

Macromolecular recognition at the air–water interface: application of Langmuir–Blodgett technique

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The proximate aim of this review is to investigate the specific interaction between two macromolecules, either two complementary strands of DNA or the binding of DNA with a protein. Although a lot of experiments have been done to address these issues, our aim here is different. We either create a dense brush of DNA chains at the air–water interface or orient a large protein, like RNA polymerase, such that they are amenable for specific interaction at the surface. The advantage of our system is that the macromolecules are stretched, oriented parallel to each other, and their concentrations can be made similar to those encountered in real nuclei. In this way we plan to construct an ‘artificial nucleus’. Other methods adopted so far can check for the possibility of collective behaviour and the effect of chain elongation or compaction. We have used Langmuir–Blodgett technique for the same and extensively performed FTIR and AFM experiments to monitor the L–B surface. Each macromolecule has been attached by one of its extremities to a hydrophobic buoy to keep it at the interface. Detailed thermodynamic analysis results in some interesting conclusions.

Keywords: LB films, promoter DNA, RNA polymerase, thermodynamics.

Preamble

SPECIFIC recognition between DNA and protein plays a major role in biology, with respect to spatio-temporal development of an organism. Control of gene expression in spatial and temporal manner indeed leads to awesome diversity which is very difficult to comprehend at the molecular level. Since last century brilliant efforts have been made through path-breaking experiments to understand the chemistry of DNA–protein recognition. Several thermodynamic constants have been estimated; complex kinetic pathway for the recognition has been deciphered^{1,2}. However, most of these studies suffer from one major limitation that is the experiments were carried out in bulk phase or in homogenous solution, which is far away from the real *in vivo* condition within cells. The condensed

packing of DNA, different salt concentrations, action of several proteins together in an orchestrated fashion put various challenges in front of investigators and they tried to answer these questions by designing different models.

We are attempting to address these issues in a different way. At first, we designed one of the reacting partners in such a way that they behave as condensed and arrayed molecules on air–water interface and then probe them for molecular interaction with the other partner. This approach led us to evaluate various thermodynamic constants for the system and compare with that of bulk phase. In the following section, we will describe these experiments which deal with two simple molecular recognitions: (a) hybridization of two complementary strands of DNA to form double helix, and (b) recognition of a specific promoter DNA sequence by RNA polymerase.

Historical background

The calming effect of oil on sea waves was known to the sailors since the ancient times. It was a noted phenomenon like many other natural phenomena occurring but was little studied until the recent times. Benjamin Franklin³ was the first to report the consequence of oil on water in 1774. Then, Agnes Pockels in 1891 described a technique to manoeuvre the oily films on water⁴. The technique involved the use of barrier across the surface of a trough full of water till the brim. Then Rayleigh came with the calculation of the thickness of these oily films⁵, and proposed that the films of oil on water were monomolecular in nature. Finally, during 1917 Irving Langmuir proposed the theoretical and experimental details of the insoluble monolayers⁶ which underlie our modern understanding of Langmuir monolayers. During the same period, in 1929, Katherine Blodgett⁷ developed a method to transfer the monolayer on solid substrates and from then this technique is known as Langmuir–Blodgett (LB) technique and the transferred monolayers as Langmuir–Blodgett films.

Thus, the focus was mainly on the interfacial properties and importance of the observation, reported by Franklin, took more than hundred years to be understood. In the meanwhile, other surface properties were studied in de-

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tail; in 1878, Gibbs published his work on thermodynamics of adsorption and surface tension⁸. However, it was the discovery of Pockels, which made the monomolecular layers increasingly interesting. Prior to Langmuir's theoretical explanation about these insoluble monolayers, Hardy⁹ observed that oils which were lacking polar functional group did not spread at the air–water interphase to form insoluble monolayers. Subsequent to these observations, Langmuir demonstrated that long chain fatty acids form monolayers that occupy same cross-sectional area independent of the hydrophobic chain length. He proposed that the long chain fatty acid molecules were oriented at the water surface in such a manner that the polar head group was immersed in water and the hydrophobic chain directed vertically up from the surface as shown in Figure 1.

Towards quantitation, Langmuir also devised a surface balance, a device bearing his name, which has a movable float separating clean surface from the surface covered with fatty acid molecules. The forces (i.e. surface tension or surface pressure) were measured directly from the deflection of the float and based on these principles the modern day Langmuir trough (Figure 2) is built. It has a movable barrier and to measure the forces, a pressure sensor is attached to the trough. Surface pressure is measured as shown:

$$\Pi = \gamma_0 - \gamma$$

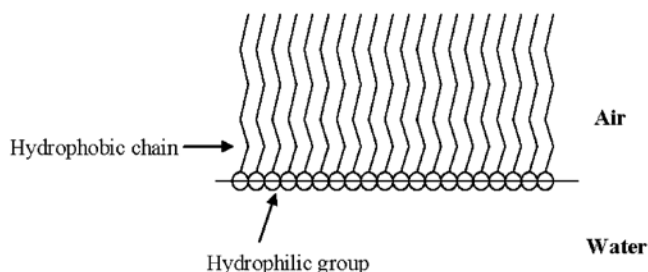


Figure 1. Schematic depiction of an insoluble monolayer formed at the air–water interphase.

