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15
Interrelationships between Protein Surface Adsorption and Bacterial Adhesion

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Bacterial adhesion

GENERAL FEATURES

A fundamental question often asked is 'why do micro-organisms stick to surfaces?' Obviously, there must be some benefit for them to do so. The prime directive of micro-organisms is to reproduce and to do so they must assimilate nutrients in sufficient amounts to ensure that the process is successful. Nearly all biological processes require an aqueous environment including the transport of nutrients into the microbial cell. Most natural marine and fresh water environments are quite limiting in terms of nutrient availability. Furthermore, the distribution of nutrients is very heterogeneous, with concentration gradients dependent on a variety of physical factors including light, temperature, and pressure. The ability to stick to a surface would immediately provide several advantages to ensure reproduction in a nutrient limiting environment. First, anchoring to a fixed surface in a microenvironment with relatively high amounts of nutrient would ensure survival. Secondly, surfaces themselves are sources of nutrients since many organic compounds freely adsorb on to surfaces. Additionally, the resulting immobilized matrix of microbial cells , their associated extracellular polymers, and adsorbed organic matter make up what is called a biofilm which offers a protective and nutritive environment for microbial replication. Surfaces amenable to microbial adhesion and colonization include nearly any inert inorganic surface of geological origin, as well as those formed through biological processes such as teeth, bone, wood and shell. Unfortunately, manufactured metallic, glass and polymeric surfaces also provide a readily colonizable surface. Microbial adhesion is not limited to hard, inanimate surfaces. Human skin, intestinal and pulmonary lining, and the urinary tract are all colonizable by micro-organisms, some of which may result in pathologies. Similarly, surfaces of botanical origin such as roots and leaves provide a suitable environment for microbial adhesion.

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ADHESION AND MECHANISMS

Adhesion requires a minimum of two surfaces to form a juncture which may or may not be freely reversible. Adhesive character relates directly to the chemical and physical properties of both surfaces. Adhesion can occur between microbial cells and between cells and inanimate surfaces. Furthermore, cells can mediate adhesive events between inanimate surfaces. The process of adhesion requires energy. Adhesion has been achieved when work is required for adhered surfaces to be separated.

Adhesion of microbial cells to surfaces is often categorized as passive or active or alternatively non-specific and specific (Busscher and Weerkamp, 1987). Passive adhesion involves physicochemical interactions between cells and surfaces. These interactions encompass Van der Waals' forces, electrostatic attractions and hydrophobic interactions. All molecules attract one another by a group of attractive forces collectively known as Van der Waals' interactions. A simplistic definition of these forces would include discussion regarding attractive forces being generated by polarizing molecular dipoles. Practically, in terms of microbial adhesion, these forces would play minor roles because of their relatively weak energies. Electrostatic forces are energetically more powerful and thus more influential. Both attractive and repulsive forces can be in play. These forces are of shorter range in aqueous solutions where most micro-organisms reside because of the high dielectric point of water. The surfaces of microbial cells possess an electric charge that is most often negative (Mozes and Rouxhet, 1991). This is easily demonstrated by a variety of techniques including particulate microelectrophoresis and isoelectric focusing which allows for a relative assignment of isoelectric points (James, 1991).

One must be careful in defining what a microbial cell surface is, which should not be confused with the cell wall or outer membrane. A microbial cell surface is most accurately described as the interface or zone between cells and their environment. The zone does not contain cell structural material but at the same time is distinct from the bulk environment as measured by refractive index (James, 1991). The DLVO theory developed in the 1940s brought forward ideas to explain the net effects of electrostatic attractions (and repulsions) and Van der Waals' forces as a function of the distance separating particle surfaces. The theory has evolved into the present day and accepted idea of the electrical double layer structure (James, 1991). Briefly, the structure encompasses two spatial regions; a relatively thin region called the Stern layer that is directly adjacent to the particle and is composed of ions held by close electrostatic attractions. An outer layer, more diffuse and heterogeneous in terms of ion content is characterized by a decrease in potential as the layer extends outwards.

The thickness of the double layer is inversely proportional to the square root of the ionic strength (Van Loosdrecht *et al.*, 1989). Hence at high electrolyte concentration, or in the presence of polyvalent counterions, the electrostatic interaction will be reduced and cell adhesion will be decreased. The influence of electrolytes on adhesion to solid surfaces was studied by McEldowney and Fletcher (1986) in order to determine the effect of electrostatic interaction. It was found that the concentration and type of electrolyte influenced bacterial adhesion to polystyrene Petri diskes (PD) and polystyrene tissue culture dishes (TCD) surfaces. This influence varied with the bacterial species and the solid surface. Marshall *et al.* (1971) found that the number of *Achromobacteria* reversibly adhered, increased with increasing electrolyte concentra-

tion or as the thickness of the electrical double layer decreased. The bacteria were observed to be reversibly adsorbed at lower concentration of the divalent electrolyte $MgSO_4$, than of the monovalent electrolyte NaCl. The concentrations at which complete repulsion occurred were approximately 5×10^{-4} M for NaCl and approximately 5×10^{-5} M for $MgSO_4$. The difference was related to the greater compression of the double layer in the divalent system at comparable concentration. With both types of electrolyte, bacterial repulsion was observed when the theoretical thickness of the diffuse double layer exceeded about 200 nm.

Husmark and Rönner (1990) reported that adhesion of *Bacillus cereus* spores to hydrophobic and hydrophilic surfaces was influenced by pH. It was observed that adhesion was maximum at pH 3 which corresponded to the isoelectric point of the spore. The differences in adhesion at different pH values were explained as related to the altered charges of the spore surface. The surface charge alteration is thought to have changed the electrostatic interactions between the glass surface and/or the conformation of the spore surface. Moreover, at pH values above the isoelectric point of the spores, they observed that adhesion was less than that at the isoelectric point and the relative decrease was larger for the strongly negatively charged hydrophilic surface than for the more weakly charged hydrophobic surface. This was related to the electrostatic repulsion between the spore surface and the negatively charged hydrophilic glass surface. A similar explanation was offered when adhesion was observed to decrease at pH values below the isoelectric point of the spores.

The electrostatic models are generally most useful for non-permeable surfaces and are recognized as inappropriate for completely explaining microbial cell adhesion because of the heterogeneity of cell surfaces and their ability to mediate adhesion through active events such as polymer production. Nevertheless they serve as a foundation upon which more active adhesion events may occur.

The hydrophobic effect is a physicochemical attractive force that plays a significant role in many examples of microbial adhesion. Simply stated, hydrophobic interactions, i.e the attraction between non-polar compounds are thermodynamically favourable because the free energy of the system is reduced, entropy is increased and stability is gained. Hydrophobic interactions between surfaces largely depend on the unique properties of water itself. The hydrophobic moieties, immersed in the aqueous phase, are surrounded by structured layers of water. The water molecules in such shells have limited freedom to undergo hydrogen bonding, and are at a higher energy level than molecules in the bulk solution (Rosenberg and Doyle, 1990). Assuming that the hydrophobic moiety can not interact with water molecules, then the energy required to introduce this hydrophobic entity into the water phase is analogous to that required to make a cavity in the water which would have the size of the immersed hydrophobic moiety. This energy is also similar to that required to increase the surface area of water. Structured water molecules must assume a constrained conformation, and when they are proximal to apolar moieties, they must assume a configuration of higher energy. When two polar moieties or surfaces approach each other, the constrained water molecules can be freed into the bulk aqueous phase. This is an energetically favourable process. It is associated with an increase in entropy (Rosenberg and Doyle, 1990). Therefore, adhesion mediated by hydrophobic interaction can be seen as a process of exclusion from the water phase, i.e. minimizing an unfavourable interface. Many naturally occurring and man-made inanimate surfaces are non-polar

