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Activated Langmuir-Blodgett Films for Immobilizing Proteins on Planar Surfaces

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A densely packed array of carboxylate (or other groups) can be produced on smooth planar surfaces using the Langmuir-Blodgett technique. These carboxylates heve been activated using the well-known carbodiimide reaction, and subsequently coupled to the free amide groups found on the surface of most proteins in order to immobilize them on the planar surface. The whole operation can be completed within a few minutes. The surface loading of protein is monitored in real time during all stages in the process using optical waveguide mode spectroscopy.

1. Introduction

Analytical techniques such as solid-phase immunoassays[1] and new methods for examining proteins in their native environments, such as atomic force microscopy[2] require proteins to be immobilized, preferably covalently, on a solid surface.[3] The immobilization procedures described in the literature usually involve a lengthy series of thermochemical reactions and intermediate washing steps to remove unreacted reagents, and moreover are not generic: each particular surface (*e.g.*, noble metallic for amperometric enzyme sensors[4] and surface plasmon resonance,[5] organic-polymeric for enzyme-linked immunosorbent assays (ELISA)[1]) requires a specific chemical process. Here we describe how smooth, planar surfaces can be rapidly and controllably functionalized for immobilizing proteins using a combination of the Langmuir-Blodgett (LB) techniques[6–9] and carbodiimide chemistry.[10]

The LB technique can be used to deposit monolayers of amphiphiles onto a planar substrate. Their surface density can be straightforwardly controlled by varying the monolayer pressure. The usual deposition mode is Y-type (molecules stacked perpendicular to the plane of the substrate in consecutive head-to-head and tail-to-tail orientations); to ensure that the outermost plane (in contact with the solvent) is composed of the hydrophilic moiety of the amphiphile, an odd number of layers is required if the substrate is hydrophobic, and an even number if the substrate is hydrophilic. In general, the deposited layers are strongly adherent to the substrate and to each other.

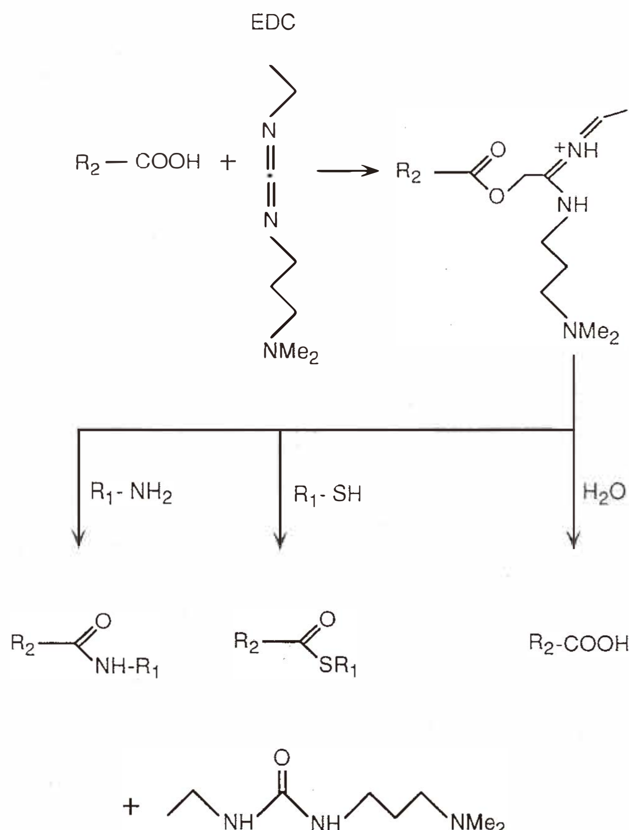
1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry[10–12] has become popular for linking proteins to solid surfaces, not least because of the mild conditions and good yields. The reaction involves activating a carboxylate group with EDC, which then reacts with a primary amine to form an amide linkage, upon which a water-soluble *O*-acylisourea is released (Scheme 1). Proteins contain residues with carboxylate and amine side groups, and the method may be applied to amino, carboxylate or thiol surfaces.[12]

In order to be able to follow all steps of coupling and regeneration, planar optical waveguides were used as substrates. The propagation of guided modes in the waveguide depends on conditions at its surface;[13–17] measurement of the effective refractive indices N for two modes allows the number of protein molecules per unit area of surface to be determined.[13–17]