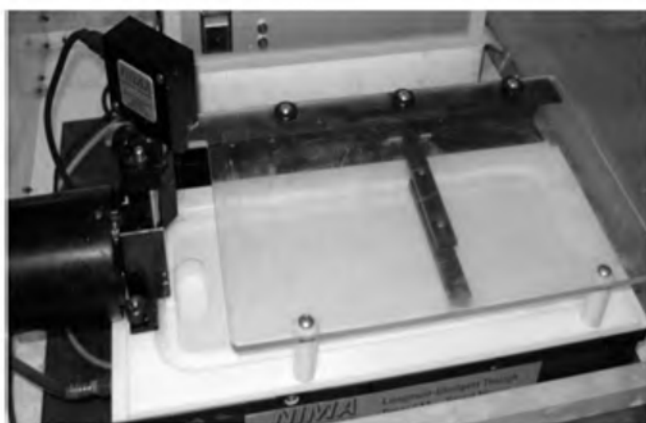


Figure 2. Langmuir–Blodgett trough.

where Π is the surface pressure, γ_0 is the surface tension of clean surface and γ is the surface tension of the surface covered with monolayer forming molecules.

The early studies were concentrated on the properties of the surface of the monolayers and not much had been speculated about the functional aspect of these monolayers. Since the demonstration by Langmuir, numerous substances had been studied. Until, the middle of twentieth century, the monolayer research had been carried out without much speculation about the practical applications of these monolayers. Blodgett developed a technique¹⁰ to transfer these monolayers on solid substrate to built films of the monolayers and which became a subject of intense interest. The second half of the twentieth century was dedicated to the stimulation of the potential application of the Langmuir monolayers. Moreover, these molecules which spread at the air–water interphase have an analogy to the membranous structure of living systems and hence biologists started showing interest on LB film.

Apart from LB technique, self-assembled monolayers (SAMs) are also widely used to generate films on solid substrate for various purposes¹¹, but application of SAMs is only in making films and not as wide as Langmuir monolayers. LB films are structurally more stable, due to their transfer technique employed to generate them, than SAMs. Additionally, one can generate uniform multilayers with the LB technique, whereas SAMs only yield monolayer. Currently, LB films are studied for its potential as sensors¹², magnetic¹³ and optical materials¹⁴; biological membrane models and for numerous chemical applications¹⁵.

Overview

A surfactant molecule is the most essential requirement to form a LB monolayer¹⁶, and these are amphiphatic molecules endowed with both hydrophobic (water hating) and hydrophilic groups (water loving). Long chain fatty acids or amines fulfil all the criteria of a surfactant molecule to form a monolayer at the air–water interphase¹⁷. These acids/ amines or any other similar molecule with hydrophilic functional groups are endowed with a long stretch of methyl groups which owing to its hydrophobic nature favours to dispose them at the air. The hydrophobic chains are made up of either saturated or unsaturated carbons and as a consequence are straight or tilted. The optimum length of chain to form a stable monolayer is that of 13 to 22 carbon atoms¹⁸. Whereas the functional group of the molecule, owing to its water loving temperament interacts with the aqueous subphase. The common functionalities include acid, alcohol, amine, nitro and sulphate groups¹⁸ and Figure 3 shows one such molecule, which has a long hydrophobic methyl chain and a hydrophilic group.

Figure 1 shows the orientation of a surfactant molecule at the air–water interphase. It is observed from Figure 1

that the surfactant molecules arrange them at the interphase in such a manner that the hydrophobic chain is positioned straight at the surface while the hydrophilic functional groups interact directly with the aqueous solution just beneath the surface. Orientation of the hydrophobic group at the interphase is not generally straight, it sometimes may be present at the subphase at an inclination and that depends on the original structure of the molecule. Equilibrium between the two oppositely oriented parts of the amphiphilic molecule is necessary to form a stable monolayer¹⁸. It is the structure of these amphiphilic molecules that allows the formation of monolayer at the air–water interphase. There is an inherent competition between the hydrophobic and hydrophilic groups of an amphiphilic molecule and the tendency to form micellar structure in the solution depends on the relative cross-sectional area of the hydrophobic and hydrophilic groups. Any shift alters the disposition of the molecule at the interphase as can be seen in Figure 4 which shows the formation of a micelle.

The interactions prevailing among the surfactant molecules are, the Van der Waals type between the hydrophobic chains; ionic, dipole–dipole and ion–dipole between the hydrophilic groups¹⁶. If the hydrophobic chain of the amphiphilic molecule has larger cross-sectional area compared to the tail or the interaction between the hydrophobic groups is stronger than the interaction of the hydrophilic group with the aqueous solution then they tend to come closer. As a result, they avoid the hydrophilic environment and form a structure, known as micelle, as shown in Figure 4. And, if the opposite happens with the hydrophilic groups interacting strongly with the aqueous solution then the molecule tend to dissolve into the subphase resulting in homogenous solution. So, the balance between the hydrophilic and hydrophobic part is an important prerequisite to form a stable monolayer. When a surfactant molecule is confined to arrange themselves on a plane (as in air–water interface), there is always a competition between both the head and the tail. Monolayer properties are also affected by the pH^{19–22} and the ionic strength of the subphase^{23,24}, the temperature^{25–27}, concentration of the surfactant molecule²⁸.

The LB monolayer formation

LB monolayers and films are generated on a LB trough, the picture of which is shown in Figure 2. The trough has

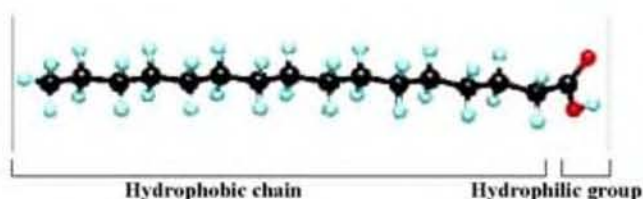


Figure 3. Stick and ball model of an amphiphilic molecule showing the hydrophobic chain in grey balls and the red sphere represents the hydrophilic functional group.

a Teflon coating to make the surface hydrophobic. It contains the subphase solution (generally aqueous) and the surfactant, dissolved in a suitable solvent, is spread on the surface of the subphase solution. The trough is cleaned first with a dilute acid to remove the inorganic impurities and then wiped with chloroform to get rid of organic impurities. After the surfactant solution is spread, some time is generally allowed for the evaporation of the solvent before any measurements are made. The trough is connected to a thermostat for the regulation of the temperature and is placed on a granite slab to isolate it from any external vibrations. The concentration of the surfactant is selected such that it spreads as a monomolecular layer over a given area.