and provide a readily colonizable plane. There are few, if any, micro-organisms that have an exclusively hydrophobic surface. Microbial surfaces have a relatively complex chemical surface architecture that is more accurately defined as amphiphatic with a heterogenic assembly of polar and non-polar surface entities. Most micro-organisms possess surface hydrophobic character to some degree, which can be measured by a variety of techniques (for review see Van der Mei *et al.*, 1991). The assays for detecting and quantifying the hydrophobic character of micro-organisms all have in common a segregation based step based on the microbial surface hydrophobicity. The MATH (microbial attachment to hydrocarbon) assay is based on the degree of microbial cell partitioning from a polar to a non-polar phase. A decrease in the turbidity of the polar phase provides a relative index for surface hydrophobicity. Contact angle measurements (CAM) of water droplets applied to surfaces is commonly used to predict hydrophobicity of homogeneous inanimate surfaces. The technique has been adapted for microbial analysis by using lawns or layers of microbial cells. There are some technical difficulties in the application of CAM with micro-organisms, nevertheless it has proved to be a useful approach for relative measurements of cell surface hydrophobicity. Other assays have employed hydrophobic interaction chromatography and the biochemical technique of 'salting out' (Lindahl *et al.*, 1981).

Physicochemical forces involved in adhesion are not independent of each other. Many studies (for reviews see Rosenberg and Doyle, 1990) have sought to find relationships between surface hydrophobicity and charge. A decrease in surface charge is often seen to be accompanied by an increase in hydrophobicity, however, just the opposite has also been observed. To summarize physicochemical forces and their role in adhesion (*Figure 1*): At distances as far as 50 nm, microbial cells can be affected by hydrophobic interactions as well as Van der Waals' forces the latter being the weaker. As the distance between the microbial cells and a surface decreases to less than about 20 nm, electrostatic attraction begins to play a significant role in the adhesion process. At distances less than 0.5 nm, specific or active interactions occur which close the distance and lead to an irreversible binding. The intricacies and heterogeneity of microbial surface components make it difficult to propose absolute models that will predict adhesive character. Physicochemical forces undoubtedly have a major influence on the adhesion event simply because of the energies involved. Active or specific adhesion promoting events which will be discussed next are also major factors which, together with physicochemical forces, are responsible for microbial adhesion leading to biofilm formation.

Micro-organisms have the ability to synthesize a variety of structural components that promote cellular adhesion. These materials have the ability to physically bridge cells to surfaces including those of other micro-organisms,biotic surfaces such a plant and animal cell tissue and a wide spectrum of inanimate surfaces.The process of infection, by definition, is initiated by an adhesion event. Adhesive materials of microbial origin include capsules, exo-polysaccharide (EPS), and the appendages: pili,fimbria, fibrils and flagella all of which are spatially located at the interface between cell membrane or wall and the bulk environment. Strongly suggestive evidence for EPS mediated adhesion has come from electron microscopy where intimate melding of surface, EPS, and cells is commonly observed (Fletcher and Floodgate, 1973, Marshall and Cruickshank, 1973).

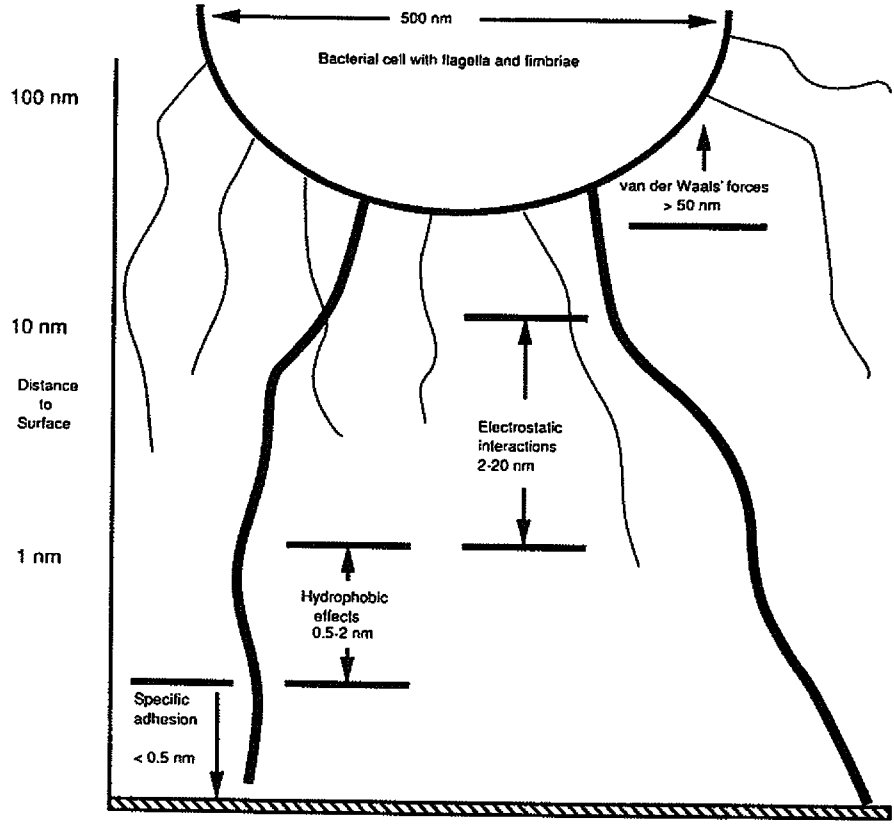

Figure 1. Spatial relationships among physicochemical forces involved in cell adhesion.

Enthalpic and entropic factors have been discussed (Christensen and Characklis, 1990) as important in polymer adhesion specifically: the recognition, that as the size of EPS increases, a greater adhesion strength is predicted. Microbial polymers are diverse in composition and certainly can contribute to the physicochemical forces of cell surface charge and hydrophobic character. Mechanical bridging to surfaces can also be mediated by cellular appendages (Hancock, 1991).

Fimbria (0.2–2 μm in length) and the smaller fibrils (< 0.2 μm) are bacterial cell surface structures that are recognized as being able to facilitate specific adhesion to mammalian cells and plant cells, likely as a preamble to the infection process. The role of the much larger flagella (up to 20 μm length) in the adhesion process is not clear although some studies exist (Belas and Colwell, 1982). Some have argued that such large structures may impart a physical barrier to adhesion. However, it is not difficult to imagine that flagella would give mechanical strength and structural stability to a biofilm matrix by virtue of their length and flexibility (*Figure 2*).

SURFACE ENERGY EFFECTS

Several studies have tried to correlate between adhesion of macromolecules and bacterial cells with surface characteristics such as surface free energy, surface wettability

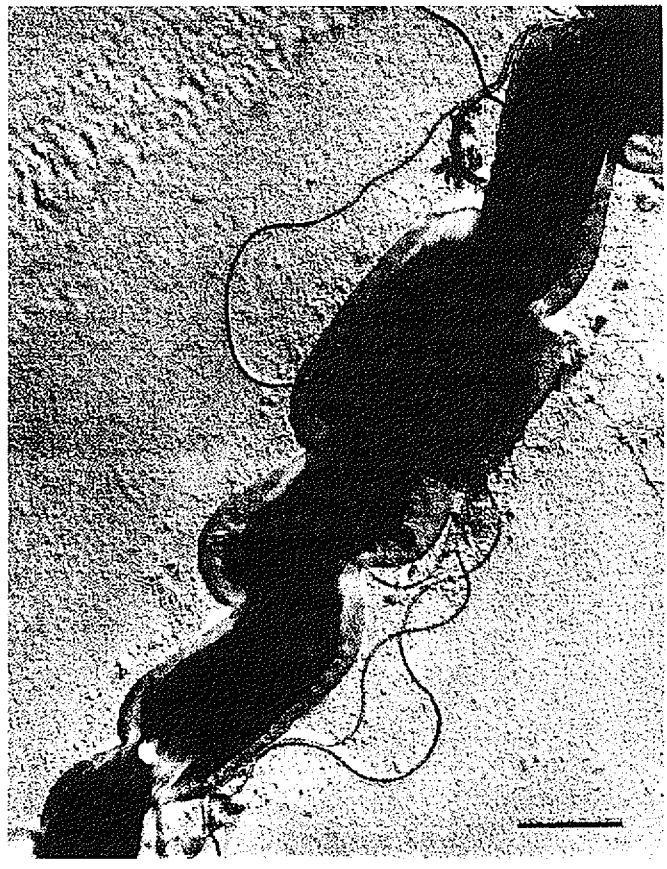

Figure 2. Surface adhered cells of *Escherichia coli* O157:H7 possessing flagella and lipopolysaccharide capsular material. Scale bar = 0.5 μm.

