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SILICONE-PROTEIN INTERACTIONS

By

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ABSTRACT

Novel organofunctional silicones have been proposed as materials for the formulation of protein delivery systems. The efficacy of such systems to deliver proteins orally, without loss of their activity has been demonstrated. Furthermore, normal (non-functional) silicones failed in exhibiting an analogous protein protective role. The functionality on these novel silicones was obviously crucial in formulating efficient protein carriers. Therefore, the interaction of these organofunctional silicones with proteins has been examined against normal silicones. The extent and strength of the silicone-protein interaction has been revealed from model surface studies at solid/liquid interfaces and also from emulsion stability studies at liquid/liquid interfaces. It has been suggested that the silicone functionality facilitates the interaction between the two species while it may also lead to self crosslinking of the silicone chains using the protein as a template.

Finally, evidence of the effect of liquid silicones on the protein biological integrity has been obtained to further prove, from a biological perspective, the suitability of liquid silicone-based delivery systems.

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Chapter 1

Introduction

General Theme

Interaction between silicones and proteins is observed in many systems. These interactions are of great relevance in various applications such as medical (biomaterials,[58-60] dental[1] and eye,[2] applications), pharmaceutical (protein delivery[3,4,50]), personal care (hair and skin formulations[20,21]), and biochemical (chromatographic separations[5] and protein crystallization supports[6,37]). In all these applications control over the silicone-protein interactions is essential. This is because the desirable magnitude of these interactions covers the whole spectrum; one desires strong, intimate interactions in cosmetic formulations but minimal interactions in biomaterials and antifouling agents.

In spite of the numerous applications involving silicones and proteins, the nature of their interaction is largely unknown both from a physicochemical and a biological perspective. Browsing the relevant literature one finds that research has explored several aspects of protein adsorption on silicone surfaces. Published work involves such topics as the amounts of protein adsorbed,[9,11,25,26,30,33,43,49] kinetics of adsorption/desorption/exchange[7-11] at a variety of conditions, protein orientation[12] and mechanisms of adsorption,[25,35] coadsorption,[13,32,34] and adsorption reversibility,[13,14] and surface-induced protein conformational[54-56] and biological changes.[3,50,51,58-60] Not all of the above issues have attracted equal attention. Most knowledge of silicone-protein interactions comes from published work on the adsorption (amounts adsorbed/kinetics) of plasma globular proteins (albumin, γ-globulin, and fibrinogen) on silicone rubbers (to a lesser extent on siliconized glasses or silicone copolymer surfaces, and negligible with silicone oils) and the change of the bioproperties of those proteins upon adsorption. Often, comparisons have been made with other synthetic surfaces.

The topic certainly needs more systematic analysis. Controversies are not rare among the various researchers who have worked in the area. This is sometimes due to the ill-defined systems used. For instance, the silicone materials used in the various studies are often of different origins and microstructures, which can affect the results.[12] It is also appreciated that the proteins more often employed in these studies have 3D-structures that are not yet well known.[54] In addition, the different conditions and techniques used for characterization may also account for the existing controversies. Furthermore, it becomes obvious that due to the diversity that characterizes the silicone-protein systems, generalizations should probably not be attempted to all silicone-protein systems from the limited number of proteins and surfaces studied so far.

For the purposes of this overview on the interactions of various silicones (elastomers, polymers, copolymers, fluids) with proteins, the focus will be on the issues of affinity between the two species and the changes of the protein bioproperties upon adsorption.

1. Interactions Governing Protein Adsorption at Silicone Surfaces

Most studies of proteins at silicone interfaces report on phenomenological aspects. Little attention has been paid to the principles that determine the protein adsorption behavior at these interfaces. However, knowledge of the underlying principles is required in order to control the interaction according to the requirements of the various applications. The interactions can fall into two categories: physical and chemical. More relevant to this review, and also more studied, are the physical interactions. Therefore, the focus of this overview will be mostly on those.

Physical Interactions

Protein adsorption on a surface is the net result of various types of interactions between all the components in the system: the protein molecules, the solvent and the sorbent surface. The origins of these interactions include Lifshitz-van der Waals forces, hydrogen bond forces, electrostatic forces, and more entropically based effects such as the hydrophobic effect and internal packing restrictions. In addition to these intermolecular interactions, intramolecular forces within the protein macromolecule are of importance in protein adsorption. When a protein interacts with a surface, structural rearrangements in the protein macromolecule[15] and dehydration of the protein occur, leading to denaturation and subsequently modification of the protein's bioproperties.

Unfortunately, comprehensive studies on silicone-protein interactions that reveal the role of these various factors involved are still lacking. However, attempts to control the interaction by modifying the system components are numerous. While all the components in the system (protein, surface, solvent) can be modified in order to obtain control over the protein-silicone surface interactions, the silicone surface modification is essentially the only approach followed to date.

In all relevant silicone-protein systems studied, if there is one common component involved, it is the siloxane part of the various silicones (polydimethylsiloxanes or PDMS). Consequently, it is worthwhile mentioning the basic interfacial properties of siloxanes. Four structural characteristics account for the silicone interfacial properties; a) the low intermolecular forces between methyl groups, b) the flexibility of the siloxane backbone, c) the high energy of the siloxane bond and, d) the partial ionic nature of siloxane bond. The former two characteristics explain the physical behavior (i.e., low surface tension, low viscosity), while the latter ones explain the chemical behavior (i.e., high thermal stability, susceptibility to nucleophilic or electrophilic attack) of silicones. The physical behavior of silicones has been mostly of concern in interfacial interactions. It is suggested that silicones could interact with substrates both through dispersion forces from the induced dipoles in the methyl groups and through permanent dipoles in the partially polar siloxane backbone. At the silicone-air interface, configurations that maximize the packing of methyl groups at the air side are adopted, but at other interfaces, the backbone dipole assumes a more important role. Thus, in general, PDMS will interact through its siloxane backbone with polar substrates, whereas with non-polar substrates interaction will be through the methyl groups. The considerable backbone flexibility of silicones allows them to adjust to the availability of reactive sites on surfaces.[16] This should hold for their interaction with amphiphilic protein molecules at interfaces.

From the above discussion it may be speculated that the hydrophobic effect and structural rearrangements of the protein molecules are the interactions that predominate during protein adsorption on silicones, due to the hydrophobic nature of silicones. However, the actual situation may be somewhat different as silicones also possess some polar character and they are remarkably flexible materials. In particular, the flexibility of the material has been found to influence protein adsorption.[42,30] Furthermore, modified silicones have additional interactions (electrostatic with aminoalkyl substituents attached to the silicone,[17] for example) depending on their specific chemical composition. In this case, maximum affinity for protein adsorption is to be observed when the charge of the protein molecule itself matches the charge of the sorbent surface.[24] Even then, though, the dominant driving force for adsorption should be hydrophobic dehydration, as it is believed that it overrules electrostatic effects.[24]

Finally, it should be noted that little is known of the silicone-protein interaction at fluid interfaces. Although at these interfaces, fundamentally, the same phenomena occur, the absence of specific binding sites for the protein (mobile interfaces) and the ability for greater protein penetration into the silicone phase may alter the overall magnitude of the phenomena.[18,19]

Chemical Interactions

Chemical reactions have been employed in order to link the two inherently incompatible species and maximize the protein-silicone interactions. Silicone-protein copolymers have been prepared in various laboratories dedicated to the formulation of personal care products. Such copolymers are very popular in cosmetics due to their advantageous properties such as silicone solubility in aqueous, aqueous/alcoholic or alcoholic solutions, film formation upon drying, enhanced substantivity to skin and hair, moisture retention, novel feel on skin, etc. For the synthesis of the copolymers, derivatization of the materials and coupling is the route often undertaken. Several reports exist on the preparation of protein[20-22] modified silicone derivatives (with phosphated silicones, or dimethicone chloroesters, for instance). Cross-linking of the protein-silicone copolymers has been employed to increase the interactions even more. In this case covalent bonding between the silicone and amino groups takes place as does the formation of cross-links between the different silicone modified protein chains. Organofunctional groups such as acyl halides, sulphonyl halides, anhydrides, aldehydes, epoxides, etc. have been used to bind silicones to proteins. Crosslinking of the protein chains was also taking place by another silicon component, a silane coupling agent, attached to the protein with the above organic functional groups. A wide range of proteins used in the preparation of such copolymers include collagen, elastin, keratin, casein, soya protein, silk protein and hydrolyzed wheat protein. At solid/liquid interfaces, bifunctional crosslinkers (e.g. gluteraldehyde) have been used for covalent immobilization of enzymes to functionalized silicone surfaces (e.g., polyaniline modified silicones).[23]

2. Amounts of Proteins on Silicone Surfaces

No matter the driving forces of the adsorption the overall phenomenon of protein adsorption on silicones is evident from the amounts adsorbed. The most common way of reporting quantity of adsorbed protein is in the form of adsorption isotherms, where the amount adsorbed is plotted against protein concentration in solution.[24] The affinity of the protein for the surface is reflected on the slope of the initial ascending part of the isotherm. Plateau values, usually corresponding to monolayers of the native molecules, are finally reached.

Several researchers have attempted either to exploit and/or evaluate the magnitude of the hydrophobic effect in order to obtain desirable amounts of proteins on the various silicone surfaces.[25,27,29,30] The findings of some of these studies follow; they are categorized by the type of silicone surfaces under study.

Silicone Rubbers

Adsorption from single protein solutions

The interaction of proteins with silicone rubbers against other materials has been mainly investigated under the hypothesis of optimal balance between hydrophobicity/hydrophilicity or between polarity and dispersion forces. Therefore, a degree of hydrophobicity or polar and dispersive components have been ascribed to the surfaces under study and they were ranked in terms of hydrophobicity or polar-dispersive component ratios. Then, protein amounts adsorbed to the surfaces were related to these terms. For example, silicone rubbers have been compared to polyether urethane, polyethylene, polyvinylchloride[25] or teflon and copoly (ether urethane ureas)[33], in terms of the amount of a specific protein adsorbed. Although, it is practically impossible to establish the influence of surface hydrophobicity (or the polar-dispersive component ratios) on protein adsorption, as it is accompanied with changes in the chemical composition of the materials, (often controversial) correlations have been reported.

In some studies the amounts of protein adsorbed have been found to decrease with decreasing polymer surface energy. In these studies (bioadhesion studies) it is argued that silicone elastomers being low energy materials, are the materials to be preferred for minimal protein adsorption.[26] Other studies have reached the exact opposite conclusion; it has been found that silicone elastomers, being the more hydrophobic surfaces, are ideal for maximal protein adsorption.[25,27,28,30,35] For example, adsorption amounts of several proteins (low density lipoprotein,[30] interleukin-2,[27] fibrinogen,[25,28] transferin,[25] bovine serum albumin,[35]) on silicone rubber appears to be increasing the more hydrophobic the surface. Moreover, surfaces of similar hydrophobicity (PDMS and polyethylene) have exhibited similar protein (fibrinogen) adsorption behavior.[19] There has been also reported that no relationship exists between the amounts of (serum) proteins adsorbed and the hydrophobicity of the surface.[29] Regarding the polar-dispersive ratios, it has been suggested that the more equal the balance of polar-dispersive forces on the polymer surface the more optimal is the interaction of the protein with the polymer surface. This was based on the finding that proteins adsorb less on hydrophobic silicone surfaces, with a polar-dispersive ratio further from unity and more to hydrophilic copoly (ether urethane ureas) (order of increasing adsorption according to polar-dispersive ratios: Teflon(0.54)<PDMS(0.76)<copoly(ether urethane ureas)(0.89-0.95)).[33] Furthermore, the microtexture of a surface possessing local differences in surface energy has been suggested to promote specific deposition patterns of proteins. Deposition studies of bovine and endogenous fibronectin and vitronectin on smooth microgrooved silicone rubber substrates concluded that the microstructured surface influences the orientation of the proteins, but a justification for any specific energetically-based hypothesis was not provided.[12] Finally, the amounts of protein adsorbed on silicone rubbers have also been related to the flexibility of the silicone. Silicone rubbers have been found to have increased amounts of adsorbed proteins (human low density lipoprotein) compared to other polymers of different hydrophobicity and elasticity.[30]

Another quite different way to probe the nature and strength of the silicone-protein interactions is surfactant induced protein elutability from the surfaces. Using chemically different surfactants (ionic, non-ionic) the amounts of protein eluted from silastic (crosslinked silicone), polystyrene, polyhydroxystyrene, teflon, polyethylene, and polyvinylchloride surfaces lead to the conclusion that ionic forces are more important than hydrophobic forces (as ionic surfactants were more effective in protein elusion) but no correlation to surface physical/chemical properties was made.[39]

Adsorption from Mixed Protein Solutions-Competitive Adsorption

In several applications, such as the development of biomaterials, adsorption of protein mixtures occurs. Therefore, studies on competitive adsorption are a significant part of the literature. Protein adsorption from blood or mixed plasma proteins has shown reduced adsorption of each protein species due to competition for adsorption sites by all proteins present. The relative amounts of each of the proteins has been found to differ from their bulk concentrations (proteins are not adsorbed in proportion to their concentrations in bulk solution) and to a different degree on the various polymeric surfaces, indicating that the physicochemical nature of the polymers influence the adsorption of protein mixtures.[31,32] Competitive adsorption of (plasma) protein mixtures (fibrinogen, albumin and γ-globulin) onto silicone rubber showed differences in amounts of proteins adsorbed; the order of increased amounts adsorbed was: fibrinogen>γ-globulin>albumin. The relevant amounts of the proteins deposited on the various polymers revealed that fibrinogen had generally the highest affinity for all surfaces (teflon, silicone rubber, copoly(ether urethane urea)) but it's affinity decreased in the order of teflon>silicone rubber>copolyether urethane urea.[32] Similar results were reported for the adsorption of blood to silicone rubber in comparison with copolyether urethane urea, teflon, biomer.

The surface energetics treatment has been applied also to account for the difference in adsorption of mixtures of plasma proteins to different polymers. Plasma protein adsorption measurements on teflon, silicone rubber, copolyurethanes showed that the degree of coverage on the various surfaces was increased when a more equal balance existed between polar and dispersive components and changes significantly with the nature of the protein.[33] Further studies indicate that time is important in these competitive adsorption experiments, as protein exchange may occur when mixtures of proteins adsorb on surfaces. An attempt to determine the time dependence of the adsorption of a protein mixture (bovine albumin, γ-globulin, and fibrinogen) on silicone rubber revealed that initially fibrinogen adsorption predominated while later it seemed to desorb to be replaced by albumin and γ-globulin. This suggests that rearrangement of adsorbed protein species occurs with time on polymer surfaces exposed to flowing blood *in vivo*.[34]

Siliconized surfaces

Some researchers have used silicone-modified surfaces either to model the silicone rubber surfaces or due to the use of such surfaces as chromatographic supports. Regarding these substrates, it should be noted that the polysiloxane coatings on silanol (glass) substrates leave an inner negatively charged (hydrophylic) surface which may influence the adsorption.[5]

A correlation between protein adsorption and hydrophobicity has also been suggested for modified glass surfaces. Bovine serum albumin affinity to PDMS-modified glass surface was greater compared to a less hydrophobic poly(methylmethacrylate)-

modified glass surface.[35] In a more detailed study, protein (bovine serum albumin, or bovine hemoglobin or lysozyme) adsorption profiles on silicone-coated porous glass indicated that adsorption was due to hydrophobic bonding between silicone residues and aliphatic protein residues rather than hydrogen or ionic bonding.[36] Protein adsorption was proposed not to be due to hydrogen binding because it was not affected by the presence of urea, which destroys hydrogen bonding. It was also not attributed to ionic bonding as in high salt concentration ionic strength had a positive effect on the amount of protein adsorbed. In a subsequent study, protein affinity for silicone-coated glasses was found to be protein dependent. Bovine hemoglobin and lysozyme showed a greater affinity than bovine serum albumin for the silicone-coated glass. Based on these findings siliconized glass was proposed for protein exclusion chromatography applications since at low salt concentrations protein adsorption was minimal.[37]

Organofunctional Silicones and Silicone Copolymers

Based on the hypothesis that hydrophilic surfaces reduce the deposition and denaturation[38] of proteins and, therefore, render the surface more compatible, silicone rubber has often been modified accordingly. Several polymers (for example, hydroxyethyl methacrylate polyvinylpyrrolidone, polyethylene glycol, polyacrylamide, methoxy polyethylene glycol-amine, hyaluronic acid, and copolypeptides)[39,40] or hydrogels,[41] grafted onto silicone rubber so that different degrees of hydrophilicity to be obtained, were compared with silicone rubber with respect to protein (blood[40] or fibrinogen/immunoglobulin[39]) adsorption. The results were in line with the expectation that the more hydrophilic the surface the less protein (from blood) was adsorbed. It was further suggested that, in general, mosaic surfaces (hydrophilized silicones (silicone copolymers)) induce minimal adsorption (of human serum albumin)[42] However, it has been proposed that there should be an equal distribution of the polar-dispersive components for adsorption to be maximal, as the non-dispersive-dispersive force balance was found to be a critical factor for binding of protein to polymer surfaces.[33]

Apart from modifying siloxane rubbers to control protein adsorption and induced denaturation new siloxanes have also been synthesized. Hydrophilic components to the silicone polymers were introduced and siloxane copolymers[43] and networks[44] have been prepared for minimal protein adsorption and denaturation. Polyethylene oxide chains have attracted great attention as the hydrophilic components due to their reputation of being protein friendly and minimizing protein adsorption.[45] Indeed, one of the most extensively used silicone copolymers, a silicone polyether, was found to keep protein adsorption to minimal levels when applied on the surface of a fabric.[43] Interestingly enough, though, the copolymer by itself had only a moderate effect on reducing protein adsorption. In another study a similar material, polypropylene glycol/polysiloxane network, has been examined for protein (fibrinogen) adsorption. It was similarly found that the amount of fibrinogen varied directly with the amount of glycol in the network; the more the glycol chains the less the protein adsorbed.[44] To enhance silicone protein affinity, cationic organofunctional silicones (usually various aminofunctionalized silicones) have been synthesized. This class of materials has a high degree of water solubility and superior affinity to proteinaceous surfaces, like hair proteins, due to favorable electrostatic attraction.[46] In a different study it has been claimed that the charge of analogous silicones played a role in protein adsoption on such surfaces.[17] Another, rather distinct way to obtain enhanced silicone-protein interactions involved the formation of a polydimethylsiloxane polymer using protein templates. For this to occur a mixture of different organofunctional silanols has been allowed to interact with a specific protein and associate with complementary amino acid residues so that silanol cross-linking to a silicone polymer occurred around the protein. After the template (protein) removal the prepared silicones were used in protein binding experiments. Each specific protein was found to preferentially bind to the silicone for which it was used as template. This specific protein-silicone affinity was taken as evidence of a complimentary organofunctional PDMS-protein interaction.[47] The same idea has found use in the coating of proteinaceous surfaces with silicone films which form with the deposition of hydrolyzed silane-containing emulsions onto the surfaces of interest.[48]

Mixed (plasma) protein adsorption (coadsorption) on silicone copolymers has also shown preferential protein adsorption and, in addition, it has been indicated that the protein adsorption varied with the sizes of both the hydrophilic and hydrophobic domains of the copolymers. For example, on triblock copolymers with poly(γ-benzyl *L*-glutamate) and PDMS segments (PDMS: 40-70 mol% middle segment) fibrinogen adsorption was found to be faster and attain equilibrium much earlier than the other proteins. Regarding adsorption at the specific polymer domains, it was found that γ-gobulin adsorption was independent of the domain sizes while BSA and fibrinogen adsorption changed by the kind of block copolymers but were not well correlated with the size of the PDMS domains.[49]

Finally, heterogeneous microgels containing silicone rubber domains in hydrogel matrixes[41] (e.g. cross-linked poly(*N*-isopropyl acrylamide)[50]) have been designed for peptide and protein delivery. Protein adsorption (loading) and desorption (release) from these microgels occurs by exploiting the swelling sensitivity of the microgel to pH and temperature.

Silicone oils

Little can be found in the open literature on the interaction of proteins with fluid silicones. As a coating, silicone oil has been shown to reduce salivary protein adsorption on surfaces.[1] Mixed with proteins, silicone oil can form emulsions. Many cosmetic formulations contain both silicones and proteins but no correlation of the function of these two components has been given. However, silicone oil in the eye tends to form emulsions and it is appreciated that proteins play a significant role in this process.[2] In addition, in a recent study, silicone oil droplets were coated with proteins (fibrinogen) to give stable "normal" silicone oil-in-water emulsions.[51] Efforts to determine the physicochemical characteristics that lead to emulsification reveal that lower molecular weight silicones emulsify with greater ease and that the ionic strength and the protein content of the aqueous solutions are critical factors in the formation of silicone oil in water emulsions.[52]

3. Structure and Properties of Adsorbed Proteins on Silicone Surfaces

a) Conformational Changes

Observed conformational changes in proteins upon adsorption are related to the ease of disruption of the protein's intramolecular network and are protein specific. Hard proteins (structurally stable) are likely to retain their native conformation while soft proteins (of low structural stability) are more subject to denaturation. The hydrophobicity of the surface is, however, also related to the degree of protein denaturation. Protein adsorption on hydrophobic surfaces is reported to be more denaturing[53] and, for many proteins, the property that is most relevant to the denaturation of proteins upon adsorption on silicones is their hydrophobicity. Once adsorbed on the hydrophobic surface proteins undergo denaturating structural rearrangements in order to develop more energetically favorable hydrophobic contacts with the surface.[24,27,55] Structural changes in the proteins could also be an entropic driving force because fully denatured proteins do not adsorb to any extent on surfaces.[55] In the case of multilayer protein adsorption, it has been found that subsequent layers are less denatured as they are less influenced by the surface-protein interactions.[25] Furthermore, conformational changes are also considered to be a function of protein-surface contact time. The shorter the protein-surface incubation time the faster the desorption and the less the induced structural changes.[55] It has been actually suggested that changes in the protein-surface interaction occur long after adsorption and it is due to increased denaturation with time that protein-surface binding becomes stronger.[9] Others have indicated, though, that subsequent unfolding may not be energetically favored which may lead to protein desorption.[55]

Probably the most comprehensive study on the structural changes of proteins on silicone has involved myoglobin and a PDMS surface as a model system. Myglobin was chosen because it is extensively characterized in solution. It was found that the native structure was disrupted following interaction with PDMS within 15 minutes of contact, which confirms the arguments that protein structure is altered upon adsorption on low energy surfaces.[54]

If hydrophobicity of the silicone surface is responsible for the protein denaturation, coated silicone surfaces and silicone copolymers could be more promising materials. Indeed, plasma proteins were not denatured upon adsorption on to block copolymers containing about 40-70 mol% PDMS. More precisely, the conformational state of plasma proteins adsorbed to triblock copolymers with poly(γ-benzyl *L*-glutamate) and PDMS segments (PDMS: 40-70 mol% middle segment) in various compositions (hydrophilicities) was found to be altered in different degrees for different proteins. Bovine serum albumin was not denatured upon adsorption on to any kinds of block copolymers. γ-globulin and fibrinogen were completely denatured upon adsorption on to poly(γ-benzyl *L*-glutamate) homopolymer and block copolymers containing more than 70 mol% PDMS.[49] In contrast, the adsorption/desorption of plasma proteins on copolypeptide coated silicone surfaces indicated protein surface denaturation, possibly driven by a need to increase hydrophobic bonding with the surface.[55] Albumin and fibrinogen sustained a marked decrease in α-helical content and γ-globulin lost most of its β-sheet structure.

Contradictory results have also been obtained regarding the protein conformational changes upon its interaction with silicone oils. For instance, there is evidence that silicone oil adsorbed antibodies retain their functional properties. On the other hand, when fibronectin and fibrinogen were stirred with octamethylcyclotetrasiloxane (low molecular weight silicone oil) for several hours, it was demonstrated that they undergo conformational changes. The denaturation was attributed to the interaction of the protein with the silicone oil.[56]

Further studies in this area are necessary. For one thing, many studies of adsorbed protein conformation have involved proteins (e.g. fibrinogen, fibronectin, serum albumin) whose structures are incompletely characterized in solution, making interpretation of adsorbed protein structure difficult.[54] Furthermore, the limitations of current structure-sensitive techniques have prevented precise examination of adsorbed protein conformational issues.[54]

b) Biological changes

Silicone rubber is known not to be particularly protein friendly. If denaturation occurs upon adsorption and the denatured proteins are unable to recover their native structure upon desorption, then the silicones would be responsible for the observed reduction of biological activity.[27] The relationship between structure and function of adsorbed proteins remains incompletely understood.[57,55] Fibrinogen is an example of a protein that although has been found to have undergone conformational changes in one study,[56] it has been found to retain its function to a significant degree in another.[51]

Enzyme activity

Adsorbed proteins typically undergo conformational changes, but probably not always to the extent that enzyme activity and antibody binding are altered significantly. There are a few examples of various enzymes that retain their biological function upon adsorption to silicones of various forms (modified elastomers, fluids). It should also be noted that surface induced denaturation is not the only reason for bioactivity loss. This could be as well explained as a consequence of loss of active site accessibility, due to its interaction with the surface.

Chymothrypsin, dispersed in silicone elastomer mixed with either silicone fluid or with some other cosolvent such as glycerol, was used in order to evaluate the biological activity of such protein delivery systems. Enzyme activity assay results indicated that the released chymotrypsin retains its activity in the media after 13 days from its initial release. The decrease of the enzyme activity after that time period was attributed either to the prolonged elevated temperature (37°C) or to prolonged interaction with the silicone matrices.[3]

To avoid silicone-induced denaturation, silicone has been modified with hydrogels. In such an application it was found that for a given heterogeneous silicone/poly(*N*-isopropyl acrylamide) hydrogel composition, 80% of amylase (a 50,000 molecular weight enzyme) activity was retained, despite the fact that the protein loading in the gel did not seem to be very homogeneous (more surface protein which could not be protected from pH induced degradation).[50]

It has been demonstrated that fibrinogen binds from aqueous media to silicone oil droplets dispersed in the aqueous media and, while adsorbed, remains functional. This was shown via macroscopic agglutination of the droplets upon stirring with thrombin (due to the fibrin formation). It was concluded that the protein, although irreversibly absorbed on the PDMS droplet (since stable emulsions are obtained), it was not so denatured as to lose its biological function.[51]

Immunological and antigenic studies

The controversy over the safety of silicone implants has led to a great number of immunological investigations of all medical grade silicones (fluids, gels, elastomers).[58,59] Although the effect of silicone biomaterials on the immune system still remains largely unknown it is believed that the denatured proteins upon adsorption to silicone materials (not the silicone materials themselves) activate somehow an immune response. It is found that the greater the surface area of the materials (e.g., gel *versus* oil or oil-gel mix) the greater the denaturation of the plasma proteins and the greater the immune response.[59] Model studies involving silicone oil and serum have also found that protein-silicone interaction yields an immunogenic moiety. This has been attributed to the denatured proteins, which have altered antigenicity, being either irreversibly attached to the silicone oil or subsequently desorbed from it.[60] In contrast with these studies, comes a diagnostic method developed to detect the presence of select proteins in liquid samples. An emulsion (silicone oil-in-water), where silicone oil droplets have been coated with a protein (antibody) or mixtures of proteins (antibody plus other non functional proteins), is used for detection of the selected proteins. These emulsions were used successfully for the detection of antigens (to the corresponding antibodies adsorbed on the droplets) when mixed with body fluids.[61]

Comparative studies of silicone elastomers with polystyrene have been done using the interaction of antibodies with these surfaces. Adsorption was found, by antigenic criteria (profound loss of bovine IgG1 and IgG2 antigenicity), to induce conformational changes in the protein. Furthermore, it was indicated that proteins adsorbed as a monolayer are denatured to a different extent and manner at the two surfaces. In contrast, secondary adsorption of the proteins indicated that they remained near to their native conformation.[62] Further work indicated that antibodies and antigens adsorbed on hydrophobic materials (silicone, polystyrene) undergo, most likely, both changes in conformation and the accessibility of antigen epitopes or antibody binding sites.[63]

Scope of the Thesis

There has been great interest in exploiting silicone-protein interactions in numerous applications over the last decades. While there are many studies covering different aspects of protein adsorption on silicone surfaces (degree, kinetics, mechanism, surface-induced protein denaturation, etc.), most of them are limited to reporting quantities of protein deposition on silicone elastomers. Only a few studies have dealt with protein adsorption on silicone copolymers and organofunctionalized silicone surfaces and even fewer with fluid silicones. A fundamental approach necessary to understand and predict silicone-protein interactions is still missing. Moreover, controversies among the existing studies, probably mostly originating from the use of different silicone-protein systems, render the topic even more confusing.

