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Surface Science 500 (2002) 28–60

SURFACE SCIENCE

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Biomedical surface science: Foundations to frontiers

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Received 1 December 2000; accepted for publication 23 July 2001

Abstract

Surfaces play a vial role in biology and medicine with most biological reactions occurring at surfaces and interfaces. The foundations, evolution, and impact of biomedical surface science are discussed. In the 19th century, the first observations were made that surfaces control biological reactions. The advancements in surface science instrumentation that have occurred in the past quarter of a century have significantly increased our ability to characterize the surface composition and molecular structure of biomaterials. Similar advancements have occurred in material science and molecular biology. The combination of these advances have allowed the development of the biological model for surface science, where the ultimate goal is to gain a detailed understanding of how the surface properties of a material control the biological reactivity of a cell interacting with that surface. Numerous examples show that the surface properties of a material are directly related to in vitro biological performance such as protein adsorption and cell growth. The challenge is to fully develop the biological model for surface science in the highly complex and interactive in vivo biological environment. Examples of state-of-the-art biomedical surface science studies on surface chemical state imaging, molecular recognition surfaces, adsorbed protein films, and hydrated surfaces are presented. Future directions and opportunities for surface scientists working in biomedical research include exploiting biological knowledge, biomimetics, precision immobilization, self-assembly, nanofabrication, smart surfaces, and control of non-specific reactions.

Keywords: Atomic force microscopy; Photoelectron spectroscopy; Secondary ion mass spectroscopy; Self-assembly; Biological compounds; Biological molecules – proteins; Solid–liquid interfaces

1. Introduction

1.1. The rationale and organization for this review

Through the first half of the 20th century physics dominated intellectual thought and discovery in the western world. With Watson and Crick's seminal 1953 paper outlining the structure of DNA, molecular biology quickly assumed a leadership role in the growth, discovery and nucleation of ideas. The surface science model, so successful in catalysis and

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0039-6028/01/$ - see front matter
PII: S0039-6028(01)01587-4

microelectronics, will find facile partnership with modern biology ideas—the outcome will be revolutionary 21st century technologies.

This article will first trace the roots of biosurface ideas. Then the contemporary surface science model will be described along with its transformation into a biosurface model (Section 2). Surface science methods will next be presented in the context of an area poised for growth, chemical state imaging (Section 3). Biomaterials and surface science have always had a close relationship so traditional and new biomaterials ideas relating to surfaces will be discussed in Section 4. Protein films have also played an essential role in the understanding of biology at surfaces and Section 5. will summarize developments enhancing our abilities to analyze these films. Finally, state-of-the-art ideas, materials and trends that will have profound implications for surface science, technology and medicine are presented in the final section.

1.2. Surfaces in biology and medicine

Biological reactions are frequently described as occurring in the solution phase, for example, the reaction of a soluble enzyme with its substrate. In fact, most reactions in biology occur, not in solution, but at interfaces. Typical interfaces of biological importance include the cell surface/synthetic biomaterial (see Fig. 1), extracellular matrix (ECM)/biomolecule, ECM/cell, hydrated tissue/air (lung) and mineral/protein (bone).

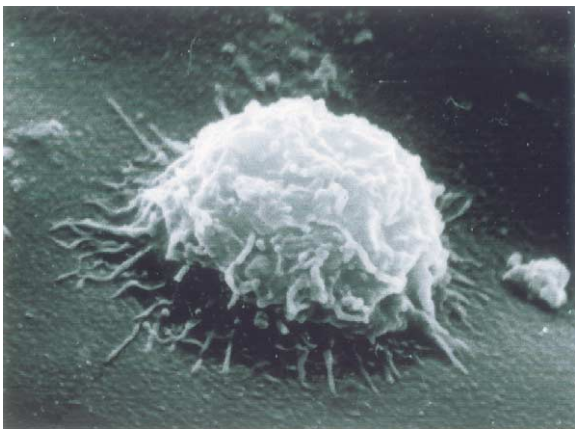

Fig. 1. A scanning electron micrograph of a myoblast cell interacting with a synthetic surface. The cell is approximately 10 μm in length.

Why would nature evolve molecular systems that exploit surfaces? We can surmise that for nature to do its work efficiently, surfaces offered the following advantages. Surfaces provide high accessibility for reaction. The low energy barrier to mobility in the plane of the surface can be used to facilitate complex reactions (clustering, conformational changes, exposure and burial in membranes). Epitaxy-like phenomena can be readily exploited at surfaces. High surface area geometries can be created to enhance reaction turnover rates. Unique organic microenvironments can enhance specific affinities and reactions. Self assembly in the plane of the interface can be used to orient and space molecules with precision. Surface energy minimization can orient specific structures to interfaces. Molecular recognition, a manifestation of both geometry and chemistry, is readily implemented at surfaces.

Beyond nature's use of surfaces, surface concepts have been adopted in medical and biological technology. Consider implant biomaterials, blood oxygenators, hemodialysis, affinity chromatography, surface diagnostics, cell culture surfaces and biosensors as examples of surface technology applied to biological problems. These applications have been largely driven by early observations that surfaces control biological reactions. Three areas, in particular, have been influential in advancing biological applications for surface science: chromatographic separations, blood compatibility and cell culture. A related, important realization was that proteins in aqueous solution rapidly adsorb as monolayers on surfaces. The thickness of the adsorbed protein monolayer (1–10 nm) is, fortuitously, in the range where most of our surface analysis instrumentation yields optimal results.

At this point it is useful to emphasize two ideas. First, surfaces are critically important to nearly all aspects of biology and biological technology. Second, the rules that govern biological surface phenomena are no different from the rules that govern the reactions at a silicon(1 0 0) surface. There is a limited set of physical laws governing this universe, and even the diversity seen in biology is constrained by these fundamental laws.

What are the differences between the surface science practiced on a platinum catalyst and the surface science practiced on a biological specimen? First, biological systems are fragile in two ways: energetic surface probes can damage organocarbon-based molecules and those molecules can be chemically and morphologically altered by the abuse they suffer with storage and preparation for surface analysis (for example, conformational changes in proteins). Second, the molecules of biology are immensely more complex than the inorganic systems used for semiconductors and catalysts (Fig. 2). Third, biological systems only function normally in aqueous media, a condition challenging for ultrahigh vacuum (UHV) surface analysis equipment. Fourth, many important biological processes occur at relatively deeply buried interfaces. Fifth, most biological surface science specimens are irregularly shaped insulators. For these reasons, special care, understanding and methodologies must be used in the analysis of biological specimens.

1.3. The evolution of biomedical surface science

The importance of surfaces has been empirically appreciated through all history as attested to by early texts such as the 1250 AD manuscript, *De Proprietatibus Rerum* (The Properties of Things), that outlined surface preparations to achieve metal–metal bonding. In the early 18th century, experimentalists such as Dobereiner and Faraday described surface-induced catalysis. By the 19th century, J. Willard Gibbs gave us a useful thermodynamics for surfaces. This early history did not directly acknowledge the relevance of these governing principles to biology. In fact, with Wohler's synthesis of urea (1828), the realization dawned that biology was not a province of matter stemming only from earlier biology, but rather a branch of chemistry, and by inference obedient to the physical laws that govern the properties of matter.

Surface science ultimately relevant to biology was developing in other contexts. Benjamin Franklin's observations on oil films on lakes foreshadowed 19th century studies such as those by Agnes Pockels [1881] on surfactant film surface tension. Pockels' work clearly hinted at modern assembled organic/biological materials.

It was probably the early colloid chemists who, in the late nineteenth century, first speculated on the relationships between interfacial phenomena and the structure and organization of living systems, especially proteins and single cells (see, for example, ''Proteins and the Theory of Colloidal Behavior,'' by J. Loeb, 1922). Many early investigators such as Faraday, Zsigmondy and Tyndall studied gold colloids, dust, bacteria particles or milk. The colloid state allowed some of the earliest appreciation of the concepts of self-assembly and minimization of interfacial energy.

In the first few decades of the twentieth century, the work of Irving Langmuir set the foundation for the surface science we have today. Some of his major contributions included methods for generating high vacuums, thin film deposition, quantitative theories of adsorption and coining the name ''plasma'' for the ionized gaseous state. Of particular relevance for this article is the development by Langmuir's technician, Katherine Blodgett, of molecular assembled films at an air–water interface that could be transferred in a compressed, monolayer state to a solid surface [1]. The analogy between these structures and the lipid bilayer surrounding living cells was clear and the films themselves suggested possibilities as a basic research tool and for novel applications. In 1946, in a paper presented at a convocation at Princeton University and subsequently published in a volume entitled ''Molecular Films, The Cyclotron and the New Biology,'' Langmuir speculated on the significance of these films for biology. The Langmuir–Blodgett invention made possible experimental systems later used by Zisman to explore fundamental aspects of organic interfaces [2] and early, exciting biomedical applications of these structures by Ringsdorf [3].

The modern characterization of organic surfaces, precursors to biomaterials, started with researchers such as Fowkes [5], Good [4] and Zisman [2] of the Naval Research labs. Zisman's investigations addressed both the preparation of surfaces and the quantitative contact angle measurements to approximate surface energy (critical surface tension). These ideas were expanded upon by

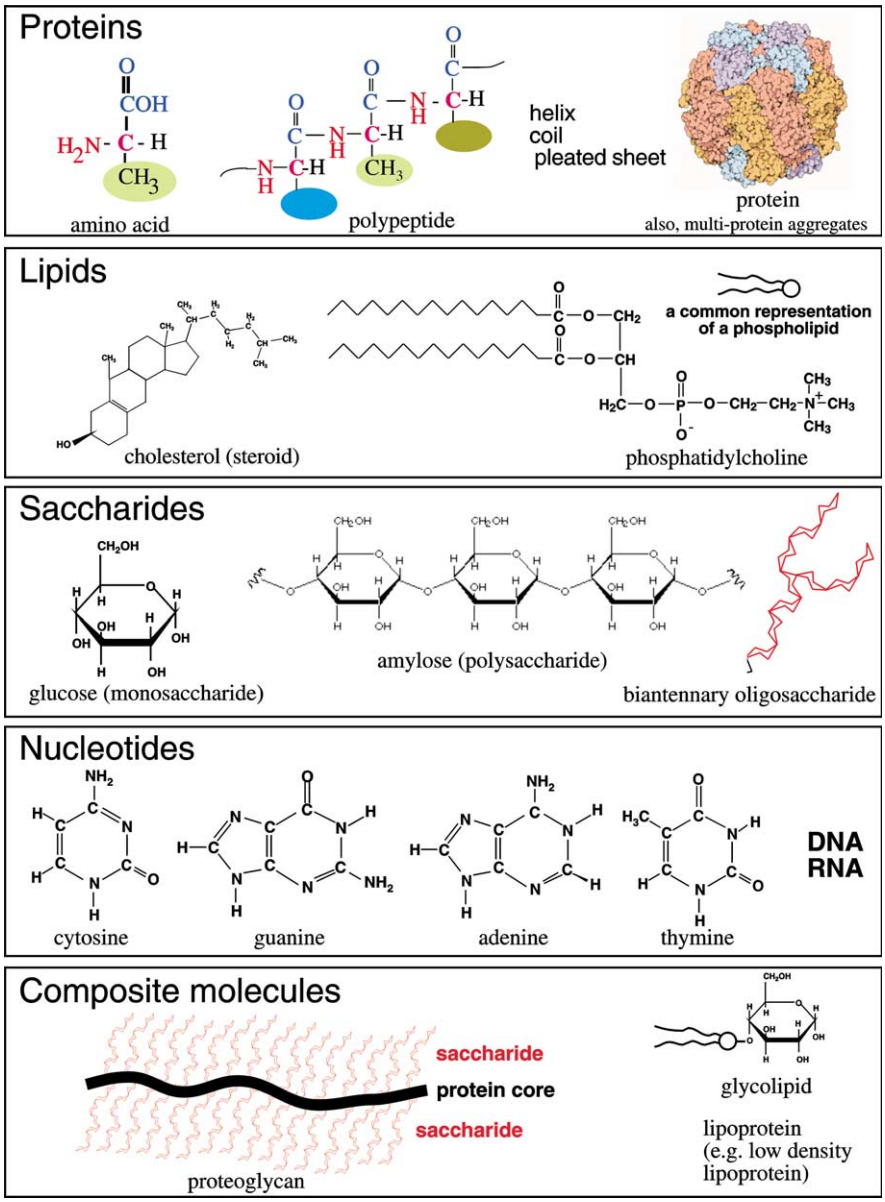

Fig. 2. The complexity of biological molecules of the type that can self-assemble into biological systems is illustrated in this figure. Semiconductor systems might involve the elements Si, O, Au, Cu, Al, Ga, As, B, P, N and perhaps a few other elements. Biology uses a substantial part of the periodic table but mostly C, O, N, P and S. The complexity arises from the many highly specific combinations with which these can be connected. For example, the 20 amino acids (complex molecules in themselves each with a symmetry-driven optical sense) making up the polypeptide chains can be ordered in almost infinite combinations. Specific arrangements yield secondary structures (random coil, helix, β pleated sheet), which then fold in a tertiary structures which then can aggregate into quaternary structures. A typical cell may have 15,000 proteins (about 2000 of those proteins are present in over 50,000 copies). These proteins are organized with sugars, nucleotides and lipids into the elegant supramolecular structure, the cell. And, the sugars, nucleotides and lipids themselves are each remarkably complex considering their structural possibilities and diversity. Protein (rubisco) image by David S. Goodsell, the Scripps Research Institute.

many investigators with a high-point likely coinciding with the development of the acid–base concept of contact angles by Fowkes [6].