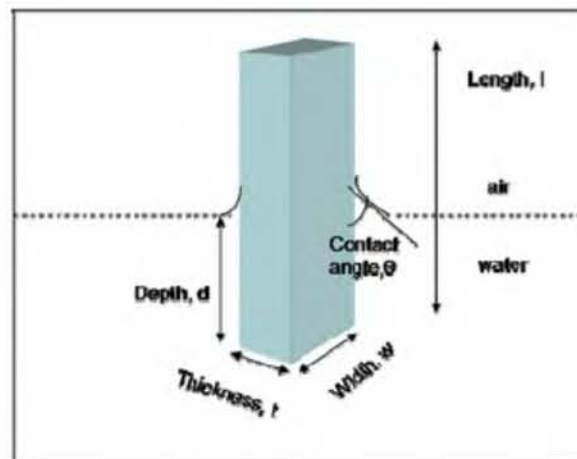
A paper plate, known as Wilhelmy plate, is dipped into the subphase solution prior to spreading of the surfactant. The Wilhelmy plate measures the surface pressure of the system, which is an important property for the characterization of any LB monolayer. This method utilizes the measurement of the vertical component of the force acting on the plate. Surface pressure is expressed as the difference of the surface tension of the subphase in the absence and in the presence of the surfactant²⁹. The set of equations followed to calculate the surface pressure is given below. Since the system is two dimensional: the surface pressure is equivalent to the surface tension at the interphase. Wilhelmy plate when suspended at the air–water interface, is pulled down into the subphase by surface tension of the aqueous solution. Hence, on a plate of dimension $l \times w \times t$ (length, width, thickness) and density ρ_p , which is immersed in subphase solution to a depth d , the total force acting downwards, is given by,

$$\text{Force} = (\rho_p l w t)g - (\rho_L d w t)g + 2(w + t)(ST)\cos\theta$$

(Force = weight – upthrust + surface tension)

(ρ_L = density of the subphase solution; g = acceleration due to gravity).

The upthrust term is eliminated as the plate is dipped at constant position always, so



Force = $2(w + t).(ST).\cos\theta$ ($\theta = 0^\circ$ as it is a paper plate)

Therefore, force = $2.(w + t).(ST)$

ST = force/perimeter; surface pressure = $ST_{\text{pure subphase}} - ST_{\text{surfactant}}$; unit of surface pressure is dynes/cm or newtons/m.

By measuring the surface pressure (P) against the molecular area ($\text{\AA}^2/\text{mol}$) at constant temperature, P–A isotherms are generated. One typical isotherm for long chain fatty acid is shown in Figure 5 *a*. Here the pressure shows an increase as a function of decrease in area, which is generated by moving the barrier over the monolayer.

The P–A isotherm in Figure 5 *a* shows two transition points, one from gaseous phase to liquid phase and the other one from liquid phase to solid phase. These two are the most important phase transitions, occurring at the air–water interphase. Liquid phase, for certain surfactant molecules, shows two regions: liquid expanded (LE) phase and liquid condensed (LC) phase (as shown in Figure 5 *b*). In most of the cases, the liquid condensed phase is only considered and liquid phase here is actually LC phase. At the gaseous phase, the molecules are far apart, randomly oriented with no interaction among the molecules and the surface pressure is nearly zero. When the barrier is moved to lower area/molecule, the surface pressure value increases. Molecules come closer and there is some interaction between the molecules but they are not arranged or properly arrayed. The molecules are present in a less compressible state and this state represents the liquid phase. As the barrier moves further towards lower area/molecule, the molecules get oriented in the closest packing possible. The interaction between the surfactant molecules is highest at this phase and behaves in an orderly crystalline fashion and is known as solid phase. Here, the molecules are in an incompressible state and upon moving the barrier further, the solid phase collapses, monolayer is destroyed, molecules move one over other and this situation is termed as ‘piling’.

There are a number of ways one can check the stability of such systems and ‘hysteresis’ is one of them. In fact, the very phenomenon of hysteresis leads one to think that

such system can be used as a memory device. Systems which show hysteresis exhibit persistent memory of the different states. With respect to P–A curves, hysteresis is generated by the repeated movement of the barrier back and forth on the surface to develop and release pressure respectively^{16,17}. The generated P–A isotherms with the concerted movement of barrier are termed as ‘hysteresis’ of Langmuir monolayers. It is often mentioned that hysteresis is the measure of equilibrium between two states and in the case of Langmuir monolayers, equilibrium will be the arrangement of molecules at the interface, when the pressure is applied and released with the help of the movement of the barrier. For Langmuir monolayers, if the gap between the successive P–A curves generated by moving the barrier forward and backward is less, the Langmuir monolayer is stable.

The arrangement of molecules at the three different phases is shown below in Figure 6. Interestingly, if a solid substrate is introduced in the monolayer at constant pressure, the monolayer can be effectively transferred on the substrate, keeping the same orientation of the molecules (LB films). The polarity of the transfer will depend on the nature of hydrophobicity of the substrate; if the plate is hydrophobic, the hydrophilic part of the monolayer will protrude out and vice versa, if the surface is hydrophilic. At the solid phase, the molecules are present in the closest possible packing and the films are very compact. Figure 7 shows the deposition process of Langmuir monolayers.