In the work included in this thesis, the focus has been on the interaction of proteins with novel, well-defined end-functionalized silicones. These silicones possess a non-ionic functionality ($(EtO)_3Si(CH_2)_3$) that can enhance the protein-silicone interaction both through hydrophilic interactions with the protein amino acids and by locking the interdiffusing chains of the two polymers together due to its ability to cross-link. The interaction of proteins with end-functionalized silicones has not been reported before. However, the concept of enhancing the silicone-protein interactions via cross-linking has found use on silane derivatized protein-silicone copolymers. The silicones used in this thesis combine the potential of cross-linkable silanes with the properties of silicones. Understanding the effect of the silicone functionality on the protein-silicone interaction was obtained by comparison with normal silicones. At solid/liquid interfaces evidence of the facilitating role of the silicone functionality on the protein-silicone interaction is given by undertaking the well-known route of model adsorption/desorption studies. Although there is no precedent for evaluating the magnitude of silicone-protein interactions at liquid/liquid interfaces such an approach has been undertaken. The correlation of the stability of surfactant-less water-in-silicone oil emulsions with the composition of the bulk phases of the emulsions served as an indication of the magnitude of silicone-protein interaction at fluid interfaces.

Finally, these novel silicones could have potential as protein delivery systems. However, there is still controversy over the issue of silicone-induced protein denaturation. Therefore, an effort to contribute to the resolution of this controversy is also part of this thesis.

Outline of the Thesis

This thesis consists of two parts. The first part is concerned with interactions at solid/liquid interfaces and consists of three chapters, while the second part is concerned with liquid/liquid interfaces and consists of two chapters. In the first chapter of each part biological aspects of the silicone-protein interaction are discussed while the rest of the chapters focus on the magnitude of the interactions.

Chapter 2 describes the preparation of the functional silicones of interest in this thesis and the formulation and properties of protein/starch/silicone microparticles. The intriguing immunological properties of the microparticles fabricated with the functional silicones aroused our interest to focus further investigations on the silicone-protein interaction involving these silicones and explore the role of the functionality.

Chapter 3 includes the first part of model studies undertaken in order to elucidate the role of the silicone functionality in the overall silicone-protein interaction. These model studies involve the modification of glass substrates with either protein or silicones (two different types) and subsequent deposition of each of the two silicones and the protein onto the oppositely modified surfaces. Silicone and protein deposition was characterized by two surface techniques (X-ray photoelectron spectroscopy and contact angle measurements) to reveal, quantitatively, the role of the functionality in facilitating the silicone-protein interactions.

Chapter 4 is a continuation of the above model studies and involves surfactant and pH-induced protein elution from the previously noted silicone-protein surfaces. Elution profiles were observed with the same surface techniques and indicate more hindered protein desorption in the presence of the functionalized silicone.

Chapter 5 marks a twist in the research interest to systems that are colloidal in nature. Liquid interfaces (emulsions) become the systems used in order to study the silicone-protein interactions. The interactions in these systems were first studied from a biological perspective due to the obvious potential of such systems in protein delivery applications where protein integrity is of paramount importance. Consequently, biological activity studies (enzymatic and immunological) have been undertaken.

Chapter 6 is an expansion of the previous silicone-protein comparative studies between the two silicones at fluid interfaces. The magnitude of the interaction at these interfaces is evaluated in terms of emulsion (water-in-silicone oil) stabilization. As emulsion stabilization was achieved only when both functionalized silicones and proteins were present at the interface the synergism was mainly attributed to the interaction of the functionality with the protein amino acids.

Contributions in this Thesis

Other researchers' contributions in this thesis are found in chapters 2 and 6. In chapter 2 the contribution of the author of this thesis involved the preparation of the functional silicones and the microparticles as well as their characterization. The microparticle characterization involved recording some of the contact angle measurements as well as the exploration of the surface roughness and contact angle relationship. The additional work done by Jianxiong Jiang and Philippa Heritage involved, except for the original preparation of the functional silicones and the microparticles, the microparticle characterization by obtaining the silicone extraction data and protein release profiles, most of the contact angle measurements, and the investigation the microparticle immunological properties. This chapter was included more because it set the stage for the majority of the thesis rather than because the author was a major contributer. In chapter 6, the contribution of the author includes everything in the chapter but the preparation of emulsions for establishing the concentration effects in emulsion stability and the observation of the stability over time, which was performed by the fourth year student Gladys Chan under the author's supervision.

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PART I

Silicone-Protein Interactions at Solid/Liquid Interfaces

Chapter 2

The Silicone-Protein Interaction at the Interface between a Functional Silicone and a Protein/Starch Microparticle

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Abstract

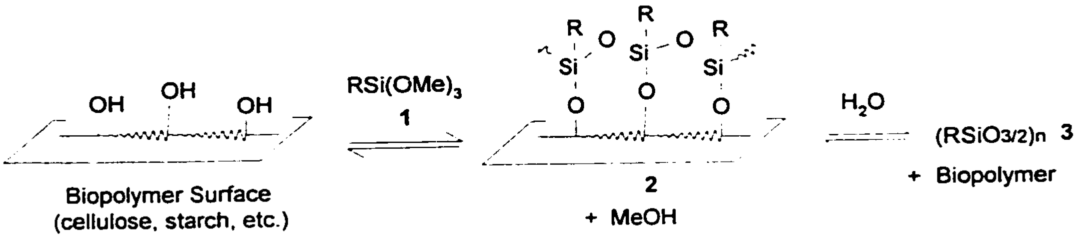
Silicone-coated starch/protein (human serum albumin, HSA) microparticles were prepared by precipitation of a starch/HSA/DMSO/water (water-in-oil) emulsion into acetone containing a silicone polymer. Two silicones were examined: unfunctionalized (trimethylsilyl-terminated, PDMS) or functionalized at the termini with $Si(OEt)_3$ groups (PDMS-TES). Microparticles were not formed in the absence of protein. Instead, the phase containing starch separated after agglomeration. Thus, stabilization/hydrophobization of the starch surface by silicone alone was not possible. However, there is a strong affinity between the silicone and the protein particularly in the case of the PDMS-TES and, simultaneously, an affinity between the PDMS-TES and the starch that leads to a stabilization of the starch surface. This could most clearly be seen from immunological data that showed that antibodies were elicited by protein in the microparticles coated with PDMS-TES following oral administration.

Introduction

In order to provide sufficient adhesion between incompatible materials, such as reinforcing glass fibers in an organic polymer, for instance, it is necessary to control the chemistry of the interface. Modifying an inorganic interface is typically done using a coupling agent, normally a silane reagent or functionalized silicone,[1] which covalently introduces surface (functional) organic groups.[2] The reaction involves the oligomerization of alkoxysilanes **1** in the presence of moisture, deposition of the oligomer on the surface of the inorganic species, and then covalent binding to the surface.[3] The organic polymer matrix in which the mineral is placed then interacts with the organic groups on the surface, from the coupling agent, rather than the inorganic support itself.

Controlling the interface between a hydrophilic biopolymer, such as protein or starch, and an organic surface is an important challenge. Protein adhesion to such surfaces, for instance, is often deleterious to the performance of implantable and topically applied materials.[4,5] On the other hand, controlling and improving adhesive interactions at such an interface could lead to the development of new compatible materials.[6,7]

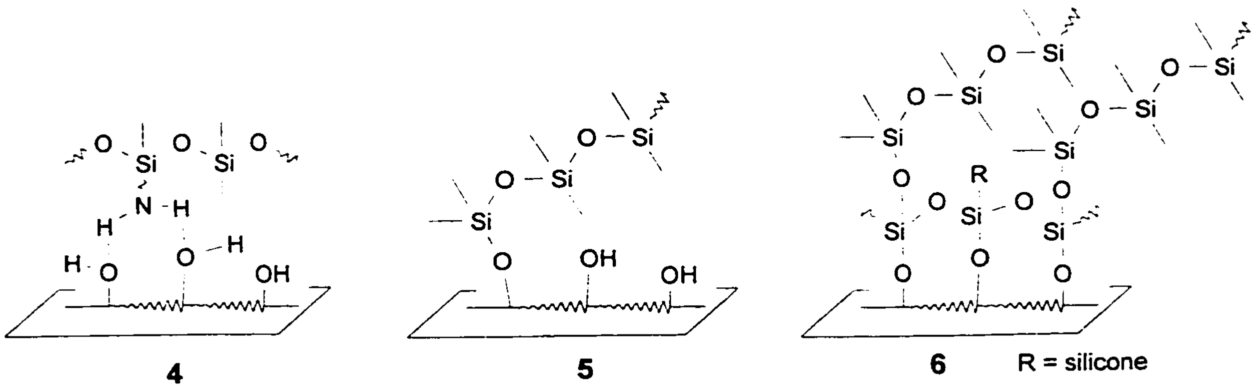
Hydrophilic biopolymer surfaces are chemically analogous to inorganic surfaces. Taking starch and silica as examples, the surfaces of both are rich in OH groups. It should be possible, in principle, to apply knowledge of the chemistry and behaviour of coupling agents[2] on inorganic surfaces to the hydrophobization of biopolymers. There are some fundamental differences in chemistry between R_3SiOH and R_3COH groups, however, which must be considered. When a coupling agent reacts with a siliceous mineral surface, a disiloxane is formed. The hydrolysis of these linkages is generally slow. However, transesterification of an organic surface **1**→**2** will lead to alkoxysilane products that remain susceptible to hydrolysis (**2**→**3**, Scheme 1): disiloxanes are thermodynamically more stable than alkoxysilanes.[8-10]



Scheme 1

Previous work with polysaccharides, wood fibers and cellulose for example, has demonstrated that silane coupling agents[11] do not confer much hydrolytic stability to the polymer matrix. It seems possible, however, that a more stable silane[12] or more hydrophobic species, for instance a long chain silicone polymer, might resist hydrolysis at the interface more efficiently.[1] Numerous examples exist of formulations in which starch (and/or cellulose) is combined with silicones for various reasons, including examples in which hydrophobization is explicitly sought. For instance, the reaction of silicones under basic conditions with starch, which should lead to C-O-Si linkages between starch and silicone, has been reported.[13] Functional silicones bearing pendant amino groups have also been used to hydrophobize starch.[14] In both cases, hydrolytic stability is controlled either by physical adhesion **4** (latter case) or the covalent binding of a single bond **5** (Scheme 2). However, situations such as these are not optimal.

We are unaware of approaches to the hydrophobic stabilization of polysaccharides, such as starch, which involve the use both of multiple coupling sites (multifunctional silane reagents that bind tenaciously to the substrate) **and** highly hydrophobic silicone chains **6** (Scheme 2). To determine the validity of this approach, we have prepared microparticles, containing both starch and a protein (human serum albumin, HSA), which are surface modified by silicone polymers. Microparticles were utilized because of their relatively high surface area, which facilitates the characterization process. The protein was initially added as a marker to determine the stability of the starch-silicone interface. However, as shall be described below, the presence of the protein played an unexpected, pivotal role in the hydrophobization of the starch surface.



Scheme 2

Experimental Section

Apparatus, methods and materials

The continuous wave ^{1}H-NMR spectra were recorded on a Varian-390 (90-MHz) spectrometer and the Fourier spectra on a Bruker-AM500 (500-MHz) spectrometer, Bruker AC-300 (300-MHz) spectrometer or Bruker AC-200 (200-MHz) spectrometer. ^{13}C and ^{29}Si-NMR, usually in $CDCl_3$, were performed on a Bruker AC-200 (at 50.3 MHz for ^{13}C) and Bruker AC-300 (at 59.6 MHz for ^{29}Si), respectively. Chemical shifts are reported with respect to tetramethylsilane as standard, set to 0.00 ppm, $CDCl_3$, set to 7.24 ppm or HOD set to 4.63 ppm. Coupling constants (J) are recorded in Hertz (Hz). The abbreviations s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, p = primary signal (some signals, including dimethylsilyl in the ^1H NMR spectrum, could be split or slightly broadened; only the main signals are quoted), $D = [Me_2SiO]_n$, $T^0 = Si(OR')_3$, $T^1 = RSi(OSi)(OR')_2$, $T^2 = RSi(OSi)_2(OR')$ and $T^3 = RSi(OSi)_3$ are used in reporting the spectra. The relative quantities of different species are reflected in the ^1H NMR signal integration. In extremely dilute solutions, the integration of the proton signals was calibrated preferably with *i*-PrOH or acetophenone as internal standards.[15]

Infrared spectra were run on a Perkin Elmer 283 spectrometer or BIO RAD FTS-40 (FTIR) spectrometer as a film on a NaCl disk. UV spectra were performed on a HP 8451A Diode Array spectrophotometer to measure the rate of release of protein (human serum albumin, HSA or HSA-FITC, *vide infra*). Advancing contact angles were measured on a NRL C.A. Goniometer (Ramé-Hart Inc.) with distilled water at ambient temperature. Optical densities (OD) in the ELISA were determined at 405 nm on a Titerteck Multiskan Plus from ICN Biomedicals.

The molecular weights of the polymers were analyzed using a Waters Gel Permeation Chromatography (GPC) equipped with a Waters 410 differential Refractive Index detector. Two Jordi mixed bed columns in series were utilized with trichloroethane as solvent flowing at 1.5 mL/min. Narrow molecular weight polydimethylsiloxane standards from Scientific Polymer Products Inc. were used for calibration of the chromatographic system.

Nomenclature

The various silicone polymers used in this study differ in their molecular weights and terminal groups. To distinguish these, we refer to silicones (PDMS, $XO(Me_2SiO)_nX$) by their terminal groups and viscosity. Silicones are typically sold by viscosity and the correlation between viscosity and molecular weight are well known.[16] The termini that have been used in this paper are the following X = Me_3Si, PDMS; Me_2SiOH, PDMS-OH; Me_2SiH, PDMS-H; $Me_2Si(CH_2)_3Si(OEt)_3$, PDMS-TES. The viscosity (in centistokes, cs) of the polymer is then appended. Microparticles are described by their coating. For example, the microparticle coated with PDMS-TES-1000 is named MP-PDMS-TES-1000.

Materials

PDMS-H (2-3 cs, 500 cs, 1,000 cs and 10,000 cs), PDMS (200 cs, 1,000 cs and 5,000 cs), PDMS-OH (1,000 cs); allyltriethoxysilane **8**, platinum catalysts H_2PtCl_6, and Karstedt's catalyst (PC075 $Pt_2[(H_2C{=}CHMe_2Si)_2O]_3$, 3.5% in vinyl-terminated PDMS, neutral, or PC072 $Pt_2[(H_2C{=}CHMe_2Si)_2O]_4$, 3.5% platinum concentration in xylene, neutral) and hexamethyldisilazane-treated silica were obtained from Hüls-Petrarch Systems (now United Chemical Technologies, Bristol PA) and used without further treatment. The purity of these materials, which was checked with ^{1}H NMR immediately before use, was >99.5%. Kiesegel 60 was obtained from Merck and CHARCOAL "DARCO" G60 was purchased from Sargent-Welch. Chloroform-*d* and D_2O were obtained from Matheson. Acetone and DMSO were purchased from Aldrich. Soluble potato starch and fluorescein isothiocyanate were obtained from BDH. A series of food grade vegetable oils (safflower, corn, sunflower, canola and Crisco™) were purchased from a local supermarket. Hexanes were freshly distilled over Na/benzophenone. HSA (Fraction V, 96-99% Albumin), Aprotinin® and phosphatase substrate tablets were obtained from Sigma. Phosphatase-conjugated goat anti-mouse IgG or IgA was purchased from Southern Biotechnology Associates. HSA-FITC was prepared by literature procedures. Female BALB/c mice, age 6-8 weeks were purchased from Charles River Laboratories Inc., Montreal.

Syringe filters (0.45 mm) were obtained from Millipore and microtiter plate wells from Costar. PE50 tubing was purchased from Becton Dickinson and Co.

The Preparation of HSA-FITC

To HSA (0.1 g) in phosphate buffer (pH 8.9, 20 mL) was added fluorescein isothiocyanate (FITC, 2.0 mg, 5.1 μmol). The pH was adjusted to 9.5 with Na_3PO_4 and the mixture was allowed to stir for 16 h at room temperature in the dark. The sample was dialyzed in 4 L of distilled water at 4 °C.

The Preparation and Quality Control of Functional Polydimethylsiloxanes

Preparation of TES-PDMS; General Procedure

A series of 3-triethoxysilylpropyl-terminated poly(dimethylsilicones) (PDMS-TES) were prepared directly by the hydrosilation reactions of PDMS-H of different molecular weights with allyltriethoxysilane under the catalysis of Pt complexes (Scheme 3).

To a mixture of PDMS-H and **8** was added the catalyst: a 0.1% *iso*-propanol solution of H_2PtCl_6;[17] or Karstedt's catalyst (PC075 $Pt_2[(H_2C{=}CHMe_2Si)_2O]_3$,[18] 3.5% in vinyl-terminated PDMS, neutral or PC072 $Pt_2[(H_2C{=}CHMe_2Si)_2O]_4$, 3.5% platinum concentration in xylene, neutral). The molar ratio of the functional polymer terminus of the polymer to the silane was 1:3-7 depending on the molecular weight of PDMS-H. If the PDMS-H was highly viscous (≥1000 cs), a 50% (v/v) of freshly distilled hexanes was also added. The mixture was stirred at ambient temperature under a N_2 or dry air atmosphere. The reaction was followed with ^{1}H NMR until the characteristic signal of the Si-H groups at 4.7 ppm totally disappeared. This took between a few hours to several days, depending on the kind and the amount of the catalyst used, the concentrations of both the catalyst and the reactants (if solvent used), the molecular weight of the PDMS-H and the atmosphere in the reactor (N_2 or air). The light yellow solutions (from residual colloidal platinum complexes) were purified by either passing the solution through a Kiesegel 60 (40-63 μ) packed column (20 mm x 150 mm), followed by an equal volume of hexane, or by treating the solution with charcoal "DARCO" G60 (activated) at 90 °C (temperature of the oil-bath) for 5 min. The former was usually applied. Distilled hexane was used to wash the chromatographic support in both cases. The solution became colorless after the treatments. Solvents and unreacted **8** were removed under reduced pressure to give clear oils in 50 - 90% yield. The purity of the products was at least 98% according to ^{1}H NMR. The only regioisomer observed in the hydrosilation were 7 (Scheme 3) in which the $Si(OEt)_3$ group has added to the terminal carbon of the alkene.

PDMS-TES ^{1}H NMR spectra consist of a set of peaks for the terminal $(EtO)_3Si$ groups (quartet near δ 3.82 and triplet near1.22, H = 7 Hz) and $(CH_2)_3$ groups (δ 1.5), and a large broad singlet for the (Me_2SiO) units in the backbone (*ca.* δ 0.65). The relative integration for the endgroups to (Me_2SiO) was proportional to the molecular weight. Ratios are given in Table 1.

PDMS-TES-3

PDMS-H (2-3 cs, 20 mL); allyltriethoxysilane (22.5 mL, 99.4 mmol); PC072 (0.2 mL, 0.35 % PC072, 0.0035 mmol); air; one week.

PDMS-TES-500

PDMS-H (500 cs, 30 mL); allyltriethoxysilane (2.6 mL, 11.5 mmol); PC072 (0.1 mL, 0.35 % PC072, 1.7 mmol); N_2; 2 days.

PDMS-TES-1000-a

PDMS-H (1000 cs, 12 mL); hexanes (20 mL); allyltriethoxysilane (0.5 mL, 2.2 mmol); PC075 (0.1 mL, 0.35% (diluted with dry hexanes), 1.7 mmol); N_2; 2 days.

PDMS-TES-1000-b

PDMS-H (1000 cs, 15 mL); hexanes (40 mL); allyltriethoxysilane (0.7 mL, 3.1 mmol); PC075 (0.06 mL, 0.35% (diluted with dry hexanes), 0.2 mmol); air; 2 weeks.

PDMS-TES-1000-c

PDMS-H (1000 cs, 46 mL); hexanes (25 mL); allyltriethoxysilane (2.1 mL, 9.3 mmol); PC072 (0.1 mL, 0.35 % PC072, 0.35 mmol); air; 1 week.

PDMS-TES-1000-d

PDMS-H (1000 cs, 450 mL); hexanes (250 mL); allyltriethoxysilane (25.0 mL, 110.5 mmol); PC072 (0.3 mL, 0.35 % PC072, 1.1 mmol); air; 2 weeks.

PDMS-TES-10000

PDMS-H (10,000 cs, 25.8 g); hexanes (50 mL); allyltriethoxysilane (0.6 mL, 2.7 mmol); PC072 (0.06 mL, 0.35 % PC072, 0.2 mmol); N_2; 8 days.

Characterization of the materials

The reactions described above could be readily followed by IR spectroscopy: the Si-H stretch in the range of 2215-2130 cm^{-1}, depending on the molecular weight of the PDMS, was quite intense. The disappearance of this signal indicated PDMS-H had completely reacted with allyltriethoxylsilane.

NMR was also used to follow and determine the progress of the reaction (Table 1). In the ^1H NMR spectra, the disappearance of the signals at 4.73 ppm (Si-H groups of PDMS) and 5.8, 4.9, 1.65 ppm (allyl groups of **8**) was indicative of complete reaction. ^{13}C NMR spectroscopy also showed the conversion of the allyl groups (at 132.8, 114.7

and 18.4 ppm) to -$CH_2CH_2CH_2$- groups (22.5, 16.9 and 14.8 ppm). In the ^{29}Si NMR, after the reaction, a 5 ppm downfield shift from (-50.5 ppm to -45.4 ppm) was observed.

Table 1:NMR spectroscopic properties of the silicon compounds[a]

Compound	^{1}H NMR	J[b]	^{13}C NMR	^{29}Si NMR
PDMS-H-3	4.73 (m), 0.18 (d), 0.08 (d)	2.8	0.47, 0.29, 0.15	-7.06, -20.07
PDMS-H-500	4.72 (m), 0.18 (d), 0.08 (bs)	3.2	3.35, 1.55, 0.78	-21.92 (m), -22.54
PDMS-H-1000	4.73 (m), 0.18 (d), 0.08 (bs)	3.0	1.49, 0.74	-21.94 (m), -22.56
PDMS-H-10000	4.73 (m), 0.08 (bs)			-21.96 (m)
AllylSi(OEt)$_3$	5.8 (m), 4.9 (m), 1.65 (d), 3.83 (q), 1.22 (t)		132.79, 114.69, 58.66, 18.44, 18.30	-50.49
PDMS-TES-3	(q), 1.22 (t), 1.57-1.41 (m), 0.74-0.60 (m), 0.07 (bs) (EtO)$_3$Si:Me$_2$SiO 2:5		58.27, 18.34, 22.54, 16.89, 14.79, 1.92, 1.18, 1.09, 0.26	6.99, (m) -21.78 (m) -22.13 (m) -45.42
PDMS-TES-500	3.81 (q), 1.23 (t), 1.58-1.41 (m), 0.75-0.60 (m), 0.08 (bs) (EtO)$_3$Si:Me$_2$SiO 1:120		58.28, 18.34; 22.52, 16.88, 14.79; 1.80, 1.06, 0.27	6.98, -21.94 (m), -45.30
PDMS-TES-1000-a	3.81 (q), 1.23 (t), 1.58-1.38 (m), 0.75-0.59 (m), 0.08 (bs) (EtO)$_3$Si:Me$_2$SiO 1:180		58.25, 18.33; 22.51, 16.86, 14.76; 1.80, 1.07, 0.32	7.00, -21.93 (m, m) -45.29
PDMS-TES-1000-b	3.81 (q), 1.23 (t), 1.58-1.38 (m), 0.75-0.59 (m), 0.08 (bs) (EtO)$_3$Si:Me$_2$SiO 1:180		58.27, 18.32; 22.51, 16.87, 14.76; 1.80, 1.06, 0.30	7.00, -21.93 (m) -45.29
PDMS-TES-1000-c	3.81 (q), 1.23 (t), 1.58-1.41 (m), 0.75-0.60 (m), 0.08 (bs) (EtO)$_3$Si:Me$_2$SiO 1:190		58.30, 18.37; 22.54, 16.90, 14.82; 1.82, 1.09, 0.35	7.00, -21.94 (m) -45.24
PDMS-TES-1000-d	3.82 (q), 1.22 (t), 1.59-1.41 (m), 0.75-0.60 (m), 0.08 (bs) (EtO)$_3$Si:Me$_2$SiO 1:180		58.28, 18.35; 22.54, 16.90, 14.82; 1.80, 1.07, 0.33	7.00, -21.92 (m) -45.44
PDMS-TES-10000	3.81 (q), 1.22 (t), 1.57-1.50 (m), 0.75-0.60 (m), 0.08 (bs) (EtO)$_3$Si:Me$_2$SiO 1:420		1.80, 1.06, 0.30	-22.94 (m)

[a] All spectra were run in CDCl$_3$ with TMS as internal standard; bs= broad singlet.

[b] $^3J_{HSi\text{-}CH_3}$

Gel Permeation Chromatography (GPC) was used to establish the molecular weights and distributions of the PDMS. The distributions of 3-triethoxylsilylpropyl terminated PDMS were related to the MW reported for the starting PDMS-H (Table 2).

Table 2: Molecular weight analyses of the silicone compounds by GPC[a]

Compound	Ret. Time	Molecular	Weight
	(min)	Theoretical	Measured
PDMS-H-3	18.12	400	400
PDMS-H-500	14.51	17500	16300
PDMS-H-1000	14.12	28000	24100
PDMS-H-10000	13.02	62000	73400
PDMS-TES-3	17.53	800	800
PDMS-TES-500	14.38	18000	18500
PDMS-TES-1000-a	14.15	28400	23400
PDMS-TES-1000-b	14.13	28400	23900
PDMS-TES-1000-c	14.11	28400	24400
PDMS-TES-1000-d	14.12	28400	24100
PDMS-TES-10000	12.95	62400	78800

[a] GPC was run using CH_3CCl_3 as solvent with sample concentrations of about 0.2% (w/w).

Preparation of Controlled Size Starch/Protein Microparticles

Preparation of the DMSO/H_2O Starch/Protein Solution

Soluble potato starch (1.0 g) was suspended in DMSO (2.0 mL) with stirring at ambient temperature in a 25 mL Erlenmeyer flask. The temperature was slowly increased to 99 °C; a clear solution was obtained which was then cooled to room temperature. HSA (or (HSA-FITC, 100.0 mg) was dissolved in distilled water (1.0 mL) with stirring at room temperature. The starch solution was added to the protein solution with vigorous stirring (500 rpm with a magnetic stir bar or using a high-speed mixer at 30000 rpm with a brush as the agitating element) at 32 °C. The mixture was allowed to stir for at least 5 min to produce an opaque solution.

Formation of the Oil-in-Water Emulsion

The starch/protein solution was added dropwise with a Pasteur pipette to vegetable oil[19] (e.g., Crisco™, 30 mL) with continuous stirring (1300 rpm with a magnetic stir bar) to produce a water-in-oil emulsion (no additional surfactant was present). The sizes of the emulsion droplets were crucial to the ultimate size of the solid microparticles. The higher the speed of the stirring and the longer the stirring time, the finer the emulsion droplet size. It was usually left to stir for at least 15 min to obtain microparticles with an average size of 2-7 μ. It was imperative to avoid large emulsion droplets prior to the final precipitation.

Precipitation of the Particles

Just prior to precipitation, the emulsion was sonicated at high power (with an ultrasonic bath) for 60 s in an ice-bath, to break any large sized emulsion particles and give a better particle size distribution. The water-in-oil emulsion was added to acetone (400 mL) with stirring (1100 rpm with a magnetic stir bar) at room temperature in a 1000 mL Erlenmeyer flask containing a coating material which consisted of: i) functionalized silicones PDMS-TES (from 0.25 - 4.0 mL, depending upon the experiment), ii) unfunctionalized silicones PDMS (0.25 - 4.0 mL), or iii) Tween-80 (2.0 mL, 1% in acetone). Solid microparticles formed as the emulsion droplets contacted the acetone solution: the acetone dessicated the droplets. The silicones or Tween-80 were crucial to the establishment of a stable dispersion. The size of the solid microparticles was directly related to the size of the emulsion droplets prior to the addition to acetone. The manner of addition of the emulsion to the acetone solution, dropwise or continuous, had little effect on the sizes of the solid microparticles formed; vigorous stirring acted only to prevent the newly produced microparticles from sticking to the glass wall of the reaction vessel. *Particles could not be prepared in the absence of proteins*; the emulsion, in this case, completely aggregated upon addition to acetone leading to a glutinous mass.