In the late 1940s and early fifties, the first biomaterials as we know them today were developed. These were used for eye lens implants (intraocular lenses), hip joint replacements and blood contacting devices (vascular prostheses, heart valves, hemodialysis). Almost in parallel with the invention of these devices, researchers began studying their surface properties, protein–surface interactions and surface modifications. Bull explored protein adsorption to synthetic surfaces starting in the 1930s and going well in the fifties. Vroman made ellipsometric and visual observations of protein adsorption and related those observations to blood coagulation [7,8]. Baier correlated critical surface tension with bioreactions and brought surface infrared methods to biosurface studies [9]. Hoffman led the way with modern surface modification methods and demonstrated how surfaces could be engineered to give desired bioresponses [10].

Andrade [11], building on the foundations developed by Siegbahn in Sweden [12] and then Clark in the UK [13,14], brought a range of modern surface characterization methods including electron spectroscopy for chemical analysis (ESCA, also called XPS), secondary ion mass spectrometry (SIMS) and zeta potential measurement to biomaterials. Benninghoven, as early as 1977, was using static SIMS to study biological systems [15]. Ringsdorf demonstrated that interfacial biology could be emulated by synthetic, organic systems to create smart systems for drug delivery, biosensing and other applications [3]. Some key reviews that were important in suggesting the role of surface science in biomaterials are cited here [11,16–23].

1.4. The impact of biological surface science

Since the introduction of modern surface methods to study and modify materials and surfaces of biological interest, contemporary surface science has had considerable impact on biology and medicine. Surface criteria were used in a pass/fail test for commercial blood vessel substitutes [24]. Parameters derived from XPS spectra were shown to correlate highly with blood platelet reaction to surfaces in an in vivo model [25]. Surface engineering and surface analysis are used to create unique cell culture products [26,27]. Gene chips and diagnostic arrays, some of the fastest growing areas in biomedicine, depend on precision surface technology [28–30]. Nanotechnology exploits surface and interfacial ideas [31,32]. Column chromatography, a separations technology critically important to the biotechnology industry, is built upon surface modifications of particulate supports [33]. Commercial surface plasmon resonance (SPR) instruments are readily available that permit biological scientists with little formal understanding of surfaces to do precision adsorption experiments in the nanometer thickness range [34,35]. Similarly, piezo (quartz crystal) balances are also used for studying surface biointeractions [36,37]. The Food and Drug Administration (FDA) routinely calls for XPS data to qualify medical devices. At least two thriving technical groups (The Surfaces in Biomaterials Foundation, www.surfaces.org, and the Biomaterial Interfaces Technical Group of the American Vacuum Society, www.avs.org) exist to promote surface concepts related to biology. Biomimetic strategies are widely used to design surfaces for medicine and biology (consider hydroxyapatite, for example—see Biomimetics in Section 6). Living neuronal circuits based on surface micropatterning are widely studied for biosensors, medical devices and neural computing [38,39]. All this evidence suggests continued strong growth and impact for surface science in biology.

2. The surface science model for biology

In many research fields such as catalysis and microelectronics, the combination of well-defined model surfaces (e.g., metallic single crystals) with sophisticated surface analysis techniques has resulted in a detailed understanding of the role that surface structure and chemistry play. An important aspect of the success of the surface science model has been the ability to reduce a complex process (e.g., refining of petroleum crude oil) into

a set of elementary steps, which then can be studied at a fundamental level with surface analysis techniques [40]. Then the results from the fundamental studies on each of these elementary steps are recombined to provide a description and understanding of the entire process. For example, petroleum reforming uses dual functional catalysts to increase product quality (e.g., gasoline octane) via dehydrogenation, hydrogenation, cyclization, and isomerization reactions. By using different single crystal surfaces of platinum with well-defined surface modifications, reactions such as the dehydrogenation of cyclohexane to benzene can be systematically studied (see Fig. 3, top) [41]. These studies have produced fundamental thermodynamic and kinetic information about the adsorption, desorption, and surface intermediate species involved in dehydrocyclization. This information can then be combined with corresponding information from the other reforming reactions to provide an overall description of the reforming process and the role surface structure and chemistry play in that process. The success of the surface science model for catalysis is due to the availability of detailed surface characterization and catalyst reactivity results that can be correlated.

The surface science model for biology and medicine is not as fully developed as the surface science model for catalysis. The ultimate goal for the biological surface science model would be to provide an understanding of how the surface chemistry and structure of a material can be used to control the biological reactivity of a cell interacting with that surface (see Fig. 3, bottom). To accomplish this goal requires understanding the cell reactivity and characterizing a complex, protein-covered surface. The increased complexity and highly interactive nature of the biological environment, relative to catalytic processes, has

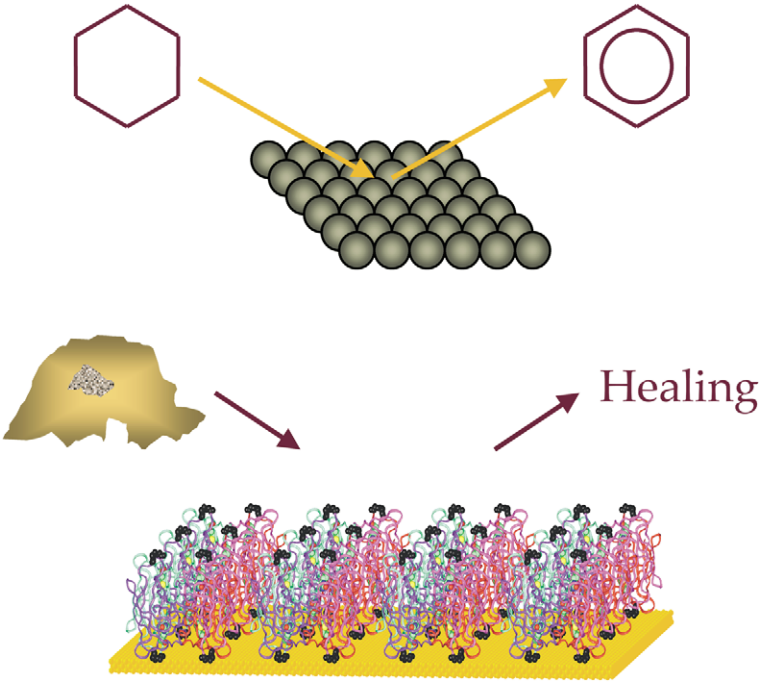

Fig. 3. The surface science model for catalysis showing the dehydrogenation of cyclohexane to benzene (top). To achieve the surface science model for biology an understanding of how the surface chemistry and molecular structure controls the biological reactivity of a cell interacting with a protein-covered surface to produce a normal healing reaction is needed (bottom).

made it difficult to isolate and study the reactivity of cells and biomolecules.

All cells communicate by the release and detection of signaling agents (cytokines) through a network made up of multiple and interactive signaling pathways [42]. The state of a cell (shape, structure, biological activity, etc.) depends on the signals it receives from its biological environment. For example, platelets normally circulate in the bloodstream in a passive state. Upon vascular injury a signaling cascade is initiated that activates the platelets for their role in healing the vascular injury. For this reason, whole blood must be treated (e.g., heparinized) when removed from the body to keep it from coagulating. Thus, the highly interactive nature of the biological environment makes it challenging to isolate a cell or biomolecule for meaningful, fundamental studies. Still, the reductionist approach to biology has been highly successful in isolating components and describing their operation. Similarly, to increase the understanding surface analysis can bring to biology, the complexity of the biological environment must be reduced where possible by employing well-defined model systems. However, to realize the surface science model for biology, the biological reactivity and the corresponding surface analysis studies will require the use of more complex systems than those used to develop the surface model for catalysis.

Significant advances have been made in molecular and cellular biology [43], material science [44], and surface analysis [45] in the past ten years that now make it possible to characterize the surface chemistry and structure of increasingly complex materials and study the biological reactivity and interactions of cells. The structure and function of many biological receptors have been determined along with their mechanism of cell binding and activation. With the combination of state-of-the-art patterning techniques and novel synthetic strategies it is now possible to prepare complex, organic surfaces with well-defined structures and chemistry [46]. The knowledge gained in the biological studies has been incorporated into the synthesis strategies for these surfaces. For example, the RGD (arginine–glycine–aspartic acid) amino acid sequence in proteins is known to play a key role in cell adhesion, so surfaces with short peptide sequences containing the RGD motif have been prepared [47]. RGD surfaces are useful and interesting models, but they only weakly emulate the multifunctionality of proteins such as fibronectin with over 2500 amino acids that contain this RGD sequence. Fibronectin comprises just part of the complex molecular structure of the natural attachment and activation substrate for cells. This example illustrates both the complexity of the problem and the surface related approaches to systematically examine aspects of this complexity (the reductionist approach).

To address the complexity of biology at surfaces, powerful surface tools are needed. Both hardware and software enhancements have been made in surface analysis techniques. The spatial resolution, energy resolution, mass resolution, sensitivity, etc. have improved. New data analysis techniques such as multivariate statistical analysis have been introduced. These combined advances in the biological, materials science, and surface analysis research fields provide the promise of realizing the surface science model for biology in the 21st century.

A major advance in materials fabrication technology during the last 10 years has been the development of self-assembly methods [48]. Self-assembled monolayers (SAMs) provide well-defined structures and chemistries that can be systematically varied (Fig. 4). Also, spatially defined arrays of SAMs can be prepared by combining self assembly with patterning methods such as microcontact printing and photolithography [49]. In addition, SAMs can be used to immobilize peptides, proteins, and other biomolecules to the surface to prepare the complex surfaces required for well-defined biological experiments. For example, a mixed biotinylated thiol/oligo-ethylene glycol thiol monolayer can be assembled onto a gold surface [50]. Since protein molecules are significantly larger in size than the thiol molecules in the SAM, the thiol molecule that contains the protein binding group (e.g., biotin) is typically diluted with a thiol that resists protein binding (e.g., oligo-ethylene glycol). Once the mixed biotinylated/oligo-ethylene glycol SAM is prepared, then the protein streptavidin is bound to it.

