Kosmotropes Form the Basis of Protein-Resistant Surfaces

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This paper presents a hypothesis relating the exclusion of a molecule (solute) from the surface of a protein in aqueous solution with the ability of that molecule to render surfaces "protein-resistant", that is, resistant to the adsorption of proteins from aqueous buffer. While few current data test this hypothesis, it does suggest that surfaces presenting groups derived from certain osmolytes—molecules synthesized by cells to relieve osmotic stress—will be protein-resistant. These predictions were tested by constructing protein-resistant, self-assembled monolayers (SAMs) based on the osmolytes betaine and taurine. Examination of data from the literature also revealed that most of the known protein-resistant surfaces are based on displays of kosmotropes—molecules that stabilize the native structure of proteins. The connection between protein resistance, kosmotropicity, and biological function as an osmolyte may illuminate all three properties.

Surfaces that Resist Protein Adsorption. Surfaces that resist the nonspecific adsorption of proteins[2,3] have (inter alia) applications in prostheses, sensors, substrates for enzyme-linked immunosorbent assays (ELISAs),[4] materials for use in contact lenses and implanted devices,[4] devices for drug delivery,[5] and materials for patterned cell culture.[6] While a number of different protein-resistant surfaces have been identified,[2,3,7−12] an understanding of the mechanism of their action at the molecular level is still incomplete.[2,3,13−22] The observation that surfaces presenting the neutral polyether poly(ethylene glycol) (PEG) resist the nonspecific adsorption of proteins has led to the extensive use of derivatives of PEG for biomedical applications.[23] PEG does have the drawback that it is susceptible to autoxidation in the presence of O_2 and transition metal ions.[24−26] Furthermore, in vivo, the terminal hydroxyl group of PEG can be oxidized by alcohol dehydrogenase to an aldehyde group; the aldehyde group may react with proteins or be further oxidized by alcohol dehydrogenase.[27,28] These factors have led to an interest in identifying additional protein-resistant surfaces.[2,3,8,11,29−31]

SAMs that Resist the Adsorption of Proteins. SAMs of alkanethiolates on gold have been useful in correlating the molecular-scale structure of surfaces with their ability to resist the adsorption of proteins. SAMs presenting oligo(ethylene glycol) (-$(EG)_n$OH and -$(EG)_n$$OCH_3$, $n = 3-6$) groups resist the adsorption of proteins well and are the standard against which new protein-resistant surfaces are judged.[3,9] These SAMs are, however, not unique in their ability to resist the adsorption of proteins; SAMs presenting other functional groups (a−l, Table 1)[2,3,7−9,11] are also protein-resistant.[32]

Other Protein-Resistant Surfaces. Chapman et al.[30] prepared polymeric films by the reaction of polyamines

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Table 1. Adsorption of Fibrinogen and Lysozyme onto Single-Component SAMs