Isolation of the Particles

The acetone-precipitated starch/protein microparticles, plus a rinsing volume of acetone (150 mL acetone, 150 mL 1% Tween 80 in acetone for microparticles without a siloxane coating), were centrifuged at 2000 rpm for 5 min at 4 °C. The supernatent acetone was decanted and the residual microparticles were dried through evaporation of acetone in air for at least three days. The solids were then collected, weighed and stored.

Characterization of the Microparticles

Surface properties: Contact angles of the microparticles

The advancing contact angles of distilled water on the silicone-grafted (PDMS-TES) microparticles were compared with those prepared from PDMS or without silicone. Silicones have very high contact angles (typically > 100°). Starch, by contrast, was completely wetted (contact angle ≈ 0°). The contact angle of HSA was dynamic: the initial angle of about 110° changed to 20° within about 6 min. As standards, the contact angles of hydrophobic surfaces silicone rubber and hexamethyldisilazane-treated silica were measured. These latter contact angles were always > 110°. The hydrophilic glass surface was wet by water with contact angles 14°. A glass surface coated with PDMS-OH (1000 cs) had an initial contact angle of 81 ± 2° which, however, dropped to 55 ± 5° within 30 min.

A film of the microparticles was prepared on a glass slide. Similar to the PDMS-OH film, these contact angles were dynamic, showing lower values with time; the values are shown in Figure 3: those measured after $CHCl_3$ extraction are shown in Figure 4. The contact angles: (i) were measured at 18 °C from spots with a thickness of the sample microparticles at least 1.0 mm; ii) out of the range of the apparatus (>110°) were approximated to 110° unless otherwise specified; iii) of the particles obtained from acetone / Tween 80 solutions (30°→15°) were difficult to establish (the particles were easily wetted and the microparticles often floated on to the surfaces of the water beads; within 6 min the water bead was covered by the microparticles.).

Silicone Release: Extraction with $CHCl_3$

Sohxlet extraction (0.5 g) with $CHCl_3$ (150 mL) for up to 240 h showed only subtle differences in the magnitude and rate of loss of silicone from PDMS-1000- and PDMS-TES-1000-coated microparticles (Figure 2).[20]

Protein Release

The UV spectra of an aqueous suspension of microparticles containing HSA-FITC was followed with time. The release of the protein from the HSA-FITC-containing microparticles could be directly observed, assuming the contribution to the UV-absorbance of the unreleased protein in the solid state was negligible. Thus, HSA-FITC-containing microparticles (about 0.21 g, 12% by weight protein) were suspended in water (10 mL). UV spectra were run after given periods of time while the solution stayed at ambient temperature. The liquid in the UV cell, together with 2.0 mL water which washed the cell, was poured back into the flask after each measurement (Figure 6). The release rates in solutions of different pH were similarly measured by suspending about 30 mg of HSA-FITC-containing microparticles in 3.0 mL buffer of defined pH water in a UV cell (Figure 7).

Immunization Protocol

Immunizations

Female BALB/c mice, age 6-8 weeks were used and allowed food and water *ad libitum*. Mice were inoculated intragastrically (IG), under ether anesthesia, on days 0, 7, 14 and 70. Animals received antigen IG in $NaHCO_3$ (500 mL, 0.2 M) using PE50 tubing.

Collection of Serum and Gut Washes

Individual blood samples were obtained via the retro-orbital plexus. Insoluble material was removed by centrifugation and sera were stored at -70 °C until used.

To detect and quantify anti-HSA IgA in the intestinal lumen, mice were exanguinated and their small intestines removed and everted over capillary tubes. The everted intestines were incubated for 4 h in ice cold enzyme inhibitor solution (5 mL) containing NaCl (150 mM), NaH_2PO_4 (10 mM), ethylenediaminetetraacetic acid (5 mM), phenylmethylsulfonyl fluoride (2mM), Aprotinin® (0.05 U/mL) and NaN_3 (0.02%). Intestines were removed and the remaining solution (operationally termed gut washes, GW) were clarified by centrifugation ($2x10^4$xg, 15 min) filtered through 0.45 mm syringe filters and stored at -70 °C until used.

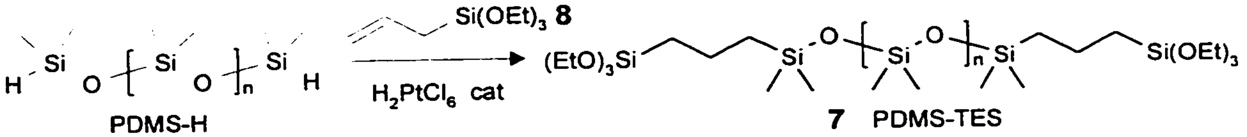
Measurement of HSA-specific Antibody Responses

An enzyme-linked immunosorbant assay (ELISA) was used to detect and quantify HSA-specific antibodies in individual serum and GW. Duplicate serial dilutions of serum and GW were examined using microtiter plate wells incubated with HSA (100 mL, 10 mg/mL in TBS) followed by incubation with gelatin (150 mL of 0.1 % in TBS, blocking buffer). Anti-HSA antibodies were quantitated by incubating wells with alkaline phosphatase-conjugated goat anti-mouse IgG or IgA (100 mL, heavy chain specific) diluted in blocking buffer. After washing, diethanolamine buffer (100 mL of 1.0 M, pH 9.8) containing $MgCl_2$ (50 mM) and *p*-nitrophenylphosphate (1.0 mg/mL, 5 mg phosphatase substrate tablets; Sigma) were added to each well and optical densities (OD) were determined at 405 nm. Normal mouse serum (NMS) or normal gut wash (NGW) pools, prepared from untreated animals, were used to establish baseline mean OD values. The results were expressed as reciprocal end-point titers representing the greatest serum or GW dilutions giving OD values exceeding 3 standard deviations above NMS or NGW mean values (Figure 8).

Results

Preparation of the Silicones

Two distinct silicones were used to modify the starch: normal polydimethylsiloxane (PDMS, $Me_3SiO(Me_2SiO)_nSiMe_3$) and polydimethylsiloxane terminated with $Si(OEt)_3$ functional groups (7, PDMS-TES $(EtO)_3Si(CH_2)_3SiO(Me_2SiO)_nSi(CH_2)_3Si(OEt)_3$, Scheme 3). Normal PDMS was obtained from commercial sources in a variety of molecular weights. The functionalized polymers PDMS-TES 7 of different molecular weights were prepared by the platinum-catalyzed hydrosilation[21] of commercially available, hydrogen-terminated silicone polymers with allyltriethoxysilane **8** (Scheme 3). Although it is possible to transesterify hydroxy- or alkoxy-terminated silicones to give analogous systems (e.g., DQ resins from $(EtO)_3SiO(Me_2SiO)_nSi(OEt)_3$), in analogy to room temperature vulcanization systems (RTV), such polymers run a greater risk of silicone depolymerization in the presence of acid or base than the analogous trialkylsilane-terminated polymers such as PDMS-TES.[22]



Scheme 3

Preparation of particles

As noted above, alkoxysilanes are susceptible to hydrolysis. Thus, an experimental design for the preparation of a silicone-grafted biopolymer must minimize exposure to water. Such conditions will promote transesterification of the TES groups with the biopolymer, rather than hydrolysis. We also wished to maximize the surface area of the system to facilitate characterization of the interface. Finally, there is the simple problem of compatibility: silicones and starch do not, in general, dissolve in the same solvents. To address these problems, a water-in-oil emulsion, with starch dissolved in an aqueous/DMSO phase, was utilized. The droplets, which possessed a relatively high surface area, were produced in a system that constrains the reaction between the biopolymer and silicone to the droplet interface. Protein was included to act as a marker for the stability of the silicone:starch interface: after suspending the microparticles in water, the rate of release of protein embedded in the starch could serve as an indication of the bonding efficiency of the silicone to the starch surface.

Therefore, an aqueous solution of a protein, human serum albumin (HSA), was mixed with a DMSO solution of soluble starch.[23] Although it was necessary to heat the starch to ~100 °C to form a homogeneous solution, this was cooled to 37 °C prior to addition of the protein solution to minimize protein denaturation.[24] The aqueous/DMSO solution was converted to a water-in-oil emulsion by adding the starch/protein/DMSO/water solution to vegetable oil[25] with sonication and/or rapid stirring (30000 rpm) in the absence of any added surfactant. The addition of the water-in-oil emulsion to acetone led to agglomerated solid starch/protein particles: acetone acts as a desiccant. Microparticles formed if a surfactant (silicone or Tween (polyethylene oxide (20) sorbitan monopalmitate)) was added to the acetone. The microparticle size could be controlled by modifying the type of agitation of the emulsion and by the nature of the added surfactants. Three types of "surfactant" systems were used in the preparation of the starch/protein particles: Tween, PDMS and PDMS-TES. The use of unfunctionalized PDMS permits the examination of the effect of physisorbed silicone on the surface. *It proved impossible to make stable, silicone-coated particles in the absence of protein.* Particle size determinations by Coulter Counter and scanning electron microscopy (SEM, Figure 1) showed that these particles typically range in size from about 2-7 μm.

Figure 1: Scanning Electron Micrograph of a PDMS-TES/HSA Starch Microparticle (black/white bars represent 1 μm)

Characterization of the Particles

Removal of Silicone: Extraction with $CHCl_3$

The particles coated with PDMS and PDMS-TES were sohxlet extracted with $CHCl_3$ (or $CDCl_3$) to estimate the proportion of bound silicones and to probe the tenacity with which the silicones were bound; the results are shown in Figure 2. A significant proportion of the silicone could be readily removed by extraction from particles made from either type of silicone. However, even after exhaustive extraction with $CHCl_3$, in no case was it possible to completely remove the silicone from the particle. The anticipated correlation between the total amount of silicone released and the presence of functional groups on the silicone (for PDMS-TES) was not observed.

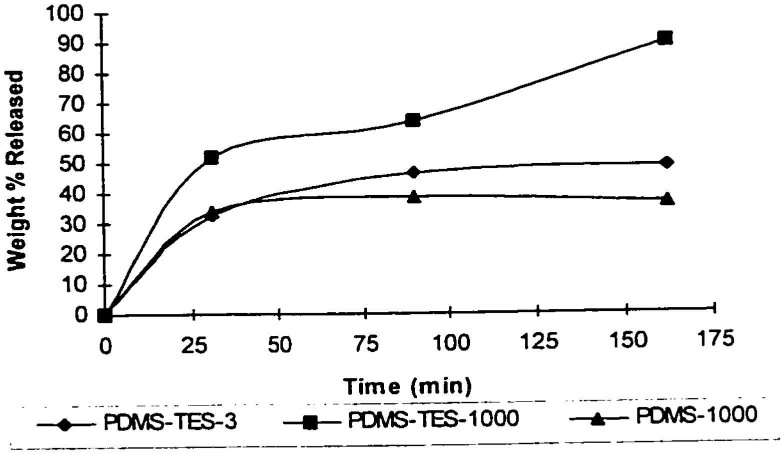


Figure 2: Plot of Loss of Silicone during Sohxlet Extraction with $CHCl_3$[a]

[a] Based on total weight of silicone present in the particle. Suffix numbers refer to silicone viscosity (see Experimental Section).

Surface Structure: Contact Angle

The advancing contact angle of silicones is extremely high when compared to other organic species; values are typically greater than 100-110°.[26] Thus, changes in contact angle serve as a sensitive probe for diagnosing changes in silicone content. The contact angles of water droplets were measured on films comprised of particles coated with PDMS or PDMS-TES. Somewhat surprisingly, these values were not static. A plot of the changes of contact angle with time is shown in Finger 3 and Figure 4. After one hour, the contact angle of the microparticle coated with PDMS-TES-1000 had dropped to

0° (Figure 4). By contrast, commercial Me_3Si-functionalized *silica* particles were found to have a high contact angle (> 110°) which did not change appreciably over the same time period.

For both the biopolymer (Figure 5) and silica microparticles, the film surfaces are far from ideal: both are very rough. It has been well established that the interpretation of surface hydrophobicity on rough surfaces must be done with extreme care.[27] Nevertheless, the trends are very compelling. Irrespective of surface roughness, more hydrophobic surfaces have higher advancing contact angles with water; the inverse is true for polar surfaces. We ascribe the dynamic nature of the contact angle values with our silicone particles to exposure of some of the underlying starch/protein and/or to migration of the particles from the glass surface to the droplet surface and concomitant exposure of the underlying glass surface to the water. That is, the surface is becoming more hydrophilic. By contrast the rough surface derived from silica particles, with their much higher density and lower mobility, showed no dynamic effects. A decrease in contact angle could also result from migration of silicone to the water droplet surface, which arises more readily from the starch/protein microparticle rather than the silica particles, due to less efficient grafting in the former case.

To a small extent, the rate of change of contact angle is dependent upon the silicone molecular weight; the values for higher molecular weight materials changed somewhat more slowly (Finger 3).

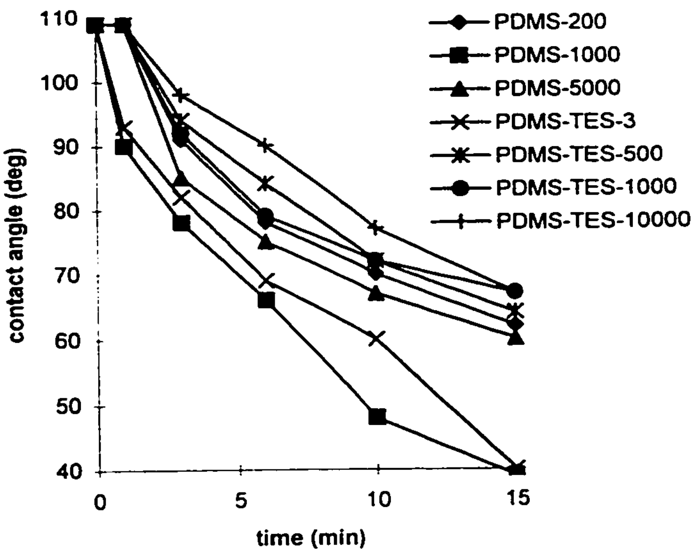


Figure 3: Plot of Contact Angles with Time for Microparticles Coated with Different Silicones[a]

[a] The contact angle of the Tween coated particles dropped from 50° to 25° over 5 min.

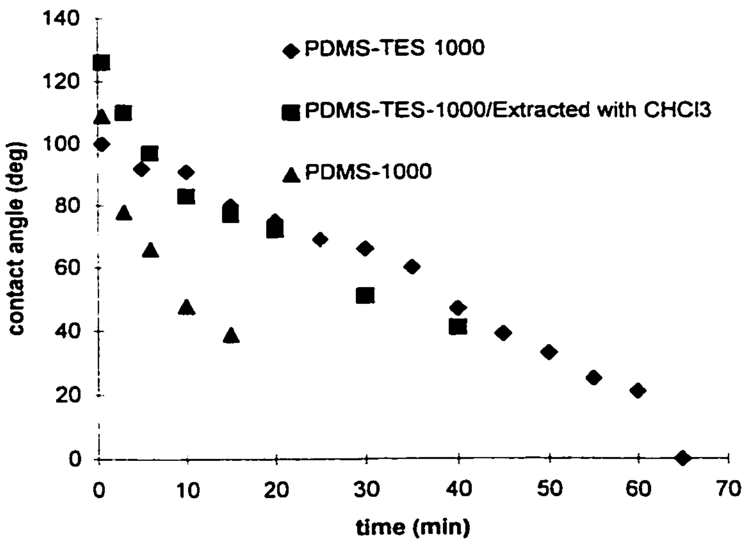


Figure 4: Plot of Contact Angle Changes with Time for Silicone Coated Microparticles

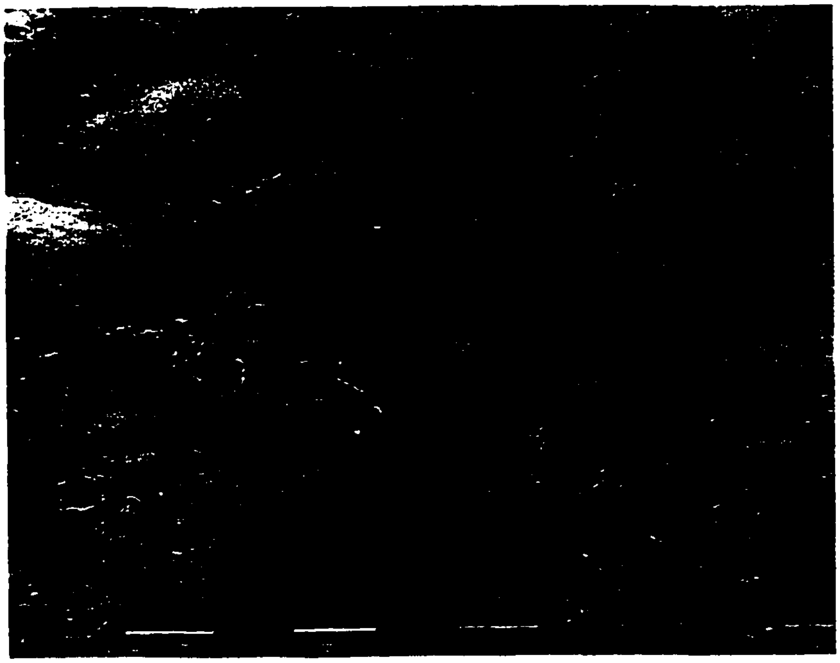


Figure 5: Scanning Electron Micrographs of Films Formed from Protein/Starch Microparticles Coated with PDMS-TES-1000 (black/white bars represents 1 μm)

Effect of Extraction

A comparison of the contact angles of particles coated with PDMS-TES-1000 before and after sohxlet extraction with $CHCl_3$ is shown in Figure 4. For both types of silicones, the initial contact angles were very high, but decreased with time. Thus, as noted above, extraction does not completely remove the silicone. This could be independently shown by ^{1}H NMR.

Protein Release: UV

The rate and degree of protein release upon exposure of the microparticles to water was measured. This allowed an independent assessment of the degree to which the starch/protein particle surface was affected by exposure to silicone polymers. Utilizing a microparticle containing a fluorescein-labelled protein (HSA-FITC, see experimental section), the concentration of protein in the aqueous supernatent could be readily assessed by UV spectroscopy (Figure 6).

The effect of molecular weight of the silicone could be more clearly seen in this experiment. An increase in molecular weight of the silicone retards the rate of protein release. There is, however, no obvious difference between PDMS and PDMS-TES modified microparticles. The silicone, even low molecule weight silicone, clearly provides a hydrophobic barrier on the surface of the particle as evidenced by the slower protein release when compared to uncoated particles. It should be noted that the UV absorbance is indicative only of the fact that the protein is exposed to or swollen with water and liberated from the particle structure, it does not allow one to discriminate between protein bonded to or free from silicone. In addition, the rate of protein release is slower than the change in contact angle (see above, Figure 3).

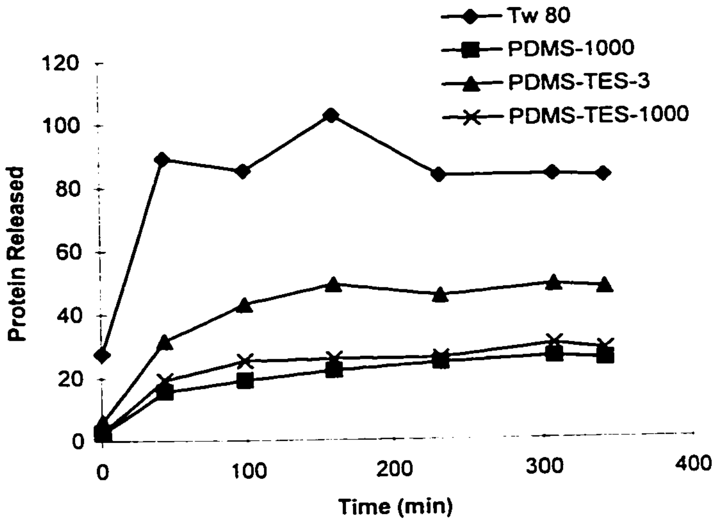


Figure 6: Effect of Microparticle Coating on Protein Release

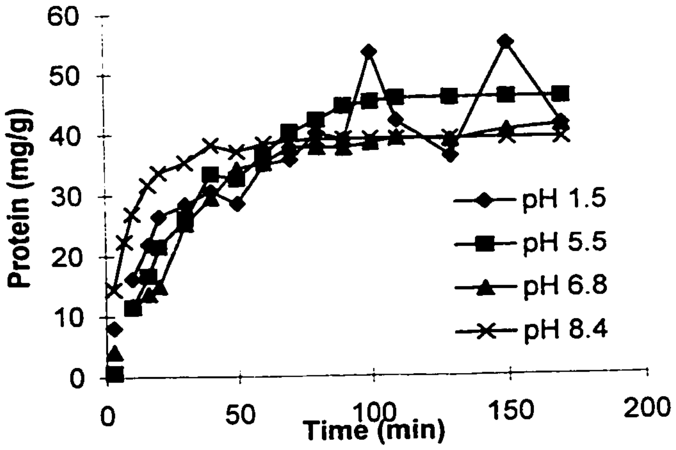


Figure 7: pH Dependence of Protein Release From Microparticles [a]

[a] The weights of the microparticles MP-PDMS-TES-1000 in the pH experiments were pH 1.5 30.8 mg, pH 5.5 29.1 mg, pH 6.8 0.0296 g and pH 8.4 29.0 mg, respectively, in 30 mL aqueous solution.

Effect of pH

Since the stability of both the protein and the silicone are affected by pH,[22] the microparticles were exposed to aqueous environments over a relatively wide pH range. The results are shown in Figure 7 and show surprisingly little effect of the pH on the protein release.

Immunological Results

As an independent means of examining the protective effect of the silicone on the starch surface, the *in vivo* immunological effect of the protein released from the silicone/starch microparticles was examined. Thus, the microparticles coated with PDMS and PDMS-TES, respectively, were introduced to mice via oral administration.[28] Animals were immunized with various doses of HSA: i) incorporated in PDMS-TES grafted, ii) PDMS-coated microparticles or, iii) in 0.2 M $NaHCO_3$. Intestinal gut washes obtained on day 28, 42 and 63 were evaluated for the presence of anti-HSA IgA using an ELISA assay. The results for oral inoculation are summarized in Finger 8. They clearly show a difference in the bioactivity of the HSA in particles made from PDMS-TES which elicited antibodies, and those made from normal PDMS which had little or no activity.

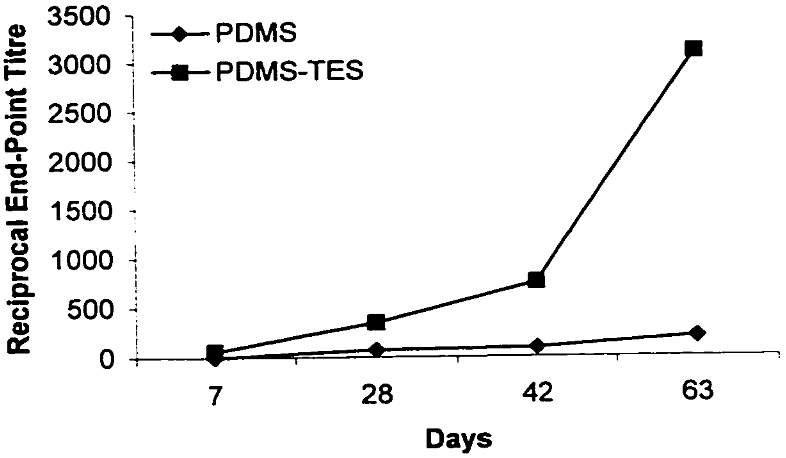


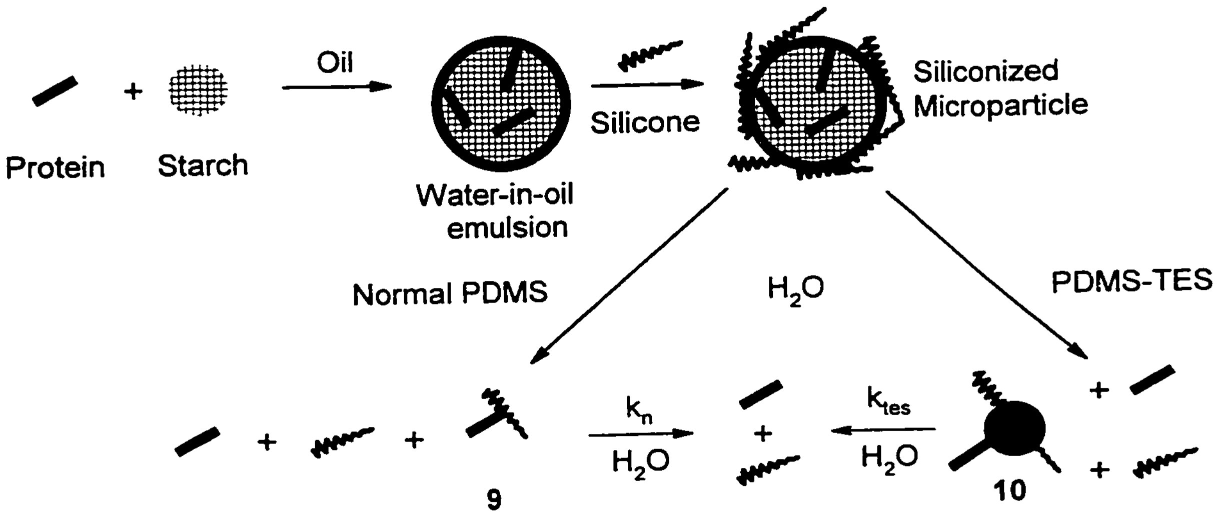
Figure 8: Antibodies raised to HSA following IG immunization with silicone-coated starch microparticles

Discussion

Nature of the Particles

In the initial stages of preparation of the microparticle, a homogeneous solution of water, DMSO, HSA and starch was dispersed in vegetable oil to give a water-in-oil emulsion. Based on the relative quantities of reagents used in the experiment, it was expected that the dominant interaction would be between the starch and silicone. However, it was not possible to make silicone-coated particles, using either silicone, in the absence of protein. Thus, in addition to its anticipated function of a marker, HSA is clearly playing another role.

Of the components present in the emulsion, the protein is best able to stabilize the oil-water interface.[29] As a consequence of their polar and hydrophobic pendant groups, proteins have surfactant properties and are frequently used as emulsifiers.[30] Upon exposure of the aqueous droplet to acetone/silicone, the HSA exposed at the oil-water interface can interact with the silicone polymer (Scheme 4). We attribute the ability to make stable microparticles, only in the presence of protein, to the rapid establishment of a silicone-protein interaction; starch itself cannot react/interact with the silicone sufficiently quickly to stabilize the particle droplet prior to aggregation. At the same time, the protein is strongly interacting with the starch, presumably through hydrophilic interactions (e.g., hydrogen bonding). There is no evidence that the protein leaches from the starch into the silicone phase. Thus, the protein is acting to glue the silicone and starch to each other.