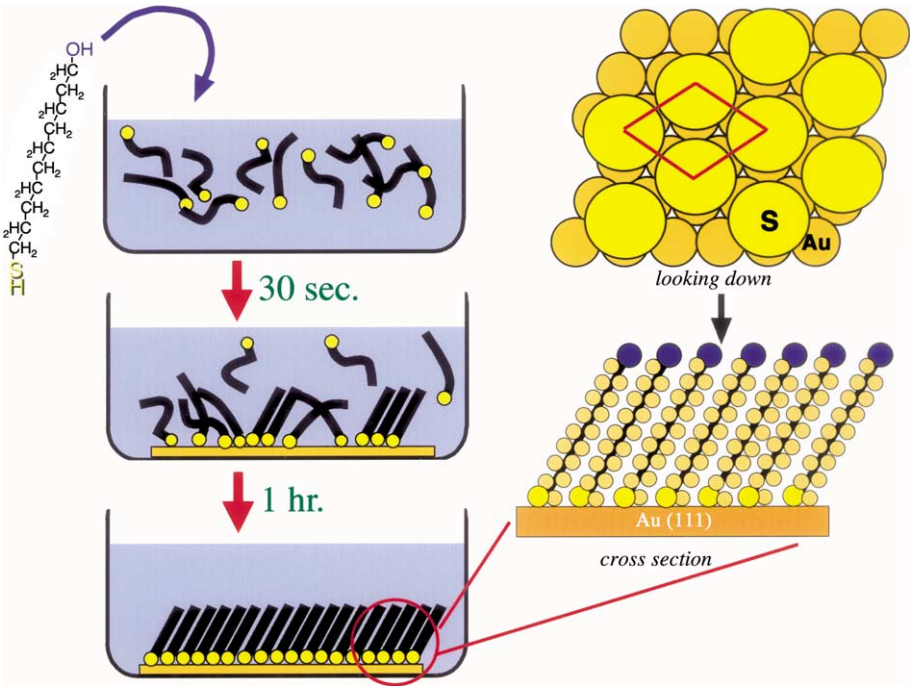

Fig. 4. The self-assembly process. An *n*-alkane thiol is added to an ethanol solution (0.001 M). A gold(1 1 1) surface is immersed in the solution and the self-assembled structure rapidly evolves. A properly assembled monolayer on gold(1 1 1) typically exhibits a $(\sqrt{3} \times \sqrt{3})R30°$ lattice.

Since streptavidin has binding pockets on opposite sides of the molecule, it can be used as a linker to bind other biotinylated molecules. A cartoon depicting the complex, multicomponent organic surfaces that can be prepared using the techniques described in this paragraph is shown in Fig. 5. Similarly, complex surfaces with immobilized peptides and proteins can be prepared using surface functionalization and polymer chemistry methods [51].

SAMs provide the model organic surfaces for use in developing the biological surface science model much the same way that metal single crystal surfaces earlier provided the model surfaces to develop the catalysis surface science model. However, organic surfaces are more fragile than the metallic single crystals since they can degrade when exposed to typical experimental and analysis conditions (elevated temperatures, X-rays, electron beams, etc.) [52]. Thus, the primary surface science techniques used for characterization of organic surfaces are XPS, static SIMS, and scanning probe microscopy (SPM) since, when used with care, they provide detailed information about the composition and molecular structure of organic surfaces without causing extensive degradation of the samples. Additional techniques such as Fourier transform infrared (FTIR), Raman, sum frequency generation (SFG), and high-resolution electron energy loss (HREELS) can be used to obtain surface vibrational spectra with relatively little, if any, damage.

Since it has been difficult to find model biomolecules that represent the reactivity of the entire complex biological environment, advancements in surface analysis techniques must be made so that increasing complex surfaces and processes can be fully characterized. A layer of adsorbed protein mediates the interaction of cells with a biomaterial when that biomaterial is placed in the biological environment. Also, future multilayered engineered surface-biology constructs may have a synthetic material, a hydrogel-like support, a SAM, a variety of tethering "hooks" and a number of oriented,

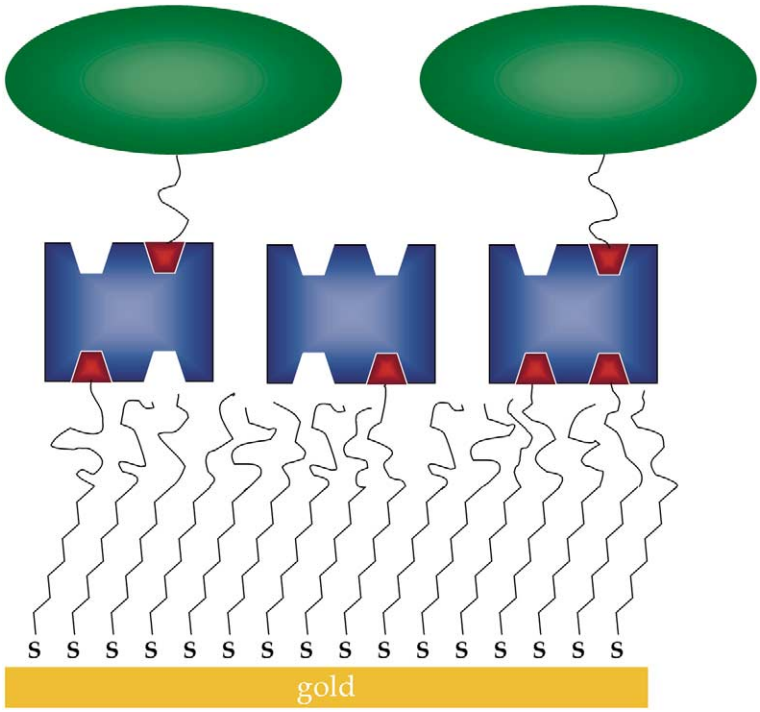

Fig. 5. A cartoon depicting how biomolecules (green) functionalized with biotin groups (red) can be selectively immobilized onto a gold surface using a streptavidin linker (blue) bound to a mixed biotinylated thiol/ethylene glycol thiol self-assembled monolayer.

organized biomolecules. Thus, it is especially important to develop surface analysis methods that can fully characterize these complex layers containing proteins and other biomolecules. An example using static SIMS with multivariate analysis to characterize adsorbed protein films is described in Section 5.

3. Surface chemical state imaging

3.1. Surface chemical state objectives

Prior to the last 5–10 years, spectroscopic analysis dominated biomedical surface science studies. The early biomaterials (silicones, polyurethanes, metals, etc.) typically were laterally homogeneous, so there was not a significant need for spatially resolved surface analysis methods. With the recent, rapid growth of methods for preparing spatially well-defined materials, the focus of biomedical surface science is now on high spatial resolution surface chemical state analysis in the x–y plane. The important objectives for developing spatially resolved spectroscopic analysis techniques are identification of all surface species present, identification of all chemically distinct regions, quantitative determination of the surface composition in each region, and optimization of spatial resolution. The driving forces for developing biomedical surface chemical state imaging techniques are addressed below.

A central goal of modern bioengineering is the development of biomaterial surfaces that direct the biological healing response [53]. These novel surfaces are envisioned to have a well-defined array of biorecognition sites designed to interact specifically with cells since many of the important functions of cells depend on the arrangement of molecules at their surfaces. Fig. 6 shows how the spatially resolved chemistry of a surface controls the shape and structure of a cultured rat bone cell [54]. New developments in surface analysis techniques are required to provide detailed surface

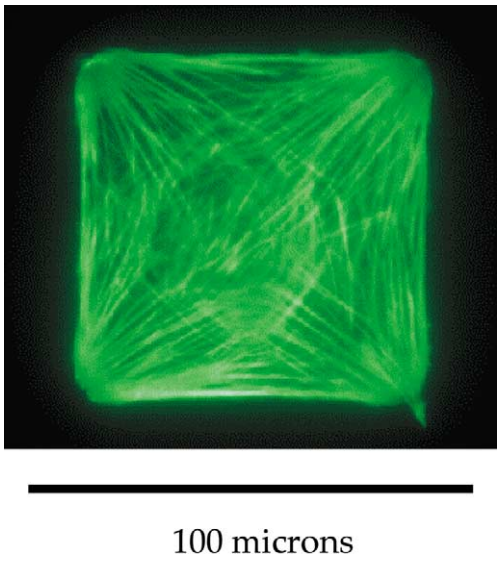

Fig. 6. An F-actin stained rat bone cell cultured on a 75 × 75 μm square region of an amino-silane functionalized quartz surface. The area around this square is functionalized with a non-fouling acrylamide/ethylene glycol copolymer. The F-actin stain shows how the surface chemistry controls the shape and cytoskeleton structure of the cultured rat bone cell. See Ref. [54] for further details.

chemical state information at high spatial resolution for mapping out the presentation of these biorecognition sites. Improving the ability of surface analysis techniques to characterize and understand the composition, molecular structure, orientation, and spatial resolution of surface species will provide the biomedical research community with the tools and information needed to develop novel biomedical devices.

3.2. Surface chemical state imaging techniques

XPS, ToF SIMS, SPM and near edge X-ray absorption fine structure (NEXAFS) each has its own strengths and weaknesses with respect to generating surface chemical state information at high spatial resolution, but together they provide a powerful set of complementary techniques (see Table 1). For example, XPS and ToF SIMS can be used to improve the level of chemical state information obtainable with SPM, while SPM can be used to improve the spatial resolution obtainable with XPS and ToF SIMS.

Table 1
Complementary surface chemical state imaging with XPS, static ToF SIMS, SPM, and NEXAFS

Technique	Current strength	Current research focus
XPS	Quantification	Spatial resolution
Static ToF SIMS	Molecular structure	Image analysis
SPM	Spatial resolution	Chemical specificity
NEXAFS	Chemical specificity	Spatial resolution

3.3. Scanning probe microscopy

SPM is the biological surface science technique that provides the highest spatial resolution [55,56]. Depending on the sample being analyzed, individual atoms can be imaged with SPM. However, the inherent chemical specificity of SPM techniques is limited. For biomedical surface science this limitation can be overcome with proper functionalization of the probe tip. Immobilizing a biomolecule onto the tip in an active state allows surfaces to be interrogated at high spatial and temporal resolution while quantifying the binding force between the tip-immobilized biomolecule and the surface [57] (Fig. 7). The SPM techniques can map surface features and measure intermolecular forces at x, y, and z spatial resolutions of 1, 1, and 0.1 Å, respectively. Additionally, rapid advances in SPM technology have resulted in the development of several different modes of operation for image generation (magnetic, electric, electrochemical, thermal, viscoelastic, frictional, adhesive, etc.).

The increasing use of SPM techniques for investigating biological problems is primarily focused in the areas of structural identification and interfacial biophysical phenomena. SPM can provide molecular resolution images of proteins, DNA, lipids and carbohydrates [58]. Cellular structure also may be identified, and some recent work has examined dynamic responses of cells to environmental stimuli [59].

The quantification of biophysical phenomena (e.g., biotin–streptavidin binding) offers exciting opportunities for developing biorecognition SPM imaging methods [60]. Most of the initial research has focused on chemical specificity, studying the

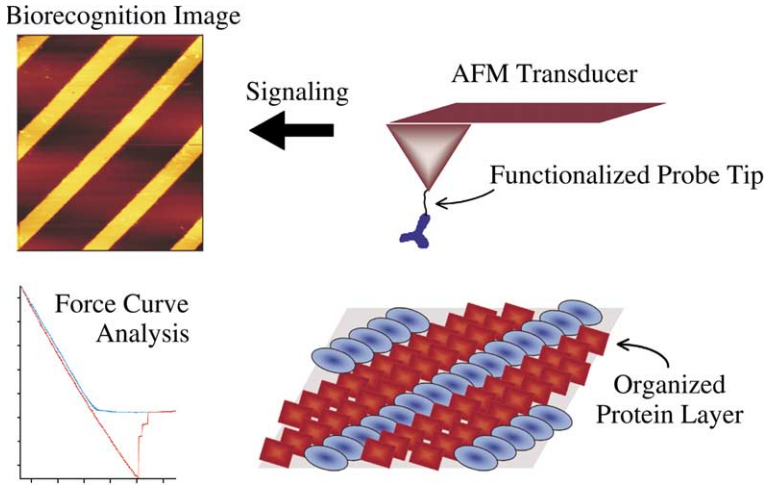

Fig. 7. A cartoon showing elements involved in biorecognition SPM imaging. A probe tip is functionalized to specifically interact with a protein on the sample surface. From analysis of the force curves taken as the tip is scanned across the sample an image of the patterned protein surface is generated.

interaction of different chemical groups on a well-defined surface with a functionalized SPM tip [61]. SPM studies typically use force curve spectroscopy, lateral force imaging, or phase shift imaging to interrogate the tip–sample binding phenomena [62].

