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Using Liquid Crystals to Report Membrane Proteins Captured by Affinity Microcontact Printing from Cell Lysates and Membrane Extracts

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The realization of general and facile surface-based methods for protein analysis has been slow compared to the development of tools used for analysis of nucleic acids because (a) proteins are chemically more diverse than nucleic acids, and (b) because amplification of protein copies and incorporation of labels (e.g., fluorescent labels) cannot be achieved as easily as with nucleic acids.[1] Transmembrane proteins, in particular, represent a particularly difficult class of proteins to analyze.[2]

In this paper, we report a simple procedure that permits detection of transmembrane proteins from crude cell lysates and cell membrane extracts. The method relies on the use of liquid crystals (LCs) to amplify and report[3] the presence of the transmembrane proteins captured by an affinity ligand. A merit of this approach is that the proteins can be imaged on surfaces without requiring the use of matched pairs of antibodies or other labels.[4] The requirement for matched pairs of antibodies complicates surface-based assays and prevents the high levels of multiplexing that are required to follow families of proteins that are involved in intracellular signaling networks. Our approach also does not require use of complex instrumentation.[2]

We demonstrate the feasibility of our approach by reporting the detection of epidermal growth factor receptor (EGFR), a transmembrane glycoprotein that possesses EGF-stimulated protein-tyrosine kinase activity.[5,6] Its overexpression and mutation have been associated with some of the most incurable cancers, including lung, ovary, colon, bladder, and head and neck.[7,8] Much effort has been devoted to the development of anti-cancer agents based on antagonizing EGFR tyrosine kinase activity or receptor interaction with other members of the EGFR family.[9,10] However, these attempts have been limited by efficient methods for assessing alterations in EGFR kinase activity and expression in tissues from treated individuals.

The success of the approach reported in this paper revolves around control of the physicochemical properties of interfaces in contact with proteins and LCs. First, we covalently immobilized a monoclonal antibody to EGFR (anti-EGFR 111.6, Lab Vision) on the surface of an elastomeric stamp molded from poly(dimethylsiloxane)[11,12] and incubated small drops (~1 μL) of cell membrane extract on the surface of the stamp. The stamp was rinsed sequentially with 0.01% Triton X-100 in phosphate buffer saline (PBS), PBS, and water, and dried under a stream of N_2. Next, we contacted the surface of the affinity stamp with a semi-transparent (thickness of 20 nm) gold film supporting a monolayer formed from 2-mercaptoethylamine. This surface was used for two reasons. First, high energy surfaces have been demonstrated to cause proteins to dissociate from the binding groups on stamps and transfer to the high energy surfaces upon contact.[12] Second, past studies have demonstrated that amine-terminated monolayers can orient nematic LCs of 4-cyano-4′-pentylbiphenyl (5CB) through hydrogen bonding and formation of electrical double layers.[13] We hypothesized that micrometer-thick films of LCs would assume orientations on amine-terminated surfaces that would be different from regions of a surface presenting EGFR, thus providing a facile means to amplify and optically detect the presence of the EGFR on the surface.

Cell membrane extracts (~1 μg/μL total protein) were prepared from either human epidermoid carcinoma cells (A431), murine fibroblasts null for the EGFR (B82L-parental), or murine fibroblasts stably expressing wild-type human EGFR (B82L-WT). Immunoblot analysis utilizing anti-EGFR (Cat # SC-03, Santa Cruz Biotechnology) confirmed the presence of EGFR in A431 and B82L-WT cell preparations and the absence of EGFR in B82L-parental samples. A431 and B82L-WT cells contain about 1 million and 100 000 EGFR molecules/cell, respectively.[14]

The gold films used in the study reported in this paper were deposited via physical vapor deposition at an oblique angle of incidence because the nanometer-scale structure of these gold films directs LCs to orient in uniform azimuthal orientations (see below).[3] The amine-terminated monolayers were treated with aqueous 1 N HCl prior to stamping and then dried under a stream of N_2. After stamping, each printed surface was paired and spaced by 13 μm (using a thin film of Mylar) from a glass microscope slide treated with octyltrichlorosilane (OTS). The cavity between the two surfaces was filled with 5CB and observed using polarized light microscopy (transmission mode).

Figure 1A shows the optical image of a film of nematic 5CB in contact with an amine-terminated surface printed with a stamp that had been incubated with a cell membrane extract containing EGFR (B821-WT). The black areas of Figure 1A correspond to the regions of the amine-terminated surface that were not contacted by the square features on the stamp. When viewed between crossed polars, these areas appeared black only when the sample was oriented such that the direction of deposition of the gold during preparation of the gold film was parallel to one of the crossed polars. The orientation of the LC was determined to be parallel to the plane of the printed surface, with an azimuthal orientation corresponding to the direction of minimum nanometer-scale topography in the gold film.[3] In contrast, the LC appeared bright green in the regions of the surface that had contacted the stamp incubated with cell preparations containing EGFR. Rotation of the sample between crossed polars revealed a parallel orientation of the LC in these regions of the surface but with an azimuthal orientation that was not dictated by the structure of the underlying gold film. To test if the patterned orientation of the LC seen in Figure 1A was due to EGFR printed onto the amine-terminated monolayer, we repeated the experiment described above using a cell membrane extract that was free of EGFR (B82L-parental). Figure 1B shows that the LC aligned uniformly in a direction dictated by the underlying gold