Scheme 4

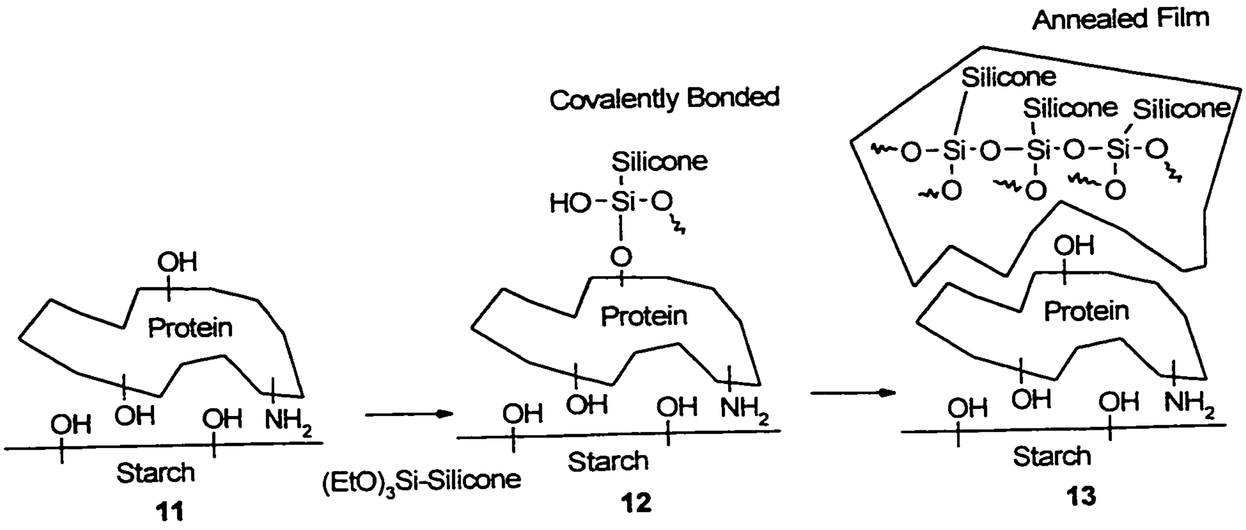
PDMS

The microparticles coated with PDMS cannot form a covalent bond between either biopolymer and silicone. Instead the silicone, known for low surface energy,[31] can wet out the starch:protein surface.[32] From the amount of silicone initially associated with the microparticles and the data from the extraction with $CHCl_3$ (Figure 4), it is clear that the initial layer is quite thick. Hydrolytic challenge, in which the microparticles were exposed to a water droplet (contact angle measurement) or suspended in bulk water, showed that the protein was, however, able to escape from the microparticle.

There are two possible types of protein: those in the particle core and those that have been exposed to silicone (**9**, Scheme 4). It has not yet been possible to distinguish between these two types of proteins; both could lead to the observed UV absorbances and ^1H NMR signals. However, the mouse immunoassay shows that viable protein is not present (or present in insignificant amounts) after oral administration, as evidenced by the absence of a significant immune response. This suggests that if there is a protein-silicone or starch-silicone association, it is insufficiently strong to survive the relatively brutal conditions found in the gastrointestinal tract; free and silicone-associated proteins are degraded: the rate of complete separation of the two polymers k_n is relatively high when compared with PDMS-TES. PDMS thus acts to provide an efficient, but only temporary, hydrophobic barrier to the protein/starch microparticle.

PDMS-TES

The comments made above are equally valid for the functionalized silicone. However, in addition to the relatively weak association between protein and silicone, a covalent bond between the two polymers can arise (**10**, ●, Scheme 4) via transesterification or, less likely, transamination processes (**11→12**, Scheme 5). To the extent that such bonds form, they will provide an additional stability to the interface between the silicone and protein. Alternatively, or subsequently, such protein-silicone bonds may hydrolyze to give a physically adhering, crosslinked silicone film (**12→13**, Scheme 5). The observed extraction, contact angle, and protein release phenomena can be explained by a combination of the factors described for PDMS and this additional bonding: the ultimate hydrolysis rate k_{tes} leading to exposed protein is much slower than for unmodified PDMS.



Scheme 5

In the experiments we have undertaken, starch was not hydrophobized by either PDMS or PDMS-TES. In the absence of protein, the interactions between starch and silicone were either insufficiently stabilizing, or so kinetically slow to form that silicone-coated starch microparticles could not be made. However, the protein clearly stabilizes the starch surface. With PDMS-TES in particular, the protein only slowly leaches from the starch. In turn, the protein is protected from the outer environment by PDMS, and, more efficiently, by PDMS-TES as shown most compellingly by the antibody production after oral administration. Finally, after removal of the acetone the starch particles are readily suspended in hydrocarbons forming non-aqueous dispersions. Thus, in a colloidal sense, we believe the silicone outer layer acts as a steric stabilizer for the microparticles.[33,34] The combination of these complimentary interactions, starch:protein, protein:silicone and the overall stabilization of the microparticles by silicone, is something we hope to exploit in the future. To do so, we will need to more clearly establish the nature of the protein silicone interaction at a molecular level by examining different silicone and protein systems.

Conclusion

In the presence of HSA, silicones hydrophobize starch surfaces. This involves a sandwich structure in which a silicone/protein layer protects the starch surface. Increasing the thickness of the silicone layer or the silicone molecular weight affords better protection to the microparticle. Functionalized (PDMS-TES) and unfunctionalized (PDMS) silicones behave remarkably similarly in physicochemical analyses that, at first glance, would suggest similar protein/silicone and protein/starch interactions in both cases. However, their behavior in a biological environment is quite different. Oral administration of the microparticles to mice demonstrated that the proteins associated with PDMS-TES retain antigenicity (or possess enhanced antigenicity) while those associated with PDMS do not. This suggests a stronger interaction between the two polymers in the former case, involving either a covalent bond between the two polymers or a physically adhering film. It is not yet possible to assess the strength of the interaction between the silicone/protein layer and the starch. However, it is clear that this layer provides surface protection to the starch.

Acknowledgments

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Chapter 3

Protein-Silicone Interactions: How Compatible Are The Two Species?

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• 5% error bars have been added to XPS graphs

Abstract

Protein-on-silicone and silicone-on-protein films were made by the sequential coating of the human serum albumin (HSA) onto silicone films on glass or vice versa. The silicones used were either trimethylsilyl-terminated poly(dimethylsiloxane)(unfunctionalized PDMS) or triethoxysilylpropyl-terminated poly(dimethylsiloxane)(functionalized TES-PDMS). Angular Dependent X-ray Photoelectron Spectroscopy (AD-XPS) and contact angle measurements (CA) were used to characterize the modified surfaces. Irrespective of the order of building the films, protein-on-silicone or silicone-on-protein, both showed essentially identical surface compositions suggesting a significant degree of mixing between the protein and silicone. The TES-PDMS was found to have a greater affinity for HSA: thicker and more homogenous silicone films were found with TES-PDMS/HSA than with PDMS/HSA films.

Introduction

The exposure of a protein solution to a solid surface results in spontaneous adsorption at the solid/liquid interface.[1] This tendency of proteins affects many natural and synthetic processes and therefore has attracted attention in various biological, medical, and technological fields (i.e., biofouling, thrombus development, emulsions).[2] Protein adsorption is the net result of the interactions between the protein molecules, the solvent and the sorbent surface. In addition to these intermolecular interactions, intramolecular forces within the protein macromolecule are important. When a protein interacts with a surface, structural rearrangements in the protein macromolecule and dehydration of the protein may occur, leading to denaturation and subsequent modification of the protein bioproperties. In particular, when the surface is hydrophobic, such as a silicone surface for example, there is evidence that proteins undergo structural rearrangements at the solid silicone/water interface.[3]

The adsorption of silicone polymers (polydimethylsiloxanes) from dilute solutions on solid surfaces has also received considerable attention.[4,5] Polydimethylsiloxanes (PDMS) can interact with substrates both through dispersion forces from the induced dipoles in the methyl groups and through permanent dipoles in the partially polar siloxane backbone. Thus, PDMS will primarily interact with polar substrates through the siloxane backbone, and with non-polar substrates through the methyl groups. The backbone flexibility of silicones allows them to adjust to the availability of reactive sites on surfaces.[7] End-functionalized siloxanes (e.g., NH_2, COOH, OH, alkoxy, epoxy), are additionally able to interact through the end groups. These siloxanes have received attention as macromolecular coupling agents since strong end group interactions with the substrate can result in greater control and reproducibility of surface modifications, while taking advantage of the hydrophobic properties of the siloxane polymers.[5-9]

Silicones have been extensively used as materials for medical applications. Numerous biomaterials used for prostheses (e.g., breast implants, finger joints, etc.) and devices for the controlled release of drugs are silicone based compounds.[10] This is mainly due to their good biocompatibility.[11] However, there have been many discussions concerning the risks that such devices may pose, such as harmful immune reactions. For instance, it has been suggested that silicones (gel, not oil[12]) introduced into the body (e.g., via a prosthetic device) have the ability to enhance the immune response of proteins (adjuvant activity), suggesting that a protein-silicone interaction yields an immunogenic moiety, [13] although the proposition has come into question.[14] A better understanding of the interaction of silicones with substances present in the body is, thus, an important focus of research.[15]

In a recent study, starch/protein microparticles that were surface modified with silicone polymers were shown, in mice, to elicit antibodies upon oral administration unlike the unmodified protein (Human Serum Albumin, HSA).[16] In the study it was shown that only the use of triethoxysilylpropyl-terminated silicone (TES-PDMS, $(EtO)_3Si(CH_2)_3SiMe_2(OSi\ Me_2)_nOSiMe_2(CH_2)_3Si(OEt)_3$) resulted in the generation of antibodies: the use of unfunctionalized PDMS ($Me_3Si\ (OSi\ Me_2)_nOSiMe_3$) resulted in no immunological effect. The role of the silicone and the nature of its interaction with the protein was not established during the course of these studies. Although physicochemical analysis suggested similar protein:silicone interactions for both PDMS and TES-PDMS: no specific effect could be found to account for the enhanced antigenicity observed only for the functionally-terminated silicone.[17]

In view of these observations, the objective of this research was to study the nature of the interaction between both types of silicone and HSA. Covalent bonding or physical adhesion between the silicone and protein could both serve to provide a hydrophobic barrier that might protect the protein on its passage through the gut. We have investigated these possibilities by studying the behavior of protein-silicone composites under a variety of hydrolytic conditions similar to those found in the body. The results of these experiments are presented in a following paper. Herein we report the preparation and characterization of model silicone-protein films prepared with both unfunctionalized and functionalized silicone polymers.

Experimental Section

Materials

Microscope slides (1 mm, 25x75 mm, pre-cleaned) and cover glass slides (18 mm^2; N° 1½) were obtained from Corning or Fisher. Human Serum Albumin (HSA, MW~67,500; Sigma) was obtained as a powder and was dissolved in phosphate buffered saline solution (0.1 M NaCl, 0.086 M, KH_2PO_4, pH=7.2). γ-Aminopropyltrimethoxysilane (Aldrich)[18] was distilled prior to use. 3-Triethoxysilylpropyl-terminated polydimethylsiloxane (1,000 cs, MW~28,000) was prepared as previously described.[16] Me_3Si-terminated polydimethylsiloxane (1,000 cs, Dow Corning), glutaraldehyde (25 wt% aqueous solution, BDH), anhydrous ethanol or methanol (Aldrich), triethylamine (Fisher), and diethyl ether (Caledon) were used as provided.

Instrumentation

A Picotron-radio frequency plasma cleaner was used for cleaning the glass surfaces.[19] Static (advancing) contact angle, θ, of a sessile drop of distilled water was measured by using a NRLCA Goniometer (Ramé-Hart Inc.). Low resolution XPS spectra were obtained on a Leybold MAX 200 XPT system.

Methods

Preparation of protein-silicone films

Silicone on Protein Films

Protein-silicone surfaces were prepared by stepwise modification of glass surfaces (microscope slides). Before modification, the slides were cleaned of any adsorbed contaminants in a Picotron-radio frequency plasma cleaner under an argon atmosphere for 10 min. After this treatment, the contact angle of the surface was <5°. When no pretreatment was applied, the contact angle of the surface varied up to 20°.

Preparation of silicone-on-protein films involved chemical immobilization of the protein on the support and immersion of the protein modified surfaces into silicone solutions. The protein immobilization was achieved by silanization of the glass slides, coupling of a linker/spacer molecule, and finally, coupling of the protein to the linker.

Silanization. The clean slides were immersed for 30 min into a 1-2% (v/v) γ-aminopropyltrimethoxysilane (APTS) solution in anhydrous methanol,[18] containing triethylamine (1-2% v/v). The slides were rinsed off with methanol and dried by heating at 80 °C for 20 h. These slides are referred to as A slides for γ-aminopropyltrimethoxysilane.

Activation for protein coupling: The A slides were immersed into the 2.5% (v/v) glutaraldehyde solution in phosphate buffered saline (PBS) for 1 h and rinsed off with PBS (Nomenclature: AG slides). Immediate coupling of the protein followed.

Protein coupling: Coupling to protein (HSA) was achieved by incubation of the modified support (AG) with the protein in PBS solution (7 mg/mL: series-I; 1 mg/mL series-II) for 1 h at room temperature. The slides were rinsed off with the PBS and dried at room temperature. These slides are referred to as AGH slides.

Silicone deposition: The protein modified slides (AGH) were coated either with TES-PDMS or Me_3Si-terminated PDMS 1% (v/v) solutions in diethyl ether, by immersion of the slides into the silicone solutions for 1 h. The slides were rinsed off with ether and dried at room temperature for at least 20 h. These slides are referred to as AGHtP (for TES-PDMS) and AGHP (for PDMS), respectively.

Protein on Silicone Films

The preparation of protein-on-silicone films involved physical immobilization of the silicone to the support and immersion of the silicone modified surfaces into aqueous protein solutions.

Silicone deposition: The clean glass slides were initially coated either with TES-PDMS or Me_3Si-terminated PDMS by immersion of the slides into the diethyl ether solutions of the above silicones for 1 h. The slides were then rinsed with the solvent and dried at room temperature for at least 20 h. These slides are referred as tP (for TES-PDMS) and P (for PDMS), respectively.

Protein adsorption: Protein was adsorbed on silicone modified slides by immersion of the P and tP slides into separate protein (in PBS) solutions (1 mg/mL) for 1 h. The slides were rinsed with the buffer and dried at room temperature for at least 20 h before characterization. These slides are referred to as tPH and PH, respectively.

Surface Characterizations

Contact Angle (CA)

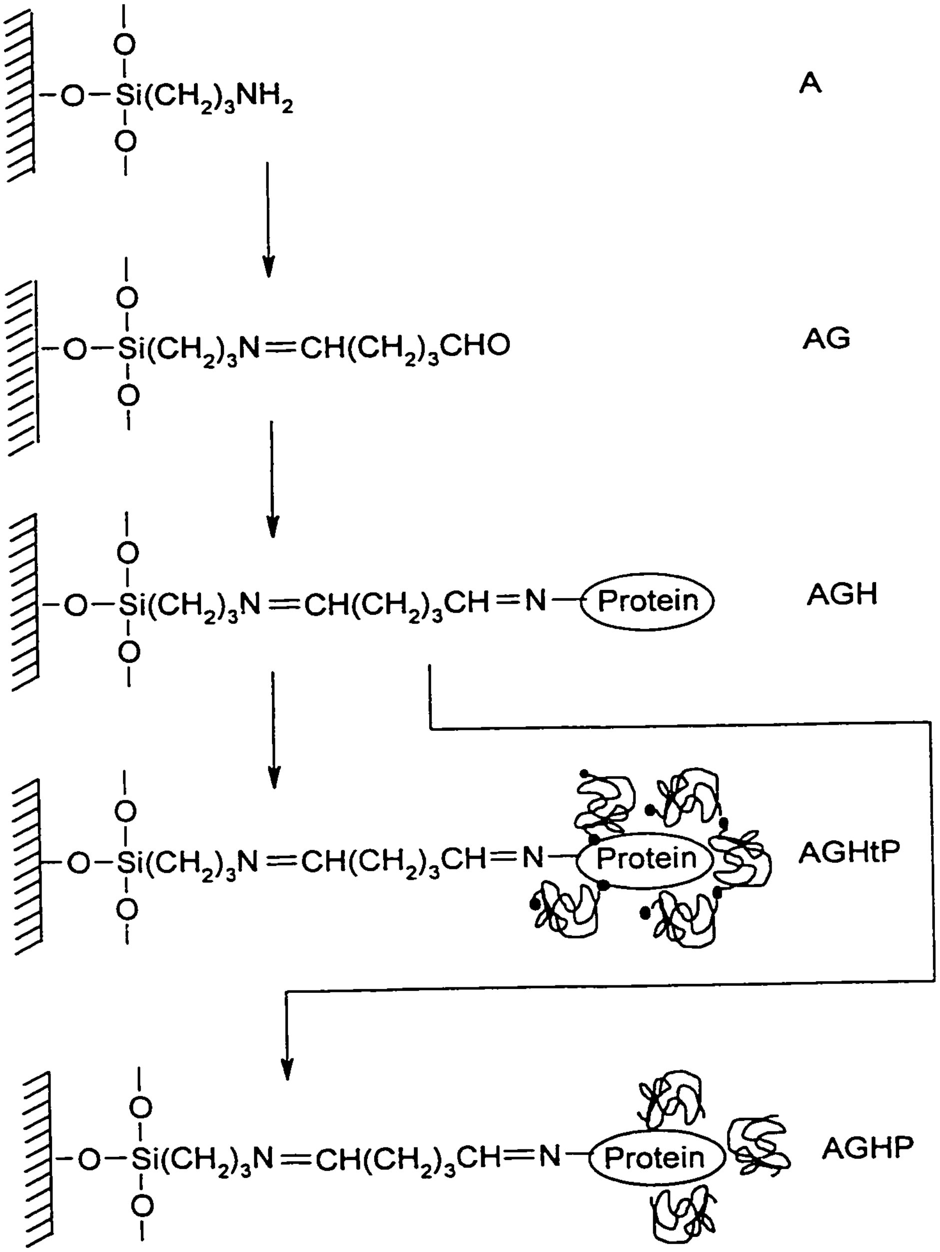
The hydrophobicity of the modified surfaces was probed by measuring the static (advanced) contact angle, θ, of a sessile drop of distilled water at room temperature (~22 °C). Each reported value for θ is the average of at least ten independent measurements at different locations on the surface.

Angular Dependent X-ray Photoelectron Spectroscopy (AD-XPS)

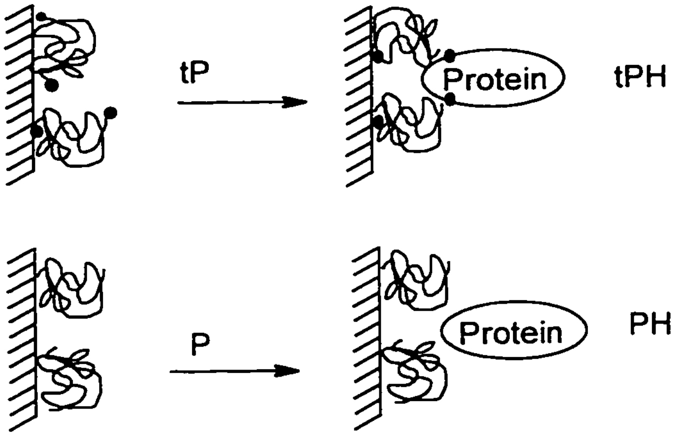
Unmonochromatized Mg K_α X-ray radiation was used as the excitation source. The source was run at 15 kV and 20 mA. Features in the resultant spectra due to excitation from the weaker X-ray satellite lines, which are also present in the non-monochromatic source, were subtracted by use of an algorithm supplied with the instrument.[20] Atomic percentages of the elements present were derived from spectra run in a low-resolution mode (pass energy=192 eV) which were normalized to unit transmission of the electron spectrometer by means of a routine provided by the manufacturer.[21] The sensitivity factors used (O 1s = 0.78, N 1s = 0.54, C 1s = 0.34, Si 2p =0.40) were empirically derived by Leybold for the normalized spectra. Binding energies and peak areas were obtained by the use of the standard provided with the spectrometer. The energy scale of the spectrometer was calibrated to the Ag $3d_{5/2}$ and Cu $2p_{3/2}$ peaks at 368.3 eV and 932.7 eV, respectively, and the binding energy scale was then shifted to place the C 1s feature present at 285.0 eV. Large-area analysis (2x4 mm or 4x7 mm) was performed so that exposure of the samples to the X-ray would be minimized while sufficient signal to noise ratios could be obtained for the spectral features.

Results

Two different types of model surfaces were prepared; a silicone-on-protein and a protein-on-silicone surface. In the first type, the protein was immobilized on a glass surface and then exposed to both functionalized (TES-PDMS) and unfunctionalized (PDMS) silicone to give AGHtP and AGHP slides, respectively. The protein immobilization involved three steps; a) silanization of the glass surface by aminopropyltrimethoxysilane (A slides),[18] b) activation of the amino functionality with glutaraldehyde (AG slides), and c) coupling of the protein (AGH slides)(Scheme 1). Protein-on-silicone surfaces were prepared by allowing two different silicone modified glass surfaces (P and tP slides) to interact with protein solutions to give PH (for PDMS) and tPH (for TES-PDMS) slides, respectively (Scheme 2). Based on the preparation methods followed, the chemical structures of the successively modified surfaces were expected to be those shown in Scheme 1 and Scheme 2.



Scheme 1: Silicone-on-protein film formation on glass slides. The silicone polymer is represented by a free line and the functional group (TES) by a dot. Grafting/crosslinking is implied when dots join two lines.



Scheme 2: Protein-on-silicone film formation on glass slides.

In principle, the build up of layers on a substrate can be followed by the changes in the surface properties and elemental composition of the surface layer in comparison with that of the substrate. Two methods were used to characterize these surface layers. Contact angle values were used as a measure of the surface hydrophobicity as each layer was added to the surface. Detection of the changes of the relevant atomic elemental percentages (rel.at.%) of the surface is an even more straightforward way to confirm the degree and type of material deposited on the surface. This was done using XPS.[22] An additional advantage with XPS is the possibility of depth profiling the surface layer by varying the detection take-off angle of the ejected electrons (AD-XPS). In a multi-step surface modification process, changes in the elemental composition as a function of distance should reflect the order of layer deposition. As the sampling depth is increased, the detected elemental composition should correspond to the earlier modification steps.

For the films under discussion, the deposition of a silicone layer on a protein substrate, for example, should involve an increase in the CA value, minimization of the nitrogen content, and a significant increase in the silicon content. The opposite should apply for a protein layer on a silicone substrate. The results of the CA measurements and the elemental analysis of the layer by layer build up of the films we prepared are discussed below.

Contact angle: To a large degree, the observed CA values of the sequentially modified surfaces (Table 1) reflect the expected hydrophobicity/hydrophilicity. For example, the silicone surfaces (tP, P slides) exhibited higher CA values than the protein surfaces (AGH slides). However, remarkable variations (large standard deviations) were observed even for surfaces obtained by a single modification step (i.e., P, or tP slides). The protein surfaces, in particular, exhibited large CA variability. The CA values reported for protein-on-silicone and silicone-on-protein slides lie in an "intermediate" area; lower than that of the silicone but higher than those of pure protein films (AGH slides).

Table 1: Average CA of modified surfaces (Series I)

Slides	Average Contact Angle (°)	Standard Deviation
A	66.2	4.7
AGH	56.9	15.2
P	94.8	3.4
tP	95.1	4.3
AGHP	64.5	7.3
AGHtP	76.5	6.7
tPH	69.8	8.5
PH	55.0	13.1

Angular Dependent X-ray Photoelectron Spectroscopy: To follow the build up of layers, the increase or decrease of the rel.at.% of the characteristic element for each layer was observed after each modification step; APTS and HSA depositions were indicated by an increase in N% (Figure 1), the glutaraldehyde coating by an increase in C%, and the silicone coatings by the increase in Si% (Figure 2). The changes in elemental composition were determined as a function of distance from the film/air interface in order to appreciate the depth of each deposited layer. The elemental composition of a homogeneous layer (of up to 80 Å) should show no angular dependence. The results of the Angular Dependent-XPS analysis are summarized in Table 2.

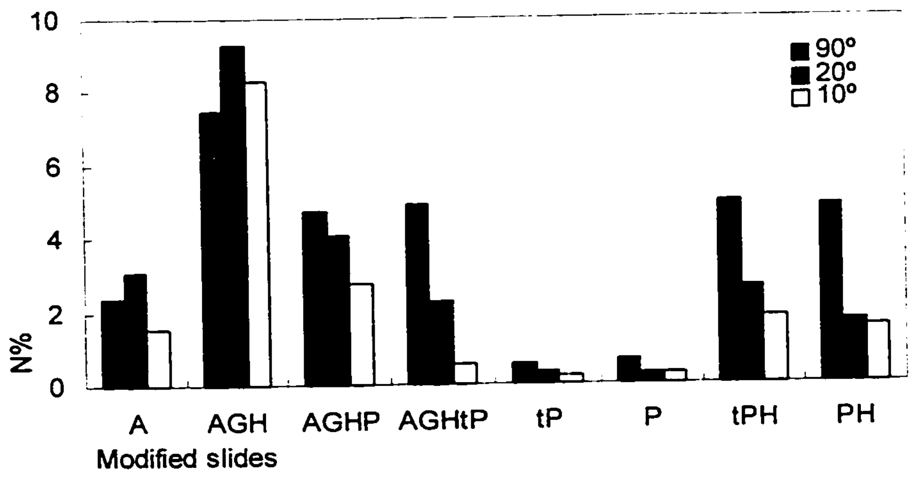


Figure 1: AD-XPS of modified slides; nitrogen content

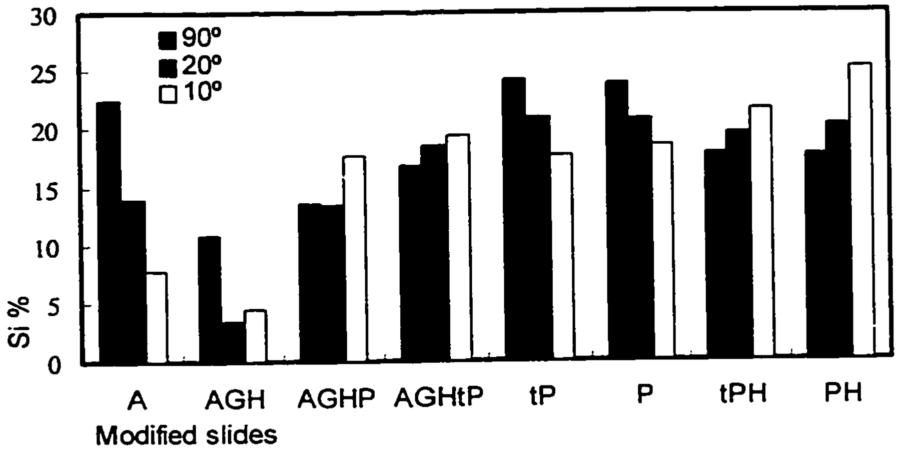


Figure 2: AD-XPS of modified slides; silicon content

Table 2: AD-XPS of modified slides (Series II)

SLIDES	ELEMENTAL COMPOSITION						
	O%	N%	C%	Si%	C/Si	C/N	Si/N
<u>90° take-off angle</u>							
A	51.7	2.4	17.2	22.5	2	7	9
AGH	34.2	7.5	40.3	10.7	3	5	1
AGHP	37.3	4.7	35.6	13.6	3	8	3
AGHtP	30.7	4.9	44.4	16.9	2	9	3
tP	49.8	0.6	19.7	24.3	2	35	43
P	58.4	0.7	10.6	23.8	2	16	35
tPH	33.8	4.9	40.1	17.9	2	8	4
PH	32.9	4.8	41.4	17.6	2	9	4
<u>20° take-off angle</u>							
A	28.2	3.1	46.0	13.9	2	15	4
AGH	20.8	9.3	61.7	3.5	6	7	0
AGHP	23.3	4.1	54.8	13.3	2	13	3
AGHtP	22.9	2.2	54.9	18.4	1	24	8
tP	32.4	0.3	43.3	20.8	2	131	63
P	34.6	0.3	41.4	20.8	2	159	80
tPH	24.4	2.6	52.4	19.6	1	20	7
PH	23.2	1.7	53.8	20.3	1	31	12
<u>10° take-off angle</u>							
A	17.5	1.6	51.6	7.7	2	33	5
AGH	18.3	8.3	62.6	4.4	4	8	1
AGHP	22.4	2.7	53.1	17.6	1	20	6
AGHtP	22.9	0.6	54.5	19.4	1	99	35
tP	25.8	0.2	53.2	17.6	1	221	73
P	24.4	0.3	54.8	18.4	1	196	66
tPH	23.7	1.8	52.1	21.6	1	29	12
PH	21.2	1.5	50.9	25.2	1	33	16

Table 3: Theoretical Relevant Elemental Composition

SLIDES	C%	N%	O%	Si%	C/Si	Si/N
Silicone	50		25	25	2	
HSA	70	15	15			0
Glutaraldehyde	70		30			
APTS	50	16	16	16	3	1
SiO_2 (Glass)			67	33	0	

The changes in elemental composition are qualitatively in line with the expected trend, as shown in Figure 1 and Figure 2. The addition of protein and then silicone to an

APTS surface (A → AGH → AGHP (AGHtP)) leads to a change in the elemental composition at the uppermost surface (10° take-off angle). The protein modified surface (AGH) is nitrogen rich and silicon poor, a trend that is reversed after the silicone is applied to the protein surface. The surface (10° take-off angle) of films formed in the opposite order (P (tP) → PH (tPH)) became nitrogen richer and silicon poorer after the protein deposition.