The force curve spectroscopic method measures the interaction force at discrete points in the image. Lateral or friction force is a scanning technique that generates an image from differential lateral twisting of the cantilever. The magnetic mode is one of most recently developed phase shift imaging methods [63]. It is especially well-suited for biorecognition imaging since it can be used in liquids and the cantilever oscillation frequency can be tuned to a value appropriate for the biological binding event being examined.

The preparation of functionalized tips is the key to successful biorecognition with SPM. A significant amount of research activity is focused on the preparation and characterization of tips functionalized with well-defined chemical and biological species [64]. For biomolecules, the goal is to immobilize them in a well-defined orientation while maintaining their full biological activity and selectivity. One strategy for maintaining the activity of a biomolecule is to tether it to the tip with a poly(ethylene glycol) (PEG) spacer [65]. The PEG tether allows the biomolecule sufficient mobility so it can selectively bind to surface biorecognition sites. It is also important that the density of immobilized biomolecules be low enough so only a single binding event is detected for each localized force curve measurement. Due to their small size, it is challenging to directly characterize functionalized SPM tips.

The most commonly used SPM method for biological recognition imaging has been to acquire an array of force curves. The biological affinity information is contained in the retraction portion of the force curve [60]. Unfortunately none of methods used to date to extract the biological affinity force from the total adhesion force provide unambiguous, quantitative results. Thus, development of an imaging mode that is based on measurement of quantitative chemical and biological recognition forces would represent a significant advancement in SPM biorecognition capabilities.

3.4. X-ray photoelectron spectroscopy and near X-ray absorption fine structure

In contrast to SPM, the spatial resolution of surface science techniques using X-rays (XPS, NEXAFS, etc.) is limited. This is because of the

difficulties associated with focusing X-rays. Until recently the smallest sample area that could be analyzed with a laboratory XPS system was 150×150 μm^2 [66]. Through a combination of improvements in X-ray focusing and lens/analyzer technology, laboratory XPS systems are now available that do real time imaging at spatial resolutions less than 10 μm. Although this spatial resolution is still orders of magnitude higher than the spatial resolutions obtained with SPM, XPS has a significant advantage in terms of its ability to quantify the surface composition of a sample. The recent construction of low-emittance synchrotrons such as the Advanced Light Source at the Lawrence Berkeley Laboratory has resulted in significant improvements in the spatial resolution obtainable with XPS and NEXAFS. Currently spatial resolutions in the 20–40 nm range are achievable using zone plate focusing elements. With further improvements in the microfabrication methods used to make the zone plates, even higher spatial resolutions should be achievable.

The theory of XPS and its application for identifying and quantifying surface chemical species is well developed [67]. Thus, the frontier for biomedical XPS is to improve its spatial resolution. However, a major challenge will be to improve the spatial resolution without introducing significant X-ray induced sample degradation. For large area analysis of organic and biological materials with monochromatized XPS, sample degradation is typically not a concern. However, as the X-ray beam is focused into increasingly smaller areas, the X-ray brightness (photons per unit area) increases and as does the potential for sample degradation. Thus, when changing from low spatial resolution analysis to high spatial resolution analysis, XPS can shift from a "non-destructive" to a "destructive" technique. To increase the spatial resolution of XPS while maintaining non-destructive analysis conditions will require advancements in methods for acquiring images with lower X-ray doses (rastering of the X-ray beam, improved sensitivity of the analyzer and detector, etc.). This need is especially critical for the high X-ray brightness achievable in micro-XPS and NEXAFS experiments done using synchrotron radiation. Fragile organic and biological samples can be completely destroyed under those analysis conditions.

3.5. Static time-of-flight secondary ion mass spectrometry

The spatial resolution of AES and ToF SIMS falls between that of SPM and laboratory XPS instruments. Most biological samples are readily degraded by the high-energy electron source used in AES, so AES is not widely used in biomedical surface science studies. Static ToF SIMS generates a mass spectrum from the outer 1–2 nm of sample, providing detailed information about the molecular structure of organic and biological materials [68]. With liquid metal sources, ToF SIMS images can be acquired at spatial resolutions down to 0.1 μm, which is sufficient for cellular resolution (1–100 μm). The mass resolution obtainable at a given spatial resolution also must be considered. The mass resolution of a Cs^+ source focused to 5 μm is degraded to the point that peaks 1 amu apart are barely resolved in the low mass range. This results in a loss of information for samples that have more than one peak at a given nominal mass. The Ga^+ source can be operated at full mass resolution ($M/\Delta M > 8000$ for conducting samples) at spot sizes down to 1 μm. This combination of high-spatial resolution and high-mass resolution is essential for imaging complex biological samples. Thus, static ToF SIMS has the capability to produce detailed molecular structural information at high spatial resolutions, making it a valuable technique for biomedical surface analysis [69]. However, many challenges need to be addressed before the full power of imaging ToF SIMS can be realized. These challenges include (1) analyzing large data sets, (2) images with low signal-to-noise ratios, (3) chemical species identification typically requires the use of several peaks and (4) distinguishing topography and chemistry effects.

With modern ToF SIMS instrumentation, a vast amount of data can be collected in a relatively short period of time. A mass spectrum can be acquired for each pixel in a 256×256 static ToF SIMS image (Fig. 8). Thus a total of 65,536 mass spectra, each containing several hundred

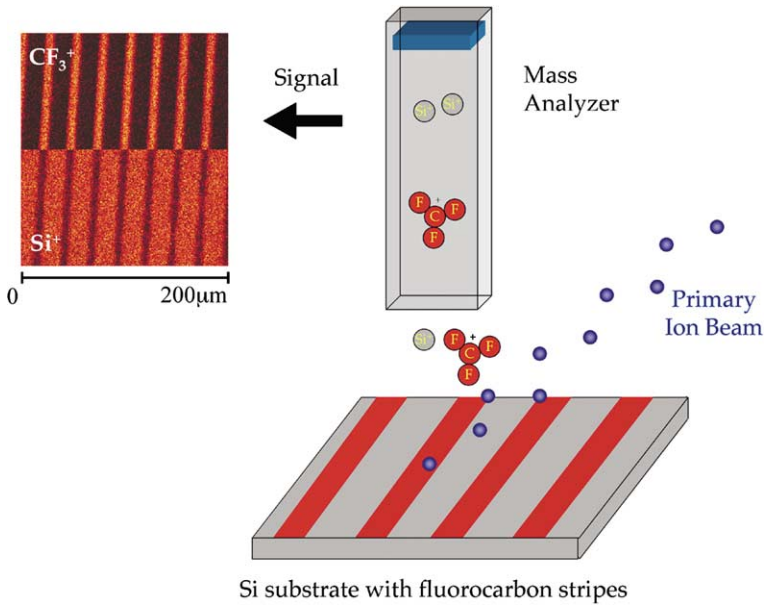

Fig. 8. A cartoon of the static ToF SIMS imaging process. A primary ion beam is scanned across the surface, which results in the ejection of secondary ions (Si^+, CF_3^+, etc.). The secondary ions are then mass analyzed to generate an image of the patterned fluorocarbon stripes on a silicon surface.

secondary ion peaks, needs to be analyzed for each image. Using the traditional ToF SIMS approach of examining images from selected individual mass peaks or the average spectrum from a selected subsection of the image can become time consuming. Even more importantly, a large portion of the acquired data is ignored and all the information present in a static ToF SIMS image is not used. Thus, improved image processing methods that use all of the data present in a static ToF SIMS image are needed to ensure all the surface chemical species are identified and quantified [69]. By examining all of the data, a set of peaks or combinations of peaks can be identified that increase the signal-to-noise ratio, improve the contrast between the chemical constituents, enhance the distinction of topographical features from chemical features, and make it easier to obtain the pure component spectra. Recognizing patterns and relationships in a set of hundreds or thousands of measured variables is a formidable task for any researcher using traditional data reduction methods. Thus, image processing methods need to be developed for ToF SIMS that permit a more efficient use of all the data in an image. Some of these methods are described below.

3.6. Image analysis methods

The analysis of static ToF SIMS images can be addressed in a three-step process. First, the raw data is denoised. Images acquired in the static mode (i.e., low ion dose) at high spatial resolution typically have low signal-to-noise ratios. Many peaks in the ToF SIMS spectra may only have a few counts per pixel. Thus, noise reduction methods that allow weak signals to be identified are important to use in the first step of imaging processing. Methods such as wavelet, median, and boxcar filtering are typically used to denoise images.

The second step is to identify the chemical species present in the sample. If one knows, a priori, what components are present in the sample this step is straightforward. This is usually not the case, so a method for extracting this information from the spectra is required. The fact that multiple peaks in the spectra can be associated with each

chemical species adds complexity to this identification. Multivariate analysis techniques such as principle component analysis (PCA) can be use to identify the chemical species present and to compress the amount of data to be analyzed [70]. It also provides information about which combination of peaks in the static ToF SIMS spectrum originate from the same chemical component. This is important since static ToF SIMS imaging is typically limited by low signal-to-noise. The counts per pixel in the image can be increased significantly by combining the counts from several spectral peaks. However, selecting the wrong combination of peaks will decrease the contrast between chemical components in the image. Thus, correct and complete identification of the chemical species present along with their characteristic mass spectrum is essential to successfully completing the third step, image construction. If each chemical component has one unique mass fragment with strong intensity then the image reconstruction is straightforward. The relative intensities of the unique mass fragments show directly how the chemical species are distributed spatially on the surface (Fig. 8). When this is not the case, then images can be constructed directly from the PCA results using the appropriate combinations of peaks to identify the spatial locations of the various chemical components. Image segmentation algorithms such as region growing can also be used for image construction. These methods are particularly useful for images with a large number of surface phases [71].

4. Biocompatibility, biomaterials and molecular biorecognition surfaces

Medical implant materials, loosely referred to as biomaterials, have played a pivotal role in bringing surface concepts to biology. The ability of these materials to save human lives and the significant economic implications of medical devices have spurred many avenues of research, including surface-biology models. This section will clarify some of the concepts surrounding modern biomaterials, especially with reference to surfaces.

A word central to biomaterials, that distinguishes them from other materials, is biocompatibility. However, biocompatibility is poorly defined. Some properties that have been suggested to correlate with biocompatibility include surface energy, negative charges, hydrogels, heparin, titanium, phosphatidyl choline, polysulfones, etc. Many of the attempts to correlate materials properties with biocompatibility invoke surface properties. Yet, to this day, there are no clear rules that can be used to design a material for biocompatibility—good evidence that we do not yet understand biocompatibility. What is biocompatibility and what route might we take exploiting surfaces to obtain a precise definition of biocompatibility?

