Direct Colorimetric Detection of a Receptor-Ligand Interaction by a Polymerized Bilayer Assembly

Deborah H. Charych,* Jon O. Nagy, Wayne Spevak, Mark D. Bednarski†

Detection of receptor-ligand interactions is generally accomplished by indirect assays such as enzyme-linked immunosorbent assay. A direct colorimetric detection method based on a polydiacetylene bilayer assembled on glass microscope slides has been developed. The bilayer is composed of a self-assembled monolayer of octadecylsilane and a Langmuir-Blodgett monolayer of polydiacetylene. The polydiacetylene layer is functionalized with an analog of sialic acid, the receptor-specific ligand for the influenza virus hemagglutinin. The sialic acid ligand serves as a molecular recognition element and the conjugated polymer backbone signals binding at the surface by a chromatic transition. The color transition is readily visible to the naked eye as a blue to red color change and can be quantified by visible absorption spectroscopy. Direct colorimetric detection by polydiacetylene films offers new possibilities for diagnostic applications and screening for new drug candidates or binding ligands.

Chemical modification of surfaces by organic monomolecular films is an attractive route to the development of new materials. Such ultrathin film coatings can dramatically alter the surface properties of the original underlying material. For this reason, the techniques of molecular self-assembly (*1*) and Langmuir-Blodgett (LB) deposition (*2*, *3*) have been widely used for coating surfaces with a well-defined, quasi–two-dimensional array of molecules.

Functionalization of these films with receptor-binding ligands has extended the field beyond the realm of materials science applications [such as wetting (*4*) and friction (*5*, *6*)] to biotechnology applications. For example, peptide or nucleotide libraries based on self-assembled monolayers offer the possibility of screening for new receptor-binding ligands (*7*). Biosensors based on LB films (*8*) can detect molecules of diagnostic significance such as glucose (*9*) or urea (*10*). Although these functionalized films have led to elegant examples of molecular recognition at an interface, the problem of transducing the molecular recognition event into a measurable signal remains. In the case of surface libraries, transduction has been accomplished indirectly by way of enzyme-linked immunosorbent assay (ELISA)–type assays (*7*). In the case of biosensor devices, detection is generally carried out by coupling the LB film to a secondary device such as optical fibers (*11*, *12*), quartz oscillators (*13*, *14*), or electrode surfaces (*15*, *16*).

In an effort to design a simple, more direct method of detecting molecular recognition events, we chose to exploit the known chromatic properties of polydiacetylene LB films. Diacetylenic lipid monomers such as compound 1 (Fig. 1) are readily polymerized in monolayers by ultraviolet irradiation to form a conjugated polydiacetylene backbone of alternating ene-yne groups (*17*, *18*). These materials change color from blue to red with an increase in temperature or changes in pH due to conformational changes in the conjugated backbone (*19*–*22*). We have developed a polymerized bilayer assembly (Fig. 2A) composed of a self-assembled monolayer of octadecyltrichlorosilane (OTS) and a LB monolayer of functionalized polydiacetylene. This film was designed to undergo a similar color transition from blue to red solely due to receptor-ligand interactions occurring at the surface of the bilayer. Therefore, the bilayer assembly incorporates both a molecular recognition site and a detection element. This simple color-based sensor enables rapid, qualitative detection of binding by visual inspection of the film or quantitative detection by visible absorption spectroscopy.

Our initial investigations focused on the binding of the influenza virus to sialic acid as a model system for colorimetric detection. Lipid monomer **2** (Fig. 1) contains a carbon-linked sialic acid head group that provides a molecular recognition site for the viral lectin, hemagglutinin. A carbon glycoside was used instead of the naturally occurring oxygen glycoside to prevent hydrolysis by the neuraminidase, which is also present on the surface of the virus (*23*). We have shown in earlier work that this modification does not alter the binding affinity of hemagglutinin (*24*). The films were prepared by a modified LB technique in which the carbohydrate head group is presented at the surface of the bilayer (shown schematically in Fig. 2A). Mixtures of 2 to 5% of glycolipid monomer **2** and matrix lipid monomer **1** were spread on the water surface of a standard LB trough. The matrix

