μmol/m^2 (*5*). Increased hydrogen bonding occurs at high-

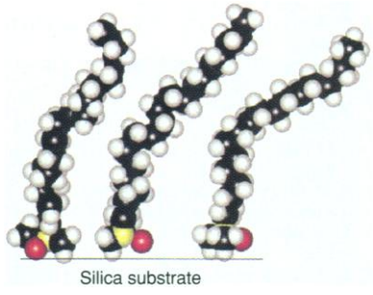

Fig. 1. Schematic illustration of C_{18} alkyl chains (dimethyloctadecylsiloxane) covalently bonded to a silica surface. Random aggregation of alkyl chains leaves sites on the silica surface exposed to the mobile phase, allowing unreacted surface silanol groups(SiOH) to deprotonate. Color assignments: black = carbon, white = hydrogen, red = oxygen, and yellow = silicon.

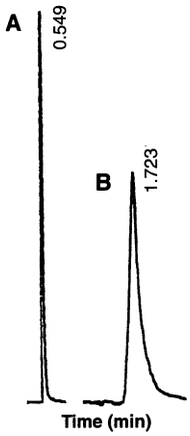

Fig. 2. Chromatograms of an organic cation, $Ru(bpy)_3^{2+}$, at (**A**) pH 2 and (**B**) pH 8 using a dimethyloctadecylsiloxane monolayer (Fig. 1). The chromatograms plot detector response as a function of time. In (A), protonation of the silanols keeps the surface nearly neutral, whereas for (B), the higher pH allows deprotonation of the silanols, giving a strongly charged surface, which slows the mobile phase. The arrival times of the zones at the detector are (A) 0.549 and (B) 1.723 min.

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er silanol concentrations, lowering the acidity of the surface, as shown by Fourier transform infrared spectroscopy (6). The least acidic silica is thus pure and fully hydroxylated, corresponding to a silanol concentration of 8 μmol/m^2 (7). A second tactic is simply to increase the ionic strength of the mobile phase, and a third is to operate at a sufficiently low pH that the silanols remain protonated. However, acid-catalyzed hydrolysis of the bond between the surface and monolayer eventually dissolves the monolayer. Fourth, in extreme cases, a sufficient concentration of adsorptive cation, such as an organic amine, is included in the mobile phase; its adsorption to the surface reduces the magnitude of the surface charge. Taken together, chromatographic separations in the pharmaceutical and biotechnical industry (i) typically use fully hydroxylated, highly pure silica, (ii) often use buffers at high ionic strength and low pH, and (iii) sometimes use amine additives.

The advent of self-assembled monolayers (SAMs) (8, 9) has inspired the idea that chromatographic surfaces might be designed from first principles to tailor the separation and eliminate unwanted electrostatic interactions. The organosiloxane monolayer has a powerful influence on surface acidity. A monomeric C_{18} monolayer (Fig. 1) reduces the charge density on the silica surface by an amount inordinately greater than the number of silanol groups consumed by the bonding: the number of SiOH groups is cut in half, while the number of SiO^- groups is reduced by approximately 80% (10). Experiments measuring the exchange of hydrogen and deuterium ions between the silica and the mobile phase show that at any one time, only 15% of the sites on the silica surface can exchange protons with the water, but over a longer time, all sites exchange (11). Furthermore, the acid dissociation constant does not change upon attachment of the alkyl groups to the silica surface; instead, the concentration of acidic sites is reduced (12). The alkyl monolayer thus reduces the acidity by reducing the area of exposed silica. Alkyl monolayers as thin as one methyl group are sufficient to block acid dissociation of silica. One methyl group must be sufficient to block acid dissociation because further reaction of the C_{18} surface with chlorotrimethylsilane reduces acidity. This post-reaction is called endcapping, and the smaller reagent groups are able to bond where steric hindrance prevents further C_{18} attachment. Endcapping decreases tailing despite the fact that it lowers silanol concentration because it reduces the area of exposed silica. Endcapping does not reduce surface charge sufficiently for demanding applications, and its benefits are only temporary because of hydrolytic degradation of the trimethylsiloxane bonds to the surface.

The ideal monolayer would achieve close-packed alkyl density, eliminating contact between the silica and mobile phase, but would also not bond to surface silanols, minimizing the acidity of silica near inevitable defects in the monolayer. This seemingly contradictory demand of high coverage without attachment requires a new concept in the organization of monolayers.