label	alkanethiol	% monolayer[a] fibrinogen	% monolayer[a] lysozyme	ref	structurally similar kosmotrope: name	structurally similar kosmotrope: molecular formula
	Protein-Resistant					
a	$HS(CH_2)_{11}(EG)_6OH$	1	1	10	PEG	$HO(CH_2CH_2O)_nH$
b	$HS(CH_2)_{11}O(Man^b)$	1	2	11	mannitol	$HOCH_2(CH(OH))_4CH_2OH$
c	$HS(CH_2)_{10}C(O)N(CH_3)CH_2(CH(OCH_3))_4CH_2OCH_3$	1	*c*	2		
d	$HS(CH_2)_{11}N(CH_3)_3{}^+Cl^-/HS(CH_2)_{11}SO_3{}^-Na^+$ (1:1)	1	1	8	taurine	$H_3N^+(CH_2)_2SO_3^-$
e	$HS(CH_2)_{11}N(CH_3)_2{}^+CH_2CH_2SO_3^-$	1	1	this work	taurine	$H_3N^+(CH_2)_2SO_3^-$
f	$HS(CH_2)_{15}C(O)Pip(NAc)$	2	*c*	2	DMA[f]	$CH_3C(O)N(CH_3)_2$
g	$HS(CH_2)_{11}N(CH_3)_2{}^+CH_2CO_2^-$	3	7	this work	betaine	$(CH_3)_3N^+CH_2CO_2^-$
h	$HS(CH_2)_{11}O(Malt^d)$	3	*c*	9	maltose	Glc-α(1,4)-Glc
i	$HS(CH_2)_{15}C(O)(N(CH_3)CH_2C(O))_3N(CH_3)_2$	4	*c*	2	DMA[f]	$CH_3C(O)N(CH_3)_2$
j	$HS(CH_2)_{11}N(CH_3)_2{}^+CH_2CH_2CH_2SO_3^-$	4	1	8	taurine	$H_3N^+(CH_2)_2SO_3^-$
k	$HS(CH_2)_{10}C(O)N(CH_3)CH_2CH_2N(CH_3)P(O)(N(CH_3)_2)_2$	4, 39[e]	*c*	2	HMPA	$O{=}P(N(CH_3)_2)_3$
l	$HS(CH_2)_{11}(S(O)CH_2CH_2CH_2)_3S(O)CH_3$	5	*c*	7	DMSO	$O{=}S(CH_3)_2$
	Nonresistant					
m	$HS(CH_2)_{10}C(O)NH_2$	30	5	61		
n	$HS(CH_2)_{11}(EG)_1OH$	35	10	10		
o	$HS(CH_2)_{11}OH$	43	1	61		
p	$HS(CH_2)_{11}OCH_3$	75	10	61		
q	$HS(CH_2)_{11}CF_3$	100	100	61		
r	$HS(CH_2)_{10}CH_3$	100	100	61		

[a] The uncertainties in these values are ±10% (relative error)[1] for values of % ML ≥ 5. For values of % ML < 5, ±0.5% ML (absolute error) represents a conservative estimate of the uncertainty. [b] Man = mannitol. [c] Not determined. [d] Malt = [Glc-α(1,4)-Glc-β(1)-]. [e] % ML = 4 for the adsorption of fibrinogen on mixed SAMs presenting hexamethylphosphoramide groups, and % ML = 39 for adsorption of fibrinogen on single-component SAMs presenting hexamethylphosphoramide groups.[1,2] [f] DMA = dimethylacetamide

(such as poly(ethyleneimine)) with SAMs presenting interchain carboxylic anhydride groups. The subsequent functionalization of the free amino groups with acetyl chloride, or with acyl chlorides that were derivatives of oligo(ethylene glycol), resulted in films that resisted the adsorption of proteins. Some surfaces derivatized with carbohydrates also resist the adsorption of proteins.[9,12,33] Osterberg et al.[12] reported that derivatives of cellulose grafted to polystyrene were nearly as effective as PEG in their ability to prevent the adsorption of proteins. The covalent functionalization of poly(ethyleneimine) (PEI) that had been allowed to adsorb noncovalently to polystyrene with carbohydrates resulted in a decrease in the extent of protein adsorption to the polymer surface.[33] Several groups have also reported that surfaces presenting phosphorylcholine derivatives resist the adsorption of proteins.[33−39]

Mechanism of Protein Resistance. Andrade and de Gennes developed a model to rationalize the protein resistance of surfaces grafted with PEG on the basis of ideas derived from the colloid stabilization literature.[22] The conformational flexibility of the grafted PEG is an important component of their model. Their model is applicable only to surfaces grafted with *long* polymer chains and does not explain the protein resistance of SAMs presenting short oligo(ethylene glycol) chains (-$(EG)_n$OH or -$(EG)_n$$OCH_3$, n = 3−6). Szleifer et al.[18−20,40] claimed that, by using single-chain mean field theory for the polymer chains, it was also possible to rationalize the protein resistance of surfaces (e.g. SAMs) presenting a high density of short $(EG)_n$OH chains (n < 7). Grunze et al.[13−17] proposed that the interaction of water with the surface of SAMs presenting oligo(ethylene glycol) groups is a more important determinant of protein resistance than the steric stabilization provided by the terminal oligo(ethylene glycol) chains. These theories[16,18,19,21,22] are attempts to explain the protein resistance of surfaces displaying PEG or oligo(ethylene glycol) groups; it would be desirable to explain or rationalize the protein resistance of others of the known protein-resistant surfaces and to provide leads to new protein-resistant surfaces.

