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Short Review

Problems and approaches in covalent attachment of peptides and proteins to inorganic surfaces for biosensor applications

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SUMMARY

Some of the fundamental problems in covalent attachment of peptides and proteins to putative biosensor surfaces are reviewed and specific approaches to these problems discussed. In addition, selected aspects of our recent work utilizing self-asssembled monolayer (SAM) systems designed to react selectively with the thiol side chain of Cys in proteins are presented. Uniform attachment of a 21-amino acid peptide antigen through a single Cys residue with retention of biological function (antibody binding) has been attained. Further work with this system may lead to solutions for some of the problems which currently prevent the development of reliable biosensors for industrial and medical use.

INTRODUCTION

It has been recognized for many years that the specific recognition properties of proteins (primarily enzymes and antibodies) might be used to construct electronic or optical sensors which would be useful for medical, industrial, and environmental applications [4,7,9,12]. For microbiologists, sensors capable of real time measurements of components in culture media, concentrations of antibiotics, or the secretion of specific products by cultured cells can be envisioned.

Certain features are essential if such biosensors are to be of practical use. They must be assembled by reproducible processes which can be easily adapted for large scale procedures. They must be stable to storage and must have a reasonable lifetime in use. They must be adaptable to different environments and the biological molecules must retain structure and function while remaining tightly associated with the sensor surface. Thus, although many biosensors of different capabilities have been described, their practical application has lagged and relatively few biosensors are available in the marketplace.

One of the major challenges in constructing practical biosensors is the necessity for tight, essentially irreversible, attachment of the biologically active molecule (protein or peptide) at the sensor surface in a fashion which favors retention of full biological activity, uniform structure, and long term stability. The preponderance of the sensors described in the literature utilize polymer trapping or membrane barriers to hold the protein near the sensor surface. However, all such systems are subject to loss of activity through such processes as diffusion or aggregation of the protein.

An appealing alternative approach to the attachment of proteins or peptides to putative sensors is through covalent linkage. This approach can avoid the problems of instability, diffusion, aggregation, or inactivation of proteins which are trapped on sensor surfaces by polymer matrices, or adsorbed on surfaces or Langmuir–Blodget films. It has been used with some success for a number of biosensor systems described in the literature [3,5,6] but very little work has been done to design chemical schemes which are capable of producing uniform layers of active protein. Indeed, most of the chemical approaches which have been described are virtually certain to generate irregular surfaces with many partially or totally inactivated protein molecules presented in a variety of orientations. In addition, the most commonly used methods are also predicted to generate cross-linking of the protein itself. In this paper we describe our recent work addressing these and other problems associated with production of optimal surface structures for biosensor applications.

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SOME FUNDAMENTAL PROBLEMS

Construction of an ideal biosensor surface presents many challenges. Four of the most apparent ones are: (1) ideally, the protein molecules should all be attached through the same locus; (2) the attachment site and chemistry must not interfere with the structure or accessibility of the active site in the protein molecule; (3) the attached proteins or peptides must not be denatured or inactivated at the surface during or following attachment; and (4) the attached protein should be stably bound at the surface through linkages which are essentially irreversible and are not susceptible to disruption by hydrolysis or other interactions with species in the bulk phase.

The first difficulty to be addressed is the fact that proteins have multiple functional groups of the type which are most convenient for attachment to sensor surfaces. The two dominant functionalities in most proteins are amino and carboxyl groups, and these are also the most commonly used for attachment to surfaces. Thus, glutaraldehyde is used for attachment of proteins to silicon or glass surfaces modified with aminosilanes. However, this reagent is also known to cross link amino groups in proteins. Furthermore, since most proteins are rich in amino functionality, the individual species are likely to be randomly attached to the surface in many different orientations. Use of carbodiimide for cross linking through carboxyl groups presents the same problems. Figure 1 presents a schematic illustration of the difficulties which arise under these circumstances. If a protein molecule has three potential sites (A, B, and C), for chemical attachment to the inorganic surface and if one such site (e.g. C) is the biologically active domain in the protein, attachment at these sites may have a different impact on the function of the resulting structure. Attachment through site A will leave the active site open and accessible, but attachment at site B produces a steric blockage, and attachment at the active site itself will inactivate the protein. If the protein species are cross linked to one another through sites A, B, or C, this not only produces a multilayer of protein at such sites, but also has the potential for blockage or inactivation of the active site. Thus, it is clear that a surface which can react with a single site on the protein, a site which is distant from the active site, is a prerequisite for production of an optimal biosensor surface, and future work directed toward that goal is required.