The type of deposition shown in the Figure 7 is known as Y-type deposition, in which the monolayer gets transferred both during upstroke and the downstroke movement of the substrate. Apart from this common type, there are two less common types of deposition: X-type of deposition and Z-type of deposition. In the later two types of deposition, the transfer either takes place during the upstroke movement (Z-type) or downstroke movement (X-type) of the solid substrate. For most of the molecules studied, the Y-type deposition is followed and in very few cases, either X-type or Z-type depositions are seen to be occurring^{30,31}. Figure 8 shows the X-type and Z-type of deposition. This type of irregularity in the deposition patterns can be due to many factors, like the nature of the molecule forming the monolayer, the solid substrate onto which the monolayer is to be transferred and also the dipping speed. Y-type of deposition is head–head tail–tail deposition, where the tail of one amphiphile will interact with the tail of another amphiphile and head will interact with another head. This happens as transfer of the monolayer takes place during upstroke as well as downstroke movement of the substrate. The X-type of deposition, on the other hand, is head-to-tail deposition which occurs mostly on hydrophobic substrates (Figure 8 *a*). In this case, the transfer of the monolayer only takes place with the downstroke movement of the substrate and hence the hydrophobic tail interacts with the hydrophilic group

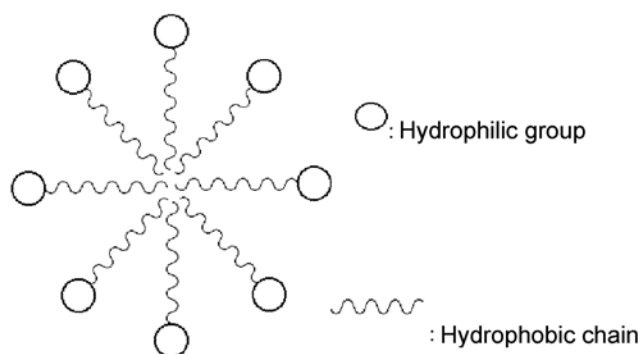


Figure 4. A schematic presentation of a surfactant forming a micelle on the surface of an aqueous solution.

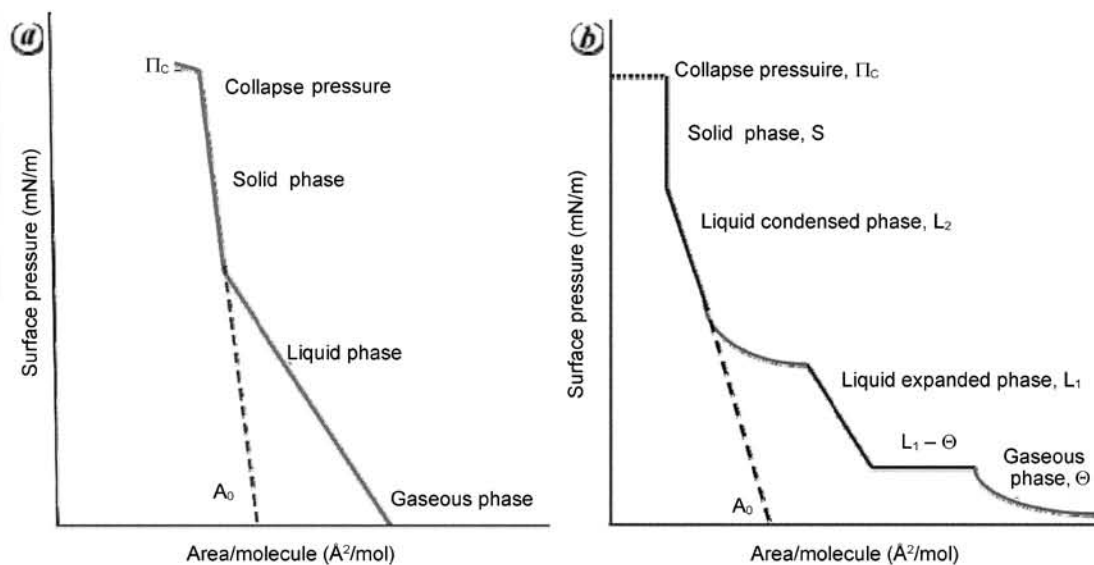


Figure 5. *a*, P-A isotherm of a long chain fatty acid; *b*, Typical isotherm of an amphiphilic molecule (taken from ref. 16).

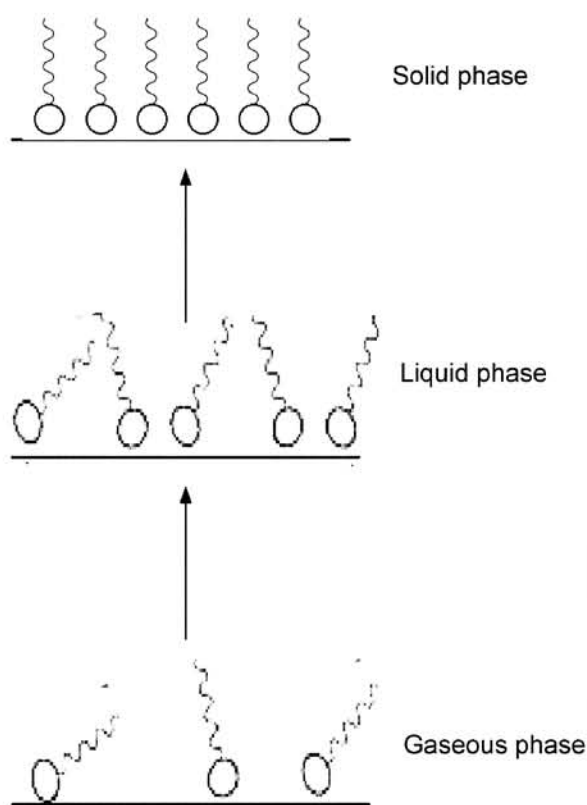


Figure 6. Orientation of surfactant molecules showing the arrangement at different phases.