Millions of medical devices are implanted into humans each year with reasonable levels of success (Table 2). The FDA and other regulatory agencies "stamp" our medical devices as biocompatible. So, why is this word poorly defined? Consider the following two ideas. First, smooth materials that do not leach biologically reactive substances will heal in the body in a manner now considered biocompatible. Are all non-leaching materials equally biocompatible irrespective of surface properties? Second, the body reacts similarly to nearly all materials that we call biocompatible and walls them off in an avascular, tough, collagenous bag, roughly 50–200 μm thick (Fig. 9). This reaction is

Table 2
Medical implants used in the United States

Device	Number/year	Biomaterial
Intraocular lens	2,700,000	PMMA
Contact lens	30,000,000	Silicone acrylate
Vascular graft	250,000	PTFE, PET
Hip and knee prostheses	500,000	Titanium, PE
Cathether	200,000,000	Silicone, Teflon
Heart valve	80,000	Treated pig valve
Stent (cardiovascular)	>1,000,000	Stainless steel
Breast implant	192,000	Silicone
Dental implant	300,000	Titanium
Pacemaker	130,000	Polyurethane
Renal dialyzer	16,000,000	Cellulose
Left ventricular assist devices	>100,000[a]	Polyurethane

[a] Since inception.

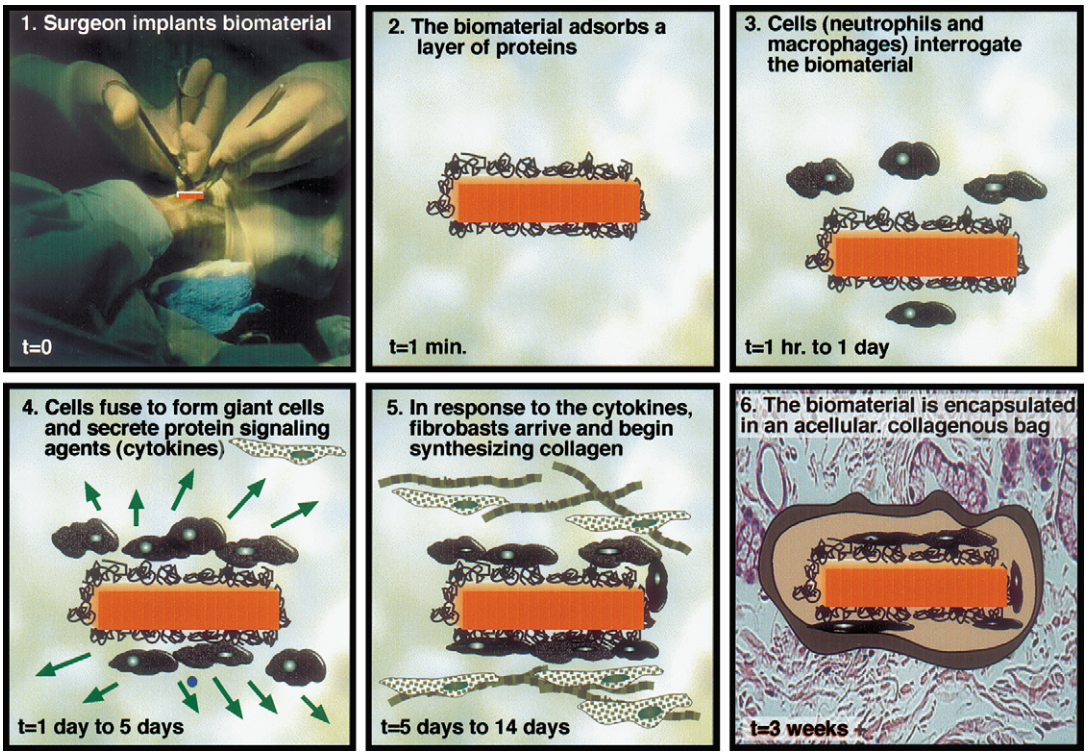

Fig. 9. The foreign body reaction is the normal reaction of a higher organism to an implanted synthetic material and is schematically illustrated here. (1) A surgeon implants a biomaterial in a surgical site (an injury). (2) Quickly, the implant adsorbs a layer of proteins, the normal process for a solid surface in biological fluids. (3) Cells (neutrophils and then macrophages) interrogate and attack the "invader," i.e., the biomaterial. (4) When the macrophages find they cannot digest the implant, they fuse into giant cells to engulf the object. However, it is too large to completely ingest. The giant cells send out chemical messengers (cytokines) to call in other cells. (5) Fibroblast cells arrive and begin synthesizing collagen. (6) The end stage of the reaction has the implant completely encased in an acellular, avascular collagen bag. There are macrophages between the collagen sac and the implant.

referred to as the foreign body reaction. Surprisingly, the accepted regulatory definition of biocompatibility revolves around this reaction of the body to rid itself of "biocompatible" biomaterials.

What are the concerns with today's biomaterials and how they heal? Uncontrolled biological encapsulation directly confounds the performance of many implanted devices. Consider, for example, implant electrodes, drug delivery systems, and breast implants. The presence of this capsule seriously degrades their performance by preventing intimate contact between device and tissue. The reaction associated with this foreign body response (long term, low level inflammation and macrophage activation) may also inhibit the luminal healing of vascular grafts, trigger capsular opacification found with intraocular lenses, lead to the extrusion of percutaneous devices, exacerbate device calcification, induce contact lens discomfort and generally lead to complications and less than desirable outcomes associated with today's medical devices. In contrast, our body has an excellent capacity to heal wounds and injuries with healthy, vascularized tissue. Could this normal healing be wrong? Why do "biocompatible" implants shut off normal wound healing? We already know how to get devices to heal with a foreign body capsule. So, what's next? Can we go beyond this aberrant healing? These questions and comments require clarification and justification.

Given a list of 10 common materials used in medicine (for example, gold, polyurethane, silicone

rubber, polytetrafluoroethylene (PTFE), polyethylene (PE), poly(methyl methacrylate) (PMMA), poly(2-hydroxyethyl methacrylate) (PHEMA), poly(ethylene terephthalate) (PET), titanium, alumina) materials that are hydrophilic, hydrophobic, hard, soft, polymeric, ceramic and metallic are represented. Yet, after one month implantation in mammals, they are all found to heal essentially identically. On the other hand, each material will be found, in vitro, to adsorb different proteins, and to show substantially different cell attachment and cell growth behavior. The origin of this striking difference between in vivo and in vitro represents one of the pervasive problems in biomaterials science.

The commonality among the ten materials in the previous paragraph is that they adsorb a complex, non-specific layer of proteins. Each will have a different protein mixture at its surface, but all the materials will quickly acquire a layer that contains many proteins (possibly comprised of 200 or more proteins) in many states of orientation and denaturation [72]. Nature *never* uses such non-specific layers—nature's use of proteins as signaling agents comes from one (or a few) specific proteins in fixed conformations and orientations so they optimally deliver signals. A hypothesis has been developed suggesting that the body views this non-physiologic proteinaceous layer as something with which it has no experience and reacts to it as an unrecognized foreign invader that must be walled off [53,73]. One of the authors (BDR) refers to these non-specific layers as "the enemy." For progress to be made, we must go beyond this ill-controlled reaction, i.e., defeat the enemy. Hence, surfaces must be developed that control the conformation and orientation of proteins with precision so that the body will specifically recognize them.

In a normal wound, the macrophage cell responsible for "orchestrating" wound healing is activated. In the presence of an uncomplicated wound, the macrophage turns on the pathways leading to normal healing by first cleaning up the wound site and then secreting the appropriate cytokine messenger molecules. These soluble messengers activate processes in the cell types needed for healing (fibroblast, keratinocyte, osteoblast, etc.).

The surfaces of today's biomaterials, if present in the wound site, turn this normal healing process off. The macrophages adhere to the biomaterial. They do not recognize it and spread on its surface as they try to phagocytose it. They cannot digest or engulf this large mass, so, to increase their effectiveness, they fuse together to form multinucleated giant cells. Of course, these cells still cannot engulf a macroscopic medical device. The giant cells signal to the body that there is a large foreign body to be walled off. The fibroblasts arrive and generate the collagen capsule, most likely guided by the macrophages. Although there is not complete consensus on how the body reacts to implanted synthetic materials, most experts would agree with the general outline described above.

There are a number of steps that must be taken to realize "biomaterials that heal." First, a serious investment must be made in the study of the basic biology of normal wound healing, in contrast to wound healing with a biomaterial present. This basic study will tell us what molecular and cellular pathways to turn on and what pathways to turn off. Second, the non-specific adsorption of proteins and other biomolecules must be inhibited. Finally, the surfaces of biomaterials should be synthesized to present to the body the same signaling groups as the surface of a clean, fresh wound.

This hypothesis on healing and the foreign body reaction opens many opportunities for surface scientists. Indeed, the basic biology studies are best left to the biological researchers. However, once the biological discoveries are made, the ability to inhibit non-specific interactive events on surfaces and the intellectual challenges of delivering the specific biological signals opens exciting frontiers for surface scientists.

Many strategies to inhibit non-specific protein adsorption (non-fouling surfaces) have been developed [74–82] (Table 3). How resistant to protein pickup can such surfaces be made? Why are they resistant to protein adsorption? How long can they remain resistant to protein fouling? Can they be functionalized with organic groups permitting the immobilization of active biomolecules on a bland background? These questions drive research in this area. A number of recent issues of *Journal of Biomaterials Science: Polymer Edition* (Volume 11,

Table 3
Strategies to achieve protein-resistant (non-fouling) surfaces

Surface strategy	Comments	Reference
PEG[a]	Effective but dependent on chain density at the surface; damaged by oxidants	[74]
PEG-like surfaces by plasma deposition	Applicable for the treatment of many substrates and geometries; highly effective	[76]
PEG oligomers in self-assembled monolayers	Highly effective; applicable for precision molecularly engineered surfaces; durability to elevated temperature is low	[80]
PEG-containing surfactants adsorbed to the surface	A simple method for achieving non-fouling surfaces; durability may be low and high surface densities are hard to reach	[78]
PEG blocks in other polymers coated on the surface	May provide a relatively low density of surface PEG groups	[77]
Saccharides	Nature's route to non-fouling surfaces; some successes but much territory remains to explored	[148,149]
Choline headgroups (phosphatidyl choline)	Has shown good success in many applications	[79]
Hydrogen bond acceptors	Possibly, this principle imparts non-fouling properties to PEG surfaces; this is leading to new discoveries of surface functional groups for non-fouling	[134]
Adsorbed protein layer	A pre-adsorbed protein layer resists further adsorption of proteins; this approach, long used by biologists, is easy to implement but of low durability	[150]
Hydrogels, in general	PEG is in this class; many other hydrogels have shown non-fouling behavior	[151]

[a] Also called poly(ethylene oxide) (PEO).

2000) have focused on these points. As an example, surfaces made by the RF-plasma deposition of tetraethyleneglycol dimethylether (tetraglyme) have been explored in our group. These surfaces have been characterized by modern surface techniques [83] revealing a crosslinked PEG-like structure and have been shown to have extremely low protein pickup (Fig. 10).

Within the University of Washington Engineered Biomaterials (UWEB) program (a National Science Foundation Engineering Research Center), key molecules that turn on and off normal healing have been explored [84,85]. Can these molecules retain their effectiveness when bound to surfaces? What strategies might be used to immobilize them in a precise manner?

Surfaces that interact with precision with biological systems will be complex—multicomponent, multilayer, orientated, patterned. Given the complexity of the molecular structures that make up the individual biomolecules comprising these surfaces, fabrication and characterization of such surfaces will push the skills of surface scientists to their limits.

In the future, tissue engineering (coupled with truly biocompatible scaffolds), stem cell technology, control of regeneration and the knowledge of the human genome will completely change the way we work with biomaterials and medical devices [86–91]. But, before these revolutionary technologies replace today's biomaterials, we still probably have 30 years during which biomaterials as we know them today will be of increasing importance. Thus, there is strong impetus to evolve the surface strategies needed to control biological interactions.

