Chapter 7
Specific Immobilization of Rotaviruses for Atomic Force Microscopy Using Langmuir Antibody Films Based on Amphiphilic Polyelectrolytes

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Abstract The Langmuir–Blodgett technique is a useful and suitable tool for fabrication of affinity layers. Langmuir antibody films based on amphiphilic polyelectrolytes have been used for specific immobilization of rotaviruses for atomic force microscopy (AFM) analysis. AFM has been used for structural analysis of virus particles and their identification based on specific interactions of antibodies with rotaviruses. Virus-containing samples were investigated in the atomic force microscope using contact and tapping mode. Monoclonal mouse antibodies against viruses were used for film deposition. Antibodies were deposited on different substrates using Langmuir–Blodgett and Langmuir–Schaeffer techniques. A comparison of the size of virus particles obtained by AFM and electron microscopy has been done.

Keywords AFM · Specific immobilization · Langmuir films · Viruses · Antibodies

Nomenclature

AFM atomic force microscopy
BSA bovine serum albumin
COVID-19 coronavirus disease 2019
DNA deoxyribonucleic acid
MGEs mobile genetic elements
LB Langmuir–Blodgett
MERS-CoV  Middle East respiratory syndrome coronavirus
PHE  public health emergency
PHEIC  public health emergency of international concern
RNA  ribonucleic acid
SARS-CoV  severe acute respiratory syndrome coronavirus
STM  scanning tunneling microscopy

7.1 Introduction

All cellular life-forms, except some intracellular bacterial parasites, host distinct repertoires of viruses and other mobile genetic elements (MGEs). Viruses appear to be the dominant biological entities on our planet, with the total count of virus particles in aquatic environments alone at any given point in time reaching the staggering value of $10^{31}$, a number that is at least an order of magnitude greater than the corresponding count of cells (Rohwer 2003; Edwards and Rohwer 2005; Suttle 2005; Rosario and Breitbart 2011; Suttle 2007; Rohwer and Thurber 2009; Koonin and Dolja 2013; Suttle 2007; Suttle 2005; Suttle 2007; Rohwer and Thurber 2009; Koonin and Dolja 2013). Accordingly, lytic infections of cellular organisms, primarily bacteria, by viruses play a central role in the biological matter turnover in the biosphere (Suttle 2007; Rohwer and Thurber 2009; Koonin and Dolja 2013; Chow and Suttle 2015; Cobián Güemes et al. 2016; Danovaro et al. 2016). The genetic diversity of viruses is harder to assess, but beyond doubt, the gene pool of viruses is, in the least, comparable to that of hosts. The estimates of the number of distinct prokaryotes on earth differ widely, in the range of $10^7$ to $10^{12}$ (Curtis et al. 2002; Amann and Rosselló-Móra 2016; Locey and Lennon 2016; Vinatzer et al. 2017), and accordingly, estimation of the number of distinct viruses infecting prokaryotes at $10^8$ to $10^{13}$ is reasonable. Even assuming the lowest number in this range and without attempting to count viruses of eukaryotes, these estimates represent vast diversity. Furthermore, the genomes of most viruses accumulate mutations much faster than genomes of cellular organisms due to both the typically low fidelity of the virus replication machinery that stems, in part, from the absence of proofreading activity in many viruses and the strong selection pressure on virus populations (Holland et al. 1982; Drake et al. 1998; Sanjuán et al. 2010; Sanjuán and Domingo-Calap 2016; Geoghegan and Holmes 2018; Domingo and Perales 2019). Thus, viruses encompass an enormous pool of rapidly evolving genes that appears to continuously contribute to the emergence of new genes in cellular life-forms through the exchange of genetic material between cells and viruses. However, despite the rapid short-term evolution of viruses, the key genes responsible for virion formation and virus genome replication are conserved over the long term due to selective constraints (Szathmáry and Maynard Smith 1997; Takeuchi and Hogeweg 2007; Takeuchi and Hogeweg 2012; Iranzo et al. 2016; Koonin et al. 2017; Berezovskaya et al. 2018).

