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ViriChip: a solid phase assay for detection and identification of viruses by atomic force microscopy

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Abstract

Bionanotechnology can be viewed as the integration of tools and concepts in nanotechnology with the attributes of biomolecules. We report here on an atomic force microscopy–immunosensor assay (AFMIA) that couples AFM with solid phase affinity capture of biological entities for the rapid detection and identification of group B coxsackievirus particles. Virus identification is based on type-specific immunocapture and the morphological properties of the captured viruses as obtained by the AFM. Representatives of the six group B coxsackieviruses have been specifically captured from 1 μ l volumes of clarified cell lysates, body fluids and environmental samples. Concentration and kinetic profiles for capture indicate that detection is possible at 10³ TCID₅₀ μ l⁻¹ and the dynamic range of the assay spans three logs. The results demonstrate that the melding of a nanotechnological tool (AFM) with biotechnology (solid phase immunocapture of virus particles) can create a clinically relevant platform, useful for the detection and identification of enterovirus particles in a variety of samples.

1. Introduction

The detection of a pathogen's antigen by immunoassay, or genomic fragments by polymerase chain reaction (PCR) procedures in clinical and other samples is taken to indicate the presence of the infectious agent. However, without other data such as infectivity studies or electron microscopic analysis, it cannot be concluded that pathogens are present in the samples. For example, *Chlamydia trachomatis* DNA is present in urine of humans for up to two weeks after the infectious agent has been cleared by antibiotic treatment [1]. Viral RNA of measles may be found in brain tissue years after the paramyxovirus has been cleared from extra-neural sites [2, 3]. Post-polio syndrome has been associated with the finding of viral nucleic acids but no infectious virus particles [4]. In occult type

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B hepatitis infections, wherein infectious virus and genomic materials can be detected, surface antigens of the hepadnavirus may not be detected in blood [5].

Methods are needed for the direct visualization of virus particles in virus detection assays. Currently, the only standard methods by which viral particles are directly detected are scanning and transmission electron microscopy (EM), with or without immune enhancement [6]. These methods have led to the discovery of a number of new viruses including the Norwalk-like caliciviruses and the rotaviruses that cause human gastroenteritis [7–10]. However, EM procedures remain somewhat cumbersome, time consuming and dependent upon expensive equipment. Furthermore, staining and metal coatings destroy the biological function of the particles and often prevent further analyses of the material by immunological or genomic (PCR) means. Despite these

drawbacks, electron microscopy remains a benchmark assay for the detection of non-cultivatable viruses and EM has been proposed as a rapid diagnostic for infectious agents in emergent situations [11].

We have exploited the rapid, label-free surface characterizing capabilities of the AFM [12] to create a technology platform for direct detection and characterization of viral particles [13]. The affinity substrate used in these studies is termed the 'ViriChip'. The ViriChip contains type-specific antibody domains capable of capturing viruses [13]. The integration of AFM with the ViriChip has resulted in the development of an atomic force microscopyimmunoassay (AFMIA). The AFMIA combines two key features: specificity determined by antibody capture, and a label-free AFM readout that offers the additional benefit of providing topographical/morphological information to corroborate affinity-based virus identification.

In this paper, we describe the development of the AFM-immunoassay (AFMIA) system for the rapid detection and identification of all six types (B1-B6) of the group Coxsackievirus infections cause a B coxsackieviruses. wide range of serious illnesses including acute, transient, flaccid paralysis, pleurodynia, epidemic myalgia, aseptic meningitis, newborn enteroviral disease, hand-foot-and-mouth disease, pancreatitis, myocarditis and pericarditis [14-16]. The group B coxsackieviruses are aetiologically associated with inflammatory cardiomyopathies and pancreatitis with an estimated 20%-25% of all such cases having evidence of group B coxsackievirus infection [15]. Also, the group B coxackieviruses, with the exception of coxsackievirus B6, have been among the 15 most commonly reported enterovirus serotypes in the USA between 1993 and 2001 [17-19]. With the discovery of new anti-enteroviral drugs such as pleconaril, rapid diagnosis of enterovirus infections can potentially save lives [20, 21].

The methodology described herein is a melding of biotechnology (immunocapture) and nanotechnology (AFM) to create a first line of action in detecting, identifying and characterizing viral particles. The methodology was found to maintain the integrity and infectivity of the viral particles, so that the results could be confirmed by follow-up studies using established methods. The AFMIA could play a major role in the rapid detection of viruses in epidemiological, clinical, environmental and biodefence contexts.

2. Experimental arrangement

2.1. Substrate preparation

Substrates were prepared from polished silicon wafers cut into 4 mm squares. The squares were ultrasonically cleaned in water and in absolute ethanol (30 min each). The polished surface of each substrate was sputter coated with 5 nm of chromium and 10 nm of gold using a dual-gun, ion beam sputterer operating at 4 mA and 7 keV (IBC 2000, South Bay Technologies, CA). Target areas (600 μ m diameter) were created using copper EM grids (Electron Microscopy Sciences) as masks during sputtering. The gold-coated, patterned substrates were removed from the sputterer and immediately immersed in a freshly prepared alkanethiolate solution to allow

self-assembling monolayer (SAM) formation. Amine-reactive surfaces were created by incubating the patterned gold surfaces in 0.5 mM solutions of dithiobis-succinimidyl undecanoate (DSU) (Dojindo, Japan) in 1,4-dioxane (Sigma, MO) for 3 h in sealed jars at room temperature. The substrates were rinsed in 1,4-dioxane and blown dry with dry argon. The target areas were covered with recombinant protein A/G (Pierce, IL) at 1 mg ml⁻¹ in PBS (10 mM phosphate buffer, 137 mM NaCl and 2.7 mM KCl) and allowed to react for 60 min at room temperature. The substrates were immersed in Viriblock, a non-specific blocking reagent (BioForce Nanosciences, IA), for 30 min to block unreacted succinimide groups. Each chip was rinsed in deionized, 0.2 μ m filtered water, blown dry with argon and stored at -20 °C until used.