The possibility that surface-bound proteins can lose their native conformation and thus their biological activity is well known [2,11]. This appears to be the result of multiple interactions of the different side chains in individual protein molecules with both hydrophilic and hydrophobic structures on the surface. It is a complex phenomenon which will not be reviewed here. However, the physical forces which produce non-specific interactions and denaturation of proteins at surfaces can be effective whether the protein is covalently linked to the surface or not. Thus, as indicated in Fig. 2, proteins bound to a surface with their active site intact and accessible may still be inactivated by either partial or total distortion of the active site due to non-specific adsorption and spreading of the protein molecules. There do not appear to be any data which allow estimation of the extent to which this phenomenon occurs in current biosensors. Quantitative evaluation of the activity of individual molecules on the surfaces is difficult and demonstration of *some* biological activity does not establish that all attached molecules are active; indeed it appears probable that in most systems a mixture of active, partially inactive, and totally inactive species is present. Thus, it appears that surfaces which are designed to have minimal non-specific adsorption of proteins in general should be used to tether proteins through single sites as described above. This requires

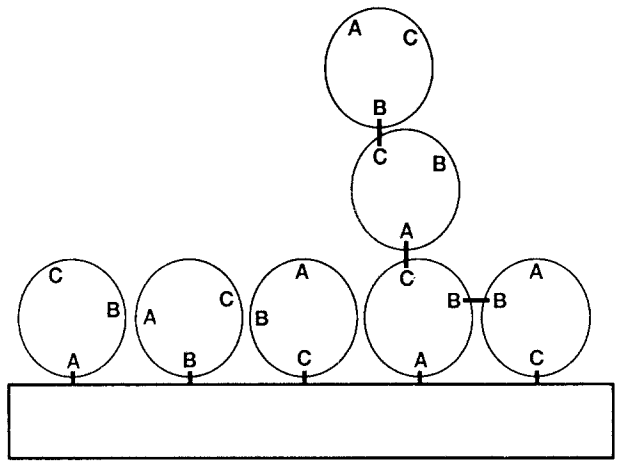

Fig. 1. Illustration of the theoretical result of attachment of peptides or proteins to surfaces through multiple sites. The illustration shows three potential attachment sites which are equivalent chemically (e.g. amino or carboxyl groups) and which can be linked to the surface or to each other by the attachment chemistry employed. In this illustration, if C is an active site, only two of the seven such sites are accessible for interaction with molecules in the bulk phase.

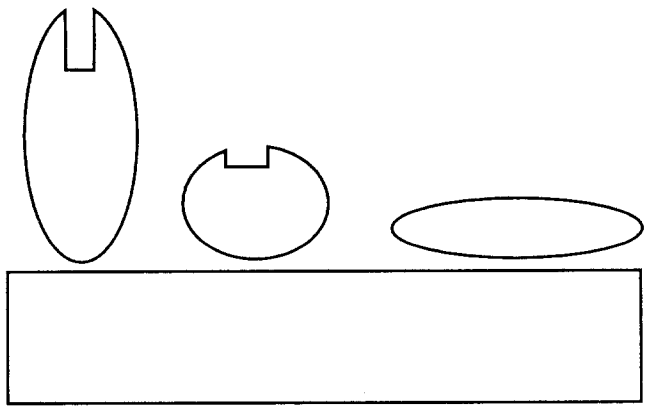

Fig. 2. Illustration of surface unfolding of a protein and subsequent distortion or loss of a specific binding site. Interaction of the protein through the surface is through non-specific hydrophobic and polar interactions which are known to occur with many proteins. Any protein attached to the surface through specific linkages is also subject to this denaturation.

the concurrent presence of a specific chemical reactivity at the surface and molecular structures which have minimal affinity for the various side chains of protein molecules.