deposited in previous stroke. Z-type of deposition follows tail-to-head interaction. In Z-type of deposition the transfer of the monolayer takes place during the upstroke movement (Figure 8 *b*). It is the hydrophilic headgroup which always gets immobilized facing the hydrophobic

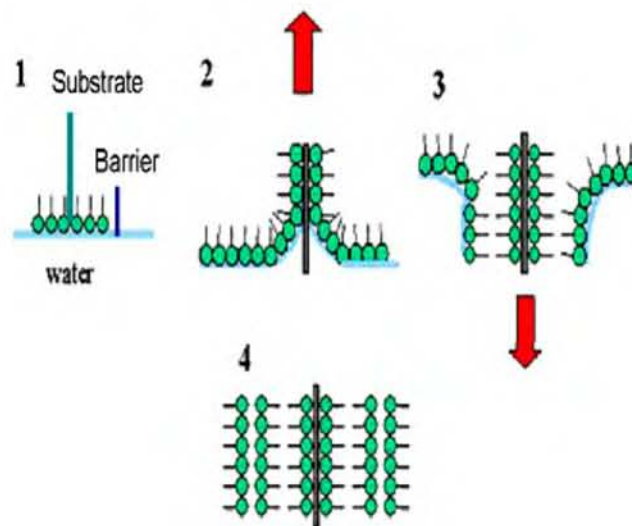


Figure 7. A representation of deposition of LB films on solid substrate.

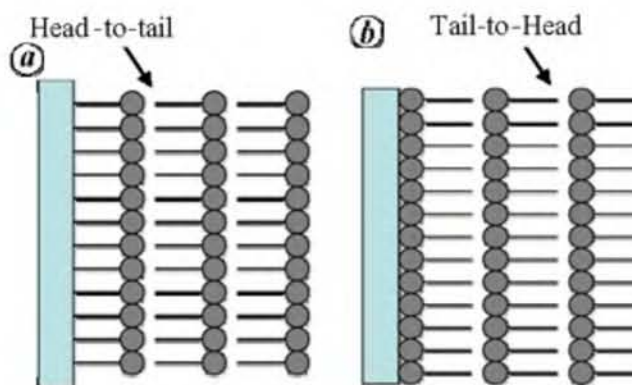


Figure 8. A representation of (a) X-type deposition and (b) Z-type deposition.

tail of the amphiphilic molecule transferred during the previous deposition. Both the Z-type and X-type of deposition are extremely unstable. However, in some cases, Z-type of deposition give rise to stable LB films³¹.

The morphological changes associated with the compression/expansion of the monolayer can be visualized by BAM (Brewster angle microscopy)^{32–34} and fluorescence microscopy^{35–37}. The fluorescence microscopy utilizes the incorporation of fluorescent probes into the monolayer at very low concentrations so that it dissolves in the liquid phase. When the barrier moves over the monolayer, the condensed phase can be visualized as bright objects. Earlier, the coexistence of phases was only inferred from the P–A isotherms and perhaps from surface potentials, till the use of fluorescence was documented^{35,36}. However, the only limitation with fluorescent microscopy is the concentration of the fluorescent probes which is very critical as it sometime creates artifact due to the concentration factors. This drawback is overcome by the use of BAM, which allows direct visualization of the monolayer without the addition of any probes. The principle of BAM is given in details elsewhere³², but when a monolayer is present on an interphase, it affects the Brewster angle condition and hence the reflectivity. So, the regions of monolayer differing in molecular density and orientation will be seen due to the optical contrast.

Fourier transform infrared (FTIR)³⁸ and Raman spectroscopy³⁹ have been used to study the conformational defects in the LB films. IR (FTIR/ATR–FTIR) spectroscopy is extensively used to establish the orientation of molecules at the monolayer^{40,41}. The morphology and molecular packing of the LB films have been determined by transmission electron microscopy (TEM)^{42,43} as well as by atomic force microscopy (AFM)^{44,45}. Interestingly, AFM also has been modified to image soft LB films, constituted of biological samples^{46,47}. Similarly X-ray diffraction (XRD) is used to check the morphology of the LB films^{48,49}. X-ray photoelectron spectroscopy (XPS)^{50,51} and UV–visible spectroscopy^{52,53} are among other methods utilized for the visualization of the LB films.

Langmuir monolayers and LB films

Two kinds of monolayers, namely SAM and Langmuir monolayer, are much in use now to monitor the properties of the molecules that assembled and oriented in a precise way. Langmuir monolayers deposited on solid substrates, as demonstrated in the previous section, generate LB film. Initial studies of Langmuir monolayers were only done with some aliphatic acid or similar such groups and the LB films obtained from those monolayers generally lacked the stability. Later, Blodgett pioneered the concept of introducing metal ions in the subphase^{7,10,54} to increase the structural stability of the LB films. SAMs, as the name suggests, are monolayers formed by the self-arrangement

of the molecules on a solid substrate when it is dipped in a solution of the compound. LB films have an added advantage over SAMs, that the molar ratios in a mixture of the components could be precisely controlled^{55,56}, which is not the case when the films are generated by SAMs. Although there is a diversity of function which associates with the LB technique, the real interest has only surfaced with the advent of LB films.