Hypothesis Relating the Preferential Exclusion of a Solute to Its Ability To Render Surfaces Resistant to the Adsorption of Proteins. To make predictions about the extent of protein adsorption on a surface displaying a given molecule, we consider an analogous ternary system—one composed of protein, water, and that molecule (Scheme 1). In such a ternary system, the molecule (solute) can be considered to partition itself between two domains:[41−44] a "local domain"[45] in the vicinity of the protein surface and a "bulk domain". If the concentration of the solute in the "local domain" is lower than the concentration of the solute in the "bulk domain", the solute is said to be preferentially excluded from the protein surface; under these conditions, the protein is considered to be preferentially hydrated; that is, the concentration of water in the "local domain" is greater than the concentration of water in the "bulk

(32) The amount of protein adsorbed (ΔRU) was determined by subtracting the surface plasmon resonance (SPR) response units (RU) prior to the injection of the protein solution from the response units 5 min after the injection of the protein had ceased. The value of ΔRU was converted to % ML (% of a monolayer) using the equation % ML = $(\Delta RU/\Delta RU_{CH_3}) \times 100$),[2,3] where ΔRU_{CH_3} refers to the value of ΔRU for the adsorption of protein on a SAM of hexadecanethiolate (presenting methyl groups).

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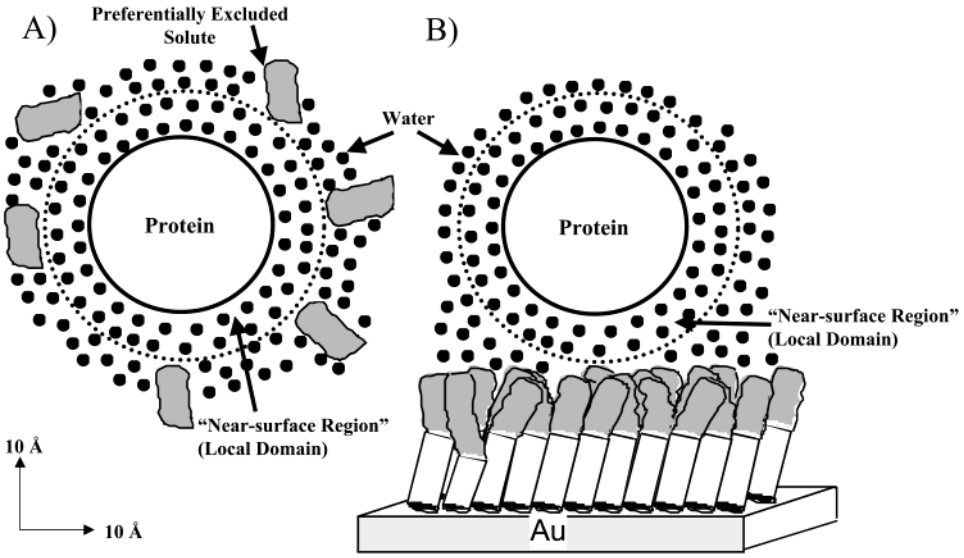
(45) The local domain can be considered to consist of one to three solvation shells around the protein.[43,44]

Scheme 1. (A) Schematic Illustration of a Solute That Is *Completely* Excluded from the Surface of the Protein (the Local Domain) and (B) Schematic Illustration of a Protein That Does Not Adsorb onto a Surface[a]



[a] In case A, there are no solute−protein contacts. In case B, the surface (to which the preferentially excluded solute is attached) is completely excluded from the surface of the protein (the local domain) and there are no surface−protein contacts. The components of the system are drawn approximately to scale.

domain".[41,42,44,46−53] We hypothesized that the molecular determinants of the partitioning of a solute between the vicinity of a protein and the "bulk" solution might be related to the determinants of the partitioning of a protein between "bulk" solution and the vicinity of a surface presenting that solute (Scheme 1). This hypothesis suggests that solutes that are well excluded from the protein surface[42,50] (i.e. solutes that effect a substantial preferential hydration[62] of the protein) should offer leads to good protein-resistant surfaces.