Even if ideal surfaces selective for a single site on a protein are obtained, there is a concern that the surfaces will be unstable or subject to long term hydrolysis from aqueous analyte solutions. The short chain silanes commonly used to modify silicon and glass for cross linking to proteins appear to be susceptible to this concern. As indicated in Fig. 3(A), these short chain species do not form compact hydrocarbon films over the silicon dioxide surface and both the attachment site for the original alkylsilane and the linkage of the protein may become open to hydrolysis. However, this problem can be overcome if long chain silanes are used to coat the surfaces (Fig. 3(B)). With chain lengths greater than eleven carbons a compact film will form, the 'self-assembled monolayer' (SAM). Thus, the synthesis of SAM-forming materials with chemical reactivity directed toward specific protein side chains is an important aspect of the effort to design surfaces with the molecular structure which is optimal for biosensor applications.

EXPERIMENTAL APPROACH AND RESULTS

General approach

We have utilized the SAM approach to address some of these problems. Specifically we have focused on long-chain alkyl trichlorosilanes which produce uniform and compact monolayers on silicon, glass, and other oxide-rich surfaces. Compounds which have specific chemical functionality directed toward the functional groups found in proteins have been prepared, with an emphasis on highly electrophilic compounds intended to react selectively with the thiol side chains of Cys residues in proteins. The SAMs formed from these compounds produce exceptionally uniform and stable surfaces, and the probability of forming stable covalent linkages with minimal impact on biological structure and activity is enhanced by use of the thiol-directed chemistry. For covalent attachment of biologically active molecules we have worked with synthetic peptides which are antigens for specific antibody interactions, but are smaller and less complex than most naturally occurring proteins. The accessibility of the antibody binding site has been assessed to examine the impact of the surface chemistry on the biological function.

Generation of surfaces which react with specific functionalities in proteins

Proteins contain many potential reactive sites for attachment to surfaces. Amino groups (Lys and *N*-terminus) and carboxyl groups (Asp, Glu, and *C*-terminus) occur with the highest frequency in naturally occurring proteins, and specific attachment at a defined site is very difficult with these functionalities. However, because of their abundance, and because of the ease of chemical attachment through these sites, they are the ones most frequently utilized. For example, it is common to coat silicon surfaces with short chain amino silanes (either the trichlorosilanes or trialkoxysilanes) to produce a surface enriched in amino functionality. This surface can then be used to attach amino groups on the surfaces of proteins through cross linkers such as glutaraldehyde, or the bisimidates. Alternatively, the surface amines can be linked to carboxyl side chains through carbodiimide chemistry. However, as outlined above, it is