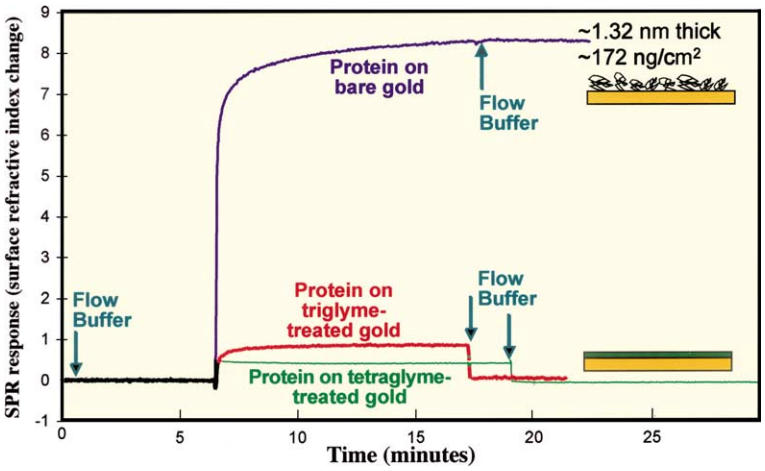

Fig. 10. A SPR experiment on protein (bovine serum albumin, 1 mg/ml) adsorption to three surfaces. Protein adsorption is rapid and high on a bare gold SPR element (blue). When buffer is flowed through the system at about 18 min, essentially no protein desorbs from the gold. If the gold is treated in an RF plasma under the vapors of triglyme (red) or tetraglyme (green), a treatment that deposits a tightly bound poly(ethylene glycol)-like layer, little protein is noted to adsorb. Protein that does adsorb is washed away when the buffer flow commences. (Data of Mar et al. see Ref. [76] for further details).

5. Characterization of complex biological surfaces

5.1. Adsorbed protein films

The adsorption of proteins onto a biomaterial surface from the surrounding fluid phase is rapid, with the surface properties of the biomaterial determining the type, amount, and conformation of the adsorbed proteins [92]. The composition of the adsorbed protein layer (i.e., the type and concentration of the proteins present in the adsorbed film) can differ from the fluid phase composition and can change with time adsorbed. This is shown schematically in Fig. 11 using three different proteins (red, green and blue). Initially the surface concentration of "red" and "green" proteins is higher than their solution concentration. With time the red and green proteins are displaced from the surface by the "blue" protein. In addition to the time-dependent compositional changes, each absorbed protein can undergo conformational and orientational changes, as shown schematically in Fig. 12. Upon adsorption, a protein can retain the conformation or structure it has in the biological environment or it may conformationally change in response to local environments. The nature of the surface strongly influences the composition and recognizability of the adsorbed protein layer, which in turn affects the subsequent cellular interactions. Thus, to understand the biological response to a material, especially in vitro, one must fully understand the nature of the adsorbed protein film that forms on that material.

The extremely high analytical sensitivity of static ToF SIMS, its sampling depth of 1–2 nm, and the molecular information it provides about the chemistry of the adsorbed protein film and the substrate offer the potential to use static ToF SIMS to gain a detailed understanding of the composition, conformation and orientation of adsorbed proteins. For an intact protein adsorbed in its native conformation, the static ToF SIMS spectrum will represent only the amino acids present on the surface of the protein since most proteins have dimensions between 4 and 10 nm, which is significantly larger than the static ToF SIMS sampling depth. For proteins with a heterogeneous distribution of amino acids across the three dimensional domain of the protein molecule, the relative intensities of the amino acid fragments detected in the SIMS spectrum will be sensitive to the orientation of the adsorbed protein and its degree of conformational alteration [93]. As a protein adjusts to the surface and changes its

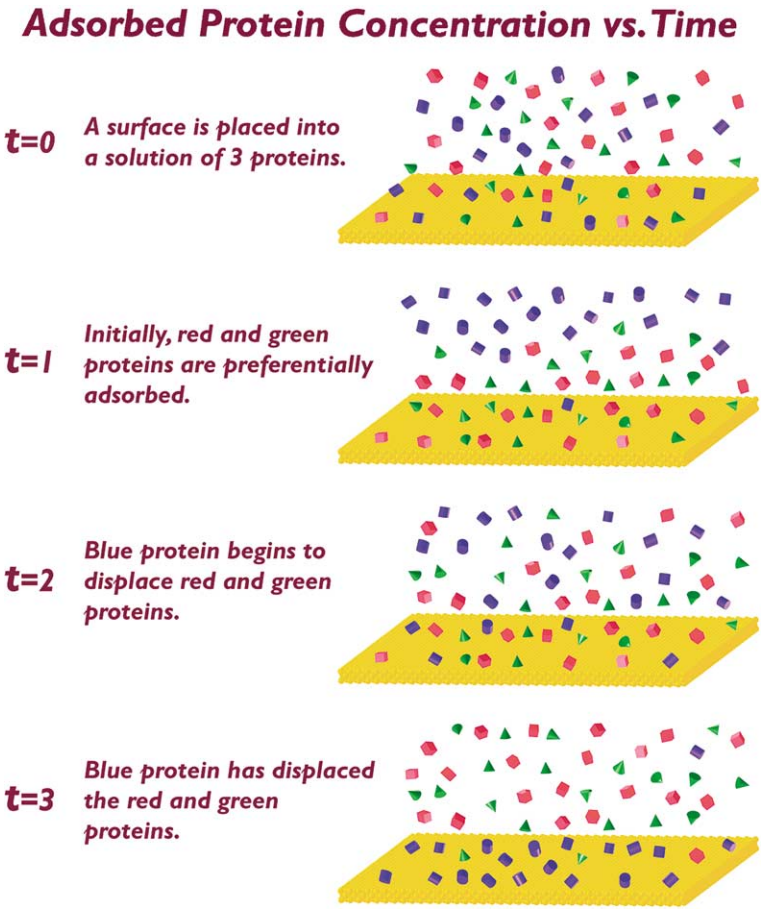

Fig. 11. A surface placed in a protein mixture will be covered with a layer of adsorbed proteins in a matter of seconds. The concentration of proteins in the adsorbed film is typically different from their solution concentration and can change with time. In this example, initially the "red" and "green" proteins are preferentially adsorbed. With increasing adsorption time (minutes to hours) the red and green proteins are displaced by the "blue" protein.

conformation or orientation, new regions of the protein with different amino acid compositions will be exposed to the static SIMS beam. This means that static ToF SIMS has the potential to provide a microscopic, chemical glimpse of changes in the protein conformation (see Fig. 13). Furthermore, during the denaturation process, the ratio of bare substrate to protein may change as the protein unfolds and spreads over the surface.

The use of static ToF SIMS for characterizing adsorbed protein films has shown the potential to probe protein conformation, assess surface coverage, measure protein concentration with extreme analytical sensitivity, map protein distributions and identify different proteins (see below). In addition, it can measure contamination and analyze synthetic substrates and binding chemistries. Thus, static ToF SIMS can make important contributions to biomaterials development. In vitro, static ToF SIMS should play an important role in the development of cell culture surfaces, biosensors, protein and DNA diagnostic arrays, immunoassays, non-fouling surfaces and chromatographic supports. In vivo, static ToF SIMS will be used to characterize surfaces engineered with specifically immobilized signal molecules, measure uncontrolled fouling, and relate surface structures to blood interactions. An example of the power of

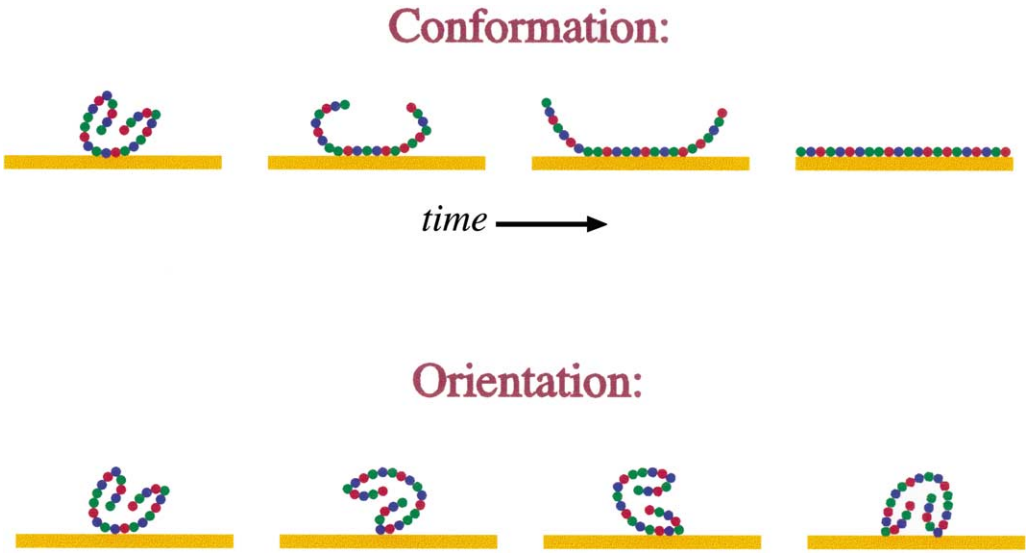

Fig. 12. The conformation and orientation of adsorbed proteins depend on adsorption conditions and surface properties. The top schematic shows a protein denaturing with increasing adsorption time. The bottom schematic shows a protein adsorbing to the surface in different orientations.

static ToF SIMS combined with multivariate analysis for analyzing the complex protein films formed on surfaces, so important for the many applications mentioned, will now be presented.

Static ToF SIMS spectra of adsorbed protein films are complex and contain peaks from all of the amino acids. Since the same 20 amino acids are present in all proteins, it is the relative intensities of the amino acid fragments in the ToF SIMS spectra that contain the information needed to identify adsorbed proteins. To do this identification efficiently for a large number of proteins requires the use of a pattern recognition method such as PCA [70]. PCA is an unsupervised classification method that can be used to reduce the dimensionality of the complex static ToF SIMS protein spectra, making it straightforward to identify proteins and also to develop an understanding why static ToF SIMS can make this identification. For example, static ToF SIMS with PCA has successfully identified 13 different proteins adsorbed onto mica from pure protein solutions [94]. It was determined that PCA was distinguishing the adsorbed proteins based on their different bulk amino acid compositions. Static SIMS with PCA is also able to distinguish albumin proteins from different species (human, cow, pig, chicken, and turkey). In addition to identification of protein type, the combination of PCA and static ToF SIMS can be used to quantify the amount of adsorbed protein present in mixed films [94]. To date, it has been successfully applied to binary protein mixtures. A challenge for the future will be to determine how much the complexity of the protein mixture can be increased (i.e., how many proteins can be present in the adsorbed film) while still retaining the ability to identify and quantify all of the proteins. It has been shown that using a PCA model built from the pure protein data set, it is possible to draw qualitative conclusions about the protein composition of films adsorbed from 1% bovine plasma [94]. It was found that initially the protein film is enriched in fibrinogen. With increasing adsorption time the fibrinogen concentration of the film decreases.