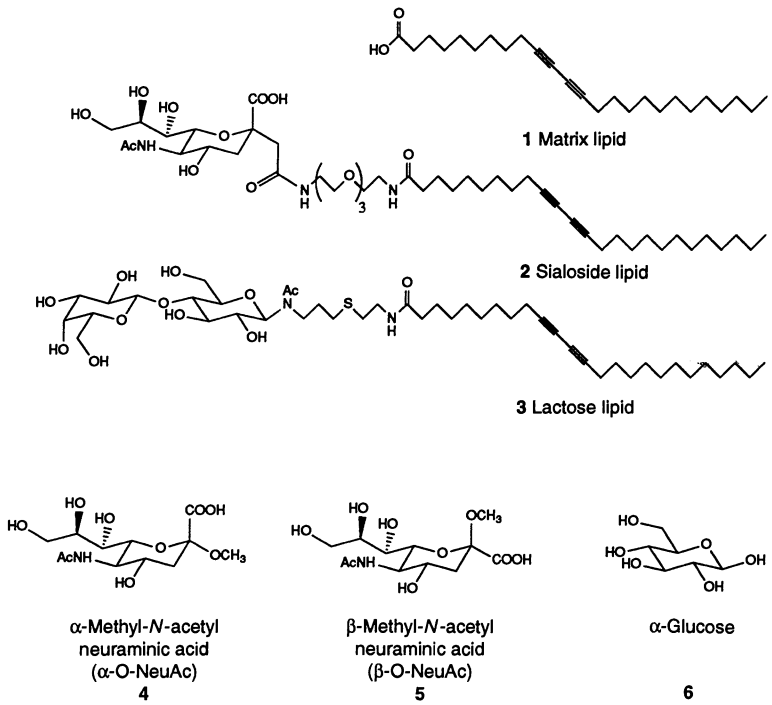

Fig. 1. Compounds **1** to **3** used in LB film formation and compounds **4** to **6** used for competitive inhibition experiments. The synthesis of compound **2** is reported in (*24*), and the synthesis of compound **3** will be reported elsewhere.

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lipid uniformly disperses the sialoside lipid, which allows optimum binding of the virus (*25*). The mixed monolayer was compressed and polymerized on the water surface. The floating polymerized assembly was lifted by the horizontal touch method onto a glass slide previously coated with a self-assembled monolayer of OTS (*26*). The resulting bilayer assembly presents an array of carbohydrate ligands at the surface. The tetraethylene glycol spacer in sialoside lipid **2** serves to extend the carbohydrate ligand beyond the carboxylic acid head groups of the matrix lipid **1**.

Films prepared in this manner exhibit a high degree of order over a macroscopic range (50 to 150 μM) as evidenced by optical microscopy with the use of crossed polarizers (*27*) (Fig. 2B). The films were further characterized by angle-resolved x-ray photoelectron spectroscopy (XPS) and ellipsometry. The XPS results indicate that the amide nitrogen atoms and the carbonyl carbon atoms of the head groups are localized at the surface relative to the methylene carbons of the lipid chains, demonstrating that the sialoside head group is presented at the surface of the film. Ellipsometric analysis of the polydiacetylene monolayer coated on HF-treated silicon indicates a film thickness of ~40 Å, in agreement with the expected value based on molecular modeling.

The bilayer assembly has a visible absorption maximum of 620 nm and appears as a blue film. When the film is incubated with X31 influenza A virus [PBS (phosphate-buffered saline) buffer, pH 7.4], the binding of the viral hemagglutinin to the sialic acid residues on the surface results in a blue to red color transition (Fig. 3A). No color change is observed when the blue film is incubated with a blank solution of PBS buffer. This result demonstrates a polydiacetylene color transition arising from affinity binding (affinitychromism) rather than thermal annealing (thermochromism). Previous studies have shown that LB films composed of lipid **1** undergo a blue to red color change when heated at 70°C, which corresponds to the endothermal transition for lipid chain melting (*28*). Lipid chain disorder and tangling decrease the effective conjugation length of the polydiacetylene backbone. Similarly, Fourier transform infrared (*28, 29*) and resonance Raman spectroscopy (*29*) as well as x-ray data (*30, 31*) demonstrate that lipid chain packing of the red form of the polymer is different from that of the blue form. Thus, conformational changes in the lipid chains affect the optical properties of the polymer backbone. Binding of the viral hemagglutinin to the sialoside bilayer assembly appears to affect the lipid chain conformations in a manner analogous to thermal annealing.