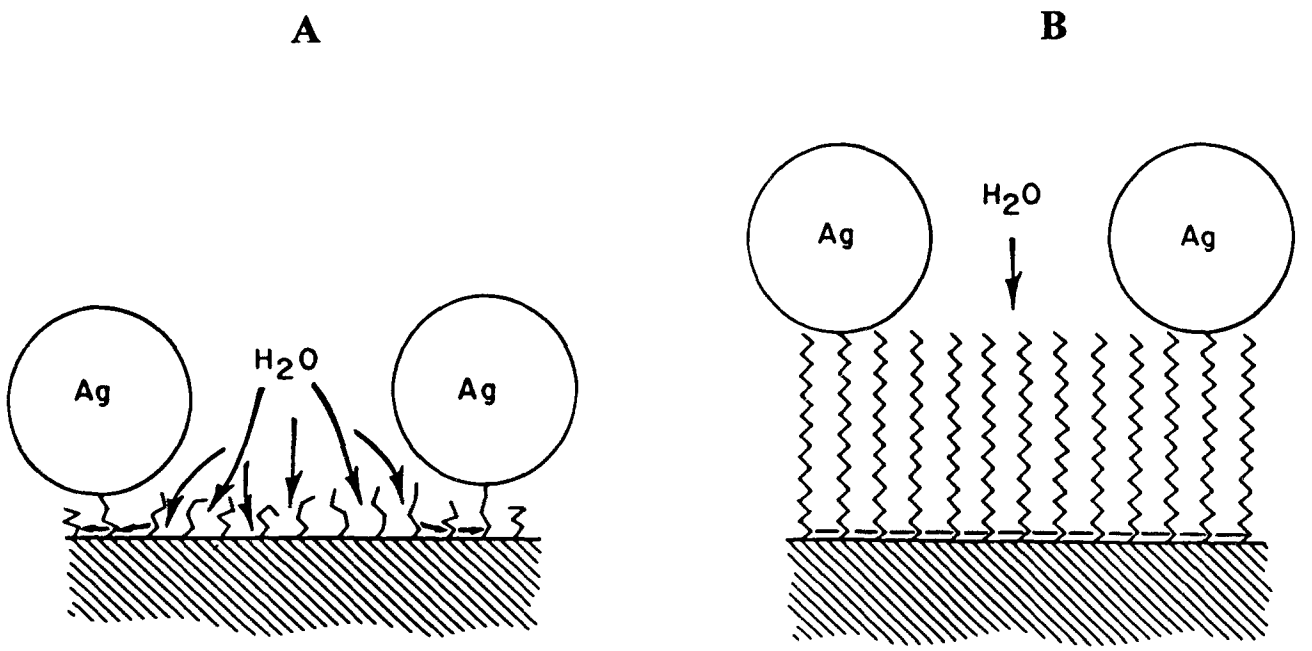

Fig. 3. (A) Surface hydrolysis of short chain silanes and attached peptides (Ag) resulting from accessibility of the silicon surface to the bulk phase. (B) Protection of the surface from hydrolysis by a self-assembled monolayer of long chain hydrocarbon with attached peptide (Ag).

apparent that these approaches must produce surfaces which are highly heterogeneous with regard to the site of protein attachment and the presence of oligomers or polymers of the protein. Little chemical characterization of such surfaces has been done, but it is apparent that they are far from ideal for biosensor applications.

An ideal surface would have the protein attached through a single site. To approach this goal, one must focus on the amino acids which occur in lower abundance in proteins, but which have the chemical property of being reactive under non-denaturing conditions. Of the possibilities in naturally occurring proteins, the sulfhydryl group of cysteine is the most obvious candidate. There are many specific and mild reagents for thiol modification, and it is not uncommon for proteins to have a small number of reactive Cys residues. An example of this is the Fab' fragment of IgGs. This fragment is generated by reduction of the disulfide bond in the bivalent (Fab')2 fragment (Fig. 4) and thus has a single free thiol. Theoretically, this protein could be attached to sensor surfaces through this single free thiol site, and generation of a uniform and biologically active surface would be expected. Indeed, Bright and coworkers [5] described a sensor in which attachment of the Fab' fragment to glass fibers was used to produce an immunosensor. However, the tresyl chemistry used did not allow totally selective attachment through the thiol, and some reactivity with amino groups was apparently obtained at pH values above 5.

Our approach to the directed attachment through thiols has been to synthesize long chain alkyl trichlorosilanes which have electrophilic functionalities with predicted specificity for thiols [8]. Three classes of compounds have been prepared: alkyl halides, benzyl halides, and α-haloacetyls (Fig. 5). Thiols are predicted to react with these compounds to varying extents through displacement of the halogen, and for each class of compound it was expected that the reactivity would increase in the sequence, chloro < bromo < iodo. As indicated in Fig. 5, this reactivity was confirmed with model thiols and Cys residues in peptides examined in solution and in SAMs. The most reactive compounds (benzyl bromide, benzyl iodide, and α-iodoacetyls have been shown to produce selective reaction with thiols in proteins, and their range of electrophilic character may allow flexibility of use for different proteins or peptides. We have demonstrated thiol-specific attachment for short peptides using the model peptide glutathione which contains both amino and thiol side chains, and using a nonapeptide Laminin fragment which contains other reactive side chains found in proteins.