Depth profiling of the elemental composition starting from the film/air interface also showed the expected trends. Profiling of the A slides showed a gradual increase of the Si% and O% (glass elements) from 8% to 22% and from 17% to 52% , respectively, as the sampling depth was increased. The most dramatic change in composition of the A slide was observed at a 20° take-off angle indicating a thickness of *ca.* 30 Å for the APTS layer. The AGH slides showed a slight N% decrease from 8-9% to 7% and significant Si% increase from 4% to 11% with increasing take-off angle. Based on the angular dependence of the silicon content, the thickness of the protein layer is also estimated to be *ca.* 30 Å. The AGHP and AGHtP slides showed an increase of their N% from 1-3% to 5% and a slight decrease in the Si% with increasing take-off angle (as one probed deeper into the built up layer from the outer surface). The angular dependence of the silicon content is also indicative of a silicone layer thickness of at least 30 Å. Finally, the PH and tPH slides showed an increase in Si% from 18% to 22-25% and a decrease of N% from 5% to 2% as the take-off angle is decreased.

The primary focus of this study was the silicone/protein interface. The Si%/N% ratios of the modified slides should best describe the transition from one layer to the other. The angular dependence (take off angle of the XPS electrons) of the Si%/N% ratio should be indicative of the layer deposition order. Thus, the protein-on-silicone and the silicone-on-protein surfaces should exhibit inverse Si%/N% angular dependence. For instance, a large value of Si%/N% at lower take-off angles (sampling closer to the air interface) should be observed only for the silicone-on-protein and not for the protein-on-silicone surfaces. Surprisingly, however, *all protein/silicone surfaces exhibited analogous angular dependence*, with Si%/N% ratios increasing with the approach to the film/air interface. The same Si%/N% angular dependence was observed whether the ultimate layer was consisted of silicone (silicone-on-protein) or protein (protein-on-silicone), as shown in Table 2.

There was a sharp distinction between the films formed from PDMS and TES-PDMS. This could most clearly be seen at the 10° take-off angle for the silicone-on-protein surfaces (Table 2). The AGHtP slide showed a substantially increased Si%/N% ratio of 35 while the corresponding AGHP slide had a Si%/N% ratio of only 6. On these slides, at the same take-off angle, the C/N ratio also differed, being 99 for the former and 20 for the latter.

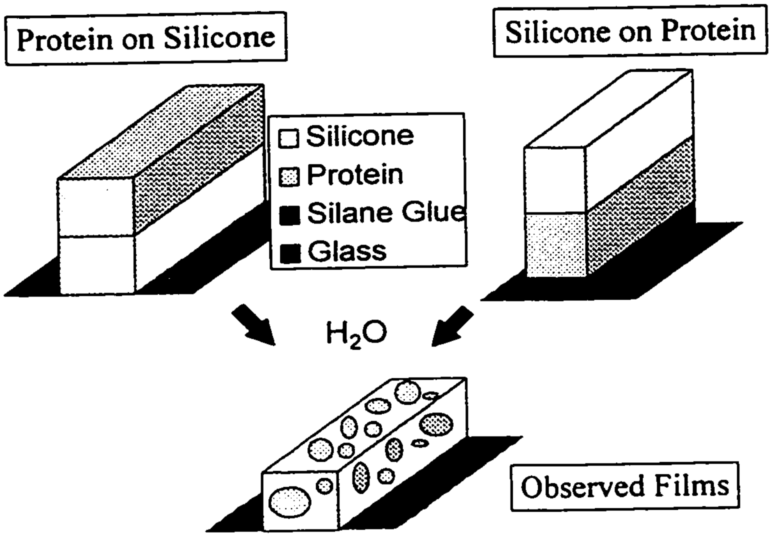
Discussion

The stepwise modification of the surface was accompanied by changes in both surface hydrophobicity (CA measurements) and the elemental composition (AD-XPS). To a degree, the results of both techniques were in qualitative agreement with the expected changes induced by the applied depositions. However, deviations from our expectations were observed. A comparison between Table 2 and Table 3 shows the difference between observed and theoretical surface elemental compositions. Moreover, the angular dependence of the elemental composition did not always indicate distinct boundaries (interfaces) between the different layers or follow the expected changes in elemental composition.

The large CA standard deviations can be attributed to a number of factors which depend on the nature of the layer deposition. For instance, the heterogeneity of the protein modified surfaces may be due to the protein adsorption in a variety of orientations and/or conformational states possibly leading to formation of distinct hydrophilic and hydrophobic surface areas.[23] Similarly, the physisorption of the silicone on glass can be inhibited by the presence of a significant amount of surface water and heterogeneous or non-complete silicone deposition is possible.[24] The surface water on these slides was not quantified.

The most surprising deviations, however, occurred with the protein-on-silicone and silicone-on-protein slides. It was anticipated that the two systems would exhibit distinctly different behaviors at the air:liquid(solid) interface (protein *versus* silicone).

However, both exhibited the same angular dependence in the elemental distribution, and comparable CAs, albeit with large standard deviations. These results indicate that both the modified surfaces are heterogeneous and/or are comprised of patchy protein and/or silicone surface layers. Although the presence of the glass substrate at the air interface cannot be excluded, these results are more likely reflective of a significant degree of mixing between the silicone and protein layers (Scheme 3).



Scheme 3: Mixing of Silicone and Protein Layers

The intermixing of protein and silicone films could have several different origins including: i) a preference of silicone for the air interface, ii) a preference of the protein for the glass surface, and/or, iii) mixing due to favorable interactions between the two polymers. Silicones certainly possess the flexibility and mobility necessary to migrate to the surface through a protein layer in order to minimize their surface energy.[25] Such migration would be particularly aided in the case of non-chemically immobilized silicones (all films derived from unfunctionalized PDMS ("P"slides)). In addition, the HSA and silicones may have a reasonable affinity for each other in that the surface of protein aggregates is stabilized by hydrophobic interactions with the silicones. Further examination of this question will be the focus of future investigations.

The two silicones used in this study behaved quite differently towards the protein. In the silicone-on-protein slides, the AGHtP slides showed a substantially increased Si%/N% ratio at the film/air interface by comparison with the corresponding AGHP slides. Moreover, the latter slides exhibited a higher C%/Si% ratio. Both of these observations are consistent with the formation of a thinner or more patchy silicone film when PDMS was used. The more homogeneous deposition of the TES-PDMS on the protein substrate suggested that the TES-PDMS interacts to a greater extent with the protein.

A stronger interaction of TES-PDMS should also be evident (silicone-type differentiation) from the protein-on-silicone films. These should exhibit lower Si%/N% ratio for the tPH slides when compared with the PH slides. Although this was not observed, it does not contradict the previous reasoning. It is possible that the TES-PDMS polar groups were oriented towards the polar glass substrate and reacted, like normal coupling agents,[9] with the glass Si-OH groups TES-PDMS prior to addition of the protein (Scheme 3).[4]

In the fabrication of microparticles as potential oral vaccines, PDMS did not serve to protect the protein whereas TES-PDMS did.[16] One goal of this study was to attempt to find characteristic differences between PDMS and TES-PDMS that would help explain these results. It is clear from the work presented above that both types of silicone interact or mix with HSA. However, TES-PDMS clearly has a greater affinity for the protein than PDMS as evidenced by the thicker, more homogeneous film which it made in combination with HSA. In the accompanying paper, we examine the hydrolytic stability of the two different protein:silicone systems to determine if this affinity translates into greater hydrolytic stability of the mixed materials.

Conclusion

Surface characterization by CA measurements and AD-XPS analysis could be used to characterized the various layers utilized to build up protein-on-silicone and silicone-on-protein films. The elemental rel.at % observed with XPS did not indicate pure layers with sharp boundaries between each other. Formation, rather, of 'mixed' layers between the protein/silicone films accounted for the observed surface properties and compositions. In particular, it seems that the silicone migrates to the film/air interface: even after coating a silicone layer with a protein, the protein becomes buried or mixed in the silicone layer and the air interface is silicone-rich. The elemental composition obtained for the silicone/protein slides was found to be similar at the film/air interface, irrespective of the order of deposition of the protein or silicone layers on the surface. TES-PDMS was found to have a stronger affinity for the HSA which may explain the enhanced antigenicity of protein microparticles when this silicone is a constituent.

Acknowledgments

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Chapter 4

Silicone-Protein Films: Establishing the Strength of the Protein-Silicone Interaction

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Abstract

Protein-on-silicone and silicone-on-protein films were prepared using either trimethylsilyl- (unfunctionalized, PDMS) or triethoxysilylpropyl-terminated (functionalized, TES-PDMS) silicones and human serum albumin (HSA). The films were exposed to aqueous solutions of different pH or containing SDS. X-ray Photoelectron Spectroscopy (XPS) and contact angle measurements (CA) were used to characterize the resulting surfaces. The film formed by coating a protein surface with TES-PDMS showed much slower protein desorption kinetics than the other slides upon challenge by SDS, acidic, neutral or basic aqueous solutions. This observation may be understood to arise from the crosslinking of TES-PDMS using the protein as a template, resulting in intimate contact between the two polymers.

Introduction

Knowledge of the degree and kinetics of protein desorption is important in applications such as the formulation of oral drug delivery systems, where the drug in question is a proteinaceous material.[1] It is desirable in these applications to control the rate/degree of protein release at gastric and/or intestinal pH's. When proteins are embedded within a polymer matrix, their release will depend upon diffusion through the matrix, which in turn is affected by the chemical potential exerted by the exterior solution. When the protein is, additionally, covalently bound into the matrix (the protein complex is a prodrug[2]), the protein release will also depend on certain physiological conditions that affect the bond cleavage such as pH-dependent hydrolytic cleavage, enzyme-catalyzed hydrolysis, etc. The retention of the biological activity of the protein is a crucial requirement that should be met by any viable delivery system.

In a recent study, starch/protein microparticles modified with silicone polymers, developed as prototypes for oral vaccines, were shown to elicit antibodies upon oral administration to mice unlike the unmodified protein.[3] The nature of the silicone was very important for the biological activity. Thus, microparticles modified with functionalized silicone (TES-PDMS, $(EtO)_3Si(CH_2)_3SiMe_2(OSiMe_2)_nOSiMe_2(CH_2)_3Si(OEt)_3$) elicited antibodies whereas those coated with unfunctionalized dimethylsilicone (PDMS, $Me_3Si(OSiMe_2)_nOSiMe_3$) led to no observable biological effect. To account for these observations, it was suggested that the protein was protected from the acidic gastric environment in the case of the TES-PDMS-modified microparticles.[3] Subsequently, the protein could be released from the matrix in the higher pH conditions of the intestine, retaining its biological activity throughout. This protection was proposed to result from a covalent protein-silicone linkage or physical adhesion of a silicone rubber film to the protein. The presence of the silicone, it was further noted, could enhance the antigenicity of the protein.

In order to establish the validity of these proposals, we developed a model system of protein-silicone films from which protein desorption could be measured. Surface analysis of this system, which is described in detail in the accompanying paper,[4] confirmed that there is a silicone-protein interaction that seems to be stronger when end-groups other than trimethylsilyl are attached to the silicone backbone.

In the present study, we have extended this work to examine the nature of the silicone-protein interaction under different pH conditions, notably those to be found in the gastrointestinal tract. We have also examined the displacement of protein from the films in the presence of an excellent surfactant, sodium dodecyl sulfate (SDS). Surface techniques (contact angle goniometry (CA), X-ray photoelectron spectroscopy (XPS)) allowed the measurement of the degree to which protein desorption occurred from silicone-on-protein and protein-on-silicone films, using the two different types of silicone, PDMS and TES-PDMS, respectively.

Experimental Section

Materials

Microscope slides (1 mm, 25x75 mm, pre-cleaned) and cover glass slides (18 mm^2; N° 1½) were obtained from Corning or Fisher. Human Serum Albumin (HSA, MW~67,500; Sigma) was obtained as a powder and was dissolved in phosphate buffered saline solution (0.1 M NaCl, 0.086 M, KH_2PO_4, pH=7.2). γ-Aminopropyltrimethoxysilane (Aldrich) was distilled prior to use. 3-Triethoxysilylpropyl-terminated polydimethylsiloxane (1,000 cs, MW~28,000) was prepared as previously described.[3] Me_3Si-terminated polydimethylsiloxane (1,000 cs; Dow Corning), glutaraldehyde (25 wt% aqueous solution; BDH), anhydrous ethanol and methanol (Aldrich), triethylamine (Fisher), and diethyl ether (Caledon) were used as provided.

The solutions used for the slide treatments were: a) dilute HCl (BDH), b) dilute NaOH (BDH), c) Milli-Q treated distilled water, and d) dilute sodium dodecylsulfate (SDS powder; BDH): for the concentrations, pH, etc., see the individual experiments. All dilutions were made with Milli-Q treated distilled water.

Instrumentation

Static (advanced) contact angles, θ, of a sessile drop of distilled water were measured using a NRLCA Goniometer (Ramé-Hart Inc.). Low resolution XPS spectra were obtained on a Leybold MAX 200 XPS system.[4]

Methods

Preparation of protein-silicone films

The preparation of these films is described in detail in the accompanying paper.[4] The main features are summarized here.

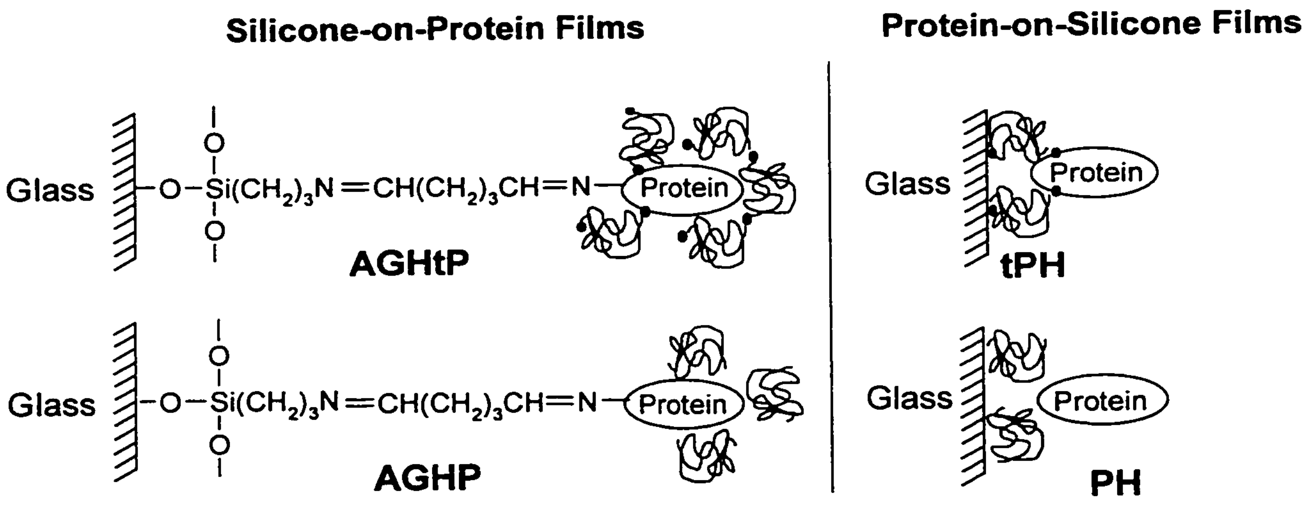
Silicone-on-Protein Films: The protein immobilization was achieved by silanization of the glass slides, coupling of a linker/spacer molecule, and finally, coupling of the protein to the linker. Typically, slides were dipped in a solution of the coating material for a period of time, rinsed with clean solvent and dried over a period of time. Before modification, the glass slides were cleaned of any adsorbed contaminants in a

Picotron-radio frequency plasma cleaner under an argon atmosphere for 10 min. After this treatment the contact angle of the surface was <5°.

Glass Silanization (**A** slides): The clean slides were immersed for 30 min into a 1-2% (v/v) γ-aminopropyltrimethoxysilane (APTS) solution in anhydrous methanol containing triethylamine (1-2% v/v); dried at 80 °C for 20 h.

Activation (**AG** slides) *and protein coupling* (**AGH** slides): **A** slides were immersed into the 2.5% (v/v) glutaraldehyde solution in phosphate buffered saline (PBS) for 1 h and rinsed off with PBS (**AG**). Coupling to protein (HSA) was achieved by incubation of the modified support (**AG**) with the protein in PBS solution (1 mg/mL) for 1 h at room temperature after which they were rinsed off with the PBS and dried at room temperature.

Silicone deposition (**AGHtP**, **AGHP** slides): The protein modified slides (**AGH**) were coated either with TES-PDMS or PDMS (1% v/v solutions in diethyl ether) by immersion of the slides into the silicone solutions for 1 h; dried at room temperature for at least 20 h (Scheme 1).



Scheme 1: Silicone:Protein Film Formation on Glass Slides. The silicone polymer is represented by a free curved line and the functional group (TES) by a dot. Grafting/crosslinking is implied when dots join two lines.

Protein-on-Silicone Films: The preparation of protein-on-silicone films involved physical immobilization of the silicone to the support and immersion of the silicone modified surfaces into protein solutions.

Silicone deposition (**tP**, **P** slides): Clean glass slides were initially coated either with TES-PDMS or PDMS (1% in diethyl ether) for 1 h; dried at room temperature for at least 20 h.

Protein adsorption (**tPH**, **PH**): **P** and **tP** slides were immersed into separate protein in PBS solutions (1 mg/mL) for 1 h; rinsed with the buffer; dried at room temperature for at least 20 h (Scheme 1).

Protein Desorption

Incubation in surfactant solution (SDS): The protein-silicone films were immersed into: a) SDS (10% w/w) for 24 h; or, b) SDS (3% w/w) for 2 h. The slides were rinsed off with Milli-Q water and left to dry overnight at room temperature.

Incubation in aqueous solutions: The protein-silicone films were immersed into different aqueous solutions covering a wide pH range (1.5±0.5, 5.5±0.5, or 9.5±0.5, respectively), at different time periods (from 10 min to 48 h), rinsed off with distilled water and left to dry overnight at room temperature.

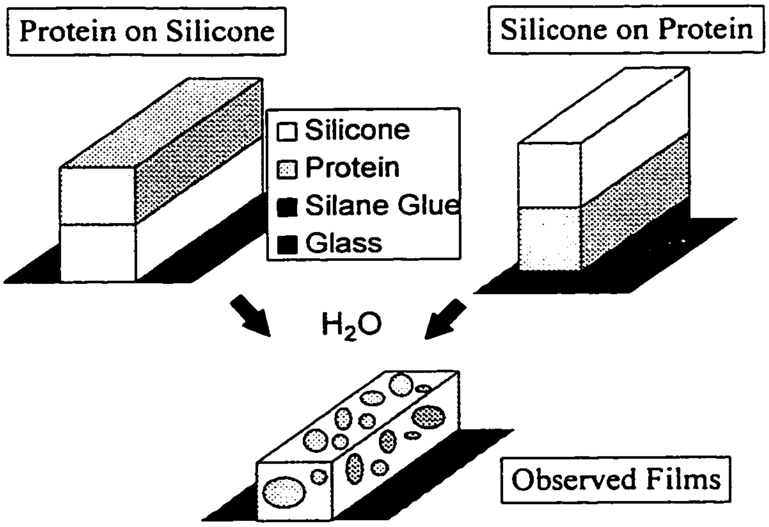
Surface Characterization

The Contact Angle (CA) and Angular Dependent X-ray Photoelectron Spectroscopy (AD-XPS) methods used are described in the accompanying paper.[4]

Results

Film Preparation

Protein-on-silicone and silicone-on-protein films were prepared by the sequential coating of the constituents on a glass slide. Unlike our initial expectations, that the surface would reflect the properties of the most recent coating, the surfaces were comprised of a mixture of silicone and protein irrespective of the order of assembling the film (Scheme 2).



Scheme 2: Mixing of Silicone and Protein Layers

Having demonstrated an affinity between the HSA and silicone, we wished to establish the strength and nature of the interaction. Therefore, protein-silicone films were exposed to: a) a surfactant solution, and; b) different aqueous solutions covering a wide pH range. The resulting surfaces of the films were characterized over time. It should be noted that there was some batch to batch variability in the slide preparation (film thickness, etc.). In the experiments outlined below, only one batch of slides was used for the hydrolysis data presented on a given Figure or Table. However, some differences may be seen in the data between different Figures.

Surfactant-Induced Protein Desorption

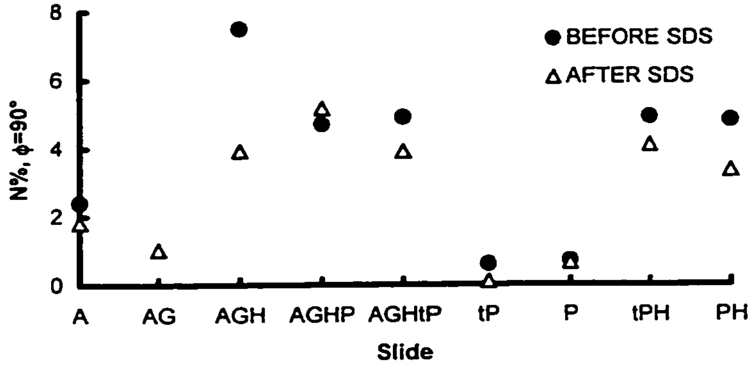
The modified slides were immersed in aqueous SDS solutions at concentrations well above the critical micelle concentration (cmc=8mM).[5] SDS solutions are well known to displace proteins from solid interfaces.[6] The effect of the surfactant on the **A**, **AGH**, **P**, and **tP** slides served as references for the protein/silicone films.

Contact Angle (CA): CA values of the different silicone-on-protein and protein-on-silicone films, and of their precursors, showed that the hydrophobicity of the slides had increased after immersion into 10% SDS solutions for 24 h: contact angles of both silicone-on-protein and protein-on-silicone films were on the order of ~100° (Table 1).

Table 1: Contact Angle Before and After Exposure to Aqueous SDS

Slides	Average Advanced Contact Angle (°)	Standard Deviation	Average Advanced Contact Angle after SDS (10%, 24h)	Standard Deviation
AGH	57	15.2	40	15.0
P	95	3.4	102	2.5
tP	95	4.3	104	1.2
AGHP	65	7.3	95	3.0
AGHtP	77	6.7	95	4.0
tPH	70	8.5	98	3.0
PH	55	13.1	102	0.8

Angular Dependent X-Ray Photoelectron Spectroscopy (AD-XPS): The results of the XPS analysis of all modified slides before and after incubation into the surfactant solution are reported in detail in the Supplementary Data. The parameters which best characterize the films are the %Si (silicon originating from the glass, APTS and silicone) and %N (protein and APTS) as a function of the take-off angle (depth profiling,[7] Figure 1). For virtually all the data to be described below, the films on top of the APTS layer were much thicker than 80 Å, and the %N values are, thus, representative of protein only. The Si rel.at.% of all hydrolyzed slides was lower at increased depth which indicates that the silicon measurement cannot be attributed to the glass, but rather to the silicone/protein layer (see Supplementary Data).



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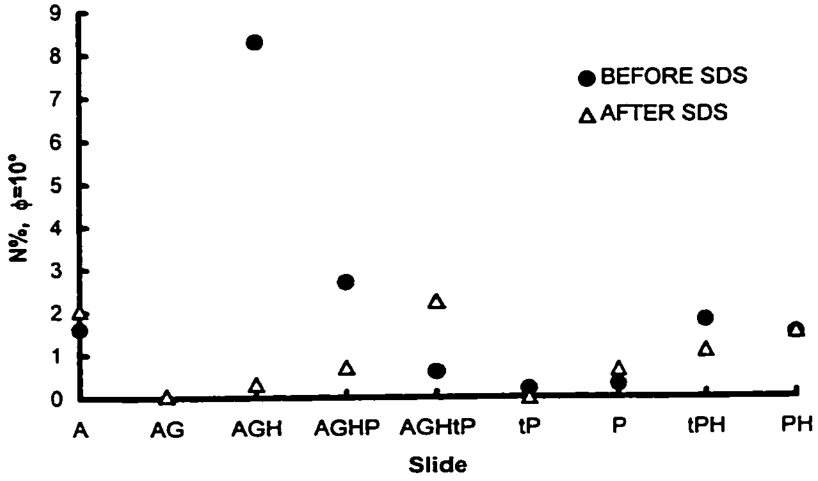


B:

Figure 1: XPS of Slides Incubated in SDS; N% at A: 10°, and B: 90° Take-off Angles.

Prior to exposure to SDS, all protein-on-silicone films show a silicone rich solid/air interface with the protein buried under the silicone layers. Based on the change in nitrogen content, both the **tPH** and **PH** slides lost protein at comparable rates. However, for the silicone-on-protein slides **AGHtP** and **AGHP**, the change in nitrogen content depended on the film. **AGHP** was shown to lose much of the surface nitrogen content unlike **AGHtP**, for which the surface N% actually increased over 3 hours (Figure 1A/B).

pH-Dependent Desorption

The modified slides were immersed into aqueous solutions covering a wide pH range. The degree of protein desorption induced by these aqueous solutions was indicated by the increase of the surface hydrophobicity, and the decrease of the nitrogen content (protein).

Contact Angle. The CA values obtained for the protein-silicone slides before (0 h) and after incubation into the aqueous solutions of pH 9.5, 5.5 and 1.5 are shown in Table 2. The contact angles of most of the samples changed relatively little as a result of exposure to the aqueous solutions. The **AGHtP** slides exhibited a somewhat larger change over 48 hours at higher pH (from 74° → 91°, Table 2). For the rest of the films, the observed contact angles were a function of the last layer to be coated onto the surface. In general, somewhat counter-intuitively, more hydrophobic solid/air interfaces were observed for the protein-on-silicone, rather than the silicone-on-protein surfaces.

Table 2: Contact Angles of Films Before and After Exposure to Aqueous Solutions of Different pH after 48 hours

Slides	t=0	pH 1.5	pH 5.5	pH 9.5
AGH	89	82	78	80
AGHP	77	78	81	81
AGHtP	76	83	71	91
PH	84	90	86	93
tPH	95	94	90	98

Angular Dependent X-Ray Photoelectron Spectroscopy: As noted above, the most notable change in film contact angle, as a function of exposure to aqueous solutions, was the case of **AGHtP** at higher pH. XPS was utilized to probe in more detail the changes that were occurring in the film during hydrolysis. The Si% and N% signals were particularly indicative of the these changes, except those slides in which there was little nitrogen content.

Preliminary experiments, which examined the rates of protein desorption from the films, demonstrated that two different time domains were followed: relatively rapid desorption from **PH** and **tPH** slides, much slower desorption from **AGHtP** and **AGHP** slides. These differences are most clear from the kinetic study of acid hydrolysis outlined below. While in both cases the protein mixes with silicone,[4] it is intuitive that protein desorption should be faster when the last layer coating the slides is protein, and slower when the silicone provides an "overlayer". For protein desorption measurements from the slides in neutral and basic media, 10 minutes for hydrolysis was allowed for the **PH** and **tPH** slides and 2 hours for the **AGHtP** and **AGHP** slides.

The behavior of the two protein-on-silicone films (**PH**, **tPH**) was rather similar during acid, neutral and basic hydrolysis, after 10 minute incubations, further from the air interface (90° take-off angle): for the acidic hydrolysis, no further change was observed after 1 hour (N%: **PH** = 1.71, **tPH** = 2.71). Nearer to the air interface, the **PH** slides became enriched in nitrogen (higher protein concentration) while the **tPH** slides showed very little residual nitrogen (Figure 2).

The **AGHP** and **AGHtP** slides behaved very differently after two hours hydrolysis. At all pH's and at all film depths, the N% content of **AGHtP** (and therefore protein content) was much higher than that of **AGHP** (Figure 3). The difference was most noticeable near the air interface.