Generally, viruses are very small living objects with the size from 0.02 to 0.3 μm, although several very large viruses with a length of up to 1 μm (megavirus,
pandoravirus) have recently been detected (Schulz et al. 2020). Living and multiplication of viruses are completely dependent on the host cells (bacterial, plant, or animal). Viruses consist of protein, and sometimes lipid, envelope, RNA or DNA core, and sometimes enzymes necessary for the first stages of virus replication. Viruses are molecular parasites or symbionts that coevolve with nearly all forms of cellular life. The route of virus replication and protein expression is determined by the viral genome type. The single-stranded DNA (ssDNA) viruses are a polyphyletic class, with different groups evolving by recombination between rolling-circle-replicating plasmids, which contributed to the replication protein, and positive-sense RNA viruses, which contributed the capsid protein. The double-stranded DNA (dsDNA) viruses are distributed among several large monophyletic groups and arose via the combination of distinct structural modules with equally diverse replication modules. Phylogenomic analyses reveal the finer structure of evolutionary connections among RNA viruses and reverse-transcribing viruses, ssDNA viruses, and large subsets of dsDNA viruses.

Viruses are classified mainly according to the structure of their genome and the type of their replication, and not depending on the diseases that they cause (Siddell et al. 2020). All viruses were divided into seven distinct classes, based on the structure of the virion’s nucleic acid (traditionally called the virus genome) (Baltimore 1971; Agol 1974; Condit 2013):

1. Double-stranded DNA (dsDNA) viruses, with the same replication-expression strategy as in cellular life-forms
2. Single-stranded DNA (ssDNA) viruses that replicate mostly via a rolling-circle mechanism
3. dsRNA viruses
4. Positive-sense RNA [(+)RNA] viruses that have ssRNA genomes with the same polarity as the virus mRNA(s)
5. Negative-sense RNA [(−)RNA] viruses that have ssRNA genomes complementary to the virus mRNA(s)
6. RNA reverse-transcribing viruses that have (+)RNA genomes that replicate via DNA intermediates synthesized by reverse transcription of the genome
7. DNA reverse-transcribing viruses replicating via reverse transcription but incorporating into virions a dsDNA or an RNA-DNA form of the virus genome

Thus, there are DNA viruses and RNA viruses; each type may have single or double chains of genetic material. Single-stranded RNA viruses, in turn, are divided into RNAs with positive polarity and RNAs with negative polarity. Typically, DNA viruses replicate in the nucleus of the host cell, and RNA viruses typically replicate in the cytoplasm. At the same time, some single-stranded RNA viruses of positive polarity, called retroviruses, use a completely different replication method (Koonin and Dolja 2014). Viruses are the causative agents of such dangerous infections as hepatitis C, herpes, and AIDS. Chronic viral infections are characterized by the prolonged, continuous release of the viruses. Viruses are spread through respiratory and intestinal secretions. Clear laboratory diagnosis of viruses is based on detection of the whole virus or viral main antigens or viral DNAs and RNAs or
antigen-specific antibodies, or visualization of viruses and combination of all these detections. It is very difficult to detect and identify viruses because of their size and because they are very small compared with bacterial pathogens. Currently, the definition and identification of viruses are based on three main approaches of polymerase chain reaction (PCR), hybridization, and immunoassays (Delwart 2007; Nicolaisen 2011; Roossinck 2011; Mokili et al. 2012).

However, because these assays depend on the reagents (antibodies, primers, or probes) developed from the characterized viruses and viroids, they are ineffective when the disease is caused by a new pathogen or a mixture of pathogens that share little or no sequence similarity with those described previously. The rapid method was offered in the use of next-generation sequencing technologies for the identification of viruses and viroids (Wu et al. 2015). Serological identification of viruses may be sensitive and specific. Sometimes a histological examination using an electron microscope can help. Viral genomes are small; the genome of RNA viruses ranges from 3.5 up to 27 kb, and the genome of DNA viruses varies from 5 up to 280 kb. Thus, partial and full genome sequencing will become an important component for specific diagnostic for virus detection and identification.

Recently, electron microscopy was used to identify the COVID-19 virus (Kim et al. 2020). As an instrument for nanoscale imaging, atomic force microscopy (AFM) has many benefits over scanning electron microscopy (SEM). While SEM must be carried out in a vacuum, AFM can be undertaken in several different environments, encompassing ambient, liquid, and vacuum. It is ideally suited to the analysis of biological samples due to its ability to image in a liquid environment. This chapter aims to test using AFM for specific visualization of viruses.