Although our work confirms specificity for thiols with these small peptides, even slight reactivity with amino groups could present difficulties with large protein molecules. Since amino groups are much more abundant than thiols in most proteins, even low reactivity of the surface functionality with amino groups could have a significant impact. Indeed, we find that in solution the reactivity of the α-iodoacetyl types of compound with amino groups is significant when the amino compound is present in very large excess (>1000-fold molar excess) over a thiol.

The reactivity of the electrophilic compounds with amino groups will be pH dependent. At higher pH values the amino groups of proteins are unprotonated and may compete with thiol for the electrophilic surfaces. However, the protonated amino group is unreactive. Normal pH values for α-amino groups vary between 7 and 8, and those for the ϵ-amino group of Lys range from 9–11. Thus, at neutral pH the Lys amino side chains are more than 99% protonated (unreactive), but more basic pH values should be avoided.

A totally thiol-specific approach is the formation of disulfide bonds between the surface and the protein. This has been reported to produce uniform layers of cytochrome *C* on glass, germanium and silicon [1]. However, specific disulfide formation between protein and substrate must overcome competing reactions between thiol–thiol interaction on the surfaces, and disulfide formation between the protein molecules. Thus, the uniformity and the extent of coverage of these surfaces may be uncertain.

Evaluation of peptide attachment and antibody binding to SAM-coated silicon and glass

We have utilized the thiol-specific SAMs to examine the nature of peptide-coated silicon and glass surfaces. Previously

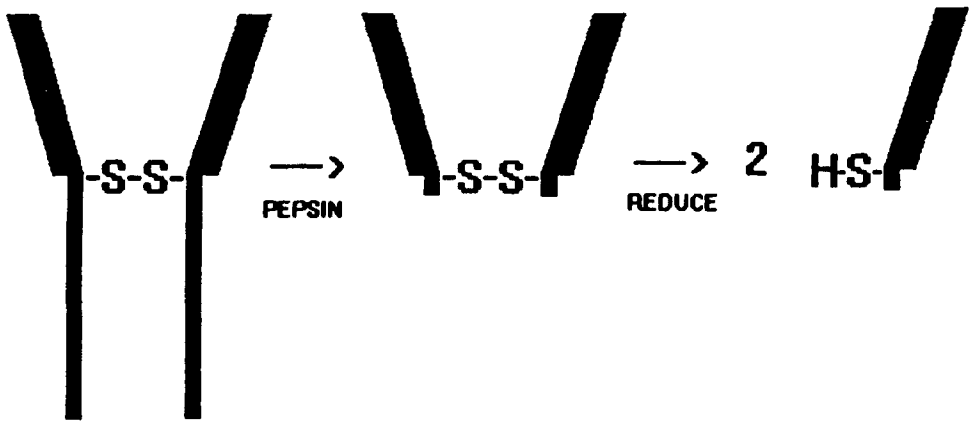

Fig. 4. Generation of an antibody fragment with a single free reactive thiol (Fab').