2. Materials and Methods

$Si(Ti)O_2$ waveguides incorporating a grating coupler (period Λ = 416.2 nm) were obtained from Artificial Sensing Instruments, Zürich, Switzerland (type 2400), and used as received. They were examined by atomic force microscopy and found to have a mean surface roughness of about 0.09 nm. A stearic acid bilayer was deposited on these hydrophilic metal oxide waveguides from a pure water subphase using standard Langmuir-Blodgett techniques[7] in order to modify the chemical nature of their surface from hydroxylated to carboxylated. The surface pressure of the monolayer during deposition was 20 mN/m, corresponding to 6.2×10^{14} carboxylate groups per cm^2. A small flow-through cuvette was then clamped over the grating region of the waveguide, which thus formed one wall of the cuvette. The assembly was mounted in an IOS-1 goniometer scanner (Artificial Sensing Instruments, Zürich) which measures the luminous power coupled into the waveguide as a function of the angle of incidence of an external He-Ne laser beam ($\lambda = 632.8$ nm) at the grating coupler. The effective refractive index N is related to the angle α of maximum coupled power according to[13–17] $N = n \sin \alpha + \lambda \ell / \Lambda$ where n is the refractive index of air and ℓ is the diffraction order. N was measured for one transverse electric and one transverse magnetic mode to allow the monomode equations[13–17] to be solved to determine the thickness d_A and refractive index n_A of the layer. These two quantities are then used to calculate ν, the surface density of protein molecules, according to[13–18]

Scheme 1. R_1: protein moiety; R_2: metal oxide surface. Note that the activated carboxylate can also react with a thiol, or be hydrolyzed back to the original carboxylate.



$$v = \frac{d_A(n_A - n_C)}{m(dn/dc)}, \qquad (1)$$

where m is the mass of a single protein molecule, n_C is the refractive index of the buffer (= 1.333972), and the coefficient dn/dc giving the variation in refractive index with bulk protein concentration has an almost universal value of 0.18 cm^3/g.[18]

Two different activation protocols were investigated: **A**, in which the carboxylate layer was activated by flowing 5 mM EDC through the cuvette for 3 min, followed by the protein solution (100 μg / cm^3); and **B**, in which the protein solution was mixed with EDC (5 mM) immediately before introducing it into the cuvette. EDC-activated carboxylate hydrolyses with a half-life of a few seconds (see Scheme 1),[11] and amide formation is favored if the

amine is present at the moment of activation, as it is in protocol **B**. On the other hand, when protein is mixed with EDC in solution, undesired intra- and interprotein crosslinks compete with bonding to the carboxylated substrate.

For comparison, we also measured the coupling yield to a conventionally fabricated aminated surface: the $Si(Ti)O_2$ waveguide was pretreated (1 min in aqua regia, 5×1 min in water, 1 min in acetone, all in an ultrasonic bath), silanized by shaking for 3 min at room temperature in a 2% solution of 3-aminopropyltriethoxysilane in 19:1 acetone:water, washed 12 times in acetone, baked for 1 h at 110°C, and finally reequilibrated with the ambient atmosphere. As controls, binding to uncoated $Si(Ti)O_2$ and stearic acid bilayers in the absence of EDC was monitored.

Protein and other solutions were drawn through the cuvette at a rate of 1.5 mm^3 / s, and the HCl solution and subsequent washing solution (i.e. buffer) at 10 mm^3 / s. The buffer used throughout was 100 mM 2-(N-morpholino)ethanesulfonic acid-NaOH (MES) at pH 5.8. All EDC solutions were prepared in buffer from dry EDC hydrochloride powder (Sigma) immediately before use. The measurement temperature was 26 ± 1°C.