A more careful kinetic profile of protein loss during acid hydrolysis was determined for the **PH, tPH, AGHP** and **AGHtP** slides, following the elemental rel.at.% over two hours using XPS. The change in nitrogen rel.at% with incubation time is illustrated with two different depth profile settings in Figure 4. At the air interface (10° take-off angle, Figure 4A), there was a rapid depletion of nitrogen for the **PH, tPH** and **AGHP** slides. There was a much slower depletion over time at a greater depth (90° take-off angle, Figure 4B). However, the **AGHtP** film showed a dramatic increase in %N at the surface over the first hour, followed by a gradual loss. A less pronounced effect was observed at greater depth (Figure 4). This is suggestive of a slower rate of protein release for the **AGHtP** slides.

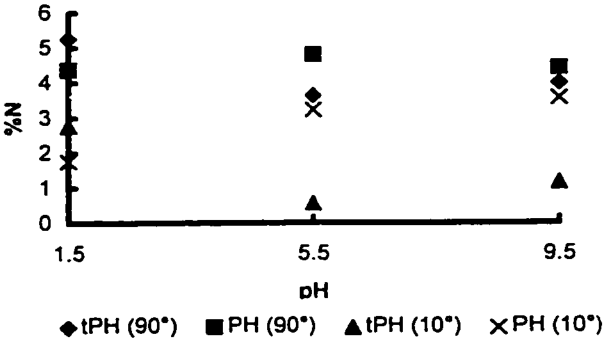


Figure 2: %N by XPS of PH and tPH Slides After Incubation (10 minutes) into Different pH Solutions

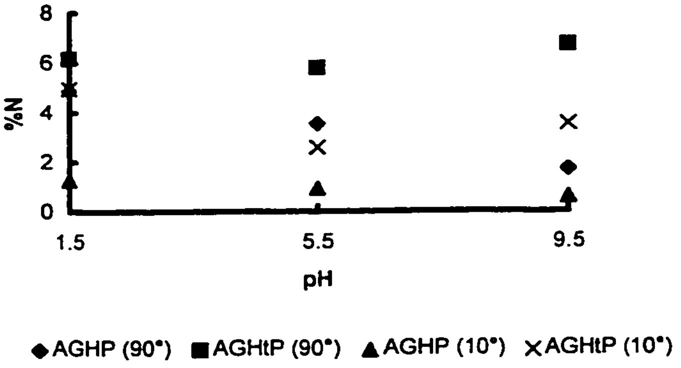
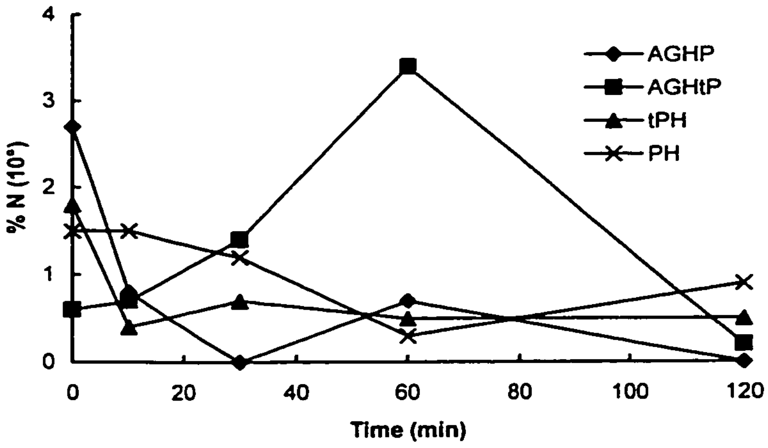
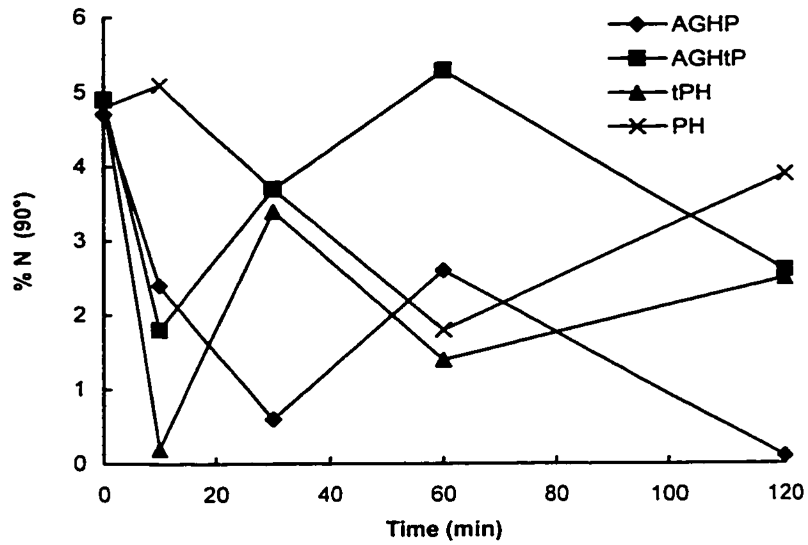


Figure 3: %N by XPS of AGHP and AGHtP Slides After Incubation (2 hours) into Different pH Solutions.

A:





B:

Figure 4: %N by XPS of Slides After Incubation in Acid; N% at A: 10° and B: 90° Take-off Angles.

Discussion

Surfactant-Induced Effects on the Modified Slides

Proteins are known to efficiently adhere to a variety of surfaces, both hydrophobic and hydrophilic.[8] They may be displaced from the surface, to a certain extent, upon exposure to different conditions, including the presence of surfactants[9,10] which help solubilize the protein, and different pH's, conditions under which the protein can undergo physical changes leading to desorption (especially on passing through the isoelectric point IP).

The purpose of the experiments described above was to establish the rate and magnitude of human serum albumin desorption, upon exposure to different aqueous media, from surfaces comprised of silicones and proteins. It should be noted that, in these experiments, the protein is initially an integral part of the surface unlike more traditional protein sorption/desorption experiments.[11] The primary focus of the experiments was to establish the role of the silicone end-groups on protein desorption. Given that the other factors which could determine protein desorption are the same for both PDMS and TES-PDMS (MW and experimental conditions were identical), any differentiation between protein release from the two silicones should be attributable to the different interactions of the respective end-groups ($SiMe_3$ *vs* $Si(OEt)_3$) with the protein.

Surfactant

Following exposure to SDS solutions for 24 hours, contact angle measurements indicated increased surface hydrophobicity for all protein-silicone slides. The results were consistent with protein desorption from the solid/liquid interface or, less likely, a burrowing of the protein towards the glass substrate: the resultant surface was silicone rich.

Additional observations were made by AD-XPS analysis following a 2 hour incubation period. We will first comment on the effect of the surfactant incubation on the reference slides (**A**, **AG**, **AGH**, **tP** and **P**).

The XPS values shown in Figure 1 indicate that the surfaces of the **A**, **tP** and **P** slides change little upon exposure to the SDS solution. The **P** and **tP** slides exhibited approximately the same Si% before and after incubation in the surfactant solutions. Similarly, the **A** slides showed no change in the N% values. By contrast, the **AGH** slides showed a great reduction of their nitrogen content, suggesting desorption of ungrafted and, possibly, grafted protein, the linkage of which had undergone hydrolysis.

The protein-on-silicone slides (**PH** and **tPH** slides) showed that about half of the original protein is present at the surface after SDS treatment, although buried protein could be seen from the nitrogen content at higher take-off angle values. Very little difference could be discerned between the films formed from the two different silicones.

The silicone-on-protein slides (**AGHP** and **AGHtP** slides) also showed increased protein content with increased distance from the surface. However, the amount of residual protein at the surface and beneath, is much higher for the **AGHtP** slides. This indicates that the functionalized silicone interacts more effectively with the protein, retarding protein desorption. Alternatively, the TES-PDMS forms a protective film through which the protein leaches only slowly.

Protein Desorption From Aqueous Solutions

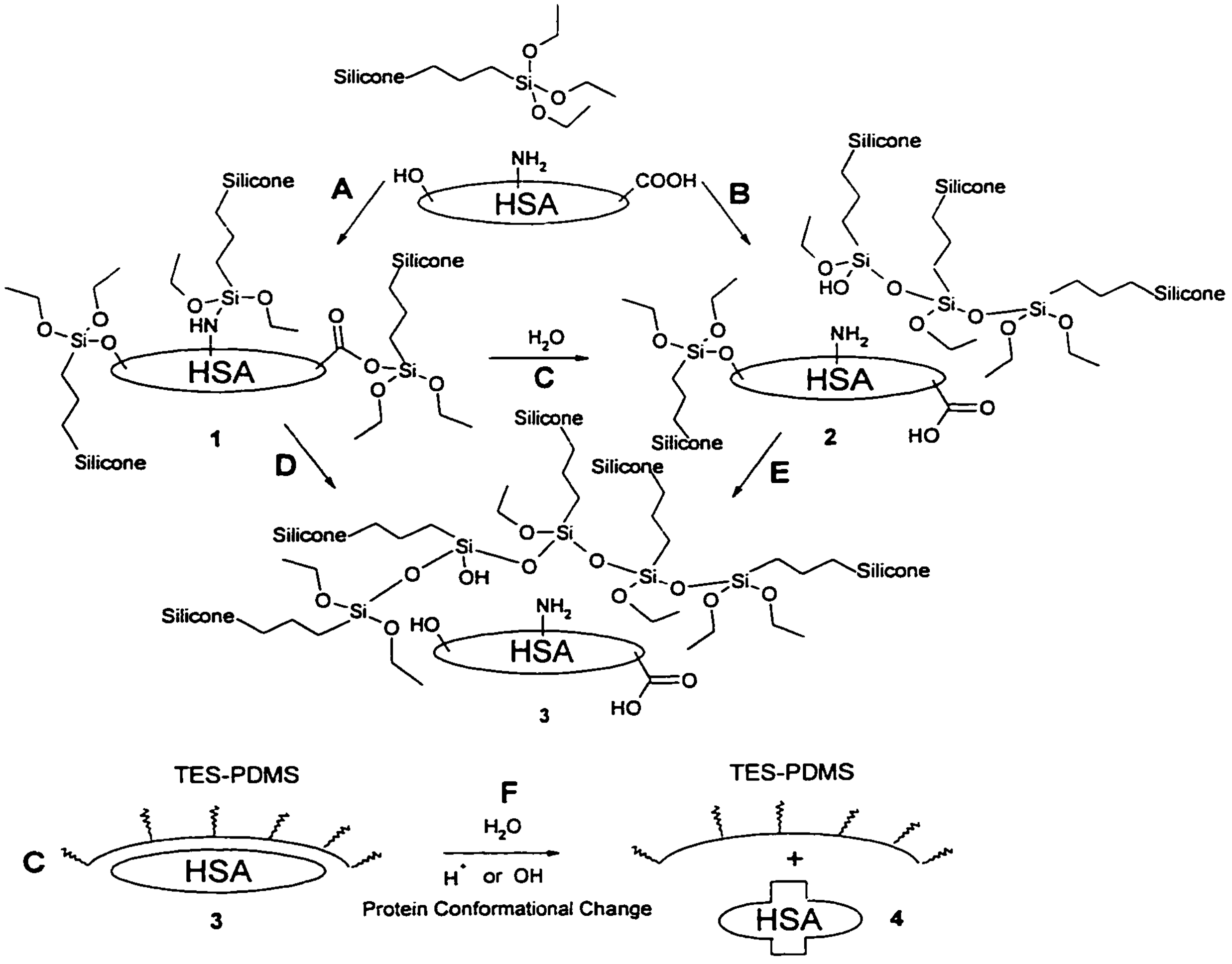
Before discussing the effect of the hydrolysis on the protein-silicone surfaces, it is useful to comment on the behavior of the individual components (protein/silicone) under a variety of hydrolytic environments.

The protein is greatly affected by different pH conditions, being denatured at pHs away from its isoelectric point. The isoelectric point for HSA was found at pH=4.7-5.2.[12] Therefore, HSA has an excess of positive net charge in an aqueous solution below pH 4-5, and an excess of negative net charge above it. Thus, electrostatic interactions of the protein with the substrate should be pH-dependent.

At the beginning of the incubation the protein was covalently bound to the solid surface via coupling agents (**AGH**, **AGHP**, **AGHtP**). However, the imine groups used for chemical binding of the protein to the APTS substrate are susceptible to acidic and basic hydrolysis.[13] The immobilization of the protein on the support initially depends on the strength of the covalent imine linkages but, after hydrolysis, depends on electrostatic interactions with the APTS-modified substrate (APTS is not easily hydrolyzed) and the degree to which the protein is entrapped by the silicone film. After hydrolysis, the net APTS-protein interactions should be repulsive in both acidic and basic pH regimes, since

APTS and HSA exhibit a similar pH dependence. The same factors will obtain for the hydrolysis of protein-on-silicone films (**PH, tPH**), except that the glass substrate was not modified by APTS groups.

Silicone polymers are known to be susceptible to hydrolysis (cleavage of Si-O-Si linkages) only under extreme acidic(if hydrolysis was taken place at pH 1.5 the tP/P wouldn't show lower ca?)or basic conditions.[14] Given the conditions used in the experiments described above, hydrolysis is not expected to arise to an appreciable extent. The TES-PDMS, however, initially bears terminal ethoxy groups which can undergo transesterification with the HSA, covalently linking the two polymers **2** (Scheme 3A,B). The alkoxy end-groups (either the intact ethoxy or protein-oxy following transesterification[15]) can also undergo hydrolysis and subsequent self-condensation to give silicone networks **3** (Scheme 3D,E). Such moisture cure silicone elastomers are well known in the silicone industry.[16] The rates of these hydrolysis and condensation reactions depend on the pH of the solution, the water concentrations and the size of the alkoxy group.[17] Hydrolysis is favored over condensation under acidic conditions with adequate water.[18] Under basic conditions, condensation is more rapid than hydrolysis: the rates of hydrolysis and condensation are relatively slow in the absence of catalysts at neutrality.



Scheme 3: Possible Interactions between TES-PDMS and HSA

Contact Angle Studies: Contact angle studies of all the slides, containing single or multiple layers on the glass substrates, were only qualitatively instructive: the error bars for these experiments are rather large.[19] With the exception of **AGHtP** slides, there was little change in the contact angles before and after exposure to any of the three different pH solutions (Table 2). The **AGHtP** slides showed a significant increase in contact angle under basic conditions (77→91° over 48 minutes). This is, of course, consistent with protein desorption which leaves behind a silicone rich air interface. Alternatively, it suggests base-catalyzed silicone crosslinking to form a hydrophobic silicone rubber at the interface.

XPS Studies: The XPS results for the **PH** and **tPH** slides (Figure 2) showed little difference between the two silicones at increased depth from the surface. However, surface protein was rapidly lost under under acidic conditions (Figure 2, Figure 4). At neutral and basic conditions there was less residual surface protein observed for the **tPH** than for the **PH** slides. However, we attribute the greater N% value to a faster diffusion of protein to the surface from the silicone layer beneath, in the latter case, rather than a higher concentration of retained protein. That is, at 10 minutes, the XPS data has captured the rapid diffusion of protein away from the **PH** surface.

There was a striking difference between the **AGHtP** and **AGHP** films. After two hours at all pH's there was much more residual protein in the former case (10° take-off angle): even the lower depths of the film (90° take-off angle) are depleted of nitrogen at higher pH for the **AGHP** slides (Figure 3). The large difference between the **AGHtP** and the other three types of slides (**AGHP**, **PH**, **tPH**) can be seen more clearly from the film hydrolysis under acidic conditions (Figure 4A). The 10° take-off angle results, which reflect the top layer of the **AGHP**, **PH** and **tPH** slides, showed rapid loss of protein. By contrast, the **AGHtP** slides initially show a buildup of nitrogen concentration with a subsequent loss. However, even the loss of protein occurred more slowly from the **AGHtP** surface than from the surface of the other films. The same effect, at lower amplitude, is shown in the 90° take-off angle results (Figure 4B). This data is consistent with retarded protein desorption from the **AGHtP** films under these conditions.

HSA can be released from the films described above by several different mechanisms. First, unbound protein may be displaced from the surface upon dilution, although this is generally considered to take place only to a limited extent.[20] Interactions with a powerful surfactant like SDS can lead to protein solubilization. Finally, the protein geometry, through which an efficient surface interaction can arise, can be distorted at pH's away from the isoelectric point of the protein facilitating desorption (Scheme 3F).

In the case of chemically grafted HSA, additional considerations are important. Clearly, if covalently tethered, the protein cannot escape the surface. However, hydrolysis of the imine tether is possible, especially under acidic or basic conditions. Since the surface bound coupling agent, APTS, will have the same overall pH characteristics as the HSA once hydrolysis of the tether has occurred, repulsive coulombic forces between the APTS and HSA in either basic or acidic regimes will favor desorption of the protein.

The studies described above clearly demonstrate enhanced protein retention in the case of the **AGHtP** slides. Two questions remain: What forces stabilize the silicone protein interaction that are not available to the normal silicone (**AGHP**)? Why isn't the same effect observed when the functionalized silicone is initially grafted to the glass (**tPH**)?

The obvious distinction between the two silicones is the presence of the alkoxy functional groups in the former case. Two distinct possibilities may be considered for these interactions: covalent and physical adhesion. Transesterification of the alkoxy group with the protein surface could lead to silicone-grafted proteins **1**, **2** (Scheme 3).[15] The subsequent rate of hydrolysis of these proteinoxysilane linkages would be slower than that of the starting ethoxysilane, based on the larger steric encumbrance at silicon.[17] We ignore in these considerations the formation of protein-based silazanes and acyloxysilanes **1** as these functional groups are quite susceptible to hydrolysis (Scheme 3C).[16,21] Protein release from covalently grafted silicone-protein copolymers would require hydrolysis of the linkage **2**→**3**→**4**.

The alternative explanation invokes efficient physical adhesion. If the silicone were to crosslink forming a network rubber along the protein surface,[22] using the protein as a template, there would be many points of contact and enhanced physical adhesion **3**. Desorption **3**→**4** would depend on factors that interrupt the matched protein/silicone interface, which may include the infusion of SDS or a conformation change in the protein caused by a pH change (Scheme 3F).

We favor the latter of these two theories. The proposal involving covalent bonding of silicone and protein is compromised by the fact that such a linkage should have been most stable in neutral conditions: both acidic and basic conditions should lead to efficient hydrolysis of the putative proteinoxysilane bonds. However, the **AGHtP** film showed stability to all pH conditions. During the preparation of the microparticles and their storage, there is always water present. In spite of the relatively slow hydrolysis, some hydrolysis of ethoxysilanes is bound to occur. This would allow the slow development of a network on the protein surface. Exposure to different pH conditions would catalyze the hydrolysis of any proteinoxysilane linkages and, simultaneously, the self-crosslinking of the silicone. In fact, the relatively rapid increase in contact angle for the **AGHtP** slides under basic conditions may reflect the formation of a silicone elastomer network. The role, if any, of the protein IP on the pH release profiles is a question we shall address in future work.

Finally, we must account for the absence of a difference between the **PH** and **tPH**, the latter of which, based on the analysis above for **AGHtP**, should also have been better bound to the protein than the non-functionalized silicone. The difference between **tPH** and **AGHtP** may be attributed to the order of assembly of the film. In the former case, the alkoxysilane groups on the PDMS-TES were directly exposed to glass. These are coupling agents[23] that can react efficiently and, by analogy with alkoxysilanes, irreversibly with the glass surface. To the extent that this occurs, fewer functional groups will be available to interact with the protein and to undergo crosslinking, sufficiently few that efficient adhesion to the protein is not observed.

These results point to the mechanism for the antigenicity of microparticles prepared from protein and PDMS-TES when administered orally to animals.[3] The protein is protected by the silicone in the gastrointestinal tract. However, the combination of silicone and protein is sufficiently fragile that protein can be released from the microparticle and come into contact with the mucosal immune system resulting in the production of antibodies at all pH's. Thus, the microparticles are not pH erosion systems as proposed in the introduction, but rather, slow release protein delivery systems. The effect of TES-PDMS on other proteins remains to be established. Furthermore, it must be determined whether silicone accompanies protein to the mucosal immune system or if the protein is completely released.

Conclusion

Formation of protein-on-silicone and silicone-on-protein films resulted in silicone rich/protein poor surfaces irrespective of the order of the film assembly. The degree of protein desorption from these films, induced by incubation in surfactant and aqueous solutions, was found to depend on the type of silicone used. The functionalized silicone forms more intimate films with the protein which serve to retard its desorption. There appears to be little effect of pH on the affinity for protein with TES-PDMS.

Acknowledgments

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Supplementary Material

Table 3: Contact Angle Data for Hydrolysis of Protein/Silicone Slides (A Superset of Table 2).

Acid Hydrolysis	**(pH= 1.5)**									
Time	0.17 h		1 h		2 h		24 h		48 h	
Slide	CA	Av.D.	CA	Av.D.	CA	Av.D.	CA	Av.D.	CA	Av.D.
AGH	88.9	4.4	86.6	3					81.8	2.7
AGHtP	76.3	3.8	75.3	4.6	74.2	5.4	75.7	2.8	83.3	5.2
AGHP	77.1	3.7	89.2	5.0	89.2	4.6	78.0	5.4	84.8	3.2
tPH	97.1	3.9	85.7	2.7	89.1	2.4	91.4	2.8	93.6	3.8
PH	83.5	2.6	87.7	0.6	86.5	1.8	88.3	4.9	90.8	1.9
tP	-	-	100.0	0.8	97.4	2.3	96.9	2.7	93.2	3.7
P	-	-	99.4	1.0	95.1	7.4	98.7	3.4	93.3	3.3
Neutral Hydrolysis	**(pH= 5.5)**									
AGH	84.2	5.2	86.7	2.2	-	-	-	-	78.4	3.7
AGHtP	76.5	3.5	98.1	1.1	72.4	2.5	81.2	3.6	70.7	4.0
AGHP	84.1	3.9	78.6	3.1	81.4	2.7	81.2	6.4	78.9	1.9
tPH	94.5	2.0	93.4	2.3	88.8	3.0	94.8	2.9	89.5	2.8
PH	87.5	3.9	82.9	3.3	86.8	1.1	85.8	3.6	86.0	2.7
tP	-	-	97.5	3.6	102.0	1.8	101.0	0.8	90.1	1.1
P	-	-	99.7	2.6	99.4	2.5	101.0	1.1	-	-
Base Hydrolysis	**(pH= 9)**									
AGH	86.1	5.3	75.5	4.5	-	-	-	-	80.3	2.9
AGHtP	73.6	5.1	68.2	4.8	81.2	7.0	74.5	3.1	91.2	4.8
AGHP	87.5	5.1	77.1	4.8	94.0	7.0	80.6	3.1	-	-
tPH	94.2	2.1	93.4	3.4	94.2	1.1	85.8	5.5	97.5	4.6
PH	87.5	2.8	83.7	3.4	88.3	1.4	82.0	5.2	92.9	3.9
tP	-	-	99.9	0.5	100.0	2.1	100.0	2.0	97.2	0.9
P	-	-	100.0	2.4	104.0	2.5	101.0	1.2	99.0	1.4

Table 4: AD-XPS Data of Protein/Silicone Slides Prior to Exposure to Different Aqueous Solutions ("Before" Data for Figure 1 and Figure 4)

Slides	Elemental Composition					
	O1s	N1s	C1s	Si2p	K2p	NaklI
φ=90°						
A	51.7	2.4	17.2	22.5	0.4	5.9
AGH	34.2	7.5	40.3	10.7	0.0	7.3
AGHP	37.3	4.7	35.6	13.6	0.0	8.8
AGHtP	30.7	4.9	44.4	16.9	0.0	3.2
tP	49.8	0.6	19.7	24.3	0.7	5.1
P	58.4	0.7	10.6	23.8	1.1	5.5
tPH	33.8	4.9	40.1	17.9	0.0	3.3
PH	32.9	4.8	41.4	17.6	0.0	3.3
φ=20°						
A	28.2	3.1	46.0	13.9	0.0	8.8
AGH	20.8	9.3	61.7	3.5	0.0	4.7
AGHP	23.3	4.1	54.8	13.3	0.0	4.5
AGHtP	22.9	2.2	54.9	18.4	0.0	1.6
tP	32.4	0.3	43.3	20.8	0.0	3.1
P	34.6	0.3	41.4	20.8	0.1	3.0
tPH	24.4	2.6	52.4	19.6	0.0	1.0
PH	23.2	1.7	53.8	20.3	0.0	1.1
φ=10°						
A	17.5	1.6	51.6	7.7	0.0	21.7
AGH	18.3	8.3	62.6	4.4	0.0	6.4
AGHP	22.4	2.7	53.1	17.6	0.0	4.2
AGHtP	22.9	0.6	54.5	19.4	0.0	2.7
tP	25.8	0.2	53.2	17.6	0.0	3.2
P	24.4	0.3	54.8	18.4	0.0	2.2
tPH	23.7	1.8	52.1	21.6	0.0	0.8
PH	21.2	1.5	50.9	25.2	0.0	1.1

Table 5: AD-XPS Data Incubation of Silicone-on-Protein or Protein-on-Silicone Slides in Aqueous SDS Solutions (Superset of Figure 1).

Slides	Elemental composition			
	O%	N%	C%	Si%
φ=90°				
A	53.0	1.8	16.3	23.2
AG	40.6	1.0	33.1	20.9
AGH	34.3	3.9	40.3	18.6
AGHP	28.5	5.2	48.9	15.4
AGHtP	32.8	3.9	44.1	14.9
tP	32.9	0.1	38.1	26.2
P	55.9	0.6	12.8	23.1
tPH	33.6	4.0	39.9	18.0
PH	31.5	3.4	42.7	18.5
φ=20°				
A	32.0	2.7	44.7	17.9
AG	22.2	0.4	57.0	19.0
AGH	22.1	1.9	57.6	17.6
AGHP	20.8	2.3	59.9	15.6
AGHtP	20.3	3.0	62.4	12.4
tP	25.2	0	48.9	25.1
P	36.7	0.6	39.3	19.2
tPH	22.0	1.7	56.4	18.5
PH	22.3	0.9	55.2	20.7
φ=10°				
A	23.6	2.0	57.4	13.8
AG	22.6	0.1	54.7	21.1
AGH	20.3	0.3	56.2	22.1
AGHP	20.7	0.7	59.0	17.6
AGHtP	19.7	2.2	60.5	14.7
tP	27.4	0	46.9	24.5
P	25.7	0.6	53.5	17.1
tPH	20.5	1.1	55.1	21.9
PH	22.3	1.5	52.7	22.1

Table 6: AD-XPS Data for Acid Hydrolysis (10 min) of Protein-on-Silicone Slides (Superset of Data shown in Figure 2)

Acid Slides	Elemental composition					
	O1s	N1s	C1s	Si2p	K2p	Nakll
φ=90°						
tP	54.1	0.9	16.6	25.6	0.4	2.5
P	55.6	0.5	16.5	23.8	0.4	3.2
tPH	35.6	5.3	40.9	16.7	0.1	1.4
PH	36.7	4.4	39.6	18.1	0.0	1.4
φ=20°						
tP	35.3	0.8	40.1	23.1	0.0	0.7
P	33.1	0.7	45.9	18.7	0.0	1.5
tPH	22.3	3.8	58.1	15.5	0.0	0.3
PH	24.0	2.8	56.4	16.3	0.0	0.4
φ=10°						
tP	26.7	0.7	49.9	22.5	0.0	0.3
P	25.4	0.0	56.0	17.0	0.0	1.7
tPH	21.0	2.8	58.5	0.2	0.0	0.2
PH	23.8	1.8	55.8	18.1	0.0	0.6

Table 7: AD-XPS Data for Basic Hydrolysis (10 min) of Protein-on-Silicone Slides (Superset of Data shown in Figure 2).

Base Slides	Elemental composition					
	O1s	N1s	C1s	Si2p	K2p	NaklI
φ=90°						
tP	58.3	0.7	17.6	18.2	0.7	4.5
P	49.1	0.8	26.5	20.1	0.3	3.1
tPH	32.0	4.0	44.9	17.5	0.0	1.7
PH	43.4	4.4	31.3	17.9	0.4	2.6
φ=20°						
tP	35.0	0.3	41.7	21.6	0.0	1.5
P	26.9	0.1	52.9	19.5	0.0	0.7
tPH	21.8	1.9	58.1	18.0	0.0	0.3
PH	25.1	4.6	55.1	14.1	0.0	1.1
φ=10°						
tP	26.8	0.7	54.1	16.6	0.0	1.8
P	22.3	0.0	54.1	23.6	0.0	0.0
tPH	21.3	1.2	57.9	19.5	0.0	0.2
PH	21.1	3.6	57.0	17.8	0.0	0.6

Table 8: AD-XPS Data for Neutral Hydrolysis (10 min) of Protein-on-Silicone Slides (Superset of Data shown in Figure 2).