### 7.2 AFM in Virology

Nanobiotechnology is a discipline in which tools from nanotechnology are developed and applied to study biological phenomena. The number of virus particles on Earth is frequently reported in the scientific literature and in general-interest publications as being on the order of $10^{31}$ (Mushegian 2020). AFM probes surface-adsorbed samples at the nanoscale by using a sharp stylus of nanometric size located at the end of a micro-cantilever. AFM can be used to explore the topography of viruses and protein structures. AFM is not limited to imaging and allows the manipulation of individual viruses with force spectroscopy approaches, such as single indentation and mechanical fatigue assays. These pushing experiments deform the protein structures to get their mechanical information and can be used to monitor the structural changes induced by maturation or the exposure to different biochemical environments, such as pH variation (de Pablo 2019). A characteristic structural element in viruses is the protein capsid, which combines multiple functions, including packaging of the viral genome and recognition of the target cell. These protein cages or shells can be defined as a structure built out of protein subunits enclosing a cavity at the nanometer scale. Although viruses illustrate the definition of a protein
cage, non-viral structures, such as bacterial microcompartments (Cheng et al. 2008), vault particles (Querol-Audí et al. 2009), clathrin cages (Fotin et al. 2004), and artificial virus-like structures (Wimmer et al. 2009; Worsdofer et al. 2011; Lai et al. 2014) are other examples. Virus protein capsids are built up of repeating protein subunits (capsomers) that pack the viral genome (Flint et al. 2004). Viral particles are endowed with meta-stable properties that permit fulfilling each task of the viral cycle sequentially (Mateu 2013). These capacities have induced using viral capsids as protein containers of artificial cargoes (drugs, polymers, enzymes, minerals) (Douglas and Young 1998) with applications in materials and biomedical sciences. 

AFM requires the immobilization of the specimen to study on a solid surface (substrate). Viral cages are normally physisorbed on the substrate, using polar, non-polar, and van der Waals forces (Muller et al. 1997). Physisorption traps protein cages on the surface without creating chemical bonds that might alter their structure. Each type of protein shell has individual features such as local charge densities and/or hydrophobic patches (Armanious et al. 2016), which can be employed for adsorption, via electrostatic and/or hydrophobic interactions, on different substrates, such as glass, mica, and HOPG (Highly Oriented Pyrolytic Graphite) (Moreno-Madrid et al. 2017).

AFM has opened a new way regarding the investigation of the physical properties of viruses and virus mechanics. The first imaging attempt regarding viruses was performed using scanning probe microscopy. In scanning tunneling microscopy (STM), imaging the sample has to be electrically conductive, and the particle was covered with a metallic layer that is far from its physiological conditions. This limitation has been overcome using AFM, which does not require electrical conductivity. AFM has been widely used for the topographic property determination of viruses, partially disassembled viruses, viral capsids, nucleic acids, etc. Also, AFM can be used to investigate the viral infection in the live-cell process and for the determination of the interaction forces between viruses and other molecules. A different approach for the determination of the mechanical properties of viruses is based on the consideration of the virus particle and the AFM cantilever. One could say that each type of virus has a preferred substrate since each kind of protein structure exposes different residues (Tetreau et al. 2020). Virus particles and other protein-based supramolecular complexes have vast nanotechnological potential. However, protein nanostructures are “soft” materials prone to disruption by force. Whereas some non-biological nanoparticles (NPs) may be stronger, for certain applications, protein- and virus-based NPs have potential advantages related to their structure, self-assembly, production, engineering, and/or inbuilt functions. Thus, it may be desirable to acquire the knowledge needed to engineer protein-based nanomaterials with a higher strength against mechanical breakage (Medrano et al. 2019).