and critical surface tension (Absolom *et al.*, 1993; Baier, 1980, 1982; Dexter *et al.*, 1975). There is a zone where adhesion is minimal and it is usually referred to as the non-adhesive zone despite the presence of an abundant but loosely organized overlaying material or conditioning film. This zone is observed to range between 20 and 30 dynes/cm (Baier, 1982). Tenacious bioadhesion is correlated with critical surface tensions converging in the zone between 30 and 40 dynes/cm, as a result of the tighter, more coherent overlying films bound to such surfaces. When a variety of solid surfaces were exposed to a warm coastal seawater (Bryers, 1987) for periods of times ranging between 20 and 384 h, the number of attached cells was a function of the existing critical surface tension. More cells adhered to high energy surfaces, than to low energy surfaces. From such observations, it was concluded that adsorbed organic layers change initial values of critical surface tension towards a narrow range of values that appear to promote bacterial adhesion.

Absolom *et al.* (1983) developed a thermodynamic model that predicts adhesion to surfaces at various conditions of free energies of the substrate, the bacterial cell surface, and the suspending medium. The model predicts that the change of free

energy of adhesion would increase as the substrate surface free energy increases, provided that the suspending medium has a surface tension less than that of the bacterial cell surface. The free energy of adhesion would decrease with increasing substrate surface free energy if the suspending medium has a greater surface tension than the bacterial cell surface. Experimental data obtained by using various substrates and various bacterial, strains both with a range of surface tensions, including *L. monocytogenes*, agreed with the predicted model. *L. monocytogenes*, a hydrophilic bacterium (contact angle of 26.1° and a surface tension of 66.3 ergs/cm^2), has shown that it would adhere in greater amounts to substrates with low surface free energy, and adhesion was less to substrates with higher surface free energy in a suspending medium of constant surface tension (i.e. at surface tension of 72.8 ergs/cm^2). Adhesion of this bacterium was also observed to go down as the substrate free energy increased, using suspending mediums with various surface tensions.

Adsorption of soluble macromolecules on to solid substrates may affect adhesion. Soil surfaces, in aquatic environment for example, will quickly adsorb organic molecules forming a conditional film. It is well established that adsorption of soluble macromolecules such as proteins to solid surfaces can affect the adhesion of bacteria and other cells. In general, the surfaces that come into contact with protein containing mixtures tend to become quickly occupied by proteins. This can and often does lead to profound alterations in the properties of the material–fluid interface. Adsorbed proteins may remain in the interface to inhibit or facilitate attachment of cells, or to be replaced by other surface active species. Some micro-organisms, including *Listeria*, are capable of synthesizing EPS which can serve to anchor them to contact surfaces (Herald and Zottola, 1988). Though adhesion is often mediated by these, it is likely that the polymers would still have to adhere to a preadsorbed film (Baier, 1982). Several studies have directly investigated the effect of preadsorbed protein films on adhesion and the results obtained were, in some cases, protein and surface dependent. Fletcher (1977) investigated the effect of bovine serum albumin (BSA) and several other proteins on adhesion of a marine pseudomonad to polystyrene Petri dishes. She found that BSA, gelatin, fibrinogen and pepsin impaired cell attachment through adsorption to the dish surface. Protamine and histone were found not to markedly inhibit adhesion.

Meadows (1971) studied the effect of four proteins on adhesion. The attachment of *Aeromonas liquifaciens*, *Escherichia coli* and *Pseudomonas fluorescens* to glass slides was followed after their suspension in phosphate buffer of pH 7.0 containing the proteins salmine, BSA, casein or gelatin. The results indicated that the presence of salmine and BSA markedly reduced the number of cells adhered, as compared to adhesion from buffer. However, casein and gelatin promoted adhesion except for the effect of gelatin on *E. coli* where adhesion was reduced. The effects were related to the glass, bacterial surface and molecular properties of each protein.

Al-Makhlafi *et al.* (1995) studied the effect of milk proteins adsorbed on to surfaces on the adhesion of *L. monocytogenes* to hydrophobic and hydrophilic silica surfaces. Effects on adhesion were observed to be protein specific, with albumin contributing the least effect in promoting bacterial adhesion. Surfaces that support biofilm formation are generally conditioned with a protein film. Whether cells adhere and a biofilm is formed is likely to depend on how the protein film is formed and its molecular orientation on the surface.

SUBSTRATUM EFFECTS

Fletcher and Loeb (1979) studied the influence of substratum characteristics on attachment of a marine *Pseudomonad* to solid surfaces. The hydrophobicity of the substrates was measured by the contact angle method. The substrates were divided into three groups in relation to their hydrophobicity. The first group included non-polar polymers polytetrafluoroethylene (PTFE) (teflon), polyethylene (PE), and polystyrene (PS). The second group represents polymers with polar groups, such as nylon 6.6, epoxy resin, and polyethylene terephthalate (PET). The third group include inorganic, hydrophilic materials such as glass, mica, germanium discharge treated in air and platinum. The results showed a linear relationship between bacterial attachment and water wettability in the series of polymers. Cell adhesion increased with increasing contact angle. However, adhesion to hydrophilic surfaces were inconsistent. The authors related that to the surface characteristics. For example, glass and mica had a low number of adhered bacteria. This was related to the negative charge of the surfaces as determined by electrokinetic measurement. On the other hand, platinum, which had a higher number of attached bacteria was found to be electrokinetically positive in organic-depleted seawater. Such data illustrate the importance of charge on hydrophilic substrata and the importance of hydrophobic interaction on polymers on bacteria adhesion.

Biofilms

The foregoing discussion has addressed the physical, chemical and biotic factors involved in microbial adhesion. More often than not, adhesion is a continuing progressive event that results in the formation of a biotic-abiotic matrix, termed the biofilm.

Charackalis and Marshall have (1990) provided succinct and essential definitions pertaining to the nature of biofilms.

'A biofilm consists of cells immobilized at a substratum and frequently embedded in an organic polymer of microbial origin.'
'A biofilm is a surface accumulation, which is not necessarily uniform in time and space.'
'A biofilm may be composed of a significant fraction of inorganic or abiotic substances held together by the biotic matrix.'

Biofilm formation is a complex process that can begin with the attachment of a single bacterium. In the food industry, the time required for bacteria to attach to surfaces and form hazardous biofilms can be relatively short (Mafu *et al.*, 1990). Cells associated with biofilms have been shown to have definite growth and survival advantages over planktonic cells, especially in regard to an increased resistance to sanitizers. Since the resistance of bacteria in biofilms has been found to increase with time, and since biofilms often involve pathogenic organisms, methods of bacterial attachment have been extensively studied. LeChevallier *et al.* (1988) demonstrated that attachment to surfaces has an impact on the bacterial resistance to disinfection. Theoretically, the physical hindrances of a surface could affect the ability of a disinfectant to approach the cell membrane. A freely suspended organism is susceptible to a disinfectant from

all sides and at all angles, while an organism attached to a surface is susceptible only from one side. This is supported by findings reported by Frank and Koffi (1990) who reported that removing adherent cells of *L. monocytogenes* from the surface increased their susceptibility to sanitizers to be equivalent to that of planktonic cells. Another effect observed was an increase in the resistance as the age of the biofilm increased. As an analogy from medical biotechnology it has been observed (Costerton *et al.*, 1985) that, when medical devices such as cardiac pacemakers are colonized by bacteria, very high levels of antibiotics are needed to eliminate the bacteria.. Common food contact surfaces such as glass, stainless steel, polypropylene, and rubber, even when new, may have fissures and crevices large enough to harbour bacteria. These features coupled with physicochemical and active adhesion forces and readily available nutrients provide a most conducive environment for biofilm formation.