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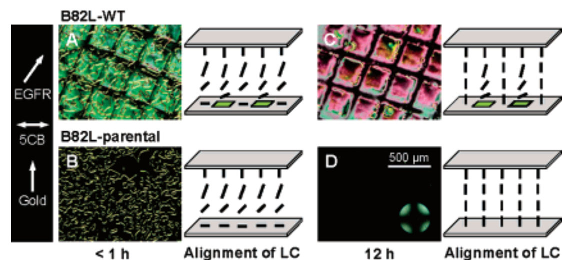

Figure 1. Optical images (crossed polars) of nematic 5CB supported on amine-terminated monolayers contacted with affinity stamps that had been incubated in membrane extracts containing EGFR (A and C) and null of EGFR (B and D). The white arrows on the left image indicate the direction of deposition of the gold film (gold), the azimuthal orientation of the LC on the regions of the surfaces free of EGFR (5CB), and the azimuthal orientation of the LC on the regions of the surface supporting EGFR (EGFR). Each image was acquired at room temperature after annealing the samples at 36 °C for the time indicated.

film when using the cell membrane extract free of EGFR, thus providing support for the conclusion that the LC in Figure 1A reports the presence of EGFR in the cell membrane extract by masking the influence of the underlying gold films on the orientation of the LC.[3,15] We note that the defect lines seen in Figure 1A,B correspond to boundaries between domains of LCs with different splay/bend distortions between the top and bottom surfaces.

Upon annealing at 36 °C, we observed the LC to undergo time-dependent changes in orientation on both the amine-terminated monolayers and the regions of the surface presenting EGFR, leading to high optical contrast between positive (Figure 1C) and negative controls (Figure 1D) and elimination of defect lines. Inspection of Figure 1C reveals that the LC on the regions of the surface presenting EGFR has transformed over 12 h from a green to a pink color, corresponding to a tilting of the LC away from the plane of the surface. On the amine-terminated regions of the surface (free of EGFR), we observed the 5CB to transform over time to an orientation that was perpendicular to the surface (homeotropic orientation).[3] Homeotropic alignment of LC on B82L-parental samples was confirmed by conoscopy. The rate of the transition is dependent on the presence of ionic impurities within the LC[16] and can be accelerated by UV illumination of the LC (known to increase the ion content of the LC).[17] This homeotropic anchoring is consistent with a dipolar coupling between the LC and the electric field of the electrical double layer formed through the dissociation of surface-immobilized salts.[13] The optical contrast evident in Figure 1C persisted for 1 week.

We obtained further support for the above conclusions regarding the influence of EGFR captured from cell membrane extracts on the orientations of LCs by using ellipsometry. We immobilized the 111.6 anti-EGFR antibody on a silicon wafer using the methods described above and incubated the surface in cell membrane extract (A431) followed by a secondary antibody (Clone 199.12, Lab Vision). The 199.12 and 111.6 anti-EGFR antibodies bind to different epitopes of EGFR. We observed that the optical thickness of the film of the 199.12 antibody captured by surface-bound EGFR from A431 cells to be ~1.1 nm. The control experiment with B82L-parental cells showed no increase in thickness after treatment with the 199.12 antibody. We also demonstrated that cell membrane extracts prepared from the A431 cell lead to results similar to those shown in Figure 1 with WT cells.

We quantified the intensity of light transmitted through the LCs on the stamped surfaces using image processing software (Adobe Photoshop). The cell extracts containing WT EGFR (1 μg/μL total protein) generated high optical responses compared to samples (also 1 μg/μL total protein) prepared from parental cells null for EGFR

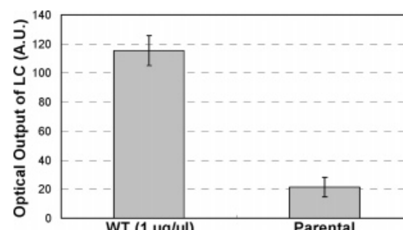

Figure 2. Optical response of 5CB to EGFR affinity-captured from cell membrane extracts containing WT EGFR and null for EGFR (parental).

(Figure 2). We also tested our method for the detection of EGFR from 0.5 to 0.1 μg/μL total protein concentration. Our result showed that the LC signal gradually decreased with the reduction of total cell protein concentrations. The loss of LC signal supports our hypothesis that the patterned orientations of the LCs shown in Figure 1A,C are caused by affinity-captured EGFR printed on the surface. Finally, we have also demonstrated detection of EGFR from crude cell lysates prepared from B82L-WT cells.

In summary, we have demonstrated a simple method for detection of a membrane protein from cell membrane extracts and crude cell lysates. The method does not require matched pairs of antibodies, as is required for surface-based fluorescence assays,[4] nor does it require the complex instrumentation associated with methods, such as mass spectroscopy[2] or surface plasmon spectroscopy.[18] Because LCs can be used to image regions of surfaces that have micrometer dimensions,[19] high sensitivities are theoretically possible using the methods reported in this paper. We calculate the areal density of EGFR molecules giving rise to the response in Figures 1 and 2 to be $\sim 10^3$ molecules/μm^2. We note that our method also allows the detection of activated (phosphorylated) EGFR in cell membrane extracts by using phosphospecific anti-EGFR.

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(11) The surface of the PDMS stamp was plasma oxidized (Plasma Etch PE-200, 8 sccm, 20 s, 100 W) and then immersed into an aqueous solution containing 10% 3-aminopropyltriethoxysilane at 80 °C for 1 h. A subsequent nucleophilic ring opening reaction between the amines on the surface of the stamp and 0.1 M succinic anhydride in *N*,*N*-dimethylformamide (room temperature, 10 min) produced a carboxylic acid-terminated surface. Anti-EGFR 111.6 (1 mg/mL) was then immobilized on the carboxylic acid-terminated surface using standard NHS-EDC protocols.
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