The characterization of viral structures has required high-resolution techniques such as AFM. AFM has been widely used in the field of biological science. Cells, their structures, and molecules have been extensively studied using this method. The AFM has also been applied within the field of virology. As the structures of many viruses have been determined by electron microscopy and X-ray crystallography, the most appropriate role for AFM is the surface topography of viruses under
many different conditions (in the air and, for the most important biological applications, in solution). The AFM has been used in the high-resolution image of many viruses. Viruses are not easy to characterize, but the capacity to predict the surface charge of viruses opens new possibilities for vaccine purification and making gene therapy too (Michigan Technological University, 2020). Before viruses can be imaged using AFM, they should be immobilized on the surface. There are two methods of immobilization—specific and non-specific. In the non-specific method, samples were put on the surfaces and analyzed after drying. In this case, it is difficult to analyze images, especially in the presence of contaminants. Antibodies are usually used to increase the sensitivity of immobilization. The standard formation of the antibody monolayers for immobilization of viruses is achieved by direct covalent attachment to the surface. It is known that monolayers of pure antibodies or enzymes can lose their specificity and activity (Babitskaya et al. 1997). Amphiphilic polyelectrolytes and lipids have been used to protect antibodies and enzymes from inactivation during the formation of monolayers. In this study, we report on the use of Langmuir films of antibodies based on amphiphilic polyelectrolyte for specific immobilization of rotaviruses for visualization by AFM.

It is possible to analyze the virus morphology in high resolution under physiological conditions (in water medium) (Kuznetsov and McPherson 2011). AFM can be used for nano-sized manipulation using cantilever (Rief et al. 1997; Muller et al. 2002; Schaap et al. 2006). Using cantilever, it is possible to analyze the packing of the genome (Carrasco et al. 2006, 2008; Roos et al. 2009; Hernando-Perez et al. 2012; Snijder et al. 2013; Zeng et al. 2017), virus maturation (Carrasco et al. 2011; Roos et al. 2012; Ortega-Esteban et al. 2013), site-directed mutagenesis (Carrillo et al. 2017), and protein engineering of capsids (Llauro et al. 2016). AFM can be used for the study of phage genome translocation into the bacterial cell (Purohit et al. 2003; Gonzalez-Huici et al. 2004). In addition, protein shells of non-viral origin, encapsulin, and the internal pressure of phages can be measured by AFM (Smith et al. 2001; Hernando-Perez et al. 2012; Llauro et al. 2014; Snijder et al. 2016).

### 7.3 Specific AFM Visualization

Rotavirus is a very contagious virus that causes diarrhea. It’s the most common cause of diarrhea in infants and children worldwide, resulting in over 215,000 deaths annually. Before the development of a vaccine, most children in the United States had been infected with the virus at least once by age 5. Although rotavirus infections are unpleasant, you can usually treat this infection at home with extra fluids to prevent dehydration. Occasionally, severe dehydration requires intravenous fluids in the hospital. Dehydration is a serious complication of rotavirus and a major cause of childhood deaths in developing countries. Good hygiene, such as regular washing hands, is important, but vaccination is the best way to prevent rotavirus.
infection https://www.mayoclinic.org/diseases-conditions/rotavirus/symptoms-causes/syc-20351300.

Rotaviruses SA 11 (obtained from Chumakov Institute of Poliomyelitis and Viral Encephalitides of Russian Academy of Medical Sciences) were grown in rhabdomyosarcoma cells in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Sigma-Aldrich, USA). After cultivation, these cells with viruses were destroyed by 3-time freezing/thawing cycles for rotaviruses isolation. Then the suspension was centrifuged to remove cell fragments. The supernatant was transferred with a syringe to clean test-tubes containing 10% sucrose. After the centrifugation, the virus particles were suspended in the phosphate buffer containing NaCl. Formalin was added to the suspension for virus disinfection.