Biofouling is the unwanted accumulation of biofilm material on various surfaces of industrial design including tanks, pipes, filters and other fabrications which have continual or intermittent contact with fluid materials. Biofouling has many costly outcomes, including reduced heat transfer efficiency, corrosion, plugging or reduced flow rates in conduits, and providing an environment for micro-organisms of public health concern.

Control of unwanted biofilm formation can be divided in to two approaches; prevention of the initial microbial adhesion / solute adsorption events or mechanical (cleaning) removal of accumulated biofilm. Over the years, several studies have been conducted to test different surfaces and surface treatments, but these have not generated adequate control procedures to prevent microbial fouling of heat transfer surfaces. Micro-organisms that are adhered to surfaces have been found to be much less susceptible to the killing effect of sanitizers commonly used in the food industry (Mosteller and Bishop, 1993). Therefore, alternative approaches need to be developed which will prevent initial adhesion of microbial contaminants to food contact surfaces rather than trying to detach them once they are adhered.

Abiotic materials, when impregnated with biocides or antibiotics, resist bacterial colonization for as long as the antibacterial agents are released from the surfaces. For example, antifoulant paints have been used to protect the hulls of ships from fouling. These paints prevent organisms from adhering to surfaces by releasing small amounts of active ingredients (Clark, 1987).

Some surface-active compounds have also proven effective in preventing microbial adhesion. Whitekettle (1991), treated stainless steel and wood surfaces with non-ionic or anionic compounds of differing chemical nature. With stainless steel, the results indicated an excellent efficacy with many of the non-ionic surfactants, i.e. the DP-1200 series dispersants. These materials all gave better than 90% inhibition of microbial adhesion as compared with untreated controls. The anionic compounds did not inhibit adhesion. Cationic compounds such as quaternary ammonium salts, also resulted in excellent inhibition of adhesion; but it was due to its toxicity against planktonic cells.

A variety of polymer plastics are susceptible to biodegradation by bacteria and fungi that metabolize plasticizers. An attempt to prevent plastic degradation was made by incorporating some toxic and nontoxic inhibitors. Price *et al.* (1991) studied the effect of an insoluble antimicrobial quaternary amine complex in plastics against bacteria and fungi adhesion. The compound called Intersept®, a relatively non-toxic

inhibitor, was processed into the matrices of ethylene vinyl acetate (EVA), polystyrene and low-density polyethylene (LDPE). The EVA–LDPE containing 3% (w/v) Intersept was found to significantly reduce adherence of *Pseudomonas aeruginosa* as compared to the EVA-LDPE polymer without Intersept. Statistical differences in degree of adherence of *P. aeruginosa* to the polymers with less than 2% Intersept was not observed. The observed effect was related partially to the possible changes in the hydrophobicity of the plastics with incorporation of the inhibitor, as well as the biocidal effect on the adhered bacteria.

Surface modification has been suggested by several authors. Rönner *et al.* (1990) studied adhesion of *Bacillus* spores to glass and stainless steel in relation to the spore hydrophobicity. The spores were found to be hydrophobic and therefore adhered to both hydrophobic and hydrophilic surfaces more than did the vegetative cells. Since stainless steel surfaces are hydrophobic and they are most often used in the dairy industry where *B. cereus* is a causative agent for microbial contamination, the authors suggested that steel surfaces may be replaced or treated so that they yield more hydrophilic surfaces and thereby minimize the risk of colonization by *Bacillus* spores. However, since dairy foods may contain different bacteria with different hydrophobicity and hydrophilicity characteristics, it is difficult to change stainless steel surfaces to be just hydrophilic. Van der Mei *et al.* (1993) characterized the cell surface properties of the thermophilic dairy Streptococci and they found them to be hydrophilic. The authors suggested that, to prevent fouling in the pasteurization process, the heat exchanger plates of the pasteurizer should be rendered more hydrophobic since the hydrophilic strains should adhere minimally to hydrophobic surfaces.

Adhesion of bacteria and other cells is generally observed to occur on surfaces that have been conditioned with a preadsorbed film of organic molecules including proteins. Our laboratories' approach to preventing undesirable biofilm formation on surfaces has been to explore the use of surface adsorbed protein based antimicrobials to pre-empt the initial microbial adhesion events. What follows is a basic discussion of protein adsorption phenomena and how to utilize it as an application in controlling microbial adhesion.

Protein adsorption

Protein interactions with solid surfaces have been studied for decades, and several thorough reviews are available (Andrade, 1985; Norde, 1986; Horbett and Brash, 1987; Brash and Horbett, 1995; Andrade *et al.*, 1996). The following are some generally well-understood, important results that have provided a basis for meeting the challenges facing researchers in this area.

GENERAL FEATURES

Surface-induced conformational changes

Once adsorbed, proteins exist in multiple 'states' on a surface (Andrade *et al.*, 1984; Jönsson *et al.*, 1987; Elwing *et al.*, 1988; Kondo *et al.*, 1991, 1992; Norde and Favier, 1992; Billsten *et al.*, 1995). These states can be identified by differences in occupied area, binding strength, propensity to undergo exchange events with other proteins, and

catalytic activity, or function. Horbett and Brash (1987) reviewed a large body of evidence supporting the existence of multiple states of adsorbed protein. There have been numerous observations indicating the presence of weakly and tightly bound proteins: for example, rinsing a surface after protein contact does not remove all of the protein, yet some of the remaining protein is removable during a second, longer rinse. Also, decreases in surfactant-mediated elutability of proteins from an adsorbed layer are observed as the protein–surface contact time increases.

Elwing *et al.* (1988) used ellipsometry to study conformational changes experienced by complement factor 3 on hydrophilic and hydrophobic silica surfaces. Results were consistent with the very important general observation that greater values of adsorbed mass are found on hydrophobic as opposed to hydrophilic surfaces. Protein molecules are assumed, in general, to change conformation to a greater extent on hydrophobic surfaces relative to hydrophilic surfaces. This is due to the presence of hydrophobic interactions between the solid surface and hydrophobic 'pockets' in the protein molecule. This interaction can give the molecule an extended structure, covering a relatively large area of the surface. The repulsive force normally acting between native protein molecules is probably decreased for such conformationally changed molecules (Elwing *et al.*, 1988). On a hydrophilic surface, forces acting between the surface and the molecule may be smaller in magnitude. The resulting conformational change would likely be smaller, preserving a greater repulsive force among adsorbed molecules. The packing of adsorbed protein is apparently such that a smaller number of adsorbed molecules are found on a hydrophilic surface, with each molecule occupying a smaller area on the surface. Lu and Park (1991) reported that the extent of conformational change experienced by adsorbed fibrinogen increased with contact surface hydrophobicity, with some loss of α-helix content.