3. Results

Measurements of N were carried out every 30 s to determine the kinetics of protein deposition. Figure 1 shows a typical result. We characterized the immobilization using three parameters, $\mathcal{M}$, $\mathcal{R}$ and ϕ_κ. $\mathcal{M}$ is a measure of the proportion of adsorbed protein removed simply by flushing with pure buffer, and is defined by

$$\mathcal{M} = 1 - \frac{\nu_{\text{after flushing}}}{\nu_{\text{before flushing}}}. \qquad (2)$$

Since it is commonly found that proteins adsorbed without any covalent links cannot be desorbed simply by washing with buffer,[19] washing was followed by brief (3 min) flushing with 0.3 M HCl, which effectively disrupts noncovalent bonds but does not hydrolyze the amide linkage. $\mathcal{R}$ is a measure of the efficiency of this regeneration, i.e., the proportion of proteins removed by flushing with HCl, and is defined by

$$\mathcal{R} = 1 - \frac{\nu_{\text{after regeneration}}}{\nu_{\text{before regeneration}}}. \qquad (3)$$

For perfect immobilization, $\mathcal{R} = 0$. Finally, the yield ϕ_κ of the coupling reaction is defined as

$$\phi_\kappa = 1 - \frac{\mathcal{R}_{\text{EDC}}}{\mathcal{R}_{\text{control (no EDC)}}}. \qquad (4)$$

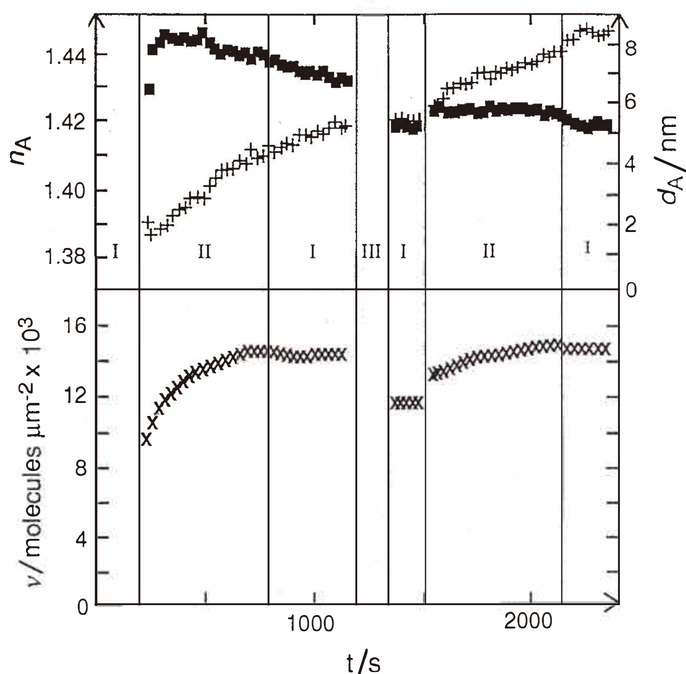


Fig. 1. Typical results for two immobilization cycles according to protocol **B**. The upper panel shows the refractive index (+) and thickness of the protein adlayer; the lower panel shows these quantities combined (eq. 1) to give the surface density of bound molecules. The time zones are as follows: I, buffer flowing at 1.5 mm^3/s; II, 100 μg/cm^3 purified antibodies from preimmune serum[19] in 5 mM EDC flowing at 1.5 mm^3/s; III, 0.3 M HCl flowing at 10 mm^3/s. We found little difference in the kinetics of adsorption between the different protocols.

The results of the measurements are given in Table 1.

4. Discussion

From these results, three types of bonding to the surface can be identified.

1: weak, reversible with respect to dilution (washing). The fraction of type **1**, equal to $\mathcal{M}$, was found to remain constant at a few percent regardless of the type of treatment, and possibly corresponds to proteins weakly attached to other proteins adsorbed on the substrate.

2: noncovalently bound, irreversible with respect to washing, released by dilute HCl, fraction given by $\mathcal{R}(1 - \mathcal{M})$.

3: covalently linked to the surface by EDC, resistant to regeneration with HCl, fraction

Table 1
Washing efficiencies ($\mathcal{M}$), regeneration efficiencies ($\mathcal{R}$), and coupling yields (ϕ_κ) for the various protocols. Results are given for purified antibodies from preimmune serum.[19] Similar results were obtained from purified antibodies to promastigate surface protease[19] and ovalbumin.