The Langmuir–Blodgett (LB) technique is a way of making ultrathin nanostructured films with a controlled layer structure and crystal parameter, which have many envisioned applications in technology for optical and molecular electronic devices as well as in signal processing and transformation. LB films have a unique potential for controlling the structure of organized matter on the ultimate scale of miniaturization, and must surely find a niche where this potential is fulfilled. The LB technique, a unique bottom–up approach, can produce ultrathin films on a monomolecular level (Bashar et al. 2019). Glass and gold were used as substrates for films. Before deposition, the surfaces were washed by ethyl alcohol and rinsed with distilled water. Antibody films were deposited by the following method. On the first stage, suspension of antibodies was mixed with Tris-HCl buffer. The buffer solution was poured Langmuir–Blodgett trough. Then alkylated polyethyleneimine was added to the surface of the air–water interface. It is believed that polyethyleneimine-based sorbents containing numerous positively charged amine groups (Vasilieva et al. 2018) are electrostatically attracted to the surface structures of the substrate providing steady attachment of cells. In the first stage, polymer macromolecules form a monolayer on the surface of the interface. At the second stage, protein molecules come closer to the surface due to electrostatic interactions. Different substance concentrations and conditions were tested for polymer monolayer film formation on the surface of the air/water interface. The roughness of the resulting films served as criteria for the fabrication of the optimum films. In the case of poly-benzyl-histidine, it became possible to fabricate films with a 1.5–2 nm thickness and a 0.2 nm roughness, which can increase the affinity of the fabricated polymer/antibodies films. This film is formed on the surface of a drying drop due to the self-assembly of polymer macromolecules on the surface of the interface. After 15 min, antibody–polymer complex monolayers were compressed using a compression barrier until the pressure reached 40 mN/m. Then the obtained film of antibody–polymer complexes was transferred to the surface of the substrate using Langmuir–Schaeffer technique (Lafuente et al. 2019). The value of the surface pressure was tested to obtain a maximal density of antibodies. The obtained films of antibodies were stored in a refrigerator at +5°С in a phosphate buffer.

Samples of antibody films were deposited on freshly cleaved mica or glass or gold. Imaging was performed using a Nanoscope IIIa (Digital Instruments, Santa Barbara CA, USA), operating in contact and tapping modes. Commercially
available tips (Veeco NanoProbe tips RTESP7 for tapping mode and NP-S20 for contact mode) were used. Data were analyzed using soft programs for Digital Instruments and NanoScale Explorer program developed in the Institute of Theoretical and Experimental Physics (Moscow, Russia).

The adsorption and binding of biomolecules and microorganisms to the surfaces is a central problem in fundamental studies, but also in biotechnological applications. AFM is a useful tool for such type of analysis. In this study, viruses were specifically immobilized using Langmuir antibody films based on amphiphilic polyelectrolytes for AFM studies. Antibodies on the basis of amphiphilic polymers were used to produce films for specific immobilization of rotaviruses. Amphiphilic polyelectrolytes were used to stabilize the protein conformation of antibodies to improve their affinity. Application of amphiphilic polymers protects the conformation of a protein globule from inactivating effects on the surface of the air/water interface and leads to the magnification of interactions between components of Langmuir film. Alkylated polyethyleneimine was used as an amphiphilic polyelectrolyte.

Immobilization of antibodies is a crucial step for fabricating high-quality affinity surfaces since after immobilization the activity of antibodies should remain high and binding of antigen should occur in a manner that reduces interference. The conventional methods for immobilization of biocomponents and antibodies include physical adsorption, covalent binding, entrapment, etc.; however, they suffer from a poor spatially controlled deposition. Comparing to these methods, Langmuir–Blodgett (LB) technique is considered as a desirable immobilization method owing to the following advantages: making uniform, ordered, and ultra-thin organic films; controlling the number of bio components by the number of deposited layers; and, in addition, preserving the activities and specific recognition properties of biocomponents. The stability of the mixed monolayer is very important because it is related to the transfer efficiency and the quality of deposited LB films. The pure amphiphile monolayer is unstable because of its partial solubility in the subphase. To prepare stable antibody monolayer, we used amphiphilic polyelectrolyte. The electrostatic force between antibodies and amphiphile molecules is expected to be an important factor for the adsorption process and increasing sensitivity and stability of affine surfaces.

The obtained films of antibodies were studied in contact mode AFM using the permissible minimal force of tip–sample interaction to avoid possible damage to the sample surface. Images of films of antibodies on the surface of gold and glass after blocking with bovine serum albumin (BSA) (Fig. 7.1) were obtained. In immunological detection, blocking is used to inhibit non-specific binding reactions. To determine the thickness of the antibody film, a relatively small surface region (1500 × 1500 nm²) was scanned with a large force of tip–sample interaction several times, which resulted in the destruction of the antibodies film in this region (Fig. 7.1). The obtained height difference between damaged and undamaged surfaces was interpreted as the thickness of the film. It varied from 8 to 12 nm depending on the selected region and the substrate material. The obtained films of antibodies were
stored in a refrigerator at +5° C in the phosphate buffer for 1 month without change of their immune activity.