In addition to determining the composition of an adsorbed protein layer, it would also be desirable to determine the accessibility and location of binding sites on a protein molecule. For example, the aggregation of fibrin proceeds by the staggered overlap of the fibrin molecules, where the central portion of one molecule interacts with the terminal

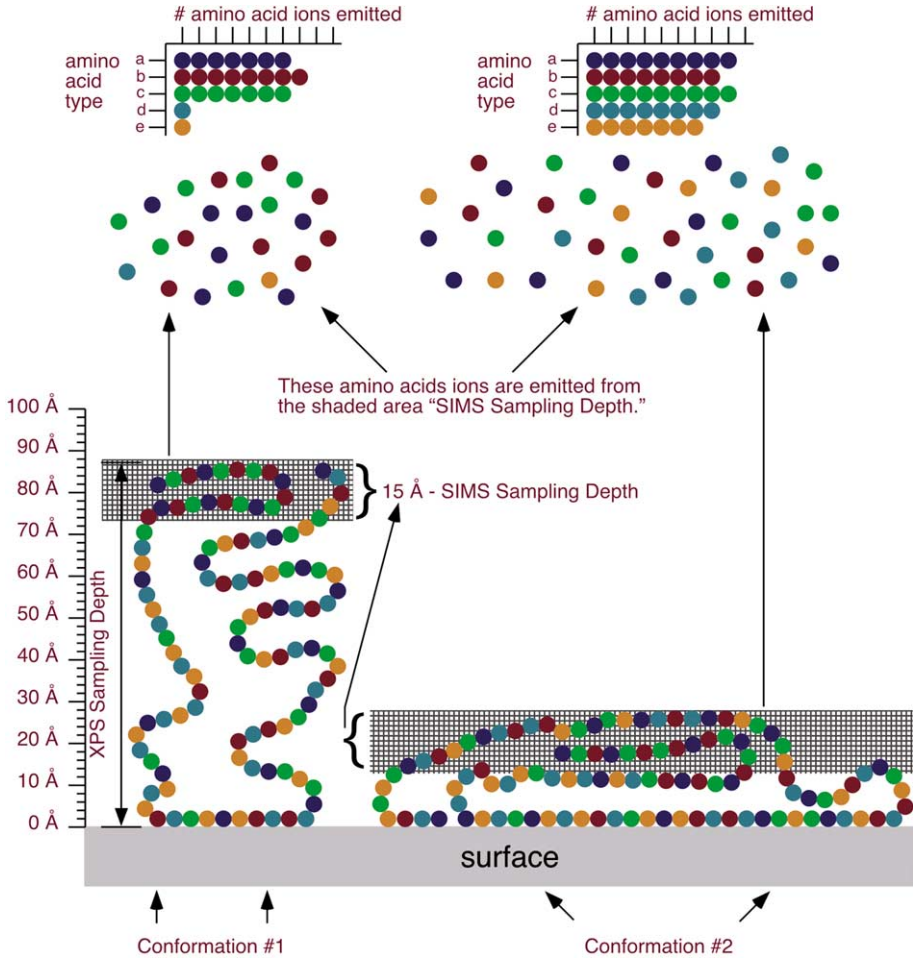

Fig. 13. A schematic showing the sensitivity of static ToF SIMS to protein conformation. Both proteins have the same bulk amino acid composition, but since the amino acid composition is not uniform across the protein molecule, conformation #1 will produce a different intensity pattern of static ToF SIMS fragments than conformation #2.

regions of neighboring molecules. Peptides are known to bind to these polymerization sites in fibrin [95], so peptides containing ^{13}C, ^{15}N, or F labels can be used as a probe for fibrin polymerization sites. Labeled peptides are necessary to generate unique SIMS amino acid fragments from the peptides that can be distinguished from the large number of unlabeled amino acid fragments that would originate from the protein. This approach should be generally applicable to protein binding reactions since the fibrin polymerization process is prototypical of the binding between specific, localized peptidic sites on two protein molecules.

5.2. Hydrated surfaces

Hydrated surfaces are challenging for UHV surface science, but normal and relevant for biology. Methods applicable to the solid–aqueous interface, and therefore relevant to biological surface studies include contact angle measurements, second harmonic generation (SFG), Brewster angle microscopy, X-ray reflectivity, SPM, frozen-hydrated UHV techniques (XPS, SIMS, etc.), environmental scanning electron microscopy, attenuated total reflectance IR (subtract out the water signal), ellipsometry, SPR and neutron

scattering. Most of these methods work in aqueous environments without appreciable interference from the water. Unique aspects associated with UHV biological surface science are addressed below.

UHV XPS studies of wet surfaces have been performed using frozen, hydrated specimens for electrochemistry [96] and biomaterials [97,98]. In biological and biomaterial XPS studies, a specific protocol has been adopted based on the following rationale. The wet sample, when frozen under atmospheric pressure conditions, will adsorb a layer of adventitious contaminants. After freezing, if the temperature is kept below −160 °C, water will not sublime from the sample. Once the sample temperature is raised above −100 °C, water will quickly sublime from the frozen ice layer "blasting off" the contaminant layer. The objective is to stop the ice sublimation when an ice layer approximately 1–2 nm thick remains so XPS can see through the ice layer to analyze the frozen, hydrated sample. To stop the ice sublimation and stabilize the thin ice overlayer, the sample temperature is rapidly lowered to −160 °C. In this way, a hydrated, frozen specimen can be studied under UHV.

The specific University of Washington protocol for UHV frozen-hydrated analysis is as follows. First, the preparation chamber stage and the analytical chamber stage in the XPS instrument are cooled to −160 °C while the specimen to be examined is hydrated by placing a drop of water on its surface. Second, the hydrated sample is cooled below −100 °C in the preparation chamber under an atmosphere of dry, purified nitrogen gas. Third, the preparation chamber is pumped down to UHV while keeping the sample temperature well below −100 °C. Fourth, the sample temperature is now raised to typically −90 °C (above the sublimation point of ice but below the polymer glass transition temperature). The sample temperature is quickly lowered below −120 °C to stop the sublimation process when a 2 nm ice overlayer remains. Fifth, the sample is quickly moved from the preparation chamber to the pre-chilled analytical chamber stage at −160 °C and XPS data is acquired, frequently at multiple photoemission take-off angles. Finally, XPS data from the "dehydrated" sample is obtained by returning the sample to the preparation chamber and bringing it to room temperature to liberate the remaining frozen water. The dehydrated sample is then returned to the analytical chamber at −160 °C for analysis. A Surface Science Instruments (SSI) X-probe XPS instrument was adapted for these cryogenic studies by adding liquid nitrogen cooled stages to the entry/preparation chamber and to the analytical chamber. Further details have been described elsewhere [98].

An example illustrating the powerful influence of the sample environment on the surface chemistry is taken from the literature [97,98]. In these studies a silicone elastomer film covalently grafted with the hydrophilic polymer PHEMA was examined both in the frozen, hydrated state and in the dehydrated state. In the frozen, hydrated state, a spectrum resembling PHEMA was observed. In the dehydrated state, the spectrum had the characteristics of silicone rubber. The data suggest that when the sample is wet, the hydrophilic PHEMA chains dominate the surface to reduce the interfacial energy between solid and water. When in air, the hydrophobic silicone chains dominate the specimen, reducing interfacial energy. Thus, for a sample of this type with polar and non-polar moieties, and considerable polymer chain mobility, it must be studied dry and wet to fully characterize the surface.

5.3. Photon in/photon out techniques

Although the standard electron and ion based surface science techniques (XPS, static SIMS, etc.) provide excellent surface sensitivity and detailed information about composition and molecular structure, they cannot be used to directly examine a biomaterial surface in an aqueous environment. The strong interactions of electrons and ions with materials provide surface sensitivity. However, these strong interactions also require that these probes be used in an UHV environment since low-energy electrons and ions cannot penetrate the aqueous-based biological environment surrounding an implanted biomaterial.

The previous section discussed how the aqueous environment can be simulated using a frozen,

hydrated method, allowing the structure of the hydrated surface to be examined under UHV conditions. Although this method provides information about the composition and structure of hydrated surfaces, it would be preferable to directly examine the surface of biomaterials in aqueous environments. Then, changes in surface composition and structure could be monitored in real time.

One method for investigating the properties of the solid (biomaterial)—liquid (biological environment) interface is to use photon in/photon out techniques since photons have longer mean free paths than low-energy electrons and ions. However, in many cases the surface sensitivity of the technique is compromised. For example, fluorescent X-rays can be detected instead of electrons in NEXAFS experiments. This allows NEXAFS experiments to be done at significantly higher pressures, but also results in the sampling depth increasing from 5 nm (electrons) to 200 nm (soft X-rays) [99]. If the surface species under investigation has a unique spectroscopic feature (e.g., adsorption peak) then the surface specificity can be regained since the signal from the surface species only contributes to that particular spectroscopic feature.

Another method for regaining surface specificity is to use total external reflection experimental conditions (grazing incident and reflection angles) [100]. This can be done for photon energies from the IR to hard X-ray regions, but requires large, flat surfaces. However, if the bulk of the material contains similar species as the surface and a suitably flat surface cannot be prepared, then most photon in/photon out techniques will not provide the needed surface sensitivity to characterize the hydrated biomaterial surface.

One optical technique that can directly examine the structure of the solid–liquid interface is SFG [101]. SFG is a second order non-linear optical process where a pulsed visible laser beam (ω_{vis}) is overlapped with a tunable, pulsed IR laser beam (ω_{ir}) to generate a signal at the sum frequency (ω_{sum}). Emission of the sum frequency light does not occur for the bulk phase of most materials. However, the symmetry of the bulk phase is broken at a surface or interfaces, so surface species do produce sum frequency signals. Thus, SFG provides both surface sensitivity and direct interrogation of the structure at the solid–liquid interface.

The SFG intensity plotted versus the frequency of the IR laser provides a vibrational spectrum of the surface species with submonolayer sensitivity. By using different polarization conditions (e.g., s-polarized sum frequency, s-polarized visible, and p-polarized IR) the orientation of surface species can be determined. Typically, it takes a few minutes to acquire a SFG spectrum over a few hundred wave number range, so by monitoring the changes in a given spectral region (e.g., C–H stretch), time dependent changes in the surface structure can be monitored with a resolution of minutes. The time-dependent restructuring of polymeric materials (migration of end groups, copolymer components, etc.) that occur upon hydration and dehydration have been determined with SFG [102].

Future opportunities for improving the SFG technique include decreasing the time resolution from minutes to seconds and expanding the vibration frequency range of the SFG spectrum. Typically SFG spectra are acquired in the range from 2500 to 3600 cm^{-1}. Expanding that range to cover 1000–4000 cm^{-1} would significantly increase the number of vibrational bands that can be accessed with SFG, thereby expanding the molecular structure information that can be determined with SFG. For example, expanding the range below 2000 cm^{-1} would allow the structure of adsorbed proteins to be determined using the amide bands. The major limitation of SFG to date has been the fact that the concentration of the surface species detected by SFG cannot be quantified. However, SFG used in combination with frozen-hydrated XPS and static ToF SIMS may provide a method for overcoming this limitation.

There are other photon in/photon out techniques that can provide information about the thickness (ellipsometry) and amount (SPR) of a deposited species, but the chemical composition and molecular structure information provided by these techniques is limited. Both ellipsometry and SPR detect changes in the refractive index, which only provides indirect information about chemical

and biological species. Thus, these techniques need to be used in combination with other techniques such as XPS and static SIMS which can provide direct information about surface composition and structure.

6. Future directions of biomedical surface science

Opportunities are plentiful in biomedical surface science. Start-up businesses based on biological surface science abound. New discoveries in biology beg for application. Surface analysis instrumentation steadily improves. Self-assembly ideas have made the routine synthesis of nearly perfect organic surfaces a reality. What paths might one take to get involved at the cutting edge of this exciting field?

A few themes will dominate the future of biomedical surface science: learning from biology, biomimetics, precision immobilization, self assembly, nanofabrication, control of non-specific reactions, and smart surfaces. We will briefly address each of these.

6.1. Biological knowledge

Biological systems use surfaces with precision. The analysis of these surfaces and their emulation (biomimetics) represents an important avenue to improved, functional biosurfaces for basic research and technological applications. Examples will be given based on cell membranes, biominerals, and the extracellular matrix.

Fig. 14 highlights the basic components of a cell membrane. This precision supramolecular structure is much more than a protective barrier. It inhibits non-specific interactions, recognizes specific ligands, performs enzymatic (catalytic) functions, pumps ions and reconfigures its topography and geometry. Surface researchers are now attempting to model cell surfaces with supported lipid bilayer membranes [103–107]. These surface assemblies lightly tether a lipid bilayer film onto a hydrated, hydrogel support conferring mobility and order. Such synthetic structures are roughly analogous to the elegant supramolecular structure pictured in Fig. 14.