LB films could be made functional with the introduction of various groups, having different functions, in the monolayer. Recently, a lot of studies are being carried out with the functional LB films. There are numerous schemes by which the functional LB films could be generated. The functional groups are introduced into the amphiphilic molecules which are spread at the air–water interface and then the LB monolayer formed is transferred onto solid substrates to create the functional LB films. Another approach is to mix the functional amphiphiles with another monolayer forming molecule and then form a mixed monolayer. This will incorporate functionalities in the monolayer, although randomly. Variety of functionalities had been used to synthesize amphiphiles which generate different LB films like cyanine dyes^{57,58} and azo dyes^{59,60}.

Recently, the LB films with metal ions are much in use as monolayers^{61,62}. Introduction of metal ions is achieved by adding the metal salts into the subphase solution and then the amphiphile, which interacts with that metal ion through the charge interaction, is spread at the surface. The other way is to have the preformed monolayer with the amphiphile first and then the metal ions are injected in the subphase. Moreover, metal ions can also react with other functional ligands which are injected into the subphase⁶³. Hence, introduction of metal ions help in the formation of functional LB films in more than one way. We will soon show how such a system can be utilized to monitor macromolecular interaction in LB films through the participation of metal ions.

Metal ions interact with long chain organic molecules having negative charge at their extremities. It is reported that the introduction of divalent cations stabilize the LB monolayer of fatty acids like arachidic acid, stearic acid^{62,64}, whereas in the presence of trivalent ions the monolayers become very rigid⁶⁵. Depending on the nature of the metal ion introduced into the monolayer, certain properties are conferred onto the monolayer, like semi conducting, magnetic, optical and catalytic properties. Due to all these properties of metal ion–organic molecules interaction, it becomes technologically significant. Additionally, it could be of biological importance as well, as metal ions interact with many biologically important molecules like lipids⁶⁶, DNA⁶⁷ and even certain bacterial enzymes⁶⁸. Among the entire metal ion–functional group interactions, the interaction with the carboxylic group is very significant. There are many facets of metal ion–COOH interaction, it is used to remove toxic metals⁶⁹; it is also

used as a high quality absorbent for metal ions⁷⁰ and hence of immense relevance in the field of catalysis. Protein molecules, also contains COO^{--} group and so the metal ion interaction becomes biologically relevant⁷¹.

Of late, a lot of studies are being carried out and many interesting systems are being generated in the area of LB films⁷². Apart from the interaction of divalent metal ions, trivalent as well as monovalent metal ions have also been monitored⁷³ and there is dissimilarity in the behaviour of interaction of metal ions from separate groups. Divalent metal ions are widely studied and even among the divalent metal ions, inconsistency in the geometry and binding strength is also observed. The behaviour of divalent metal ions towards the carboxylic functional group is influenced by the ionic radii and electronegativity of metal ions^{64,74}. Interactions of numerous divalent metal ions with long chain fatty acids have been explored^{64,75}, like Pb^{2+} , Cu^{2+} , Ca^{2+} , Ba^{2+} , Co^{2+} and it has been observed that the metal ion- COO^{--} interaction does not follow similar rules at the air-water interface as they do so in solution phase. There are a lot of reasons for this discrepancy, like the pK_a of amphiphile is not the same at the interface as in solution or maybe the available geometry to interact with metal ions is not satisfactory. Detailed study on the chemistry and physics of the interaction of metal ions-carboxylic group is required for the understanding of the system and also for the potential applications of this class of interaction.

LB films and macromolecular recognition in biology

DNA plays a key role in all cellular functions like replication, recombination or transcription. It is, therefore, of considerable interest for scientists to study this molecule with detailed insight. Here, we will describe our attempts to follow biological reactions in a condensed phase or LB films and we termed such a system as 'artificial nucleus'. So, our goal is two fold: first, we want to construct a large scale artificial cell nucleus and second, we intend to use this model system to investigate fundamental issues in biology such as DNA transcription. To the best of our knowledge the concept of an artificial nucleus which we are proposing is new. It should open new ways to probe into various elementary mechanisms in DNA-related biology. In other words, we are motivated to develop a system which allows studying processes such as DNA transcription on a large collection of DNA molecules and in conditions as close as possible to the *in vivo* situation. The problem which always surfaces while working with high concentrations, which is often unrealistic, is the repulsive interaction between the neighbouring molecules. This problem is evaded if the formation of an artificial nucleus is achieved by first tethering low concentration of DNA chains at the air-water interface with the help of

Langmuir technique and then compressing them with a mechanical barrier until a dense brush is formed.

Although a lot of single molecule experiments have been performed to study the interaction between a single molecule of DNA and DNA-binding proteins^{76,77} *in vivo*, DNA is not isolated. Realization of this fact led us to study DNA and DNA-related functions in a system closer to reality. The general idea of our approach is to form a monolayer with a T7 A1 DNA at air-water interface and then to compress the monolayer till the density reaches the density inside the real cell nucleus. This system is advantageous firstly because excessive amounts are not required to achieve dense brushes of DNA and secondly, it can be made analogous to the true cell by adding necessary proteins and enzymes into the subphase which will interact with the DNA at the interface hence, mimicking the biologically relevant mechanisms. DNA will itself not form a monolayer as it is highly soluble in water and so to form a stable monolayer at air-water interface, it has to be made partially hydrophobic. To achieve this, it was thought to attach water-insoluble moiety to one of the ends of DNA molecule, so that the resulting species remains bound to interface via the hydrophobic group and some part of it also dangles into the solution due to the DNA chain. One major point to keep in mind while creating such amphiphiles is that the hydrophilic-hydrophobic balance should be correct for the formation of a stable monolayer at the air-water interface.

