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TOTAL INTERNAL REFLECTION FLUORESCENCE IN BIOLOGICAL SYSTEMS

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Fluorescence of labeled proteins near a solid/liquid interface can be excited by the evanescent field of total internal reflection to study reversible adsorption kinetics and surface diffusion.

1. INTRODUCTION

Many biochemical processes occur at interfaces, including: the binding of hormones, neurotransmitters, and antigens to cell membrane receptors; blood coagulation at foreign surfaces; electron transport in oxidative respiration; photosynthetic reactions; and anchoring and motion of bacteria, algae, and animal cells on solid surfaces. In addition, biochemistry at liquid/solid interfaces has industrial and medical importance in areas such as trace detection of blood serum antibodies and enzymes by immobilized antigens and substrates, and the manufacture of biochemical products by surface-immobilized enzymes.

Central to the study of biochemistry at interfaces are: (1) identification of the molecular species in proximity of the surface; (2) quantitation of surface concentration; (3) measurement of the average time of residence of a molecule reversibly adsorbed at a surface before it desorbs back into bulk solution; and (4) detection of surface diffusion that may play a role in two-dimensional surface chemistry. Total internal reflection fluorescence (TIRF)[1,2] offers an experimental approach for selective detection of specific fluorophores (usually fluorescence-labeled proteins) in a microscopic surface area.

In TIRF, a laser beam totally internally reflects at a solid (usually glass)/liquid (usually salt water) interface, thereby setting up an evanescent field in the liquid. This field, which exponentially decays within a fraction of a wavelength of the surface, excites fluorescence from fluorophores in the proximity of the surface while leaving those farther out in the solution unexcited. Depending on the application, the laser beam may be focused to an area of several square microns, and the fluorescence emitted into the solution is observed through a conventional high aperture microscope objective. In combination with other fluorescence techniques, the molecular dynamics of the surface-bound fluorophores can be studied. Reviewed here are various

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applications of TIRF to biological or biochemical systems that have been employed in our laboratory.

2. CELL-SUBSTRATE CONTACTS[3]

Numerous types of cell surface specializations occur at contact regions between two biological cells or between a cultured cell and the glass or plastic on which it grows. One example of such a specialization is an aggregation or cluster of acetylcholine receptors (AChR) on developing muscle cells in culture at the cell-substrate contact regions. AChR is a membrane protein that naturally clusters at neuromuscular junctions. It can be visualized by fluorescein-labeled α-bungarotoxin, which binds irreversibly to AChR. A key question is what cellular molecules or structures are responsible for immobilizing AChR in a cluster. Protein filaments which might be suspected of connecting to AChR can be visualized by rhodamine-labeled antibodies. These filaments can then be observed by TIRF without exciting fluorescence from the whole depth of the muscle cell, and any spatial correlations between clustered AChR and certain submembrane filamentous structures immediately underlying them can be seen. In collaboration with R. Bloch, we have thus far found a significant *anti*-correlation between the spatial distributions of clustered AChR and of filamentous vinculin, filamin, smooth muscle myosin, and α-actinin. Where AChR is clustered, those types of protein filaments tend to be excluded.

3. BIOMOLECULAR DYNAMICS AT ARTIFICIAL SURFACES[4,5]

For measuring the desorption rate of reversibly adsorbed fluorophores in chemical equilibrium at a glass surface, TIRF can be combined with either fluorescence photobleaching recovery (TIR/FPR) or fluorescence correlation spectroscopy (TIR/FCS).

In TIR/FPR, the evanescent light is very briefly increased in intensity by a factor of several thousand in order to irreversibly photobleach only those fluorophores adsorbed to the surface. Subsequently, as these photobleached fluorophores desorb and are replaced by unbleached fluorophores adsorbing from solution, fluorescence is excited by a re-attenuated evanescent field. The rate of recovery of observed fluorescence is a measure of the desorption kinetic rate. In applying TIR/FPR to study the adsorption of bovine serum albumin to glass at equilibrium, we found a range of surface residence times ranging from around 5 sec to more than one hour, probably due to several classes of binding sites. By varying the sharpness of focus of the incident laser beam, surface diffusion (which also contributes to the post-bleach recovery) can be distinguished from adsorption/desorption kinetics. We found that the most weakly

adsorbed class of serum albumin apparently diffuses on the order of two microns on the surface before it desorbs.

The kinetics of immunoglobulin adsorption to protein-coated glass was measured by TIR/FCS.[6] In TIR/FCS, no bleaching flash is employed; a continuous evanescent illumination excites fluorescence from adsorbed fluorophores. Only fluorescence originating from within a small area on the surface, defined by an image plane diaphragm, is recorded. As molecules adsorb and desorb at random in equilibrium, the resulting statistical number fluctuations give rise to fluorescence fluctuations which can be autocorrelated on-line by a computer. The decay rate of the autocorrelation function is a measure of the desorption rate. In the case of immunoglobulin adsorption, again a range of desorption times was noted, with the shortest time less than 5 msec, limited by the rate of bulk diffusion away from the surface.

4. MOLECULAR DYNAMICS ON BIOLOGICAL MEMBRANES

The possible non-specific adsorption and surface diffusion of hormones on cell membrane surfaces is of some interest in cell biology because of the possible enhancement of the hormone's reaction rate with discrete hormone receptor sites that may result from it.[7] We have studied the equilibrium kinetic rate of adsorption/desorption of fluorescein-labeled epidermal growth factor (EGF, a small peptide hormone) to the surface of erythrocyte membrane, using TIR/FPR. This study necessitated overcoming two technical problems: (1) how to flatten the erythrocyte membrane on glass so that its exterior face lies submerged in the evanescent field; and (2) how to avoid observing the large non-specific adsorption of EGF to the glass itself. For solving the first problem, we covalently attach poly-L-lysine to glass, allow erythrocytes to stick to it, and then hemolyze them. The result appears to be a completely flattened membrane, some portions of which appear to be one bilayer thick with an exposed cytoplasmic face and the remainder of which appears to be two bilayers thick with an exposed exterior face. For solving the second problem, we pre-coat the glass with a 200 Å thick layer of aluminum (by vacuum vaporization). The aluminum layer is then covalently bound with poly-L-lysine and erythrocyte membranes are attached as on uncoated glass. Fluorescence from EGF adsorbed directly onto the aluminum layer is then effectively quenched by the metal.[8] However, fluorescence from EGF adsorbed onto an erythrocyte membrane (and thereby held away from the aluminum layer by about 100 Å) is not completely quenched but is still excited by the evanescent wave. Preliminary results with this preparation indicate that non-specifically adsorbed EGF remains associated with the membrane for a time on the order of one-half second

before desorbing. The physical significance of such surface residence times for possible reaction rate enhancement depends on how fast the non-specifically adsorbed hormone diffuses on the surface and how far it must go on the average before it finds a specific receptor.

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