Multilayer vs. monolayer adsorption

Some workers have reported that protein can adsorb on to a surface in more than one layer. Arnebrant *et al.* (1985) studied adsorption of β-lactoglobulin and ovalbumin on hydrophilic and hydrophobic chromium surfaces using ellipsometry and potential measurements. On clean hydrophilic surfaces their results showed that a thick, highly hydrated layer is obtained, which can be partially removed by aqueous buffer rinsing. They suggested that the protein adopts a bilayer formation on the surface, with the bottom layer unfolded and attached by strong polar bonds to the surface. Rinsing experiments showed that the upper protein layer is loosely attached, which indicates that the outer layer has a structure closer to that of its native state. This adsorption behaviour was described in terms of surface-induced conformational changes and charge interactions between the protein molecule and surface. In particular, there are always some polar amino acid side chains that can interact strongly with a surface, even if both the protein and surface are negatively charged. Such binding is expected to result in unfolding of the protein, and irreversible adsorption. The consequence of this would be the probable exposure of hydrophobic loops into aqueous solution; adsorption of a second protein layer can therefore reduce the interfacial free energy. In the case of protein in contact with a hydrophobic metal surface, recorded values of adsorbed mass were found to be consistent with formation of a monolayer. Arnebrant

and Nylander (1988) reported possible bilayer formation upon adsorption of oligomeric units of insulin as well.

Some observations from adsorption equilibrium experiments

A great deal is known about how various conditions affect the adsorbed mass of protein. Numerous protein adsorption isotherms have been constructed and compared on the basis of temperature, pH, ionic strength, conformational stability of the protein in solution, and solid surface charge and hydrophobicity. The effects of protein conformational stability and solid surface properties are perhaps best revealed with reference to pH and ionic strength influences.

In general, the effect of pH and ionic strength on protein adsorption is dependent upon which type of interactions predominate (e.g. electrostatic, hydrophobic, or Van der Waals' interactions). Consider a negatively charged surface in contact with a protein solution. If electrostatics comprise the major interactions, adsorbed mass should be greater at pH values below the isoelectric point relative to pH values above it. Below the isoelectric point, the protein and surface are of opposite charge, while both the protein and solid surface are negatively charged at pH values greater than the isoelectric point. As ionic strength increases, the electrostatic interaction is reduced due to 'shielding' by counterions; consequently, increasing the ionic strength should decrease adsorbed mass at pH values less than the isoelectric point and increase the adsorbed mass at greater values of pH. The relationship between adsorbed mass and changes in pH and ionic strength becomes intimately tied to protein conformational stability. In general, pH and ionic strength conditions that lead to a less stable conformation for the protein in solution will lead to an increased adsorbed mass, assuming that the protein molecule is more stable on the solid surface (Luey *et al.*, 1991).

Another observation of importance is that protein adsorption is often an apparently irreversible process. The adsorbed mass remains constant or decreases very little when the solution in contact with the solid surface is depleted of proteins. This irreversibility is more pronounced as protein–surface contact time increases.

Some observations from adsorption kinetic experiments

In considering the kinetics of any interfacial process, the question of transport vs. reaction control must be addressed. Protein adsorption at the interface is controlled not only by the intrinsic kinetic rate which is a function of protein, solution and surface properties but also by the rate of transport of macromolecules from the bulk solution across the concentration boundary layer near the interface.

Proteins are large molecules, and can include a number of different side chains. Diffusion coefficients may vary widely among proteins depending on their concentration and the electrostatic condition of the solution (Cussler, 1989). The initial adsorption rate of protein molecules on to a solid surface can be transport limited either at low or high concentration. The diffusion limitation exists as long as there is a significant concentration gradient near the solid surface. With careful design of an experimental system to minimize the transport-limited period, however, an intrinsic kinetic rate can be estimated.

In any event, changes in adsorbed mass as a function of time under various conditions have been plotted, and explanations have been proposed. Still, relatively little is known about the nature of the adsorbed layer, and predictive models to describe any aspect of adsorption as a function of protein and interfacial properties are lacking. Protein adsorption is characterized by the likely presence of a time dependence in the development of bonds with the surface, a time dependence in the lateral mobility of the protein molecules, and time-dependent conformational changes; it is thus very difficult to describe mathematically.

Many experimental observations have indicated that a major portion of the final adsorbed amount had been adsorbed within the first few minutes of contact (Andrade *et al.*, 1984; Soderquist and Walton, 1980). Soderquist and Walton (1980) proposed that there are three distinct processes contributing to the kinetics of uptake of protein on polymeric surfaces. First, rapid and reversible adsorption of the proteins occurs in a short period of time. Up to 50–60% surface coverage there is a random arrangement of adsorbed molecules, but then some form of surface transition occurs that is probably in the direction of surface ordering, thereby allowing further protein uptake. Secondly, each molecule on the surface undergoes a structural transition as a function of time that occurs in the direction of optimizing protein–surface interaction. Thirdly, the probability of desorption decreases with an increase in the period of incubation, and protein slowly adsorbs more or less irreversibly.

COMPETITIVE ADSORPTION: MOLECULAR STRUCTURE AND INTERFACIAL BEHAVIOUR

Perhaps the most important work in this area has focused on the composition of adsorbed layers formed from complex mixtures (see, for instance, Brash, 1987), identification of molecular factors relevant to the sequential and competitive adsorption behavior of proteins, and the conformational state of adsorbed protein. Study of molecular influences on protein adsorption has received much attention in particular due to its relevance to better understanding of adsorption competition in complex mixtures. Important contributions to current understanding of molecular influences on protein adsorption have evolved from several comparative studies of protein interfacial behaviour, in which similar or otherwise very well-characterized proteins (Arai and Norde, 1990a,b; Shirahama *et al.*, 1990; Wei *et al.*, 1990), genetic variants (Elbaum *et al.*, 1976; Horsley *et al.*, 1987; Xu and Damodaran, 1993) or site-directed mutants (Kato and Yutani, 1988; McGuire *et al.*, 1995a,b; Billsten *et al.*, 1995) of a single protein had been selected for study. A number of factors are known to affect protein adsorption, and these studies have stressed the importance of protein charge, hydrophobicity and structural stability in interfacial behaviour.

Hen lysozyme, ribonuclease A, and α-lactalbumin adsorption to hydrophilic and hydrophobic, polystyrene-coated silica (both negatively charged surfaces) was studied by Shirahama *et al.* (1990). Concerning adsorption to hydrophilic silica, they found that adsorbed mass increased with increasing charge contrast between the surface and protein. At the hydrophobic surface, they found electrostatic interaction to have a lesser effect on the outcome of their experiments in that the adsorbed mass was not clearly related to charge contrast between the surface and protein. Arai and Norde (1990a) described adsorption from single-component solutions of hen lysozyme, ribonuclease A, myoglobin and α-lactalbumin to four different materials, varying in

surface charge density and hydrophobicity. Results of that work led to the major conclusion that, at a given surface, adsorption of a globular protein is related to its structural stability, i.e. proteins of high stability behave like 'hard' particles at a surface, with the interactions governed by hydrophobicity and electrostatics, while adsorption of proteins of low stability ('soft' proteins) may be influenced by structural rearrangement, allowing adsorption to occur even under conditions of electrostatic repulsion.

Horsley *et al.* (1987) made a comparison of isotherms constructed for hen and human lysozyme at silica derivitized to exhibit negatively charged, positively charged, or hydrophobic surfaces. Knowledge that human lysozyme contains one less disulphide bond and is less thermally stable than hen lysozyme, along with visualization of these proteins with molecular graphics, enabled them to explain some of the differences in adsorptive behaviour observed between the two lysozyme variants. Xu and Damodaran (1993) compared adsorption kinetic data measured for native and denatured hen, human, and bacteriophage T4 lysozymes at the air-water interface. Their results showed substantial differences in adsorption dynamics among the three variants, as influenced by their structural state, and the physical and chemical nature of the protein and surface.