We first attempted to realize our goal by tethering DNA onto submicron-size latex beads^{78,79}, as shown in Figure 9a. A lot of studies have been done on this possibility of coupling DNA on latex beads^{80,81}. DNA recognition is first and most important for biological conditions and so DNA on the beads was tested for its biological activity. It was found to be transcriptionally active⁷⁸ and even could be used for hybridization with short oligonucleotides. From the rate of hybridization we could also estimate the value of activation energy from temperature dependence studies (Figure 9b). Interestingly, a single base mutation in the recognition sequence of DNA did not allow the formation of the double helix.

Hence, it was perceived that this generated amphiphile can be used to develop a monolayer at air-water interface for the purpose to generate 'DNA-brushes' which could then be utilized for the study of biologically relevant mechanisms. However, we noticed that the bead bound DNA cannot form stable monolayer or LB film and tend to disintegrate when we tried to pick them up on solid substrate. Perhaps, if the plate was sufficiently hydrophobic to capture latex beads, the monolayer would be stable. All our attempts in this direction failed but still we have been able to establish a hybridization method which is a successful strategy for the grafting of double stranded (ds) DNAs on the surface of polystyrene microspheres. The DNAs remain end grafted and hence accessible for biological reactions. Moreover, the kinetics of hybridiza-

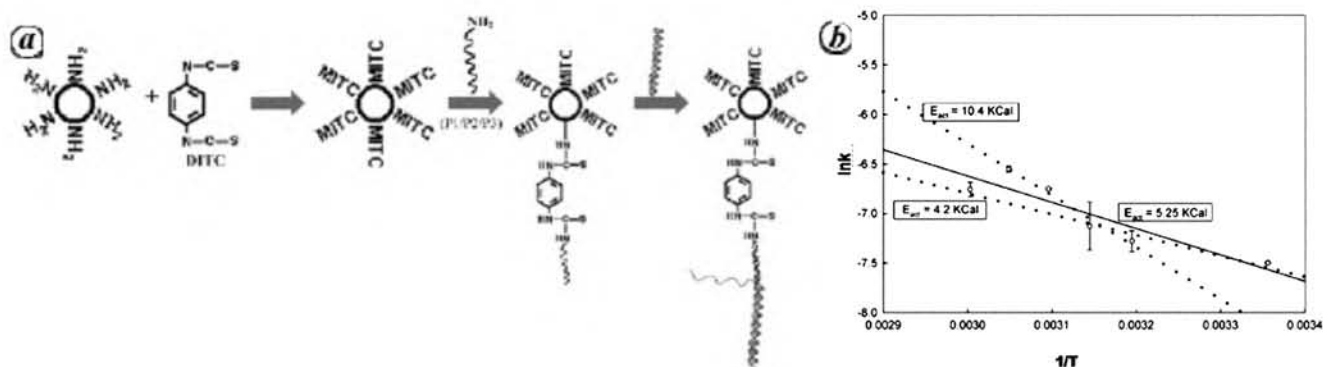


Figure 9. *a*, Sequence of reactions followed for grafting a DNA to a polystyrene microsphere (with permission, taken from D. Ghosh *et al.*, *Langmuir*, 2003, **19**, 5830–5837). *b*, Plot of $\ln k$ (k = rate constant) versus $1/T$ (T = temperature in kelvin), giving the activation energy for the hybridization kinetics of T7A1 DNA with primer P1 in the heterogeneous phase (P1 attached to the polystyrene micro-spheres (O)). The range of error associated with the experimental data is also shown. The dotted lines show the maximum range to which the activation energy values can vary (with permission, taken from D. Ghosh *et al.*, *Langmuir*, 2003, **19**, 5830–5837).

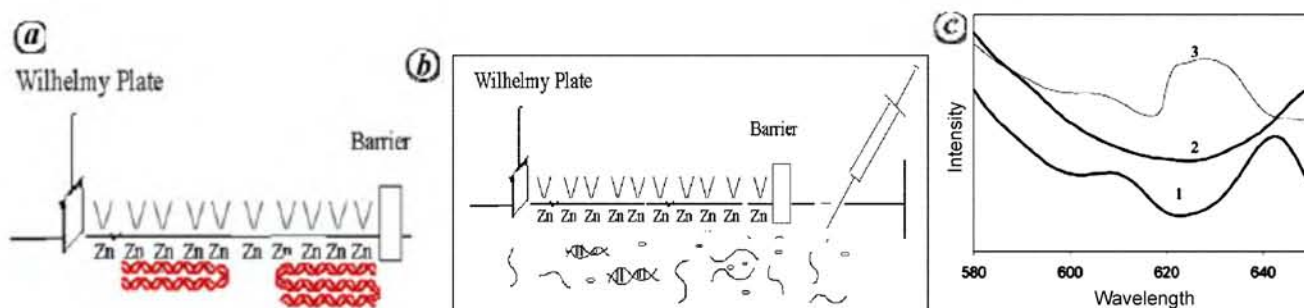


Figure 10. *a*, Schematic representation of Zn-arachidate Langmuir monolayer to which DNA is bound. *b*, A cartoon representation showing the hybridization phenomenon occurring at the air-water interface. *c*, Fluorescence spectra of 19 layers of the ssDNA1-Zn-arachidate layer (curve 1), ssDNA1-ssDNA3-Zn-arachidate (curve 2), and ssDNA1-ssDNA2-Zn-arachidate (curve 3). The excitation wavelength was kept at 280 nm (with permission, taken from A. Bhaumik *et al.*, *Langmuir*, 2004, **20**, 5891–5896).

tion of a ds-DNA to an oligonucleotide in heterogeneous phase was also monitored.