Substrate	Solution	$\mathcal{M}$	$\mathcal{R}$	ϕ_κ	$\mathcal{R}^c$	ϕ_κ^c
Si(Ti)O_2	Protein	0.02 ± 0.02	0.75 ± 0.05	–	–	–
Stearic acid	Protein	0.03 ± 0.02	0.7 ± 0.2	–	0.7 ± 0.2	–
Stearic acid + EDC	Protein[a]	0.01 ± 0.01	0.44 ± 0.02	0.4	0.44 ± 0.02	0.4
Stearic acid	Protein + EDC[b]	0.01 ± 0.01	0.3 ± 0.1	0.6	0.07 ± 0.01	0.9
—NH_2	Protein	0.04 ± 0.02	0.8 ± 0.1	–	–	–
—NH_2 + EDC	Protein	0.03 ± 0.02	0.5 ± 0.05	0.4	–	–

[a] Protocol **A**.
[b] Protocol **B**.
[c] After a second cycle of immobilization: for protocol **A**, reexposure to the protein solution; for protocol **B**, reexposure to the protein solution mixed with EDC.

given by $(1 - \mathcal{M})(1 - \mathcal{R})$. The fraction of type **3** is expected to be 0 for untreated surfaces and ideally should increase to 1 for EDC-immobilized proteins. In practice, however, it is usually higher than 0 for the noncovalently bonded controls,[19] and lower than 1 for the EDC-immobilized proteins.

The former discrepancy may partly be a kinetic phenomenon; 3 min flushing with HCl was sufficient to achieve a quasi plateau in ν in the covalently bonded runs, but not in the noncovalently bonded controls. The latter discrepancy may arise because not all orientations of the protein with respect to the activated surface result in the same number of covalent bonds, since the amine-bearing amino acids (arginine and lysine) are generally not uniformly distributed over the protein surface. Only one orientation (or group of orientations) will result in a sufficient number of bonds to the activated carboxylates to result in type **3**. Other orientations will be "nonspecific" (type **2**) and reversible with respect to HCl. During deposition, however, their presence on the surface prevents the regions they occupy from being occupied by orientations leading to the desired type **3**. By carrying out cycles of regeneration with HCl and re-exposure to the protein, these unwanted type **2** molecules can be removed and progressively replaced by type **3** molecules (Table 1). As expected, yields are increased by this procedure only when using protocol **B**; in the case of **A**, any EDC remaining uncoupled after the first exposure to protein is hydrolyzed. From the upper panel in Fig. 1 it can be seen that the thickness of the protein layer does not increase during the second immobilization cycle, which means that the fresh increase of mass is not due to the bonding of proteins to the first layer, but results from the filling of gaps in the first layer (increase of refractive index).

The competition between noncovalent and covalent bonding results in the selection of

a particular orientation or group of orientations: the one with the greatest number of amide links. There is independent evidence, *e.g.*, from the EDC cross-linking of enzyme pairs to produce active, functional complexes, that the EDC reaction is specific for a small subset of possible orientations.[20–23]

It has recently been shown[24] that electrostatic interactions between a charged surface and a multipolar protein should result in a preferred orientation of approach to the surface. Yields of type **3** bonds should be significantly enhanced if the electrostatically preferred orientation corresponds to that favoring the highest number of covalent bonds.

The sensitivity of covalent bonding to orientation is expected to be enhanced by a link of zero length.[25] A longer linker should increase coupling yields, but at the expense of less rigorous orientational selection.

Hydrolysis of EDC-activated carboxylates competing with primary amine attachment will also reduce the fraction of type **3** bonds. The hydrolysis can be partly countered by prereacting the EDC-activated carboxylate with *N*-hydroxysulfosuccinimide (NHS).[26] The resulting higher efficiency of covalent linking, however, increases the likelihood of intra- and interprotein cross-linking, and therefore the EDC-NHS procedure should only be applied to protocol **A**. In fact, the coupling yields obtainable by the simple one-step protocol used in this work will be adequate for many applications. After 9 min of protein deposition in the presence of EDC, under the conditions specified (Table 1), 0.34 $\mu g / cm^2$ of antibody are deposited, of which 70% correspond to type **3** (covalently bonded). Therefore, the final surface density of immobilized antibody is 9400 molecules μm^{-2}, or about 100 nm^2 per molecule. We have not addressed the question of what fraction of these immobilized proteins preserve their native conformation, and hence, in our case, their antigenic activity. The answer is likely to depend upon the particular protein and its function.

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