Kato and Yutani (1988) evaluated the interfacial behaviour of six mutants of tryptophan synthase α-subunits, produced by amino acid substitution at a single position (residue 49) in the protein's interior, using surface tension, foaming and emulsifying property measurements. The stability of these subunits, as measured by their free energy of denaturation in water (ΔG_{water}), varied from about 5 to 17 kcal/mol, depending on the residue chosen for substitution into position 49. They could attribute observed differences in interfacial behaviour to ΔG_{water} of these proteins with good success. In particular, they observed that less stable mutants are most surface active, i.e. they more rapidly adsorbed and/or more readily unfolded or otherwise rearranged at the hydrophobic interfaces studied in that work.

Multi-component systems

Concerning sequential and competitive adsorption experiments performed by Shirahama *et al.* (1990) at hydrophilic silica, they found that: (i) once an adsorbed layer of a given protein was formed, almost total displacement of that protein would occur upon introduction of a second protein to solution if the second protein had a more favourable capacity for electrostatic attraction with the surface (otherwise sequential adsorption was not observed); and (ii) in a mixture, the protein capable of the more favourable electrostatic attraction with the surface preferentially adsorbed, essentially to the exclusion of the other protein. At the hydrophobic surface, they found (i) the eventual make-up of a film adsorbed from a mixture was not related to charge contrast; and (ii) once an adsorbed layer of a given protein was formed, only partial displacement of that protein would occur upon introduction of a second protein to solution even if the second protein had a more favourable capacity for electrostatic attraction with the surface.

Arai and Norde (1990b) studied the sequential and competitive adsorption behaviour exhibited by the same four proteins tested earlier, and summarized the data with basically the same conclusion concerning 'hard' and 'soft' proteins, i.e. in sequence

and in mixtures, adsorption of a globular protein is related to its structural stability.

The elutability of adsorbed protein by surfactant has been used to provide an index of protein binding strength (Bohnert and Horbett, 1986; Rapoza and Horbett, 1990a,b; Ertel *et al.*, 1991; Wahlgren and Arnebrant, 1991, 1992; Wahlgren *et al.*, 1993a,b; Krisdhasima *et al.*, 1993; Vinaraphong *et al.*, 1995; McGuire *et al.*, 1995a,b). Elutability by dissimilar protein dissolved in a contacting solution has also been used to provide the same information. For example, Slack and Horbett (1989) evaluated the strength of attachment of fibrinogen to solid surfaces by measuring its time-dependent elutability by plasma, and modelled fibrinogen adsorption with reference to its rate of conversion from a weakly bound (exchangeable) to a tightly bound (non-exchangeable) state. Wahlgren and Arnebrant (1991, 1992) used *in situ* ellipsometry to continuously monitor the different effects of cetyltrimethylammonium bromide and sodium dodecylsulphate on the elutability of β-lactoglobulin and lysozyme from hydrophilic and hydrophobic surfaces, as well as adsorption from protein/surfactant mixtures. The elutability studies allowed postulation of four plausible mechanisms for surfactant-mediated removal of adsorbed protein. With the aim of relating surfactant-mediated elutability to protein molecular properties, Wahlgren *et al.* (1993a) studied removal of six, well-characterized proteins from hydrophilic and hydrophobic silica surfaces using a single surfactant, dodecyltrimethylammonium bromide. Some general trends regarding molecular property influences on elutability emerged from that work, but clear correlations between individual protein properties and elutability remained difficult to quantify. On the other hand, similar tests conducted with synthetic mutants of a single type of protein showed a clear correlation between protein stability and elutability (McGuire *et al.*, 1995a). One reason for inconsistency on this issue is that proteins under normal circumstances differ with respect to multiple properties.

MODELING THE PROCESS

Multiple adsorbed states and kinetic models

The concept that adsorbed proteins can exist in multiple states on a surface plays a role in interpretation of most, if not all, experiments in protein adsorption (Andrade *et al.*, 1984; Jönsson *et al.*, 1987; Elwing *et al.*, 1988; Horbett and Brash, 1987). Biophysicists rather routinely gain information relevant to protein structure in solution with circular dichroism (CD). It would be attractive to use CD in structural study of adsorbed protein as well, and adsorption experiments using CD have been performed using stacked quartz plates to increase surface area (McMillin and Walton, 1974). A recent innovation has made CD more applicable to study of structural changes during adsorption, and that is the use of ultrafine silica particles, or nanoparticles (Kondo *et al.*, 1991, 1992; Norde and Favier, 1992; Billsten *et al.*, 1995). In these tests particles have ranged from less than 10 to about 30 nm, and are small enough not to interfere with the CD spectra. Individual molecules are allowed to adsorb to nanoparticles, resulting in a 'solution' of adsorbed protein. In general, structural changes upon adsorption have been unambiguously measured, and agree with expectations for 'hard' and 'soft' proteins. Work by Billsten *et al.* (1995) represents the most direct illustration of the effect of stability on structural rearrangement at a solid surface.

Lundström (1985) presented a dynamic model of protein adsorption on solid surfaces. The model describes the fractional surface coverage of adsorbed molecules as a function of equilibrium concentration, and is based on the assumptions that a protein molecule may change conformation after adsorption and may desorb from the surface. Lundström and Elwing (1990) presented a more detailed version of earlier models (Lundström, 1985; Lundström *et al.*, 1987) to allow for bulk-surface exchange reactions among proteins in one- and two-component solutions. (Some earlier experimental observations (Elwing *et al.*, 1987a,b) indicated that adsorbed protein molecules undergo exchange with protein dissolved in solution, with these exchange reactions taking place more readily on hydrophilic than on hydrophobic regions of a surface.) The work featured manipulation of the equations describing the fractional surface coverage of protein in specific states, and simulations of total surface coverage as a function of equilibrium concentration, and as a function of time. No experimental data were presented, but the shapes of the curves were in qualitative agreement with experimental observations. Currently, however, there is no adequate experimental methodology to directly monitor changes in these fractional surface coverages. Because of this limitation, a more simplified model that can be statistically compared with the available data is needed. Such a model would enable individual rate constants to be related to contact surface, solution, and protein properties.

In summary, we know that adsorbed protein can exist in structurally dissimilar forms, and that simulated kinetic patterns based on models incorporating conformational changes agree with those of experimental data. We know that structural stability plays a large role in competitive adsorption processes in cases where structural change can be expected to accompany adsorption, and differences in structural stability can be correlated with conformational changes experienced at solid surfaces. These summary findings can form the basis for modeling competitive adsorption.

Modeling protein adsorption with a mechanistic approach

Past work with synthetic mutants of bacteriophage T4 lysozyme have involved *in situ* ellipsometry and surfactant-mediated elution (McGuire *et al.*, 1995a,b), ring tensiometry (Wang and McGuire, 1997), the interferometric surface force technique (Fröberg *et al.*, 1997), and CD (Billsten *et al.*, 1995). These studies have shown that: (i) structural alterations definitely occur upon adsorption; (ii) the extent and rate of structural change is related to thermal stability; (iii) mutants exhibited resistances to surfactant-mediated elution that were proportional to thermal stability, and consequently related to extent of structural change; and (iv) concerning a number of T4 variants, results could be explained by modelling adsorption as occurring such that molecules adopt one of only two states, each varying in binding strength and occupied area, with differences in behaviour among the molecules attributable to the relative populations in each state. However, success achieved in relating adsorption to thermal stability should not be taken to imply that $\Delta G_{unfolding}$ calculated for the whole of a complex protein is directly related to its surface activity. T4 lysozyme behaves, more-or-less, as a single-domain protein. But what has been learned with the T4 lysozyme system should apply to whole proteins that can be characterized as 'single-domain', or to the individual domains of a complex molecule. Most proteins and enzymes are best characterized as consisting of multiple subunits, or structural domains. It is realistic to