In addition to qualitative evaluation by visual inspection, the degree of color change is readily quantified by standard visible absorption spectroscopy (Fig. 3B). The blue-colored film has a strong absorption maximum at 620 nm and a weaker absorption at 550 nm. After incubation with influenza virus, a dramatic change in the visible absorption spectrum occurs. The maximum at 550 nm increases with a con-

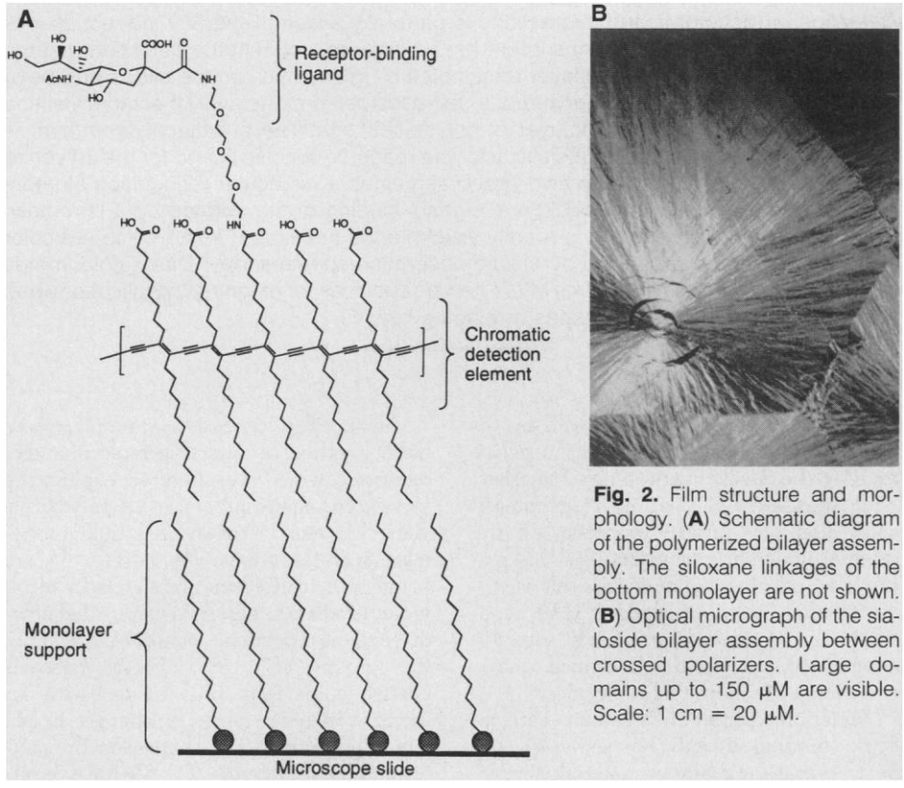

Fig. 2. Film structure and morphology. (**A**) Schematic diagram of the polymerized bilayer assembly. The siloxane linkages of the bottom monolayer are not shown. (**B**) Optical micrograph of the sialoside bilayer assembly between crossed polarizers. Large domains up to 150 μM are visible. Scale: 1 cm = 20 μM.