Mixed SAMs: The Role of Short Chains

Self-assembly of trifunctional organosilanes (13) can potentially combine the high density of alkyl chains with infrequent bonding to the silica surface because the reagent groups bond to one another, forming a polymeric network. Monolayers made from octadecyltrichlorosiloxane (C_{18}) have been formed on the thin SiO_2 layer on silicon wafers. Adsorbed water hydrolyzes the reagents, which then form a polymeric network. Infrared spectra reveal the alkyl chains to be nearly all trans in conformation, achieving a surface alkyl group concentration of 8 μmol/m^2 (9, 14). The similarity between the concentration of alkyl chains and the initial concentration of silanols is a coincidence. Octadecyltrichlorosilanes have been used extensively for chromatographic monolayers, comprising the "polymeric" stationary phases (15). These phases had not been intended to organize with the close-packed densities of SAMs; instead, they have liquid-like densities on the order of 5 μmol/m^2, exposing the silica substrate. This lower density of alkyl chains is required for chromatography, but the high density of functional groups is needed to reduce the surface charge density. Both requirements are satisfied by the mixing of long and short alkyl chains (Fig. 3). The first chromatographic experiments entailed mixed C_{18} and C_3 monolayers, which achieved the maximum density of organosiloxane groups, 8 μmol/m^2, in a molar C_3:C_{18} ratio of 1.6:1, as determined by microanalysis and ^{13}C nuclear magnetic resonance (NMR) (16). Phase separation of the long chains was discouraged with the use of an alkane solvent such as heptane. Infrared spectra indicated random chain conformations, and ^{13}C NMR relaxation measurements indicated fast motions of the chain segments, both arguing against phase separation of the C_{18} chains. Quantitative ^{29}Si NMR revealed that only 30% of the reagent groups were attached to the silica substrate (17). The mixed monolayers demonstrated high stability toward pH extremes (16).

Despite the high alkylsiloxane density of the mixed C_{18}-C_3 monolayers, they exhibited even higher electrostatic interactions with organic cations, in comparison with the conventional monomeric C_{18} stationary phase (17). This increase indicates the presence of significant defects in the SAMs. Space-filling models (Fig. 4) lend structural

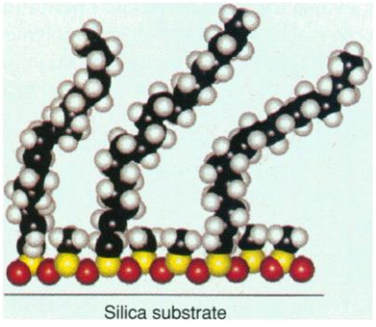

Fig. 3. Illustration of mixed long and short SAMs using space-filling models. The density is high just over the substrate where a barrier over the silica surface is needed and is low a few angstroms away, where low density is needed for chromatographic adsorption. Color assignments are as in Fig. 1.

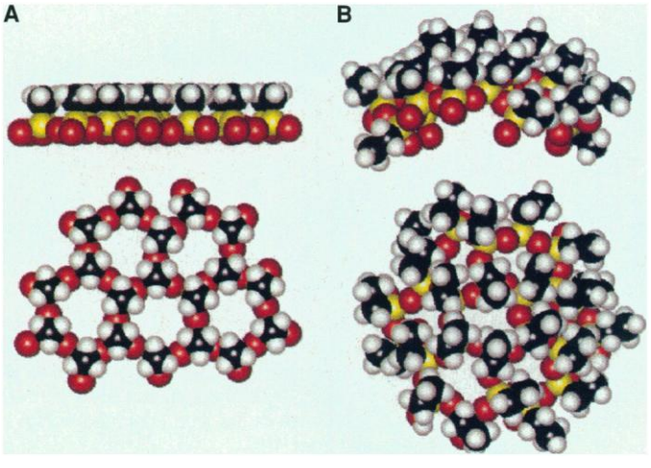

Fig. 4. Space-filling models (side and top views) of (**A**) two-carbon (ethyl) groups and (**B**) one-carbon (methyl) groups illustrate that any chain having more than one carbon is sterically hindered from cross-linking through siloxane linkages in two dimensions. Color assignments are as in Fig. 1.

insight, showing that alkyl groups can sterically hinder two-dimensional cross-linking. Methyl groups are capable of densely packing in two dimensions (Fig. 4A), being sterically compatible with flat, hexagonal structures where each reagent siloxane group is attached to three other reagent groups. Such a structure would maximize coverage of methyl groups to 10 μmol/m^2. The same bonding cannot, however, be achieved in a flat monolayer using ethyl groups (Fig. 4B) because one of the hydrogens on the second carbon sterically prevents cross-linking of one of the functional groups of the Si atom. Any alkyl group longer than a methyl group is incapable of fully cross-linking while remaining planar. The space-filling models show that one should not reasonably expect the mixed C_{18}-C_3 monolayer to form the dense structure needed for preventing dissociation of silanols. A long chain can be accommodated if all three of its neighbors are methyl groups, predicting that success is feasible with mixed C_{18}-C_1 monolayers if the C_{18} concentration is limited to 3.3 μmol/m^2 or less (Fig. 3).