Protein-Resistant Surfaces Based on Osmolytes. Yancey et al.[1] have noted the "convergent evolution" of osmolytes in a wide variety of organisms. A small number of compounds (polyols, betaine, taurine, trimethylamine-*N*-oxide) occur as osmolytes in bacteria, plants, and fish. From an evolutionary perspective, it may be favorable for an osmolyte to be preferentially excluded from the surface of a protein, because the binding of osmolyte to protein would reduce the activity of the osmolyte in "bulk" solution; the increase in osmotic pressure due to the osmolyte would therefore be smaller than that in the absence of binding.[1,42] The binding of osmolytes to the surfaces of proteins might also compromise their biological function. We decided to test surfaces based on osmolytes for their tendency to resist the adsorption of proteins.

We synthesized alkanethiols g and e,[54] which are structurally similar to the osmolytes betaine and taurine (Table 1). We chose two proteins for our studies of adsorption (Table 1): fibrinogen, a large (340 kDa) protein present in blood, which adsorbs strongly to hydrophobic surfaces and is negatively charged (pI = 6.0)[55] under the conditions of our experiments (phosphate buffered saline, pH 7.4), and lysozyme, a small protein (14 kDa), which is positively charged (pI = 10.9)[55] under these conditions. SPR sensorgrams for the adsorption of fibrinogen on different SAMs are shown in Figure 1, and the experimental results are summarized in Table 1. Table 1 indicates that these surfaces are protein-resistant. Holmlin et al.[8] have tested the protein resistance of SAMs displaying groups that may also be considered to be structurally related to taurine (d and j, Table 1); they also demonstrated that SAMs presenting *N*, *N*-dimethylaminopropane-1-sulfonic acid groups, and mixed SAMs formed from a 1:1 combination of a thiol terminated in a trimethylammonium group and a thiol terminated in a sulfonate group, are protein-resistant (Table 1).

Many of the Known Protein-Resistant Surfaces Are Based on Displays of Kosmotropes. Yancey et al.[1] argue that the "convergently evolved" osmolytes—most

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(56) The effect of a preferentially excluded solute on the nature of a protein−solution interface[42,53] may be compared with the effect of a salt on the interface between air and an aqueous salt solution. Salts—preferentially excluded from the air−solution interface—increase the surface tension of that interface (compared to the surface tension in the absence of salt)[57] and favor a state with a lower interfacial area).
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(60) The protein resistance of SAMs presenting permethylated sorbitol groups does not contradict our hypothesis, since the kosmotropicity of permethylated sorbitol has not been experimentally tested.
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(62) Quantitative measures of the extent of exclusion of molecules from the surfaces of proteins may be provided by the preferential hydration parameter $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$[41,42,49,50] (henceforth referred to as $(\partial g_1/\partial g_2)$), where component 1 is water, component 2 is protein, component 3 is the solute, g_i is the mass of component i, and μ_i is the chemical potential of component i. The preferential hydration parameter provides a measure of the relative global affinities of solute and water for the protein surface.[42] Values of $(\partial g_1/\partial g_2)$ may be measured by equilibrium dialysis, densimetry, or osmometry.[41,42,44,46] Positive values of $(\partial g_1/\partial g_2)$ indicate that the solute is preferentially excluded from the protein surface, whereas negative values indicate that the solute is preferentially enriched in the vicinity of the protein surface.[5,42]