Nature has its own equivalent of "single crystal surface science." Inorganic crystalline structures are used throughout biology. Complex calcium phosphate crystalline phases comprise roughly two thirds of bone. Calcium carbonate crystals form into otolith structures in the inner ear responsible for balance. Mollusks synthesize calcium carbonate-based nacre (mother of pearl). Diatoms extract silicon from the ocean to make their silica skeletons. Interestingly, these crystals rarely exist in isolation. Most commonly, they are closely complexed with organic components. In fact, there are many aspects of this process suggestive of epitaxy and molecular recognition.

For much of the history of biology, the material between cells, the extracellular matrix, was thought to be uninteresting, amorphous filler. In recent years, it has been found to be organized into precise structures that control many functions central to life. Along with a mechanical function, the ECM has important roles in cell adhesion, migration, proliferation and differentiation. As an example of the elegant reactions that occur on ECM, consider protein binding to hyaluronic acid. This polysaccharide, which can have molecular weights up to 25,000,000 and forms an amorphous gel, binds specifically to a 30 amino acid peptide sequence with a molecular weight of approximately 3000. This is an elegant surface interaction engineered over approximately 7.5 nm of linear surface.

Based upon these examples of complex biological surfaces, we can begin to perceive the challenge. It is fourfold: (1) What is the biological significance of these structures (functionality and mechanism)? This is the challenge of biological discovery. (2) How can we characterize surface structures given their oriented multilayer organization and remarkable chemical complexity, mobility and fragility? (3) How can we bring the surface science model of structure and reactivity into congruence with the biological model of surface functionality? (4) How can we emulate nature's elegance and create biomimetic surfaces?

6.2. Biomimetics

Since nature uses surfaces with precision, there is clear justification in emulating nature's methods

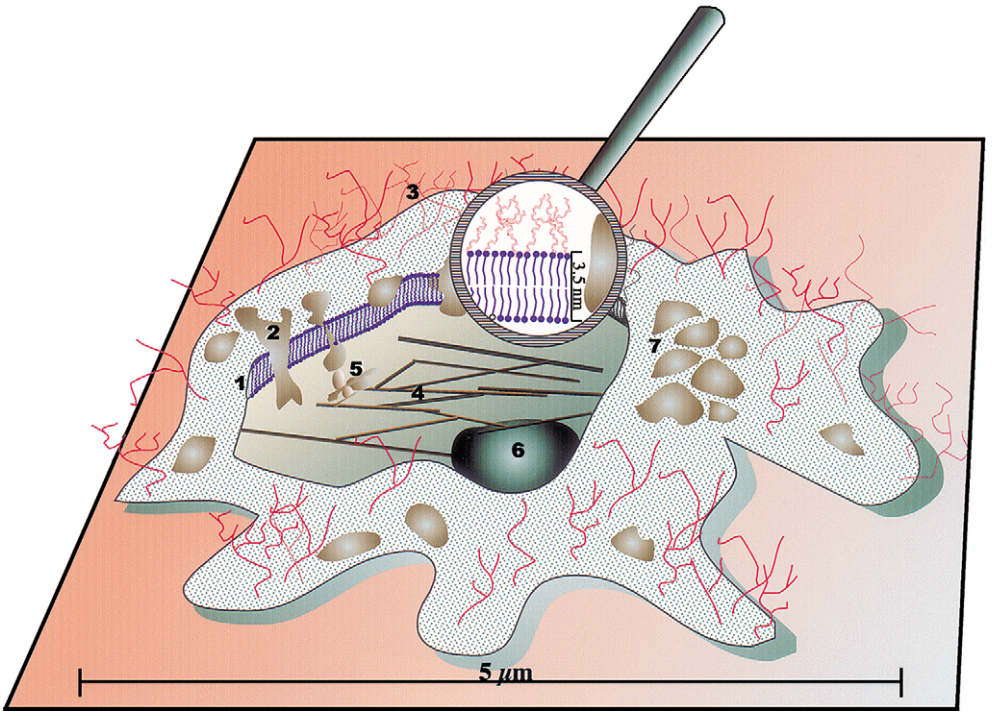

Fig. 14. The basic components of a cell membrane: (1) the lipid bilayer membrane, (2) an embedded protein through the lipid bilayer membrane, (3) saccharide chains on the surface of the cell, (4) the cell cytoskeleton, linked to a transmembrane protein through (5) a series of smaller proteins (talin, vincullin, etc), (6) the cell nucleus, (7) proteins exposed at the cell surface.

to make more functional, controllable surfaces. A variety of approaches have been developed to copy nature's way of doing things. Many of these address pharmacological strategies, which have only limited relevance to this article. The efforts directed to materials that are stronger, lighter, tougher, cheaper, cleaner to manufacture and biodegradable largely focus on bulk properties. However, these are generally multiphase, composite materials. The nature of the interface plays a key role in the ultimate properties. For example, nacre, the material lining an abalone shell, is a mechanically tough substance. The calcium carbonate that makes up 95% of nacre is a brittle mineral. The 5% protein dispersed between mineral "bricks" is sufficient to confer strength and toughness to the composite [108]. The interfacial interactions between the protein component and the carbonate platelets are central to explaining the substantial enhancement in mechanical properties. Other examples of biomimetic strategies involving surfaces and interfaces include synthetic mussel adhesive [109], nanopits for protein recognition [110], supported lipid bilayer membranes to mimic natural cell membranes (discussed in the previous section) and hydroxyapatite surfaces for bone incorporation [111]. Further examples of biomimetic surface approaches are presented in each of the following sections.

6.3. Precision immobilization

A surface skill used in nature with elegance and precision is the ability to order and organize complex molecules at surfaces. Precision immobilization typically aims to copy nature's way of organizing molecules and is thus an example of a biomimetic strategy. Such ordering permits biomolecular signals to be delivered with great precision. Biomolecules used in precision immo-

Table 4
Methods to immobilize active biomolecules to surfaces

Method	Comment
Non-specific adsorption	Little control is afforded of protein orientation or activity; low durability
Non-specific covalent immobilization	Little control is afforded of protein orientation or activity
Immobilization on an antibody surface	Using monoclonal antibodies, protein orientation can be controlled
HIS tags	Histidine sequences (HIS tags) can be specifically engineered into proteins for attachment and orientation
Biotin/streptavidin	A flexible strategy for tightly fixing protein to surfaces; in vivo biological reaction to streptavidin is a concern
Crystallized protein layers	Useful only in limited cases
Immobilization to a template structure	An evolving field
Biomimetic recognition sites	An evolving field
Incorporation in a supported bilayer	As an emulation of the cell membrane this has the possibility to stabilize fragile proteins
Nucleotide conjugation/hybridization	Many possibilities are being explored
Electrostatic	A non-specific approach to immobilizing proteins when the protein has an isoelectric point higher or lower than seven and a surface has a positive or negative charge

bilization strategies include proteins, lipids, polypeptides, polynucleotides and polysaccharides.

Possibilities for surface immobilization of biomolecules are suggested in Table 4. Two books that overview this field are cited here [112,113]. The degree of specificity (precision) in immobilization ranges from relatively low to extremely high. The characteristics of successful precision engineered biorecognition surfaces include the presence of one receptor site, an appropriate surface density of those sites, controlled orientation of the sites, some molecular mobility to enhance "docking," and stability (of the biomolecular conformation and the film integrity). The ability to inhibit non-specific reactions (in particular, protein adsorption) is also essential to succeed at emulating nature's surface signal delivery strategy. The ultimate goals in surface immobilization of biomolecules are high activity (functionality) and specificity.

6.4. Self-assembly

Self-assembly can be used to create bulk materials or ordered surfaces. Molecular mobility allows complex, often flexible molecules sufficient time and geometric opportunity to associate and assume their lowest energy state, the crystal. Self-assembled surfaces prepared from organic molecules are valuable as models (analogous to the metallic single crystal models) for exploring biological-like, hierarchical systems and also present possibilities to nanofabricate real surfaces for technological applications. SAMS were discussed earlier in this review. However, they offer so many opportunities that further elaboration is useful.

Three- and two-dimensional self-assembly are well known. Two-dimensional self-assembly is more relevant for this surface article. The commonality in systems that show 2-D self-assembly are a relatively simple molecular geometry, a driving force for interacting with a smooth surface, a lateral interactive force between molecules to stabilize them in the crystal and a chemical group that forms the outermost surface of these systems. The scientific roots of this area of study lie in the Langmuir–Blodgett deposition of lipids and surfactants [114]. The discovery in 1983 of thiol assembly on gold [115] (see Fig. 4) launched an explosion of publications and new discoveries. Self-assembly of complex organic structures on solid surfaces has been observed for phospholipids [116], silanes [117], *n*-alkyl thiols [115,118,119], porphyrins [120], nucleotide bases [121], hydrocarbons [122], proteins [123,124], and many other organic structures. An example from nature of the self-assembly of proteins on the surface of a bacterium is shown in Fig. 15 (also see Ref. [123]). Recent developments in the self-assembly of multilayer systems of polyions is also interesting in this context [125].

Fig. 15. A TEM image of a bacterial cell with an ordered S-layer protein array with square lattice symmetry. Bar = 100 nm (used with permission of Prof. U. Sleytr, http://www.boku.ac.at/zuf/sxl9.htm).

6.5. Nanofabrication

Nanofabrication, nanotechnology and nanoscience represent growth areas in research and development. However, nature has been using nanofabrication ideas since the beginnings of biological evolution. For example, the coordinated workings of receptor and enzyme mechanisms in the cell membrane suggest clever, nanoscale machines [126]. Topics already addressed here such as precision immobilization, self-assembly and biomimetics are all examples of nanofabrication. Thinking at the nanoscale does open interesting possibilities for synthesis of new structures and the interface of biology and materials. Particularly interesting work has been done using dendrimers (tree and star-like polymers) [127], rotoxanes [128] and DNA [129,130] as building components for creating nanostructures. Tools that the surface scientist can apply for nanofabrication are the atomic force microscope [32,131] and electron beam lithography.

6.6. Control of non-specific reactions

The subject of protein resistant (non-fouling) surfaces has been addressed earlier in this article. How such surfaces function, how to optimize them and how to use them in technology remains an important frontier area in biointerfaces. Non-fouling surfaces will be important for biomaterials, biosensors, medical diagnostics, heat exchangers, ship bottoms and food processing plants. Improved biofouling-resistant surfaces will become a reality when we have an enhanced understanding of why such surfaces function as they do. Theories focussing on polymer chain excluded volume, polymer chain entropy, water structure and hydrogen bond acceptors have been put forth [132–136].

6.7. Smart surfaces

The term "smart material" has been used to describe materials that go through rapid phase transitions with a small change in environmental conditions leading to a useful physical property change. Many examples of such materials have been produced—this is a branch of chemistry demonstrating creativity and promise [137–144]. When coupled to enzymes or other specific biological receptors, the smart materials are made smarter still [144,145]. Surfaces that undergo rapid shifts in surface properties with small external changes open many possibilities and present a frontier area for biosurface science [146,147].

7. Conclusions

Biomedical surface science will contribute to both fundamental knowledge and technology. From the basic science perspective, surface science models will assist in the understanding how nature does its work–chemical and biological models are insufficient to provide this knowledge without in-

voking the special properties that characterize the surface state. Technology will clearly benefit from a surface science model of biology in more functional medical implants, improved biosensors, chip-based neuronal computing, precision medical diagnostics, barnacle-resistant ships, finer biomolecule separations, biosynthetic production of plastics and chemicals and interfacially engineered biocomposite materials. Areas such as nanotechnology and smart materials hold untold promise and will certainly partner with biomedical surface science to implement novel technologies and new discoveries. Advances in surface analysis methodology will be central to these developments in basic science and technology.

Acknowledgements

The authors acknowledge generous support from the National ESCA and Surface Analysis Center for Biomedical Problems (NESAC/Bio, funded by NIH grant RR-01296 from the National Center for Research Resources) and the UWEB Engineering Research Center (funded by grant EEC-9529161 from the NSF) during the preparation of this manuscript and for some of the experimental work described herein. We also would like to thank our colleagues for many stimulating discussions over the years regarding the development of biomedical surface science.

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