AFM images of rotaviruses have been received using Langmuir films of antibodies formed on the basis of amphiphilic polymer (Fig. 7.2). The apparent diameter of rotaviruses ranged from 100 to 120 nm. These results have been compared with the real diameter of virus particles which can be obtained by electron microscopy (70 nm). Analysis of the data revealed that the diameter of viral particles received by AFM is bigger in comparison with results obtained by electron microscopy. Those distortions universally exist in AFM images due to geometrical interaction between the sample surface and the limited size tip. The distortions can cause bigger images than the real sizes using commercial pyramidal tips. The distortions of the images are affected by the shape of the AFM tip and the circumstance of the
particles. Previously, the ability to analyze specific binding of bacterial fragments with the affine surface has been shown (Dubrovin et al. 2012).

7.4 Conclusion and Future Perspectives

In conclusion, a method based on Langmuir antibody films using amphiphilic polyelectrolytes has been developed to specific immobilization of rotaviruses for AFM analysis. AFM is the only method that is presently capable of non-destructive continuous imaging with nanometric resolution. However, it is often difficult to know which object is located on the surface under investigation. From this point of view, we used antibodies for specific immobilization of viruses on the surface for AFM analysis. The new type of Langmuir antibodies films has been used for specific visualization of rotaviruses. The apparent diameters obtained by AFM of these viruses were compared with data obtained by electron microscopy.

A novel method of specific visualization of viruses is very important now. Recently, the charge-coupled device is responding for an outbreak of respiratory disease caused by a new coronavirus that was first detected in China and which has now been detected in almost 70 locations internationally, including in the United States. The virus has been named “SARS-CoV-2,” and the disease it causes has been named “coronavirus disease 2019” (abbreviated “COVID-19”) (www.cdc.gov). On January 30, 2020, the International Health Regulations Emergency Committee of the World Health Organization declared the outbreak a “public health emergency of international concern” (PHEIC). On January 31, 2020, Health and Human Services Secretary Alex M. Azar II declared a public health emergency (PHE) for the United States to aid the nation’s healthcare community in responding to COVID-19 (www.who.int). In December 2019, a cluster of patients with pneumonia of unknown cause was linked to a seafood wholesale market in Wuhan, China. A previously unknown betacoronavirus was discovered through the use of unbiased sequencing in samples from patients with pneumonia. Human airway epithelial cells were used to isolate a novel coronavirus, named 2019-nCoV, which formed a clade within the subgenus sarbecovirus, Orthocoronavirinae subfamily. Different from both MERS-CoV and SARS-CoV, 2019-nCoV is the seventh member of the family of coronaviruses that infect humans. Enhanced surveillance and further investigation are ongoing. Coronaviruses are enveloped non-segmented positive-sense RNA viruses belonging to the family Coronaviridae and the order Nidovirales and broadly distributed in humans and other mammals. Although most human coronavirus infections are mild, the epidemics of the two betacoronaviruses, severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), have caused more than 10,000 cumulative cases in the past two decades, with mortality rates of 10% for SARS-CoV and 37% for MERS-CoV. The coronaviruses already identified might only be the tip of the iceberg, with potentially more novel and severe zoonotic events to be revealed.
In December 2019, a series of pneumonia cases of unknown cause emerged in Wuhan, Hubei, China, with clinical presentations greatly resembling viral pneumonia. Deep sequencing analysis from lower respiratory tract samples indicated a novel coronavirus, which was named 2019 novel coronavirus (2019-nCoV). Thus far, more than 800 confirmed cases, including in health-care workers, have been identified in Wuhan, and several exported cases have been confirmed in other provinces in China, and in Thailand, Japan, South Korea, and the USA (Huang et al. 2020).

We suppose that method described in this study may be useful to study other types of viruses including 2019-nCoV.

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