Neutral Slides		Elemental composition				
	O1s	N1s	C1s	Si2p	K2p	Nakll
φ=90°						
tP	46.2	0.7	27.6	23.4	0.3	1.8
tPH	28.8	3.6	48.0	18.2	0.0	1.4
PH	40.3	4.8	32.7	20.2	0.2	1.9
φ=20°						
tP	26.2	0.0	50.5	22.6	0.0	0.7
tPH	22.5	0.9	54.4	22.1	0.0	0.2
PH	24.4	3.9	56.1	15.2	0.0	0.4
φ=10°						
tP	19.5	0.3	55.9	24.1	0.0	0.2
tPH	21.4	0.6	53.2	24.4	0.0	0.4
PH	21.4	3.2	56.5	18.5	0.0	0.4

Table 9: AD-XPS Data for Acid Hydrolysis (2h) of Silicone-on-Protein Slides (Superset of Data in Figure 3).

Acid Slides	Elemental composition					
	O1s	N1s	C1s	Si2p	K2p	Nakll
φ=90°						
AGH	31.9	4.4	44.9	17.7	0.0	1.1
AGHP	29.9	4.9	46.9	17.3	0.0	1.1
AGHtP	36.5	6.2	40.2	15.5	0.0	1.6
φ=20°						
AGH	23.4	1.4	54.9	20.2	0.0	0.1
AGHP	23.2	2.6	55.2	18.6	0.0	0.4
AGHtP	20.5	6.6	61.4	11.0	0.0	0.5
φ=10°						
AGH	22.1	1.7	53.2	23.0	0.0	0.0
AGHP	24.4	1.3	51.4	22.3	0.0	0.6
AGHtP	19.6	5.0	62.4	12.8	0.0	0.3

Table 10: AD-XPS Data for Basic Hydrolysis (2h) of Silicone-on-Protein Slides (Superset of Data in Figure 3).

Base Slides	Elemental composition					
	O1s	N1s	C1s	Si2p	K2p	Nakll
φ=90°						
AGHP	26.0	1.7	48.4	23.3	0.0	0.6
AGHtP	32.7	6.7	43.9	15.2	0.0	1.6
φ=20°						
AGHP	23.2	0.6	51.1	25.1	0.0	0.0
AGHtP	21.2	5.1	60.6	12.8	0.0	0.4
φ=10°						
AGHP	23.8	0.6	49.3	26.2	0.0	0.1
AGHtP	21.5	3.5	57.5	17.5	0.0	0.0

Table 11: AD-XPS Data for Neutral Hydrolysis (2h) of Silicone-on-Protein Slides (Superset of Data in Figure 3).

Neutral Slides	Elemental composition					
	O1s	N1s	C1s	Si2p	K2p	Nakll
φ=90°						
AGH	26.5	6.0	53.6	13.1	0.0	0.9
AGHP	25.6	3.6	51.6	18.7	0.0	0.6
AGHtP	25.7	5.8	56.5	11.4	0.0	0.7
φ=20°						
AGH	20.6	3.4	61.5	14.4	0.0	0.0
AGHP	22.7	1.5	54.5	21.1	0.0	0.3
AGHtP	18.6	4.1	65.8	11.5	0.0	0.0
φ=10°						
FAGH	20.4	1.9	59.5	17.8	0.0	0.4
AGHP	20.6	1.0	56.7	21.7	0.0	0.0
AGHtP	21.2	2.6	62.6	13.6	0.0	0.1

Table 12: AD-XPS Data for Slide Incubation in Acid (pH=1.5) (Superset of Data in Figure 4).

Slides	Elemental composition														
	C%	N%	Si%	C%	N%	Si%	C%	N%	Si%	C%	N%	Si%	C%	N%	Si%
		0 min			10 min			30 min			60 min			120 min	
φ=90°															
AGHP	35.6	4.7	13.6	54.9	2.4	19.3	52.4	0.6	23.2	54.6	2.6	18.8	51.2	0.1	25.3
AGHtP	44.4	4.9	16.9	53.1	1.8	21.6	58.3	3.7	13.9	59.1	5.3	12.6	54.7	2.6	18.5
tPH	40.1	4.9	17.9	51.1	0.2	25	52.3	3.4	16.9	46.2	1.4	28.0	55.0	2.5	17.6
PH	41.4	4.8	17.6	53.5	5.1	13.8	49.3	3.7	17.7	55.3	1.8	19.9	47.0	3.9	19.4
φ=30°															
AGHP	-	-	-	54.5	1.2	21.8	50.9	0	25.7	53.7	0.9	22.4	51.0	0	25.5
AGHtP	-	-	-	52.7	0.9	23.4	61.6	2.7	14.8	60.5	4.2	13.8	55.6	1.0	20.8
tPH	-	-	-	50.9	0	25.7	59.8	2.1	17.0	51.5	0.8	24.0	58.8	1.3	19.0
PH	-	-	-	62.3	3.5	13.4	57.3	2.8	17.2	55.3	0.4	22.2	55.5	2.3	19.5
φ=20°															
AGHP	54.8	4.1	13.3	54.3	0.9	22.4	51.1	0.1	25.7	53	0.6	23.6	51.3	0	25.6
AGHtP	54.9	2.2	18.4	52.3	0.7	23.9	59.9	1.7	17.6	61.1	3.6	14.1	54.9	0.5	22.3
tPH	52.4	2.6	19.6	51.5	0.2	25.5	59.0	1.4	18.7	52.0	0.6	24.4	57.1	0.9	20.7
PH	53.8	1.7	20.3	62.3	2.3	15.5	57.4	1.7	19.0	53.3	0	23.9	55.9	1.5	20.8
φ=10°															
AGHP	53.1	2.7	17.6	53	0.8	24.0	51.8	0	26.4	53.2	0.7	24.3	51.0	0	26.4
AGHtP	54.5	0.6	19.4	52.4	0.7	24.6	59.8	1.4	18.6	59.1	3.4	16.8	52.9	0.2	24.6
tPH	52.1	1.8	21.6	49.9	0.4	27.5	57.0	0.7	21.5	52.4	0.5	24.2	55.9	0.5	23.0
PH	50.9	1.5	25.2	59.3	1.5	18.3	55.8	1.2	21.8	52.5	0.3	25.1	53.7	0.9	23.3

PART II

Silicone-Protein Interactions at Liquid/Liquid Interfaces

Chapter 5

Protein-Silicone Interactions at Liquid/Liquid Interfaces

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Abstract

The enzyme alkaline phosphatase and the globular protein human serum albumin (HSA) were emulsified with low molecular weight silicone oil and a silicone/polyether surfactant to give stable emulsions. The biological activity of the proteins in the emulsions was assayed both *in vitro* (enzyme kinetics of alkaline phosphatase) and *in vivo* (immune response to oral administration of the HSA emulsion). Depending on the assay, either a small decrease or large increase in biological activity was noted. The nature of the silicone-protein interactions is discussed.

Introduction

Silicone emulsions have found wide application in the medical and pharmaceutical fields due to their desirable physical and chemical properties. For instance, silicone oil emulsions have been used for the preparation of elastomers used in controlled drug delivery systems.[1] Simple aqueous[2] and non-aqueous silicone oil emulsions (e.g., propylene glycol-in-silicone oil) and multiple emulsions[3] have become increasingly popular in the cosmetics industry mainly due to the desirable physical properties imparted from the silicone oils. These properties include a smooth, non-greasy feel, and a wide range of available viscosities, which eliminates the need to use waxes in formulations. However, despite the extensive industrial use of silicone oil emulsions, the systematic study of their interfacial properties is only in its infancy.

One of the current challenges in drug delivery is the formulation of systems for the administration of proteins and proteinaceous materials.[4] We have an interest in the use of silicones as vehicles for the oral delivery of vaccines. It is clearly important for such an application to understand the effects, deleterious or otherwise, of the silicone on the immunogenic protein. Most of the knowledge of these interactions comes from studies of protein solutions at solid silicone elastomer surfaces.[5,6] However, such studies on solid/liquid interfaces may not apply to liquid/liquid interfaces. Although fundamentally the same, these interfaces differ in interfacial properties: the former offer specific, fixed sites for protein adsorption, while the latter are homogeneous and flexible. It is accepted in general that proteins at fluid interfaces are more mobile and thus free to penetrate the non-aqueous phase. Furthermore, it is believed that proteins at fluid interfaces exist in a state between the native and the denatured state, termed the "molten globule" state.[7] The only study of silicone oil-protein interactions at liquid/liquid interfaces of which we are aware suggests that proteins undergo (detrimental) conformational changes upon long-term mixing with silicone oil.[8]

Stable silicone emulsions could serve as protein carriers for drug delivery systems. In the present study, we are concerned with the degree of retention of the biological activity of proteins in silicone emulsions. We have, therefore, evaluated the biological activity, *in vitro* and *in vivo*, of two different proteins in silicone emulsions.

Experimental Section

Materials

Alkaline Phosphatase type VII-S from bovine intestinal mucosa (EC 3.1.3.1), Human Serum Albumin (HSA, Fraction V, 96-99% Albumin), and *p*-nitrophenyl phosphate tablets 104-105 were obtained from Sigma. Phosphatase-conjugated goat anti-mouse IgG or IgA was purchased from Southern Biotechnology Associates. Octamethylcyclotetrasiloxane (D_4; viscosity 2.3 cs) was obtained from Gelest. 3225C formulation aid (a silicone-polyether copolymer) was provided by Dow Corning. Trishydroxymethylaminomethane ("Tris") and HCl were obtained from BDH. All aqueous solutions were prepared with Milli-Q treated distilled water. Female BALB/c mice, age 6-8 weeks were purchased from Charles River Laboratories Inc., Montreal.

Instrumentation

A Hewlett Packard 8452A UV-VIS Spectrophotometer with constant temperature circulating bath was used for monitoring the enzymatic reaction. A Caframo mixer was used for the emulsion formulation and a Leitz Wetzlar microscope was used for droplet size determination.

Methods

Emulsion Formulation

Emulsion Composition. Different emulsion compositions were used for the different studies:

In vitro studies: The emulsion comprised a 50 wt% aqueous phase (50/50 composition). The protein concentration in the aqueous phase ranged from 0.08 mg/mL to 0.008 mg/mL. The surfactant formulation aid was 2.5 wt% in the emulsion, which provides 0.25 wt% of active surfactant (the silicone-polyether surfactant is supplied as a 10 wt% active solution in cyclic silicones).

In vivo studies: The emulsion formulated for the immunological studies contained 25 wt% aqueous phase and 3.75 wt% of surfactant solution (25/75 composition). The protein concentration in the aqueous phase was 0.5 mg/mL.

Emulsification Conditions. Emulsification was achieved under turbulent mixing by slow admixing of the aqueous phase to the silicone oil phase containing the macrosurfactant. The mixing required to produce stable emulsions was obtained in a vessel equipped with two blades: a straight (90°) and a pitched (45°) one. The pitched blade was placed between 1/2 to 2/3 of the liquid level height while the straight blade was placed at a level equal to 1/6 to 1/3 the diameter of the vessel (both measured from the vessel bottom). The shaft was slightly tilted to minimize vortex formation. This system provides mixing tip velocity (speed at the edge of the blade most remote from the mixing shaft) of 275 m/min at an agitator speed of 3440 rpm. After completion of the addition of the aqueous phase, the emulsion was left stirring for at least as long as the time of addition (20 min).

Demulsification: Emulsion inversion was induced by increasing the volume fraction of the dispersed phase by simple addition of buffer solution. Prior to inversion, the original emulsion was concentrated to a gel-like consistency by centrifugation at 2000 rpm for 1 h. The supernatant was a clear octamethylcyclotetrasiloxane solution. Emulsion inversion and phase separation was achieved by slowly adding Tris/HCl buffer solution into the gel-like protein-in-silicone oil emulsion with dual blade, turbulent mixing at 3440 rpm.

Enzymatic Activity

In situ: The protein-in-silicone emulsions were mixed either with an aqueous phase containing the substrate or another silicone oil emulsion containing an aqueous substrate solution. The enzymatic activity was qualitatively observed by the development of a yellow color, characteristic of the product formation (*p*-nitrophenoxide from *p*-nitrophenyl phosphate), in emulsions containing alkaline phosphate at concentrations as low as 0.008 mg/mL.

In Solution (recovered aqueous phase): The hydrolytic activity of alkaline phosphatase was quantified by following spectrophotometrically at 404 nm the formation of *p*-nitrophenoxide. The molar absorption coefficient of *p*-nitrophenoxide at 404 nm, in Tris/HCl buffer at pH=8.0, is 18000 M^{-1} cm^{-1}.

The kinetic experiments were performed in disposable plastic 4-mL (1 cm^2 x 5 cm) cuvettes, containing a magnetic stirrer. The cuvettes were filled with the substrate and were equilibrated at 25 °C before the enzyme addition. The enzymatic reaction was started by the rapid addition of 10 μL of alkaline phosphatase to 2.99 mL of a range of *p*-nitrophenyl phosphate concentrations in Tris/HCl buffer solution at pH=8.0. The concentration range of *p*-nitrophenyl phosphate was 1-6 μM. The absorption signal was recorded every 2 sec for 30 sec. Initial velocities were derived from the velocity values that corresponded to the linear part of the Michaelis-Menten equation, by extrapolation to t_0.

Immunological Studies

A group of five mice was immunized orally with HSA-containing silicone oil emulsions (25/75 wt% composition). The animals received 50 μg of protein per dose, three times in seven day intervals. The collection of individual blood samples was performed as described elsewhere 9 at days: 7, 21, 28, 35 and 48. An enzyme-linked immunosorbent assay (ELISA) was used to detect and quantify the HSA-specific antibody responses in the individual serum samples. The ELISA assays were performed as described elsewhere.[9]

Results

Emulsion Characterization

Droplet Size Measurements. Microscopic viewing of one part of the emulsions, diluted in three parts of silicone oil, showed that the average droplet size was 5 μm and 2 μm for the 50/50 and 25/75 emulsion compositions, respectively.

Stability Observations. In 50/50 and 25/75 compositions of buffer solutions in D_4, sedimentation was observed due to viscosity differences. Emulsions made with other more viscous internal phases (such as glycerol/buffer) at 50/50 ratios were gel-like and no creaming was observed after several months.

Enzymatic Activity

Alkaline phosphatase retains at least some of its enzymatic activity while being entrapped in silicone oil over several days. This was clear from the development of the yellow color characteristic of *p*-nitrophenoxide upon addition of *p*-nitrophenyl phosphate-containing solutions or emulsions to alkaline phosphatase-containing emulsions. However, the quantification of the degree of the retention of the enzymatic activity was not possible *in situ*. Therefore, the aqueous phase was recovered from the emulsions and an enzymatic assay was run in solution. The alkaline phosphatase solution that had been exposed to silicone in the emulsion was compared to an aqueous solution that had not been so emulsified. The Lineweaver and Burk equation[10] was used to determine the kinetic parameters, K_M and V_{max}, of the enzymatic reactions:

$1/U = K_M/V_{max}S + 1/V_{max}$

where U: initial velocity, K_M: Michaelis-Menten constant, V_{max}: maximum velocity; and S: concentration of the substrate *p*-nitrophenyl phosphate (PNP)

A graph of 1/U against 1/S gave a straight line of slope K_M/V_{max} and ordinal intercept $1/V_{max}$. The K_M values were 1.34 10^{-5} M and 1.99 10^{-5} M, while the V_{max} values were 60 and 74 μM/min for the emulsified and non-emulsified alkaline phosphatase solutions, respectively (Figure 1).

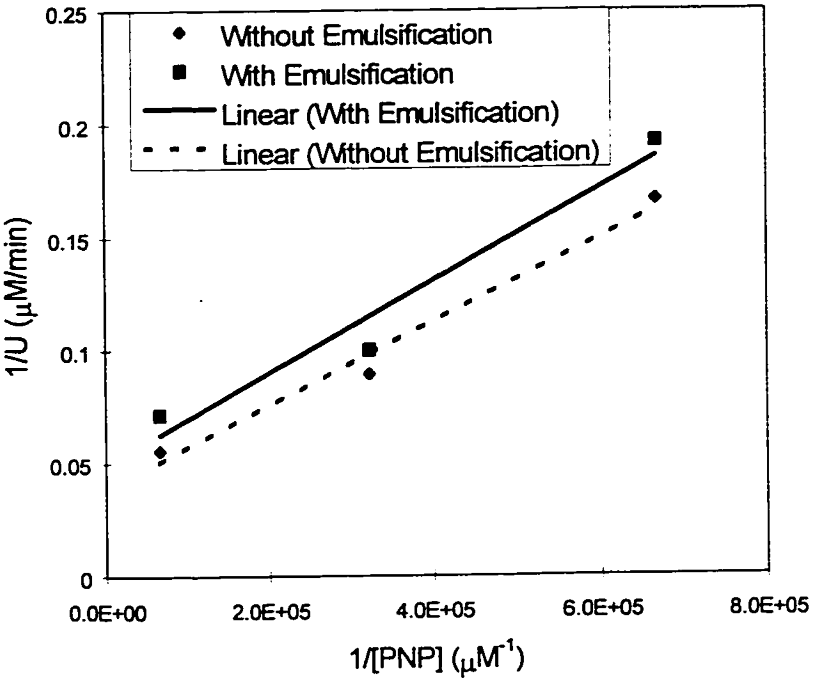

Figure 1: Activity of Alkaline Phosphatase Without ◆and With ■ Emulsification in Silicone Oil

The turnover number, which is a measure of the enzymatic efficiency, can be derived from the V_{max} values. The turnover number is defined as the ratio of V_{max} to the total enzyme concentration. While the enzyme concentration is less for the solution recovered from the emulsion due to the buffer added during demulsification, the turnover numbers for the emulsified and non-emulsified enzyme solutions should be similar within experimental error if enzyme degradation does not take place, as was observed (60 and 74, respectively). The determination of enzyme concentrations is not trivial at such low concentrations and work is ongoing to ensure that the results presented here are accurate and reproducible.

Immune Response

HSA was emulsified in silicone oil using the same conditions as for alkaline phosphatase. The emulsion was orally administered to mice as previously described for HSA-containing starch microspheres.[9] The immunogenicity of the protein contained in the emulsion was reflected in the blood antibody titre assayed using ELISA. The results are shown in Figure 2 along with the microparticle assays for the purposes of comparison. In both cases, the protein dose to the animal was identical: 50 μg.

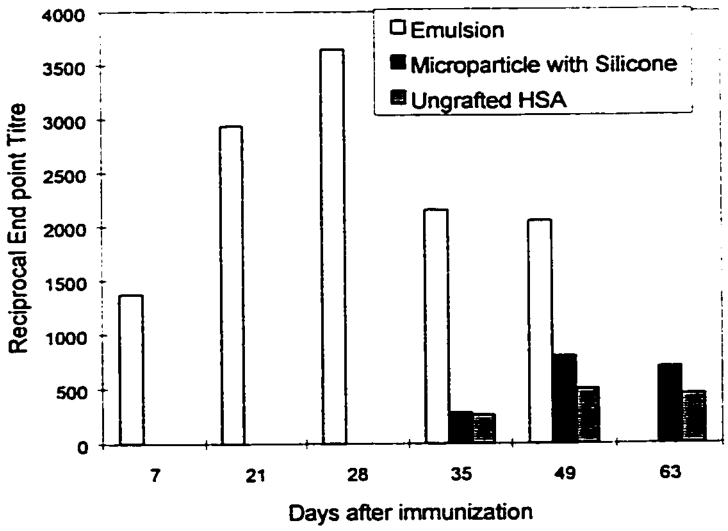

Figure 2: Immunogenic Activity of HSA-in-Silicone Oil Emulsions and in Starch Microparticles Coated with Functionalized Silicone

It can be seen that the immunogenic activity of the HSA administered in a silicone emulsion is higher than the same amount of protein entrapped in a microparticle. We note that these results are preliminary and have not been repeated with the necessary controls in place.

Discussion

Emulsion Stability: Silicone emulsions formulated with the silicone-polyether emulsifiers used in the present study have been found to be stable.[11] This fact opens the door to a wide range of applications that exploit the desirable silicone oil properties. To date, protein/enzyme entrapment in such emulsions has only been proposed.[3] In the current study proteins were entrapped in silicone oil emulsions (Figure 3) without any observable, detrimental changes in the emulsion properties. We are as yet unable to comment on the ability of the protein to enhance emulsion stability.

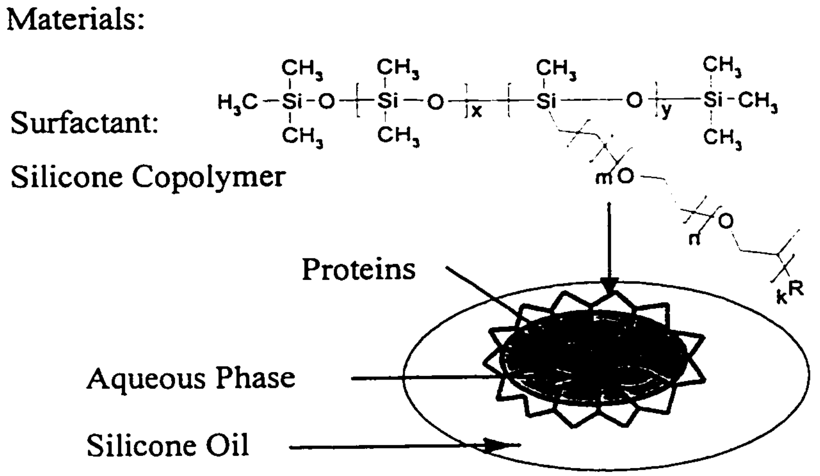

Figure 3: Structure of Protein-in-Silicone Oil Emulsions

Protein Stability: The focus of the present study was the determination of the degree of retention of biological activity of two different proteins, alkaline phosphatase

(MW≈140 kDa) and human serum albumin (MW≈70 kDa), after emulsification in silicone oil.

In vitro, alkaline phosphatase emulsified in silicone oil was found to be enzymatically active. Furthermore, the enzymatic activity was retained even after breaking the emulsion at levels similar to the enzyme solution that was not exposed to silicones.

In vivo, human serum albumin emulsified in silicone oil was found to lead to an immune response following oral administration to mice. This indicates that at least some of the protein was effectively protected through passage via the gastrointestinal tract of the animals (the oral administration of protein in water or in combination with unfunctionalized polydimethylsilicone oils and starch , ungrafted HSA, led to very minimal or no immune responses[9]). That is, the protein in the silicone oil emulsions was effectively protected from enzymatic degradation or decomposition due to the extreme pH conditions in the stomach. However, at least some of the protein was released from these emulsions (possibly in the intestine), in a form recognizable to the immune system, since HSA-specific antibodies were raised. A comparison of the magnitude of immune response to HSA entrapped in microparticles with the current HSA-silicone oil emulsion carrier system suggests the emulsion system to be promising direction for oral drug delivery.

It should be noted that silicones can behave as adjuvants. That is, the immune response of a given protein can be enhanced when the protein is emulsified in silicones (or other hydrophobic materials).[12] This effect is known for emulsions administered by injection. However, such an effect could also arise for the emulsions considered here. That is, the amount of protein required for the observed immune response may be less as a result of the presence of the silicone emulsion. The question of silicone adjuvancy in these orally administered emulsions needs to be clarified.

These preliminary results demonstrate that the biological activity of proteins may be retained in silicone oil droplets even under the relatively harsh emulsification conditions used. This cannot be attributed to the conformational stability of the proteins studied as the HSA is known to be a "soft" globular protein that can easily denature.[13] These findings are somewhat in contrast with previous work at silicone oil/protein solution interfaces,[8] where protein denaturation was ascribed to its interaction with the silicone oil, and with other work that attributed protein denaturation to emulsification processes.[14] There are many possible explanations for the contradiction between these studies and the current results.

The retention of enzymatic activity in the presence of silicone oil may be explained in four different ways. First, a significant amount of the protein present in the emulsion may remain in the water phase and not be affected by contact with silicone oil or the surfactant. Any degradation of protein at the interface would not affect the protein in the water bulk phase, from which the biological activity results.

Second, the proteins we have examined may not be affected by exposure to silicone oil, or conformational changes of the enzyme may indeed occur but not to the extent that the active site is completely and irreversibly altered. To test this possibility, silicone oil was mixed with protein in buffer solution in the absence of surfactant: some enzymatic activity was observed but at a much lower level.

A third explanation is that the surfactant present in these emulsions is playing an important role in the maintenance of biological activity. The surfactant we used, Dow Corning 3225C, is a rake copolymer consisting of a siloxane backbone and block polyethylene oxide/polypropylene oxide chains. It is possible that the protein never has the opportunity to interact directly with the silicone oil, or with air that could also lead to conformational changes, due to steric protection at the interface provided by the polyethylene oxide/polypropylene oxide chains: it is well known that proteins are very compatible with such polyethers.[15]

Finally, the protein may find its way to the interface where it acts as a cosurfactant. With favorable interactions with the surfactant, which restrict the flexibility/mobility of the protein at the interface, it is possible that denaturation is minimized. In this case, the orientation of the protein between the two phases may be very important. To investigate these possibilities we are examining the biological activity of a series of different proteins over a range of concentrations.

Conclusion

Preliminary *in vitro* and *in vivo* studies on protein-silicone interactions at fluid interfaces demonstrate that proteins entrapped in silicone oil emulsions retain their biological activity despite relatively harsh emulsification and demulsification conditions. Although still too early to reach any concrete conclusions, it can be said based on these studies that silicone emulsions could be developed into protein delivery systems.

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Chapter 6

Protein-Silicone Synergism at Liquid/Liquid Interfaces

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Abstract

In previous studies non-ionic organofunctional silicones were shown to enhance the intimacy of protein-silicone interactions at solid-liquid interfaces. In the current studies the same phenomenon was shown to manifest itself, even more clearly, at liquid-liquid interfaces. Proteins or silicones by themselves were unable to impart stability to otherwise surfactant free water-in-silicone oil emulsions. However, stable water-in-silicone oil emulsions resulted from the simultaneous adsorption of a protein and a (triethoxysilyl)propyl-functionalized silicone at the interface from the corresponding bulk phases. Similarly, combinations of proteins and functional silicones lowered the interfacial tension of water-in-silicone oil emulsions more efficiently than either of the surfactants on their own. This clearly implies effective protein-silicone synergism at the interface, possible reasons for which are discussed.

Introduction

Mixed surfactant systems are of scientific interest and have great technological value.[1] Surface active compounds are used in numerous applications including foam control, emulsification, controlled release, etc. The production of single-component surfactants is generally expensive, usually less biofriendly, and with properties that are rarely better than those of mixtures.[1] Thus, mixtures of surface active compounds are generally employed in a variety of commercial products. The cosmetic industry, for example, frequently combines silicones with proteins in formulations for topical applications.[2] In spite of the increase in research activity on surfactant mixture behavior, it remains a challenge to understand the interactions between different surface active components.

Having demonstrated in earlier studies[3] an enhanced affinity between proteins and silicones at solid/liquid interfaces, when the latter polymer is end-functionalized with (triethoxysilyl)propyl groups, we wished to extend our studies to liquid/liquid interfaces. In the present study the mixed adsorption of polymers (silicone and protein) at the silicone oil/water interface from both bulk phases was explored and compared to the separate adsorption of the individual components. The emulsion stability and interfacial tension (IFT) were studied at various bulk concentrations for different ‘silicone-protein mixtures’ and the molecular interactions between the various components were considered.

Experimental Section

Materials

Octamethylcyclotetrasiloxane “D_4”, and PDMS (MW≈28,000 and 500) were obtained from Dow Corning. Mercaptopropylmethyl(2%)-dimethylsiloxane copolymer “PDMS-P-SH” (200 centistokes; MW≈10,000) was obtained from Petrarch Systems, Inc. (Triethoxysilyl)propyl-terminated polydimethylsiloxane (MW≈28,000) “PDMS-T-TES” and (triethoxysilyl)propyl(3%)-dimethylsiloxane copolymer (MW≈13,000) “PDMS-P-TES” were prepared as previously described.[4]

Human Serum Albumin "HSA" (Fraction V, 96-99% Albumin) was obtained from Sigma. Lysozyme "LZ", from hen egg white (crystallized as hydrochloride), was purchased from Boehringer-Mannheim. Trishydroxymethylaminomethane "Tris" and HCl, used in the buffer preparation, were obtained from BDH. The aqueous solutions used in emulsification experiments were prepared with Milli-Q treated distilled water. Deuterium oxide, purchased form Cambridge Isotope Laboratories Inc., was used rather than water to prepare the buffered aqueous phase used for the interfacial tension experiments. This was done in order to increase the density difference between the two phases and, therefore, the accuracy of the interfacial tension measurements.