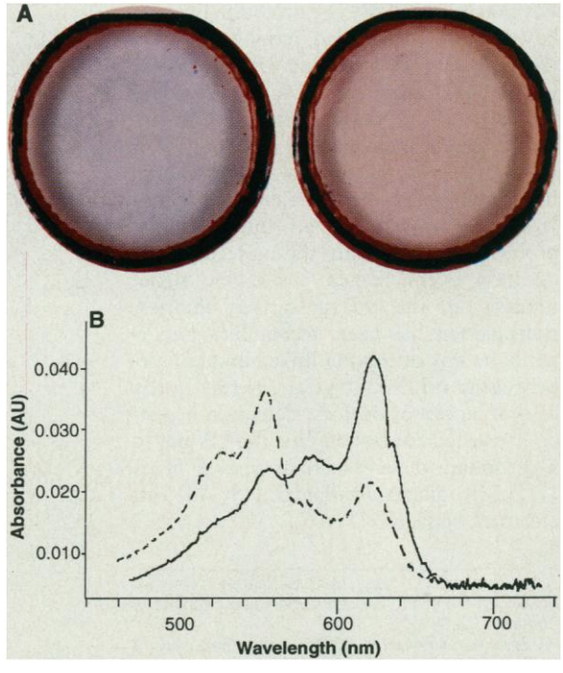

Fig. 3. Colorimetric detection of influenza by sialoside bilayer assembly (2% sialoside lipid **2** and 98% matrix lipid **1**). (**A**) The colorimetric response of the film, supported on a glass microscope slide, is readily visible to the naked eye for qualitative evaluation of the presence of virus. The film on the left (blue) has been exposed to a blank solution of PBS. The film on the right (red) has been exposed to 100 HAUs of virus (CR = 77%, see text). A colorimetric response of ~15% can be observed visually. (**B**) The visible absorption spectrum of a bilayer assembly prior to (solid line) and after (dashed line) viral incubation. The bilayer assembly was inserted into a quartz cuvette containing PBS buffer (pH 7.4), and the absorption spectrum was obtained. Addition of influenza virus in PBS buffer (pH 7.4) resulted in a chromatic transition following a 30-min incubation period. (Although the film color begins to change within seconds after exposure to virus, 30 min was found to be the average length of time required for the CR to reach a plateau value in a nonstirred solution). These spectra represent a CR of 50%.

current decrease in the maximum at 620 nm, resulting in a red-colored film. In order to quantify the response of a film to a given amount of virus, the visible spectrum of the film before exposure to virus was analyzed as

$$B_o = I_{620}/(I_{550} + I_{620}) \quad (1)$$

where B_o is defined as the intensity of absorption at 620 nm divided by the sum of the absorption intensities at 550 nm and 620 nm. After exposure to influenza

$$B_v = I_{620}/(I_{550} + I_{620}) \quad (2)$$

where B_v represents the new ratio of absorption intensities after incubation with the virus. The colorimetric response (CR) of a film is defined as the percent change in B upon exposure to virus

$$CR = [(B_o - B_v)/B_o] \cdot 100\% \quad (3)$$

The colorimetric response is directly proportional to the quantity of influenza virus [Fig. 4A, measured in hemagglutinating units (HAUs), where 1 HAU is defined as the highest dilution of stock virus that completely agglutinates a standard erythrocyte suspension (*32*)]. Saturation of the colorimetric response occurs at ~80 HAUs. Incubating the red film with a buffer blank (no virus) for 1 hour did not result in a return of the blue color. Thus, the structural changes which result in the color change appear to be irreversible under these conditions.

The specific nature of the interaction between the influenza virus and the sialoside film surface was confirmed by competitive inhibition assays (Fig. 4B). The known inhibitor of influenza hemagglutination, compound **4**, has a dissociation constant K_d of 2 mM as determined by a standard hemagglutination inhibition assay (HAI) (*33*). Incubation of the sialoside bilayer assembly with influenza virus in the presence of the known binding inhibitor **4** results in no CR (CR < 0.5%) and the film remains blue. This result demonstrates that the inhibitor effectively competes with the sialoside surface for binding to the virus. When the blue film is exposed to the same quantity of influenza in the presence of a non-inhibitor (compound **5**, K_d > 50 mM, or glucose, compound **6**), the color change is identical to a film exposed to influenza alone.

In order to test the capability of the film to predict the value of K_d for an inhibitor, the CR was measured for a series of inhibitor concentrations. The CR increases in a linear fashion (r^2 = 0.995) with decreasing concentrations of inhibitor **4**. Extrapolation of this plot to CR = 0% gives the inhibitor concentration that completely prevents binding of the virus to the surface. This value represents the minimum inhibitor concentration required to effectively compete with the sialoside surface. The value obtained, 2.5 ± 0.83 mM per 4 HAUs of virus, is in agreement with the value of 2 ± 1.1 mM obtained by a standard HAI assay (*33*) and 2.8 ± 0.30 mM as obtained by nuclear magnetic resonance spectroscopy (*34*). The inhibition assay described here is direct and easy to perform. This approach avoids the need for red blood cells, which are used in the standard HAI assay. In addition, the subjectivity of reading microtiter plates in the standard HAI assay is replaced by a quantitative spectrophotometric method (*35*). This methodology could be applied to screening for new drug candidates or binding ligands.

In order to assess the CR due to nonspecific adhesion, two experiments were performed. In the first experiment, films incorporating lactose lipid **3** were incubated with influenza virus. Lactose is not a ligand for the hemagglutinin lectin. Incubation with 100 HAUs of virus, which is a concentration corresponding to a maximum response in the sialoside films, shows only a small effect (CR of 2 to 4%). In the second experiment, films containing sialoside lipid **2** were exposed to concentrated solutions of bovine serum albumin. Again, the same small CR was observed (*36*). These results indicate that nonspecific adsorption of virus or protein to the film surface does not produce the dramatic color change observed from specific receptor-ligand binding.