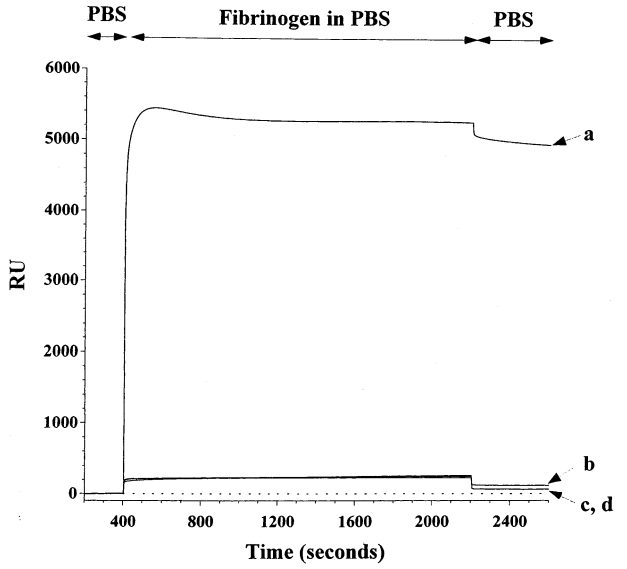

Figure 1. Surface plasmon resonance sensorgrams for the adsorption of fibrinogen (1 mg/mL) in PBS (pH 7.4) on (a) SAMs presenting methyl groups, (b) SAMs formed from alkanethiol g (Table 1) presenting groups that are structurally similar to betaine, (c) SAMs formed from alkanethiol e (Table 1) presenting groups that are structurally similar to taurine, and (d) SAMs presenting oligo(ethylene glycol) groups. The adsorption is reported in response units (RU).

of which are electrically neutral—must share a common ability to provide environments compatible with macromolecular structure and function. These osmolytes stabilize the native structure of proteins.[42] Timasheff et al.[42,47,48,50] have related the preferential exclusion of solutes to their effect on the stability of proteins in aqueous solutions. On the basis of extensive studies in the past few decades, Timasheff noted that kosmotropes (e.g. PEG and betaine, Table 1) are preferentially excluded from the protein surface at 20 °C.[42] He explained the stabilization of the native structure of proteins by preferentially excluded kosmotropes in terms of Le Chatelier's principle. Since the exclusion of solutes from protein surfaces is entropically unfavorable, a preferentially excluded solute drives the equilibrium between the native and denatured states toward the native state—a state of the protein that usually has a lower surface area exposed to water than the denatured state.[56]

We tested the hypothesis that kosmotropes should form the basis of protein-resistant surfaces by examining data from the literature (Table 1).[2,3,7,8,11,42,47,48,50,51,58,59] Table 1 demonstrates that many of the known protein-resistant SAMs are based on displays of kosmotropes, or molecules that are structurally similar to known kosmotropes.[60] The results for SAMs presenting hexamethylphosphoramide (HMPA) groups are intriguing; while mixed SAMs presenting the kosmotrope HMPA are protein-resistant, single-component SAMs presenting HMPA groups are not protein-resistant.[2,3] Ostuni et al.[2] speculated that the lack of protein resistance for single-component SAMs presenting HMPA groups may result from unfavorable steric interactions caused by the bulkiness of the alkanethiol, with possible accompanying exposure of methylene groups.

Discussion

On the basis of a survey of ~50 organic functional groups, Chapman et al.[2,3] noted that several different types of organic functional groups can form the basis of surfaces that resist protein adsorption. The observation that most of the known protein-resistant surfaces are based on displays of kosmotropes is compatible with the results of this survey. The molecular basis for kosmotropicity is also probably not the same for every solute; several organic solutes (e.g. zwitterionic osmolytes such as betaine, and neutral polymers such as PEG) are kosmotropes.[2,3,8,42,50] For instance, the conformational flexibility of PEG may contribute to its preferential exclusion and kosmotropicity,[42] and to the protein resistance of surfaces functionalized with PEG;[9,21,22] conformational flexibility is, however, *not* a prerequisite for kosmotropicity of a solute, and is also not *required* for protein resistance of a surface,[2,3] although it may be important in certain cases. Our proposed link between kosmotropicity and the protein resistance of surfaces suggests that understanding the various mechanisms responsible for kosmotropicity will shed light on the mechanisms responsible for the resistance of surfaces to the adsorption of proteins.

Conclusions

The hypothesis that molecules that are excluded from the protein−water interface (kosmotropes) themselves exclude proteins from surfaces to which they are attached is intuitively plausible. The data that are currently available with which to test this hypothesis are few, and there are substantial differences between molecules in solution and molecules attached (particularly at high density) to a surface. It is possible that the packing or orientation of the attached molecules may influence the protein resistance of the surface. Nonetheless, these data are sufficient to suggest a connection between these complex properties—protein resistance, kosmotropicity, and biological function as an osmolyte—that may illuminate all three.

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Supporting Information Available: Procedures used to synthesize alkanethiols e and g are described. This material is available free of charge via the Internet at http://pubs.acs.org.

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