The cross-linking of C_{18}-C_3 and C_{18}-C_1 monolayers predicted by the models can be tested experimentally by ^{29}Si NMR spectroscopy because the chemical shift of the Si atom changes measurably depending on whether the reagent Si atom is linked to another reagent group or terminated in an –OH group (*18*). The ^{29}Si NMR spectra of the two monolayers (Fig. 5), each having C_{18} concentrations of 3.0 μmol/m^2, are in agreement with the predictions of the models (*19*). For a C_{18}-C_1 monolayer, the signal is primarily in the −66 parts per million (ppm) band (Fig. 5A), which corresponds to fully cross-linked reagents. Only about 10% of the intensity in this largest band is attributable to covalent bonds to the silica surface. The spectral signal is distributed over three bands in the case of C_{18}-C_3 (Fig. 5B), with the largest band corresponding to reagent groups terminating in an –OH group rather than being cross-linked. Microanalysis reveals higher density for the C_{18}-C_1 monolayer, totaling 10 μmol/m^2, also in agreement with the space-filling models. Further, the chromatograms for aniline (Fig. 5), an organic cation at neutral pH, differ markedly for the two monolayers. The C_{18}-C_1 monolayer effects a sharper peak and a shorter elution time than does the C_{18}-C_3 monolayer, confirming the expectation that C_{18}-C_1 reduces the surface charge more effectively.

A crucial factor for preparation of these monolayers is the amount of water adsorbed to the silica gel before self-assembly. Self-assembled organosiloxanes on silicon wafers require sufficient water for reaction (*20, 21*), and different silica gels have significantly different adsorptivities toward water (*22*), presumably because the differing concentration of surface silanol groups (*23*). For fully hydroxylated silica, equilibration with 50% humidity provided optimal performance, and the weight gain revealed that 25 μmol of water was adsorbed per square meter. This approaches the stoichiometric amount of water needed for initial hydrolysis of the reagent trichlorosilanes. For an inexpensive silica gel presumed to have lower silanol concentration, a humidity of 90% was required to obtain comparable coverage and cross-linking (*22*). In both cases, too little water leaves unreacted Si-Cl groups, evident in ^{13}C NMR spectra as $SiOCH_3$ groups that form after a rinsing with methanol. Too much adsorbed water causes excess polymerization, evident in the form of microscopic polyorganosiloxane balls, which prevent uniform packing of the chromatographic column.

The question arises as to whether the methyl groups are truly self-assembled. Self-assembly occurs when attractive interactions among the alkyl groups contribute significantly to their organization; consequently, there exists a critical temperature T_c that cannot be exceeded during the preparation of SAMs (*24, 25*). The value of T_c varied from 0°C for C_{10} monolayers to 40°C for C_{22} monolayers on silica (*25*). Methyl monolayers would thus necessitate very low temperature for self-assembly. However, pure methylsiloxane monolayers prepared at room temperature on flat silica surfaces show evidence that the methyl groups are directed outward: water beads up to a contact angle of 80° (*19, 20*). This is the same contact angle observed for methylthiol on gold (*26*), where the orientation is intrinsic. The methylsiloxane monolayer is also resistant to attack by bases (*22*), consistent with the steric protection of the siloxane bonds through orientation of the methyl groups. The orientation of the methyl groups could owe to the reaction occurring at a chemical interface. Trichloroorganosilane initially hydrolyzes to form $RSi(OH)_3$, which is amphiphilic for R=methyl, and the subsequent polymerization is known to be the slow step (*27*). The amphiphiles are likely to be well oriented at the interface as they polymerize. The experiments investigating T_c for self-assembly made use of a solvent mixture of alkane and CCl_4, where the latter was added deliberately to improve solvation of hydrolyzed reagent (*24, 25*), perhaps explaining the more disordered monolayers prepared above T_c. In summary, high density and strong orientation, normally hallmarks of self-assembly, are apparently achievable through interfacial orientation of the hydrolyzed reagents during polymerization. The term "self-assembly" might only apply to methylsiloxane monolayers for historical reasons.