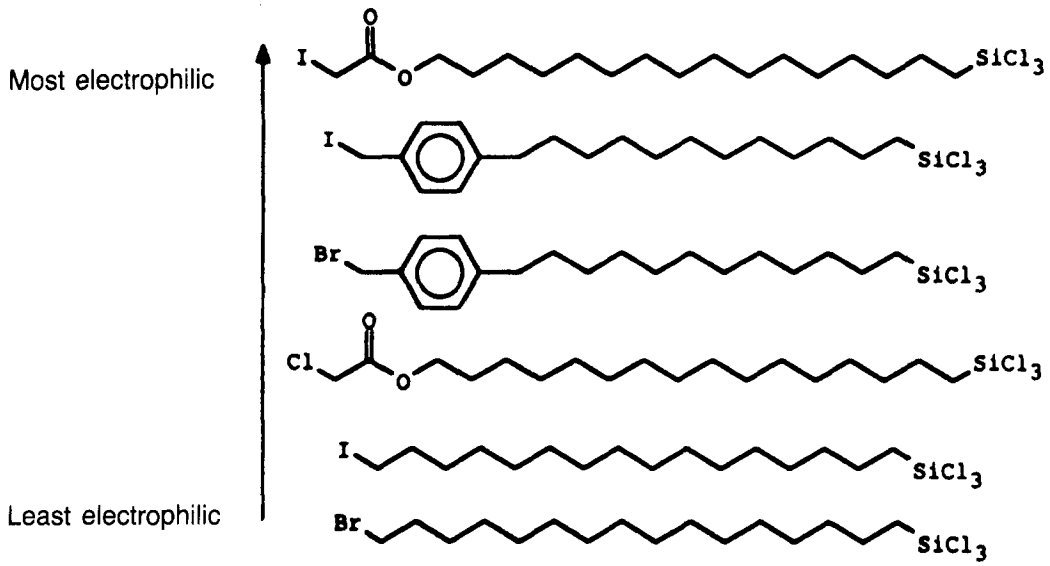

Fig. 5. Electrophilic long chain trichlorosilanes synthesized for study of peptide and protein attachment through free Cys side chains. The compounds are arranged in increasing order of reactivity with model organic thiols, and with model peptides.

[1] we have reported the attachment of short peptides and demonstrated specificity for the thiol side chains of Cys residues in those peptides. To extend this work toward the design of an immunosensor, we have further examined the attachment of synthetic peptides for which antibodies have been obtained.

A peptide which is derived from the parathyroid hormone receptor, a cell surface protein in bone and kidney cells, has been used for these studies. This peptide contains 21 amino acids with a single Cys residue at position 10 (Fig. 6), and we have raised an antiserum to it for our work with that receptor system. This peptide was found to attach to the α-iodoacetyl surfaces described previously. The surface attachment of the peptide could be followed by measurement of surface nitrogen or sulfur with Electron Spectroscopy for Surface Analysis (ESCA), determination of the thickness of the surface layer by ellipsometry, and biological activity (antibody binding). Fig. 7 shows the time course for attachment of this peptide as determined by ellipsometry measurements of the thickness of the surface layer on a SAM-coated silicon surface formed with the α-iodoacetyl compound. As was demonstrated with smaller peptides in earlier work [8], saturation of the surface as determined by ellipsometry occurred within a few hours. This time course and saturation were also demonstrated with ELISA and ESCA.

With the 21-amino acid peptide used in these experiments, the surfaces are covered with a layer which maximizes at an average thickness of 6–7 Å. Since the dimensions of a fully extended peptide are approximately 75 × 7 Å, this thickness is consistent with a geometry in which the peptide is

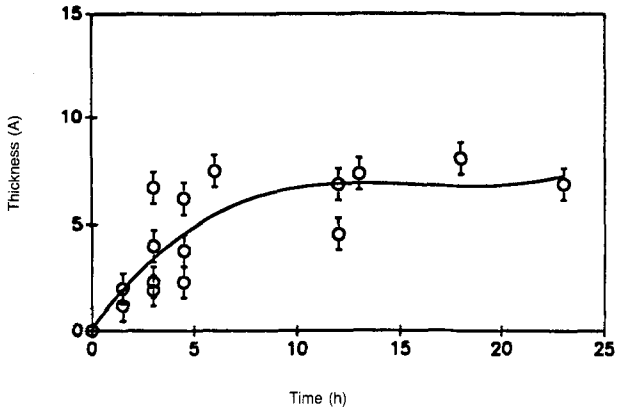

Fig. 7. Time course for attachment of PTH receptor fragment to silicon surfaces coated with the iodoacetyl SAM, and measured by ellipsometry. The 6–7 A average thickness at equlibrium indicates that the peptide molecules are probably not fully extended or totally flat on the surface.

approximately fully extended horizontally on the surface. However, since ellipsometry gives an *average* thickness, this result could also be obtained if the surface were only partially coated and the peptide molecules were more vertical in their orientation. Thus, the 6–7 Å thickness does not establish any particular geometry or density of surface coating.