An alternative strategy was therefore, necessary. Instead of attaching the DNA directly to water-insoluble group, it was proposed to first form a monolayer with an amphiphile which will form a stable monolayer and then attach the DNA with the aid of a linker. Metal ions form stable monolayers with long chain fatty acids and hence the system chosen for our purpose was arachidic acid on a subphase of ZnSO_4 (ref. 82). Zn(II) has an affinity towards DNA⁸³ and so DNA was immobilized onto the monolayer at interface via the Zn(II) ions which were bound to the surface arachidic acid molecule by charge interaction and this is schematically shown in Figure 10 *a*.

Zn-arachidate (ZnA)-DNA monolayers and LB films of same were fabricated. The ZnA-DNA films were characterized by P-A isotherm as well as AFM and fluorescence microscopy. The microscopic images confirmed the immobilization of DNA onto LB monolayers of ZnA. For any specific interaction to occur, it is indispensable to have the interacting species to be geometrically aligned. To accomplish our objective of studying DNA-protein

interaction, it was a prerequisite for the DNA to be aligned in such a manner so that the site of interaction is accessible for protein recognition. To authenticate the alignment of DNA at the LB monolayer, a hybridization experiment was performed. In this experiment a short single strand DNA was attached to the amphiphile in the monolayer through a metal linker (Figure 10 *b*) and its complementary sequence was added to the subphase.

When the barrier moved and LB film was generated, we noticed the double-stranded DNA at the interphase was produced and detected by fluorescence (Figure 10 *c*). Interestingly, non-complementarity in the recognition sequence of DNA at the level of even one base pair mismatch did not allow the formation of the double helix. The hybridization data also confirmed that the DNA is freely immobilized to the monolayer to undergo the sequence-specific hybridization⁸². Hybridization was performed with short single-stranded DNAs, contrary to the long double-stranded DNA used for AFM and fluorescence microscopy imaging which attested to the fact that long DNAs at the air-water interface get entangled or coiled. Hence, the efficiency of transcription mechanism at the

interface when the DNA is immobilized is not much⁸². At this point we cannot predict whether or not such problems are encountered *in vivo* but Langmuir monolayers open interesting opportunity to study such system.

Although the ZnA system successfully aligned the DNA and it is a promising template as a DNA chip, to our knowledge not many such reports are present in the literature. The method employed here is an improved way to immobilize long DNA, which in turn may act as an affinity surface for protein recognition. Through AFM and fluorescence images the DNA was found to be coiled. However, it still remained good substrates for cleavage reaction. It also acted as a target for hybridization through Watson–Crick base pairing.

However, due to the coiling of long DNAs, the accessibility of the molecule for protein recognition is not much efficient. We needed to overcome this predicament as our focus was to study the DNA–protein interaction and the critical point in this regard is the recognition of one of the interacting species by the other. The system which is employed for our study is two dimensional and due to the restriction in the degrees of motion, the apt orientation of the interacting molecules becomes more crucial. To resolve the crisis, we thought of immobilizing the protein and not the DNA. We engineered the system, contrary to the manner used to immobilize DNA, very specific for the immobilization of protein. The folding of DNA was inevitable as the chemistry used to immobilize the DNA onto the ZnA–LB monolayer was the nonspecific ionic interaction between the Zn^{2+} and the backbone negative charges of DNA. To circumvent this, the scheme to immobilize protein was specific for one point attachment to the monolayer so that the site to be recognized by the interacting partner is well exposed and accessed.

Hence, we exploited the well-known Ni(II)–histidine chemistry to immobilize the protein molecules on LB monolayers. The LB monolayer was constructed with

Ni(II) cations in the subphase solution and the protein was Histagged. This ensured that single protein molecule, instead of having multiple binding sites, will bind specifically to one point through the Ni(II)–histidine chemistry, thus having correct orientation of the molecule to enhance the interactions⁸⁴. Figure 11 shows the orientation of the protein molecules and its recognition with DNA after being immobilized at the surface.

This scheme for the immobilization successfully got the protein molecules attached at the surface and more so in desired orientation for further interactions. This solved our purpose to engineer a system which could be developed for the study of thermodynamics of the interaction at the crowded environment⁸⁵.

Here, we designed a system comprising a hydrophobic monolayer upon a hydrophilic subphase containing metal ion. The system was then employed for the successful interaction of biomolecules injected into the subphase. The aim was to follow macromolecular interactions at the LB surface. First, due to a reduction in dimensionality at Langmuir monolayers, it can be used suitably for macromolecular interaction. Second, one of the reactants at the surface is oriented, arrayed in a crowded fashion, resulting in a situation (we presume) closer to *in vivo* conditions and if the reactants are biological macromolecules, the thermodynamic parameters so derived will have fascinating applications. This technique is not specific for our system but it is general and it could be employed for any similar systems.

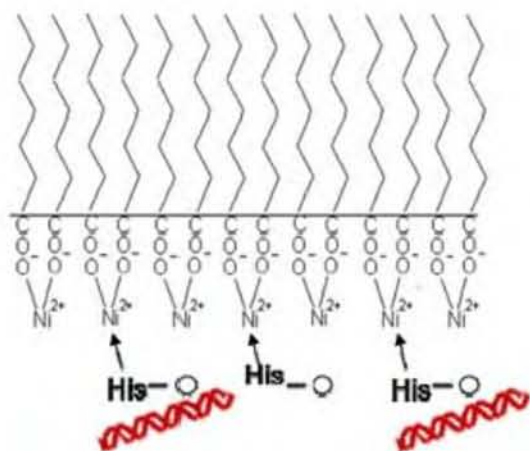


Figure 11. A schematic representation showing the one-point attachment of Histagged protein molecules onto the NiA LB monolayer.

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