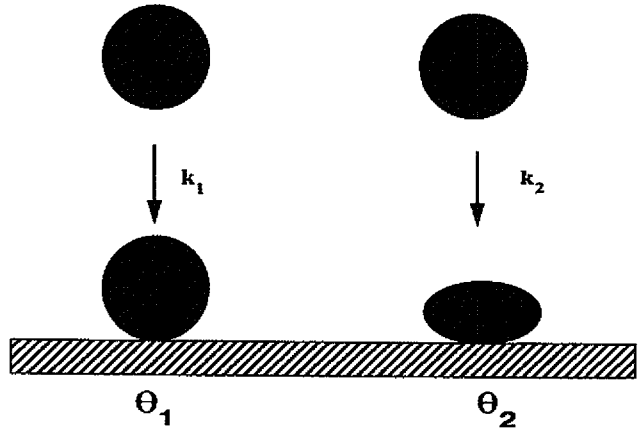

Figure 3. A simple mechanism for protein adsorption from a single-component solution, allowing adsorption into one of two states exhibiting different resistances to elution and occupying different interfacial areas. (See text for further explanation.)

think that the behaviour of these complex proteins at solid–water interfaces can be interpreted in terms of properties of individual domains relevant to their surface activity. As far as adsorption is concerned, the interfacial behaviour of a complex protein could possibly be considered as largely dominated by the interfacial activity of only one domain, or even a sub-domain (Andrade *et al.* 1990). In summary, the surface activity of single domain proteins can be explained with a two-state model, and the surface activity of complex proteins can, in some cases, be attributed to the surface activity of a single domain. Consequently, an important working hypothesis underlying one current approach to modelling is that the adsorption behaviour of complex proteins can be explained with a two-state model. If this is the case, a testable model for quantifying adsorption behaviour in mixtures could be constructed.

The simplest adsorption mechanism consistent with the fact that adsorbed proteins can exist in multiple states would include two adsorbed states. *Figure 3* shows such a mechanism. Rate constants k_1 and k_2 govern adsorption into states 1 and 2, respectively. Although the mechanism is drawn to depict molecules adsorbing directly into states 1 and 2 from solution, a more accurate and detailed mechanism might include a multi-step path to state 2. However, for modelling purposes, the actual path to state 2 is not consequential; only the different rates of generation of two functionally dissimilar forms of adsorbed protein need to be accounted for. If adsorption of practically relevant proteins can be adequately described in this way, extension to the case of competitive protein adsorption would be straightforward.

Figure 4 shows a mechanism for competitive adsorption (between two proteins, A and B) based on *Figure* 3. In each case, all associated rate constants can be determined *a priori*. The protein-specific k_1C and k_2C of *Figure 4* can be obtained from single-component kinetic data and the various exchange constants can be determined through sequential adsorption experiments, as will be described below. Expressions for the rate of change of the fractional surface coverages can be easily written, and solved for θ_1 and θ_2 of each protein as a function of time. It would be a simple matter to simulate the adsorption kinetics associated with each fractional surface coverage (and therefore

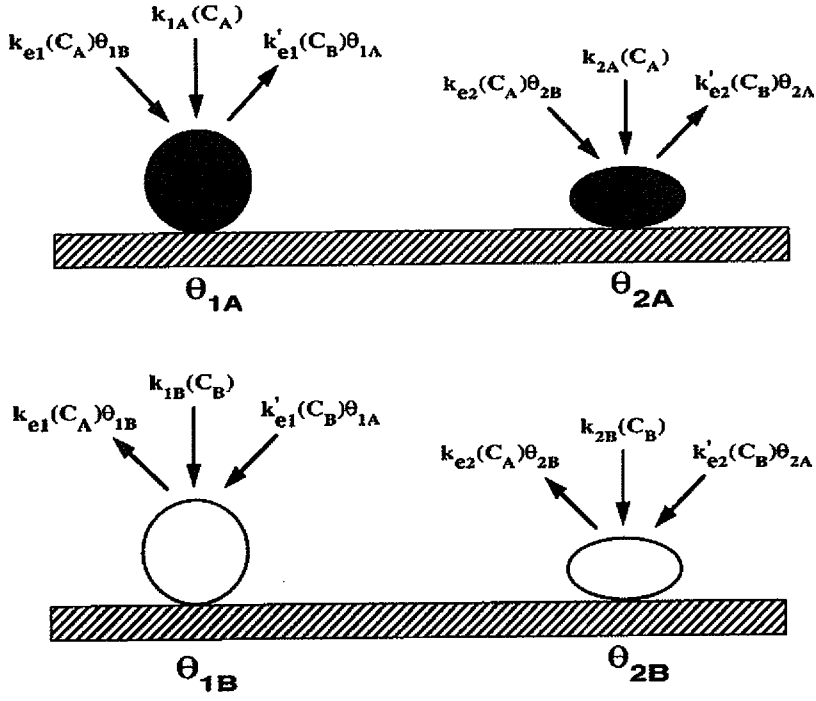

Figure 4. A mechanism for competitive adsorption between two proteins A and B, based on the single-component mechanism of *Figure* 3. (See text for further explanation.)

the total surface coverage of each protein) and these simulated patterns could be compared to experimental kinetic data for adsorption from binary mixtures. *Figure 4* can be easily redrawn to depict competitive adsorption of three proteins A, B, and C. So long as we have sequential adsorption data for each pair permutation of A, B and C, we will have an *a priori* estimate of all rate constants and their competition can be simulated.

Estimating the rate constants

Single-protein adsorption data can be analyzed in order to estimate rate constants k_1 and k_2, describing the tendency of the protein to adopt states 1 and 2, respectively. In the absence of diffusion-controlled adsorption, equations describing the time-dependent fractional surface coverage of protein in each of the two states (θ_1 and θ_2) shown in *Figure* 3 can be solved, such that:

$$\theta_1 = 1/(1 + ak_2/k_1)[1 - \exp(-k_1C - ak_2C)t] \quad (1)$$

and

$$\theta_2 = [(k_2/k_1)/(1 + ak_2/k_1)][1 - \exp(-k_1C - ak_2C)t], \quad (2)$$

where a is A_2/A_1, the ratio of interfacial areas occupied by state 2 and state 1, C is the bulk protein concentration, and t is time. The maximum adsorbed mass of molecules allowable in a monolayer can be identified as Γ_{max}, corresponding to a monolayer of

state 1 molecules. θ_1 and θ_2 are the mass of state 1 and state 2 molecules adsorbed at any time, respectively, divided by Γ_{max}. Thus at any time, $\Gamma = \Gamma_{max}(\theta_1 + \theta_2)$, and when the surface is covered, $\theta_1 + a\theta_2 = 1$. So,

$$\Gamma = \Gamma_{max}[(1 + k_2/k_1)/(1 + ak_2/k_1)][1 - \exp(-k_1C - ak_2C)t], \quad (3)$$

and kinetic data could be fitted to Eq. (3) in order to estimate the rate constants.

Now consider a solution of a protein, A, brought in contact with a surface to begin a sequential adsorption test. Assume with the mechanism of *Figure* 3 that adoption of either state 1 or 2 occurs directly upon adsorption. After adsorption is allowed to occur, the adsorbed film is contacted with protein free buffer, and protein B is introduced. It can be assumed that molecules in each state are exchangeable, but characterized by different rate constants. Since no post-adsorptive reactions occur except exchange,

$$-d\theta_{1A}/dt = k_{e1}[C_B]\theta_{1A} \quad (4)$$

and

$$-d\theta_{2A}/dt = k_{e2}[C_B]\theta_{2A} \quad (5)$$

where $k_{e1}[C_B]$ and $k_{e2}[C_B]$ are concentration-dependent exchange rate constants for proteins in state 1 and state 2, respectively. So, during the period following introduction of protein B,

$$\theta_{1A} = \theta_{1A,ti} \exp(-k_{e1}[C_B]t) \quad (6)$$

and

$$\theta_{2A} = \theta_{2A,ti} \exp(-k_{e2}[C_B]t), \quad (7)$$

where 't_i' is the time at which protein A is removed from the bulk phase by rinsing. Eqs (6) and (7) indicate that measurement of θ_{1A} and θ_{2A} as a function of time would yield the exchange constants.