The bilayer assembly described here contains both the receptor-binding ligand and the capability to signal the specific binding event. Since ligands other than sialic acid could be incorporated into the film, affinitychromism offers the possibility of a general method for the direct detection of receptor-ligand interactions. This approach exploits the conserved binding specificity of bacterial and viral receptors and avoids the need for antibodies, which bind protein epitopes that are subject to genetic shift and drift. The direct detection of binding events is an important discovery with wide-ranging applications in the areas of diagnostics and therapeutics. These films could be used for screening new drug candidates by inhibition of the CR. A combination of this methodology with a technique for preparing spatially resolved chemical libraries on the film surface would offer a powerful method for identifying new ligands.

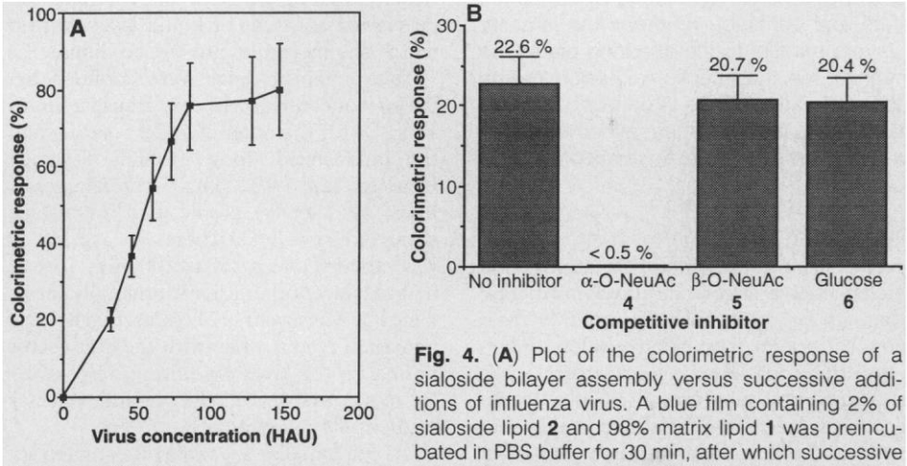

Fig. 4. (**A**) Plot of the colorimetric response of a sialoside bilayer assembly versus successive additions of influenza virus. A blue film containing 2% of sialoside lipid **2** and 98% matrix lipid **1** was preincubated in PBS buffer for 30 min, after which successive aliquots of X31 influenza A virus were added. The film was incubated for 30 min following each addition of virus, and the visible absorption spectrum was recorded. The CR is calculated according to Eq. 3. Linear regression analysis of the first six data points gives a slope of 0.93 (r^2 = 0.985). (**B**) The CR of the film can be inhibited by compounds that bind to viral hemagglutinin. Incubation of a sialoside bilayer assembly with 32 HAUs of influenza virus produces a colorimetric response of 22.6%. However, the same concentration of virus in the presence of 17.3 mM concentration of compound **4** (K_d = 2 mM) completely suppresses the CR to less than 0.5% due to competitive inhibition. The CR is not diminished in the presence of 17.3 mM concentration of compound **5** (K_d > 50 mM) or compound **6** that do not compete for binding to viral hemagglutinin.

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25. We have previously shown that 1 to 5% of sialoside lipid gives maximum binding of the virus to polymerized liposomes (*24*). Ideal mixing of the two components was determined by analysis of the Langmuir isotherms. Various ratios of monomers **1** and **2** give isotherms whose limiting areas and collapse pressures change in direct proportion to the mole fraction of **2** as expected for miscibility [see (*3*)].
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36. The above experiments define a background level of CR arising from nonspecific adhesion. Thus, from Fig. 4A, the minimum quantity of virus that is detectable and well above this background level is ~25 HAUs. According to some estimates (*35*), this value corresponds to ~200 million particles. Given a cross-sectional area of $\sim 8 \times 10^{-11}$ cm^2 per particle, the minimum film area affected is ~0.02 cm^2, or 1/100 of the sample area. Given the high film response, it is likely that each binding event results in a much longer range induced disorder.
37. We thank M. E. Schaefer and J. H. Gilbert for virus protocols, technical help, and the generous donation of X31 influenza A. Supported by the Director, Office of Energy Research, Office of Basic Energy Sciences, Division of Materials Sciences, and also the Division of Energy Biosciences of the U.S. Department of Energy (DE-AC03-76SF0098). M.D.B. thanks the American Cancer Society for a Junior Faculty Award and Eli Lilly for a Young Investigator Grant. W.S. thanks NIH for a predoctoral training grant. Synthesis of the C-glycoside of sialic acid was supported by the California Competitive Technology Program (grant C89-285) in collaboration with J. R. Murdoch and H. O. Ribi, Biocircuits Corporation.