The peptide-coated surfaces were exposed to immune and pre-immune sera from rabbits. Antibody binding was determined by ELISA and by silver-enhanced immunogold

NH_2-SER-VAL-ARG-ALA-THR-LEU-ALA-ASN-THR-GLY-CYS-TRP-ASP-LEU-SER-SER-GLY-ASN-LYS-LYS-COOH

Fig. 6. Amino acid sequence and functional group content of parathyroid hormone receptor fragment used for immunosensor studies. This fragment represents amino acids 341–361 of the human form of the receptor.

experiments [13]. Fig. 8 shows the distribution of a second antibody visualized by scanning electron microscopy of the silver-coated gold particles. It is apparent that the active antigen molecules are uniformly distributed, although the surface is not fully coated with antibody. However, deductions regarding the density of antigen packing cannot be made from this type of experiment since antibody molecules can sterically block several antigen sites, and since the silver coating is not uniform for all antibody molecules.

Further details of these experiments are described elsewhere [10]. In general, it was found that no particles were seen with surfaces exposed to a preimmune serum, and the number of particles was a function of the time of exposure to antibody and the fractional saturation of the surface with antigen. In some cases, segments of the surfaces were coated with very close-packed antibody, but this has not yet been a consistent observation. Thus, using the SAM to generate uniform and stable surfaces which are reactive against a specific single site in antigen peptides, we have been able to retain biological activity in at least a portion of the attached peptides and to obtain a uniform distribution of the biologically active molecules.

DISCUSSION

The technical challenge of controlling the interactions of proteins with surfaces has been long recognized. Indeed, in the biosensor field this challenge remains the primary barrier which impedes progress toward reliable and commercially useful biosensors of many sorts. Thus, the development of systematic approaches toward solutions to the problems outlined above is essential if progress is to occur by any means other than serendipity.

It appears that the SAM may offer some opportunities to address the problems. The control of the chemical behavior of a surface so that it will interact with proteins in desired ways is possible through approaches such as those we have described here. The surface accessibility to molecules in the bulk phase is strongly controlled, surface reactivity can be chemically determined so that specific reactivity with desired side chains of proteins can be attained, and the surface reactivity can be diluted with inert SAM-forming surfactants to control the spacing of the surface protein. Further, it may be possible to obtain SAM-forming compounds which produce surfaces with greatly reduced nonspecific interactions so that surface denaturation may be reduced.

For applications in microbiology, the specific approach described here may lead to development of sensors for antibody secreting cells. Attachment of synthetic peptides is more readily controlled and monitored than with larger proteins, and biological activity is less fragile. Synthetic peptide antigens with a single thiol can be used, and if the antigen does not contain a Cys it can be added in the synthesis. Thus, monitoring the growth of antibody-secreting cells in commercial or research applications may be possible through use of such immunosensors.

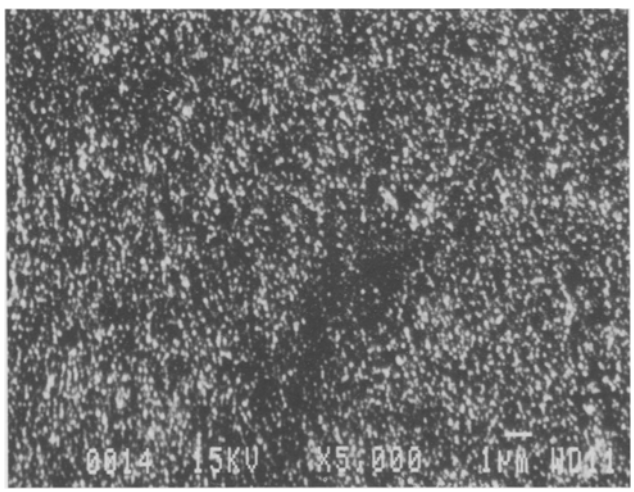

Fig. 8. Distribution of antibody molecules on α-iodoacetyl SAM-coated glass surfaces treated for 6 h with antigen. Particles represent silver-coated gold beads of 50–150 nm diameter (10 nm gold beads) attached to second antibody by protein A. Surfaces not treated with antigen or exposed to non-immune sera showed no gold.

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