All rate constants shown in *Figure 4* can thus be determined *a priori*. In each case, then, expressions for the rate of change of each of the various fractional surface coverages can be easily written, and solved for θ_1 and θ_2 of each protein as a function of time. For example, *Figure 4* shows that there are two sources for θ_{1A}: protein A can adsorb from solution into state 1 ($k_{1A}C_A$), and protein A can exchange with adsorbed protein B in state 1 ($k_{e1}[C_A]\theta_{1B}$). θ_{1A} is depleted by exchange with incoming protein B ($k_{e1}[C_B]\theta_{1A}$). So,

$$d\theta_{1A}/dt = (k_{1A}C_A)(1 - \theta_{1A} - a_A\theta_{2A} - \theta_{1B} - a_B\theta_{2B}) + k_{e1}[C_A]\theta_{1B} - k_{e1}[C_B]\theta_{1A}, \quad (8)$$

and expressions for the other fractional surface coverages can be prepared in a manner analogous to Eq. (8) (for illustrative purposes we have made the simplifying assumption that state 1 molecules are exchangeable only by state 1 molecules, and state 2 molecules are exchangeable only by state 2 molecules). It is a simple matter to simulate the adsorption kinetics associated with each fractional surface coverage, and therefore, the total surface coverage from binary mixtures. *Figure 4* can be easily redrawn to depict competitive adsorption of three proteins A, B, and C, or more. So long as there are sequential adsorption data for each pair permutation of A, B and C, for example, there will be an *a priori* estimate of all rate constants and their competition can be simulated. Such comparisons would provide a basis for design of

further experiments to better resolve molecular effects on competition in complex mixtures, subsequently enabling more quantitative prediction of adsorbed layer effects on practically relevant phenomena, such as bacterial adhesion.

Preventing bacterial adhesion through control of interfacial dynamics

Surface derivatizations and coatings

Chemical modification of high energy materials, e.g. metals and glass, can be used to yield interfaces of variable charge density and hydrophobicity. Although such derivatizations would still allow pathogens to adhere, bacterium-surface binding affinities can, in general, be substantially reduced (Wojciechowski and Brash, 1996). The goal in producing such modified surfaces is to reduce interfacial free energy, thus reducing the driving force for adsorption and adhesion.

The most common approaches to preventing fouling on structural materials have been coatings that release biocides such as organotin, copper, silver, and carbamates (Wojciechowski and Brash, 1996). Attempts have also been made to develop surfaces to which bacteria will not adhere. In the areas of food, pharmaceutical and biomedical technology, a number of organic thin films and coatings hold considerable promise in this regard. Such films can be constructed to present a variety of surface chemistries, for example, by attachment of 'anti-fouling' polyethylene oxide (PEO) chains. When a protein or any adsorbate approaches a surface grafted with terminally bound, water-soluble polymers like PEO or polyethylene glycol, the surface polymers become compressed. Compression results in decreased conformational entropy for the compressed chains, which is an unfavourable thermodynamic state. The system thus attempts to move back to the higher entropy state, repelling the adsorbate. McPherson *et al.* (1995) suggest this 'steric repulsion theory' explains the well-known ability of an adsorbed layer of serum albumin, a flexible, globular protein, to enhance the biocompatibility of blood contacting implants. Adsorbed layers of albumin have been shown to reduce the extent of adhesion of *Listeria monocytogenes* to silica as well (Al-Makhlafi *et al.*, 1994).

In any event, it is now well accepted that protein adsorption and bacterial adhesion can be greatly reduced at surfaces treated with PEO (Desai *et al.*, 1992). The efficacy of protein repulsion depends on polymer surface density and the molecular weight (length) of the PEO chains. Leachable biocides (Deitch *et al.*, 1983), bound or leachable antibiotics, and immobilized quaternary ammonium compounds (Speier and Malek, 1982) have also been prominent in the preparation of bacteria-resistant biomedical materials. To-date, however, there is no particular surface treatment that is in any way widely accepted as sufficient for preventing bacterial adhesion.

Protein antimicrobial barriers to adhesion

Modified materials based on the covalent or non-covalent attachment of antimicrobial compounds to 'standard' surfaces, or controlled release of antimicrobials, are also being developed. The efficacy of covalently bound antimicrobials may be limited because their motion is restricted, and their mechanisms normally involve disruption of the cell membrane of the target organism. Non-covalent immobilization as well as

controlled release of bactericidal agents should be effective in short-term applications, but their effectiveness must eventually vanish.

Much research in the authors' laboratories has focused on development of 'food grade' antimicrobial barriers, the premise being that, if antimicrobial activity can be maintained at the interface, sensitive bacterial cells or spores that attempt to attach would be killed. Historically, the peptide antiobiotic nisin has been used in foods as a direct additive to inhibit the growth of Gram-positive cells and spores and is approved in the USA for use with pasteurized cheese spreads. Nisin can withstand activity loss during thermal processing and exposure to acidic food environments. These characteristics and others such as non-toxicity make its use as an antimicrobial agent attractive in many food processing situations (Hurst, 1981). Similarly, hen lysozyme is a commercially available protein antimicrobial that offers application in food processing systems.

It has been shown that nisin can adsorb to surfaces, maintain activity and kill cells that have adhered (Bower *et al.*, 1995a,b). The effectiveness of adsorbed nisin was highly dependent upon the conditions under which it was adsorbed. Surfaces exposed to solutions of high nisin concentration had increased antimicrobial activities, decreased adherence of *L. monocytogenes*, and fewer viable attached cells than surfaces placed into lower concentrations. Nisin films produced when surfaces were exposed to a nisin solution for eight hours retained less activity than those made after only one hour of adsorption. Nisin films placed in phosphate buffer for 5, 10, or 15 hours, exhibited progressively less nisin activity, a greater number of attached cells, and an increase in the number of attached, viable cells. The antimicrobial activity of adsorbed nisin also depended upon surface hydrophobicity, with surfaces of low hydrophobicity retaining more nisin activity and having fewer adhered cells than the more hydrophobic surfaces.

To be of real value in practice, protein antimicrobials must be able to withstand elution by other proteins and surface-active components (Lakamraju *et al.*, 1996). The activity of adsorbed nisin can decrease significantly, for example, when either BSA or β-lactoglobulin is allowed to sequentially adsorb. The viability of adhered *L. monocytogenes* cells can increase after sequential adsorption with BSA or β-lactoglobulin as well. Schmidt *et al.* (1990) studied the activity of hen lysozyme after allowing the enzyme to non-covalently bind to alkylated silica surfaces. Using a combination of fluorescence photobleaching and excitation in an evanescent field, they concluded that lysozyme was adsorbing to the surface in multiple layers. The first layer was composed of molecules adsorbed in a tightly bound state that were not measurably elutable by identical molecules in the bulk. However, these enzymes no longer exhibited any catalytic activity. A second adsorbed layer was less tightly bound and consequently more elutable. Lysozymes in this layer retained some activity.

Thus identical protein antimicrobials in a given film may adsorb with different binding strengths. In addition, optimal application conditions for one protein antimicrobial may be suboptimal for another. For example, hen lysozyme is an enzyme, exhibiting a different mode of action from that of nisin; the effect of adsorption on its activity will also differ. There is yet the comprehensive mechanistic understanding required to apply non-covalent immobilization of protein antimicrobials in practice. But, recent advances in molecular biology would allow generation of improved forms of these and other proteins relative to their functionality in a given application. Thus

continued attention to preparation of food grade antimicrobial barriers, involving enzymatically active and/or inactive proteins, is probably warranted.

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