Methods

Emulsification

The bulk phases used in emulsification were prepared as follows. All silicone polymers were dissolved in D_4. The concentrations of the silicone polymers in D_4 ranged from 3-20 % (wt/wt). The protein concentrations in buffer ranged from 0.003-0.3 (g/mL). All protein solutions were freshly made on the day of the measurement. The protein solutions of both the albumin and the lysozyme were prepared in Tris buffer (pH 8 and ionic strength 0.1).[5]

Emulsification was achieved under turbulent mixing by slow addition of the aqueous phase containing the protein to the silicone oil phase containing the silicone polymer. The conditions of mixing have been previously described.[6]

Emulsion Stability

Phase volume separation and droplet sizes were measured over time. Droplet size was routinely measured with a Leitz Wetzlar microscope. Emulsion droplet images were obtained by using an Axioplan Universal Microscope (Carl Zeiss, Germany) at the differential interference contrast (DIC) imaging mode, with an objective lens of 40x10.75 Plan-Neofluar (Zeiss) and 3CCD color video camera (DXC-930, Sony Corp. Japan). The program used for capturing the images was Northern Exposure, ver. 2.9 (Empix imaging Inc., Canada).

Interfacial Tension Measurements

A Krüss Model DVT-10 drop volume tensiometer was used to carry out the interfacial tension "IFT" measurements. The instrument was equipped with a syringe pump to control accurately the flow rate of the drop phase and to force the drop phase through a capillary into the continuous phase. A timer was started when the first drop in each experiment detached from the capillary and was detected. The time elapsed between subsequent drops (time for droplet formation) was then measured. Since the flow rate was constant, the volume of the droplet was the product of the flow rate and the time of the formation of the droplet and was directly proportional to the interfacial tension between the two phases:

$$\gamma = V_{drop}\, g\, (\rho_h - \rho_l)/\pi d$$

where V_{drop} is the volume of a droplet, ρ_h is the density of the more dense phase (aqueous), ρ_l is the density of the lighter phase (silicone oil), and d is the diameter of the orifice, which was 254 μm. All measurements were performed at 23 °C.

Density Measurements

The densities of both the silicone and aqueous phases were measured at 23 °C with an Anton Parr DMA 48 density/specific gravity/concentration meter.

Results

Functional silicones were examined extensively as surfactants in the experiments described below; they were either modified with (triethoxysilyl)propyl groups ($(CH_2)_3Si(OEt)_3$) at several pendant positions or at the two termini. These compounds were synthesized by the hydrosilylation of commercially available or readily prepared hydrosilicones.[4] The structural variables that were examined included the number of functional alkoxygroups per silicone chain (including the parent, unfunctionalized, PDMS) and the molecular weight of the polymer. Two proteins, which differed in size, hydrophilicity and conformational stability, were examined: human serum albumin, and lysozyme.

Water-in-D_4 (octamethylcyclotetrasiloxane) emulsions were formulated using at least one polymer. This could be a protein in the aqueous phase, a functional silicone in the silicone oil phase or both. Several of the parameters that affect interfacial properties were varied and optimal conditions were determined. The parameters studied included:

the number and nature of the functionalities on the silicone polymer; molecular weight of the silicone backbone; and protein type. Synergism between a protein and a silicone at the interface was assumed to exist when a given property, either emulsion stability or interfacial tension, of a specific 'silicone-protein mixture' reached a more desirable value (improved stability, lower IFT) than could be attained by one of the surface-active components (silicone or protein) acting independently.

Emulsion Stability

The aim of the very first experiments was to investigate the extent to which protein and functional silicones could stabilize the water/silicone oil interface. Therefore, four water-in-silicone oil emulsions were made, the compositions of which are shown in Table 1. Stable emulsions were those for which no phase volume change occurred after droplet settling due to gravity.

Table 1: Silicone oil emulsion composition

Emulsion Abbreviation	Aqueous Phase	Silicone Oil Phase
Tris/PDMS	Tris	PDMS (MW≈500, 0.6 wt%)/D_4
HSA/PDMS	HSA (0.03g/ml)/Tris	PDMS (MW≈500, 0.6 wt%)/D_4
Tris/PDMS-T-TES	Tris	PDMS-T-TES (MW≈500, 0.6 wt%)/D_4
HSA/PDMS-T-TES	HSA (0.03g/ml)/Tris	PDMS-T-TES (MW≈500, 0.6 wt%)/D_4

Of these four emulsions, the Tris/PDMS was clearly the least stable; it phase separated immediately after mixing stopped. Both the Tris/PDMS-T-TES and the HSA/PDMS emulsions formed large droplets (visible by eye) that phase separated within half a day. Only the HSA/PDMS-T-TES mixture gave a milky dispersion stable for at least several weeks. The average droplet size was *ca.* 5μm.

These experiments suggested that the protein and functional silicone act cooperatively to stabilize the water/silicone oil interface. The concentrations of the two polymer constituents were systematically varied over a concentration range (in D_4) of 3-20% (wt/wt) for the silicone polymer and of 0.003-0.3 g/mL for the protein emulsion, in order to determine the optimal values. Emulsion stability was observed over time. As shown in Figure 1 and Figure 2, 6% PDMS-T-TES and 0.03 g/mL HSA were found to be the optimal concentrations for emulsion stability as judged by emulsion lifetime.

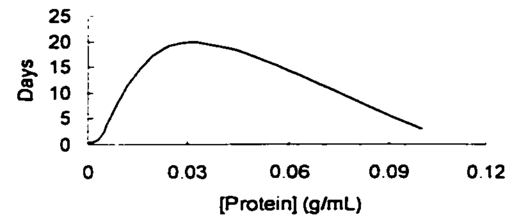

Figure 1: Emulsion stability duration *versus* HSA concentration (0.6% wt. of PDMS-T-TES)

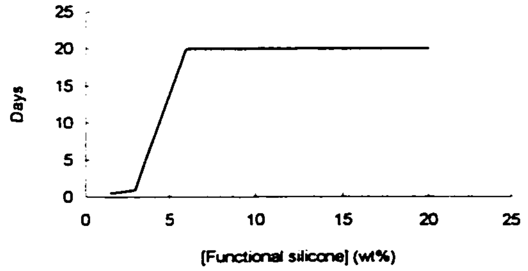

Figure 2: Emulsion stability duration *versus* PDMS-T-TES concentration (0.03 g/ml of protein)

Interfacial Tension

The experiments above provided information about the thermodynamic stability of the protein/silicone w/o emulsions. To obtain a better understanding of the role of the proteins and the functional groups on silicone in stabilizing the interface, interfacial tension measurements were undertaken.

Effect of the silicone functionality

The effect of the functional groups, pendant on a silicone backbone, on the water/silicone oil interfacial properties was initially studied. This was done by comparing the IFT values of interfaces that differed only in the chemical nature of the pendant groups of the silicone polymers dissolved in the oil phase (otherwise the silicones were of similar molecular weight and had the same number of functional groups). Measurements were taken against a series of HSA solutions (0 to 0.3 g/mL). The results of the comparison between silicones possessing terminal (triethoxysilyl)propyl (PDMS-T-TES) and methyl groups (PDMS), respectively, are shown in Figure 3. It is obvious from this graph that the (triethoxysilyl)propyl groups systematically lower IFT values more efficiently than methyl groups.

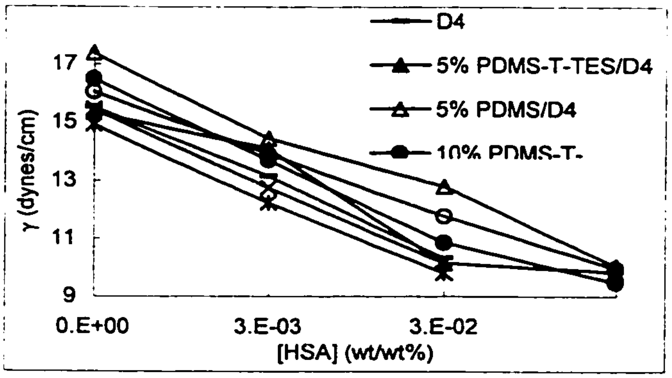

Figure 3: Interfacial tension of silicone oil-water interfaces in the presence of silicones and proteins; PDMS-T-TES *versus* PDMS

For the effect of the silicone functionality to be magnified, and thus more easily observed at a single given silicone concentration, comparison was made between the IFT properties of silicones that had same number of pendant groups but different silicone molecular weights (low *versus* high MW, end-functionalized silicones; PDMS-T-TES). The differences observed are shown in Table 2. PDMS-T-TES of MW≈500 led to a significantly lower IFT value (10.74 dynes/cm) than the corresponding PDMS-T-TES with MW≈28,000 (13.72 dynes/cm).

(Triethoxysilyl)propyl groups were compared with thiopropyl groups randomly distributed on functionalized silicones; PDMS-P-TES (MW≈13,000) and PDMS-P-SH (MW≈10,000) were examined. In this case the two functionalities were compared against both HSA and LZ at a single protein concentration (0.003 g/mL). Lower IFT values were obtained for the silicone with (triethoxysilyl)propyl groups than for the thiopropyl groups; 12.26 dynes/cm over 14.58 dynes/cm with HSA in the aqueous phase, and 13.68 dynes/cm over 14.97 dynes/cm with LZ in the aqueous phase, respectively (Table 2).

The effect of the silicone functionalities is evident in the following micrographs (Figure 4). Identically formulated emulsions containing both protein and silicones differed only in the functionality of the silicone used. When non-functionalized PDMS was used, although no emulsion was obtained, it seems that some of the PDMS was entrapped in the aqueous phase (Figure 4A). When PDMS-P-TES and PDMS-P-SH were used the effect of the functionality is clearly seen in the size differences of the droplets of the corresponding emulsions (Figure 4B, C), with much smaller droplets in the former case. It should also be noted that the stability of these two emulsions was strikingly different. The PDMS-P-SH-containing emulsion began to phase separate only a few days after preparation while the PDMS-P-TES emulsion remained unchanged for more than a couple of months.

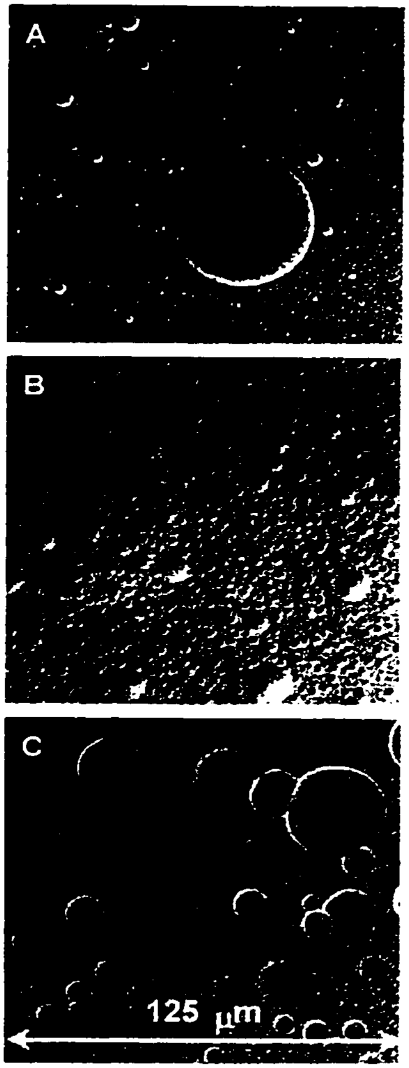

Figure 4: Microscopic illustration of the effect of the presence and nature of the silicone functionality on silicone-protein mixing: A. Mixing of PDMS and protein results in immediate phase separation. Some silicone apparently is entrapped in the protein-containing aqueous phase, shown here; B. Mixing of PDMS-P-TES and protein results in stable water-in-silicone oil emulsions of average droplet size <5µm; and C. An analogous to PDMS-P-TES polymer (with a thiopropyl functionality), PDMS-P-SH, when mixed with protein forms less stable water-in-silicone oil emulsions of larger average droplet size and broader distribution.

Effects of protein type

The emulsion stability was not only affected by the nature of the silicone, but also by the protein. Interfacial tension measurements with proteins, but in the absence of silicone polymers in the oil phase, served as reference points for the different intrinsic interfacial properties of the proteins. The decrease in interfacial tension could be seen with different silicones: PDMS-T-TES (MW≈500), PDMS-P-TES (MW≈13,000), and PDMS-P-SH (MW≈10,000) (Table 2). As shown from the IFT values in Table 2, HSA served to stabilize the w/o emulsions more efficiently than LZ. For example, PDMS-P-TES with HSA had an IFT of 12.26 dynes/cm, while with LZ 13.68 dynes/cm was found. Negligible difference, though, was observed between PDMS-P-SH with HSA (14.58 dynes/cm) and PDMS-P-SH with LZ (14.97 dynes/cm). In addition, the difference between the IFT values of the two proteins at the protein/silicone oil interface is lessen in the presence of silicone polymers. That is, while at the H_2O/D_4 interface HSA and LZ IFTs differ by 2.28 dynes/cm, in the presence of PDMS-P-TES or PDMS-P-SH the difference drops down to 1.42 and 0.86 dynes/cm, respectively.

Table 2: Interfacial tension of various silicone oil-water interfaces in the presence of silicones and proteins.

Silicone in D_4 (wt/wt %)	Protein in Tris/D_2O (3×10^{-3} g/ml)	γ (dynes/cm)	Standard Deviation	γ (LZ-HSA)
10% PDMS-TES (MW~500)	HSA	10.74	0.30	
10% PDMS-TES (MW~500)	LZ	11.59	0.40	
10% PDMS-TES (MW~500)				0.86
10% PDMS-P-TES (MW~13,000)	HSA	12.26	0.31	
10% PDMS-P-TES (MW~13,000)	LZ	13.68	0.09	
10% PDMS-P-TES (MW~13,000)				1.42
D_4	HSA	13.80	0.04	
D_4	LZ	16.08	0.79	
D_4				2.28

Discussion

Proteins are known to adhere well to hydrophobic substrates, such as silicone elastomers.[7] In previous studies the introduction of the (triethoxysilyl)propyl functionality on silicone polymers seemed to facilitate protein adsorption to silicone-modified surfaces even more than unfunctionalized silicones.[3] It was, therefore, anticipated that a similar effect, modified accordingly by the greater intermolecular penetration and molecular mobility that is expected for fluid surfaces,[8] might be seen at a liquid/liquid interface. The interfaces involved in this study consisted (in all possible combinations) of a buffer solution in water, which may also contain HSA or LZ, and either unfunctionalized or functionalized (with (triethoxysilyl)propyl groups) silicones in D_4.

Emulsion stability is indicative of the interfacial properties of the two bulk phases. For stable emulsions to be formed, two conditions should be satisfied: droplet formation should be facilitated by lowering the interfacial tension between the two liquids; and the formed droplets should be prevented from coalescing, by steric stabilization for example.[9] In the water-in-silicone oil emulsions under discussion, with protein dissolved in the aqueous phase and silicone polymers dissolved in the silicone oil phase, the protein would be expected mainly to effect a decrease in the interfacial tension, whereas the silicone polymer is more likely to stabilize the interface via steric stabilization. However, for silicones to function effectively as steric stabilizers they must be well anchored at the interface. Often weak physical molecular forces at interfaces are not enough for adequate polymer anchoring.[10] A covalent bond, or a protein-silicone complex (see below) could provide the necessary binding and, in addition, lower further the IFT.

The magnitude of interfacial tension is a direct method to quantify the extent of the silicone-protein interactions. It is one of the oldest and most popular methods for studying mixtures of surface active components.[11] In recent studies, an analysis of interfacial tension data led to the possibility of determining an interaction parameter[12] between two surfactant mixtures and of evaluating the synergism[13] of surfactant mixtures. In the present studies the drop volume method was used to obtain the interfacial tension of silicone oil/water interfaces. As this is a dynamic technique, the data obtained are unlikely to correspond to true equilibrium values, although the high polymer concentrations used will shift the values close to the equilibrium ones. We report these primarily because of the systematic trends that they reveal.

When PDMS is allowed to interact with proteins, attractive physical interactions between the two polymers likely involve dipole-dipole interactions between the polar silicone backbone and the polar protein groups and hydrophobic interactions between the pendant methyl groups of the silicone backbone and hydrophobic protein groups.[14] When (triethoxysilyl)propyl groups are attached to the silicone backbone, the above interactions are reinforced further by some kinds of interaction between the (triethoxysilyl)propyl groups and the protein. These could involve efficient hydrogen bonding of the exchangeable hydrogens on the protein (-OH, -NH) with $Si(OEt)_3$, or hydrophilic interactions of hydrated or partly hydrolyzed (e.g., $HO(EtO)_2Si(CH_2)_3$) (triethoxysilyl)propyl group with hydrophilic groups on the protein, for example. If the overall physical interactions are strong enough, a silicone-protein complex could arise. In addition to the physical interactions, another possibility exists with the (triethoxysilyl)propyl functionality. This group can undergo hydrolysis and, subsequently, condensation reactions with functional groups on the protein (e.g. $-NH_2$, -OH) leading to the formation, via transesterification, of covalent silicone-protein bonds.[15] The magnitude of such physical or covalent interactions silicone-protein interactions should be reflected in the interfacial properties of the silicone oil/water interface.

(Triethoxysilyl)propyl-modified silicones were shown to strongly interact, at liquid solid interfaces, with HSA.[3] The data obtained from emulsion stability and interfacial studies, at a liquid/liquid interface, are consistent with the previous data at the solid/liquid interface. First, it is clear that the functionalized silicone-protein mixture exhibited synergistic behavior at a water/D_4 interface. Silicone oil emulsions that were stable for several weeks to months could only be obtained if *both* the protein and the functionalized silicone co-adsorbed at the silicone oil/water interface. The independent use of either of these two surface active components did not lead to stable emulsions. Second, the nature and number of the silicone pendant functionalities were directly related to the degree of stabilization at the interface. Interfacial tension measurements on a series of silicone-protein systems also show these effects. Similarly, and not surprisingly, the nature of the protein also affects the interfacial properties.

The effect of the silicone functionality

Three different silicone functionalities were compared in order to determine which factors are important in stabilizing the silicone oil/water interface, particularly in the presence of protein. These were the methyl group (which is equivalent to "no functionality" on silicone), the (triethoxysilyl)propyl, and the thiopropyl group. The (triethoxysilyl)propyl group gave the lowest IFT values at silicone oil/aqueous interfaces of those studied. The lower IFT values of the (triethoxysilyl)propyl group compared to the methyl group (PDMS-T-TES or PDMS-P-TES, respectively, to PDMS) under many different conditions (three concentrations of silicone polymers used at three protein concentrations, Figure 4) translate to PDMS adsorption at the interface that is facilitated by the (triethoxysilyl)propyl groups. At constant silicone weight percent, functionalized silicones that differed only in their backbone length (PDMS-T-TES of MW≈500 *versus* PDMS-T-TES of MW≈28,000) showed that the IFT decrease was more pronounced the greater the number of functional groups at the interface (i.e., the smaller the backbone length), as is intuitively expected. The importance of the nature of the functionality on the silicone backbone was indicated by the comparison between the (triethoxysilyl)propyl and the thiopropyl groups (PDMS-P-TES *versus* PDMS-P-SH). Again, lower IFT values were reported for the (triethoxysilyl)propyl groups (Table 2).

The effect of protein

In these initial experiments, the effect of the protein type on the silicone oil/water interfacial properties was limited to only two, quite distinct proteins, human serum albumin and lysozyme, at a single concentration. Human serum albumin is a globular (≈ 70 kDa) protein with weak internal coherence that is known to be very surface active.[16] Lysozyme is a small (≈14 kDa) hydrophilic enzyme with high conformational stability.[17,18] The differences between HSA and LZ are depicted in their IFTs; HSA always decreased the IFT more than LZ. As expected, this difference in protein adsorption at the interface and the subsequent IFT depression is protein concentration dependent. At the optimal protein concentration for emulsion formation, the corresponding IFT depression seems to be due to the protein adsorption alone, while at lower protein concentrations the silicone adsorption has an observable effect on the IFT values.

What do these observations translate into at the molecular level? The silicone adsorption, which is facilitated at the interface, and the resulting emulsion stability due to the pendant (triethoxysilyl)propyl groups is unlikely to be due to a silicone-protein covalent bond. Silazanes ($R_3Si-NHR''$) are rapidly hydrolyzed and, were they to arise from reaction of protein amino groups with (triethoxysilyl)propyl groups, would not be expected to survive to provide any long term stability to an emulsion. The same is true for silyl esters (R_3SiO_2CR'') that could be formed from protein carboxylic acids. Transesterification could lead to proteinoxysilanes (Protein-OH + $(EtO)_3SiR$ → Protein-O-$Si(OEt)_2R$ + EtOH). Such processes are very slow in the absence of a catalyst especially at pHs near neutrality (although it is possible that the protein could serve the role as catalyst[19]) and are unlikely to occur to a significant degree within the emulsification time frame.

On the other hand, physical interactions between the (triethoxysilyl)propyl groups and the protein that are strong enough to stabilize the protein-silicone interface (e.g., complementary H-bonding between protein-OH and $(EtO)_3SiR$ or $HO(EtO)_2SiR$ clusters) and are not time dependent could give rise to long term emulsion stability. We attribute the stability of the emulsions primarily to many weak, hydrophilic interactions of the (hydrated?, hydrolyzed?) (triethoxysilyl)propyl groups with amino acids. This argument is also supported by the comparison of PDMS-P-TES or PDMS-T-TES and PMDS-P-SH. The greater interaction inferred for the (triethoxysilyl)propyl-functionalized silicones may be the result of their greater number (three ethoxy *versus* one thiol group per functional group) that allows a greater degree of interaction and/or a fundamental difference in the nature of the interaction (the TES groups will be much better able to engage in H-bonding than the thiopropyl group, for instance). Note that there will also be beneficial hydrophobic interactions between the silicone backbone and hydrophobic groups on the protein, the globular proteins in particular.

The (triethoxysilyl)propyl groups affect the IFT values even when protein is absent from the aqueous phase, although in a less consistent way than when protein is present. We attribute this to interactions between the most polar groups on the silicone, the triethoxysilyl group, and the aqueous interface. The protein, when at the interface, modifies the nature of the interface to ameliorate the interactions with these groups; this could include improved H-bonding as note above and the ability of silicone, due to its high backbone flexibility, to additionally favorably interact with any exposed hydrophobic regions of the protein (that is, to adopt a complementary interaction).

Overall, these studies point to a rather synergistic silicone-protein co-adsorption at the silicone oil/water interface, possibly due to many, though weak, hydrophilic and hydrophobic microenvironments created between proteins and silicones that possess (triethoxysilyl)propyl groups. The phenomenon is magnified with an increase in the number of the silicone functional groups.

During emulsification, proteins dominate the interfacial tension reduction at the silicone oil/water interface, although the functional silicones also have an effect. Stabilization of the emulsion is provided by the silicones, acting as steric stabilizers, that are anchored to the protein at the interface by protein-$Si(OEt)_3$ group interactions. Thus, the behavior of these emulsions can be attributed to two processes: (a) lowering of IFT, mainly due to the protein surface activity, so that droplet formation is facilitated, and (b) effective anchoring of silicone steric stabilizers, via the (triethoxysilyl)propyl silicone-protein interaction, which prevent droplet coalescence.

Conclusion

Although proteins and silicones both possess surface activity, neither can independently provide sufficient interfacial stabilization to allow the formation of stable water/D_4 emulsions. However, their ability to stabilize a water/oil interface, when combined, is improved as long as the silicones carry some weakly polar organic functionalities. Both the concentration of the silicone functionality and the type of protein can be used to tune the colloidal properties of the protein-containing silicone emulsions. Although the detailed mechanism is not yet established, our studies reveal a silicone-protein synergistic behavior, possibly due to complimentary co-adsorption at the liquid/liquid interface.

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Chapter 7

Thesis Concluding Remarks

Functionalized (PDMS-TES) and non-functionalized (PDMS) silicones have been found to behave remarkably differently at interfaces (Solid/Liquid and Liquid/Liquid) both in laboratory and biological environments suggesting different protein/silicone interactions for these two silicone polymers.

At the Solid/Liquid interface, silicone-protein microparticles and model films have been the systems that exhibited distinct interactions for the two silicones. Firslty, oral administration of silicone/protein/starch microparticles to mice demonstrated that the proteins associated with TES-PDMS retain antigenicity (or possess enhanced antigenicity) while those associated with PDMS do not. Secondly, silicone adsorption onto immobilized protein films, observed by contact angle measurements and AD-XPS analysis, has shown a greater deposition with TES-PDMS. Furthermore, the degree of protein desorption from the silicone-protein films, induced by incubation in surfactant and aqueous solutions, was found to depend on the type of silicone used. Slower protein removal from these surfaces was observed when the outmost surface layer consisted of

TES-PDMS. It became obvious from these hydrolysis experiments that the functionalized silicone formed more intimate films with the protein, which served to retard its desorption.

The distinction between the two silicones on their interaction with proteins is observed also at fluid interfaces. Water-in-silicone oil emulsions have been effectively stabilized in the absence of any surfactants but the combined presence of TES-PDMS and protein. PDMS and protein mixtures failed to form stable interfaces resulting, instead, in instant phase separation. Furthermore, in the course of these studies it was found that both the concentration of the silicone functionality and the type of protein could tune the silicone-protein interaction.

The stronger affinity for the protein obtained with the functional silicone, in all of the three cases above, undoubtedly suggested a stronger interaction between this silicone and proteins. It was initially thought that the silicone-protein interaction was covalent in nature. This could explain the enhanced antigenicity of the TES-PDMS/protein microparticles, the retarded protein release rates from the model TES-PDMS/protein films and the surfactant properties of the TES-PDMS/protein mixtures. However, the small effect of pH on the protein desorption from the model silicone-protein surfaces indicates that a TES-PDMS/protein covalent bond is unlikely to have formed. This is because a silicone-protein Si-C bond, if formed, would have resulted in much slower hydrolysis rates at neutral pH and therefore much slower protein desorption at this pH. In addition, the formulation of stable surfactant-less emulsions occurs within such a time frame that a TES-PDMS/protein bond is unlikely to form. Therefore, the strong interaction observed cannot be a result of some covalent bonding between these two polymers.

The protein-silicone affinity observed could alternatively be explained by a physically adhering silicone film being formed using the protein as a template. In this case, some protein functionalities would have provided a catalytic effect for the TES-PDMS self cross-linking. This scenario would explain both the better protective role of the protein by the TES-PDMS and the retarded protein desorption from the corresponding model silicone-protein films. However, this mechanism would not explain the emulsion stabilization achieved only with the combined presence of TES-PDMS and protein at the interface. A physically adhering silicone film has been shown not to be able of anchoring on the aqueous droplets providing the required steric stabilization. Unless the elongated silicone chain entangles in the protein chains while self cross-linking (within the very short time of the emulsification process) the improved silicone-protein interactions cannot be attributed to the TES-PDMS self cross-linking. Finally, it seems that the enhanced silicone-protein affinity observed could be best explained overall by the additional dipolar interactions offered by the silicones that carry these weakly polar organic functionalities (TES) with protein functionalities. The detailed mechanism of this interaction is not yet established and further studies are necessary. Future work could probably address the issue by probing changes in the interfacial properties of TES-PDMS/protein film under self cross-linking conditions and non-self cross-linking conditions.

Finally, preliminary *in vitro* and *in vivo* studies on protein-silicone interactions at fluid interfaces demonstrated that proteins entrapped in silicone oil emulsions retain their biological activity despite the relatively harsh emulsification and demulsification conditions. It can be said, based on these studies, that silicone emulsions could be developed into protein delivery systems, though it is still too early to reach any concrete conclusions on the topic of retention of the bioactivity in silicone fluids. Further bioactivity studies involving several different proteins of well-known surface properties and location of their active center are necessary to complete the picture.