A comparison of the surface charges of the conventional monomeric C_{18} and C_{18}-C_1 SAMs have be made from chromatograms of $Ru(bpy)_3^{2+}$, where the surface charge density was be calculated through application of Gouy-Chapman theory (*28*). The result is that the mixed C_{18}-C_1 monolayer has a surface charge density at high pH one-third of that found on the conventional monolayer (*29*). Furthermore, a C_{18}-C_1 SAM on a highly acidic silica gel containing metal impurities reduces the acidity to a level below that of the conventional C_{18} monolayer on pure, fully hydroxylated

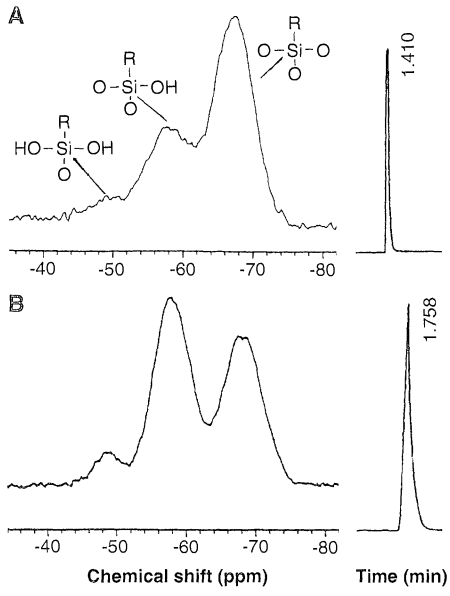

Fig. 5. Silicon-29 NMR spectra of (**A**) C_{18}-C_1 and (**B**) C_{18}-C_3 monolayers on silica. The spectral peak at −66 ppm is expected for a fully cross-linked reagent siloxane group. To the right of each spectrum is the corresponding chromatogram of aniline at neutral pH. The elution times are (A) 1.410 and (B) 1.758 min.

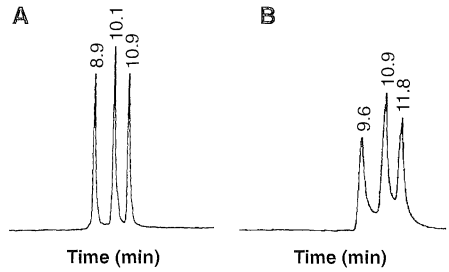

Fig. 6. Chromatogram at pH 2 of the three cytochrome c genetic variants, which emerge in the order of bovine, equine, and canine, shown for (**A**) the mixed C_{18}-C_1 monolayer and (**B**) a conventional monolayer of the type illustrated in Fig. 2. Reprinted from (*19*).

silica gel (29). These results indicate that the densely organized SAMs will play an important role in chemical separations of charged species such as biomolecules. The ability of the surface silanols to dissociate at all suggests the presence of defects, and further research is needed to understand defect formation.

Applications

In chromatographic applications of SAMs, one of the most difficult samples to separate is a mixture of closely related proteins. Cytochrome c is a protein having a molecular weight of 12 kD and a net charge of +20 at pH 2 (30). The genetic variants bovine, canine, and equine differ from one another by only 1% in molecular weight. We obtained chromatograms for these three genetic variants of cytochrome c at pH 2 for the mixed C_{18}-C_1 monolayer (Fig. 6A) and for a conventional monolayer of the type illustrated in Fig. 2 (*19*) (Fig. 6B). Separation conditions—such as flow rate, mobile phase composition, pH, and temperature—were the same, and the silica was the same. Baseline separation was obtained only for the mixed C_{18}-C_1 monolayer, and tailing was evident for the conventional monolayer. The improved resolution in this separation with the mixed C_{18}-C_1 polyorganosiloxane monolayer can ultimately translate to greater purity and greater recovery of biomolecules and expensive pharmaceuticals.

A requirement for general applicability of SAMs in protein separations is that the stationary phase must be tailored to the application. Some proteins denature or irreversibly adsorb upon contact with hydrophobic surfaces, requiring hydrophilic, "biocompatible" surfaces for adsorption. The discussion of mixed SAMs has thus far been limited to hydrophobic monolayers because orientation of the functional groups relies on amphiphilic character. It is straightforward to add chemical versatility to these monolayers by using a reactive functional group in place of the C_{18} group. Replacement of C_{18} with an allyl group [$H_2C{=}CHCH_2Si(Cl)_3$] allows subsequent reaction of the double bond to form any of a variety of functional groups, imparting polar, ionic, or chiral character. Biocompatible polymer films can also be attached to or deposited on the monolayer, again improving separations of proteins that strongly adsorb to negatively charged surfaces. The latter idea was demonstrated experimentally: a polyacrylamide film was grafted to a SAM in a silica capillary (*31*). A mixture of three proteins—cytochrome c, lysozyme, and ribonuclease A—was separated in this capillary by electrophoresis, a technique where an electric field draws the charged proteins along the axis of the capillary. A separation of high efficiency was demonstrated at pH 4.7, where the product of protein charge and surface charge were maximized, thus maximizing the electrostatic interaction. Combining research in SAMs with recent advances in the synthesis of well-defined polymers (*32*) promises to create biocompatible surfaces that have a substantial impact on the separation of highly charged biomolecules.

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