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Structure of Membrane Surfactant and Liquid Crystalline Smectic Lamellar Phases Under Flow

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Synchrotron x-ray scattering studies were performed to probe the nonequilibrium structures of two layered systems at high shear rates: the smectic-A phase of the thermotropic liquid crystal 4-cyano-4′-octylbiphenyl (8CB) and the lamellar L_α phases of surfactant membranes composed of sodium dodecyl sulfate and pentanol. Whereas the lamellar surfactant phases oriented primarily with their layers parallel to the shearing plates, as expected intuitively, in the corresponding high shear regime, the smectic-A liquid crystalline material oriented with the layers perpendicular to the shearing plates. A careful numerical study revealed that this surprising layer orientation results from nonlinear dynamics of the liquid crystal director and is caused by the flow distortion of thermal fluctuations.

The behavior of relatively simple liquids composed of small molecules, like water, under shear flow is well described by the classical "Newtonian" theory of hydrodynamics (*1*). However, many complex macromolecular fluids, such as lyotropic and thermotropic liquid crystals, colloidal suspensions, and polymeric solutions and melts, have a large-scale interior structure that can be significantly distorted by shear flow because of their relatively long relaxation times (*2*). At the macroscopic level, this distortion manifests itself as a breakdown of Newtonian hydrodynamics that requires the introduction of new stresses in the hydrodynamic equations, so-called "normal stress" effects originally elucidated by Weissenberg in polymeric and surfactant liquids (*3*). The scientific challenge remains to correlate the bulk macroscopic response of the material under stress to the underlying distorted microscopic structure. Non-Newtonian flow behavior in complex fluids plays a central role in the mechanics of many technological materials [for example, in lubricating films (*4*, *5*) and the processing of polymer films and fibers (*6*)], which for the most part occur under poorly understood conditions far from equilibrium. It is precisely in these technologically important materials, which are extremely susceptible to large deformations of their underlying structure (such as layer orientation and macromolecular conformations), that bulk studies with a direct structural probe such as x-rays and neutrons are most relevant.

An important group of complex fluids involves layered materials, which may be used as lubricating films, such as liquid crystals, block copolymers, and surfactant membrane liquids. We focus on experimental results on the dilute lamellar L_α phases of surfactant membranes and on the smectic-A (SmA) phase of liquid crystals composed of rod-like molecules (Fig. 1, A and C) and compare them with results from a numerical study of a simple theoretical model of such materials under conditions of high shear rates. An important advantage of studying these layered systems is that their equilibrium structures have been previously investigated by x-ray scattering (*7*, *8*). The membrane surfaces of the L_α phase were composed of thin water layers coated with surfactant and cosurfactant molecules (SDS and pentanol) separated by dodecane (Fig. 1C). The SmA phase we studied is thermotropic; that is, under heating it transforms into a nematic liquid that has orientational order but no translational order (Fig. 1B) (*9*). We introduce a reduced temperature $t_r = (T - T_{NA})/T_{NA}$, where T_{NA} is the critical temperature for the nematic-SmA transition. Both systems can be viewed as stacks of liquid layers within which the molecules are free to diffuse.

The complex fluids were confined between concentric cylinders transparent to x-rays, with the outer cylinder rotating and the inner fixed, in a specially designed Couette cell (*10*). This approximates a linear velocity (v) profile with a constant shear rate $\gamma = v/D$, where the gap size D was varied between 250 to 2000 μm. The a, b, and c layer orientations, originally introduced by Miesowicz (*11*) to describe flowing nematics, refer to cases with the unit vector normal to the layer $\hat{\mathbf{n}}$ pointing, respectively, along the z, velocity $\mathbf{v}$, and velocity gradient $\nabla\mathbf{v}$ directions (Fig. 2A).

In the lamellar L_α phase, we studied six mixtures with dodecane volume fractions Φ between 0.54 and 0.62, which correspond to multilayer phases with the interlayer spacing d increasing from about 118 to 160 Å (*8*). At high shear rates, the layers were oriented primarily parallel to the shearing plates, as expected intuitively (c orientation). The scattering intensity for a typical

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