2.2. Anti-viral antibodies

Monoclonal antibodies against the six types of group B coxsackievirus were purchased from Chemicon International (CA). Anti-coxsackievirus B1 (MAB944, lot 22051058) would neutralize CB1 Conn 1 strain at a titre of less than Anti-coxsackievirus B2 (MAB946, lot 068CBD) 1:16. would neutralize CB2 Ohio-1 at a titre of 1:24 000. Anticoxsackievirus B3 (MAB948, lot 21080575) would neutralize CB3 Nancy strain at a titre of 1:6000. Anti-coxsackievirus B4 (MAB941, lot 21011469) would neutralize CB4 JVB strain at a titre of 1:524 000. Anti-coxsackievirus B5 (MAB943, lot 20020331) would neutralize CB5 Faulkner strain at a titre of less than 1:16 and anti-coxsackievirus B6 (MAB945, lot 19050600) would neutralize CB6 Schmitt strain at a titre of less than 1:16. Anti-CB1, 3, 5 and 6 were obtained as purified proteins without added carrier bovine serum albumin (BSA) while the antibodies against B2 (MAB946) and B4 (MAB941) were purchased as ascites. The antibodies against B2 and B4 were further purified using a protein A/G column according to the instructions of the manufacturer (Pierce, IL). The concentrations of all antibodies were determined by BCA protein assay (Sigma, MO) using BSA as the standard. All antibodies were stored under conditions recommended by the manufacturers.

2.3. ViriChip construction

The virus chip, 'ViriChip', was constructed by placing 1 μ l of the anti-viral antibody at 0.5 mg ml⁻¹ in PBS on the protein A/G domain of a substrate at room temperature. The substrates with the antibody droplet were incubated for 60 min on wet filter paper in a sealed Petri dish to facilitate antibody binding. Unbound antibody was removed by directing a stream of filtered, distilled water over the chip surface. It was not necessary to block the antibody-free protein A/G surface since these viruses did not bind to A/G surfaces. The prepared ViriChips were washed in filtered, deionized water and dried in a stream of dry argon and either used immediately or stored for up to 60 days at -20 °C.

2.4. Virus and cells

Infectious virus stocks were obtained from the American tissue culture collection—CB1 (VR-1032) strain Conn-5, CB2 (VR-29) strain Ohio-1, CB3 (VR-30) strain Nancy, CB4 (VR-18)

strain J.V.B.-Benschoten, CB5 (VR-185) strain Faulkner and CB6 (VR-1037) strain Schmitt 1-15-21. Virus stocks were prepared by infecting buffalo green monkey kidney (BGMK) cells in either T-flasks or Blake bottles at a multiplicity of infection of approximately three after they had reached approximately 80% of confluency. Cells were maintained in RPMI-1640 medium supplemented with 7.5% foetal bovine serum (Gibco, NY) and antibiotics. Cell passaging was done at near confluency using a 0.25% trypsin-EDTA mixture (Gibco, NY). Lysates were collected 24-36 h post-infection and viruses were separated from lysed cells and debris by centrifugation and filtration $[5000 \times g, 15 \text{ min}, 4 \circ \text{C}; 0.2 \,\mu\text{m}$ polyethersulfone membranes (Corning Costar, NY)]. The viruses were collected by centrifugation (140 000 \times g, 3 h at 4 °C). The viruscontaining pellets were dislodged from the tubes by soaking in TEN buffer (10 mM Tris HCl, pH 7.8, 2 mM EDTA and 100 mM NaCl) for 12 h at 4 °C. The pellets were resuspended in TEN buffer by mixing and brief sonication at low power using a bath-type sonicator. The resuspended virus preparation was centrifuged $(14\,000 \times g, 2 \min, 4 \,^{\circ}\text{C})$ to remove residual debris and any large aggregates of virus and virus containing debris. Each virus preparation was divided into aliquots and stored at -80 °C. Virus titres, usually 10^{10} -10^{11} TCID50 ml⁻¹ (tissue culture infectious dose ml⁻¹), were determined in triplicate by the endpoint dilution method using BGMK host cells [22].

2.5. Standard assay

Experimental samples and controls in Viriblock were brought to room temperature and a small volume $(1 \ \mu l)$ of each was applied onto the antibody-coated domains. The ViriChip was incubated without mixing or agitation at room temperature in a humid environment for 60 min. At the end of the adsorption period, ViriChip surfaces were rinsed with a 3–5 s stream of deionized, filtered water from a wash bottle and rapidly blown dry under a stream of argon. Chips were mounted on metal discs and imaged by AFM under ambient conditions.

2.6. AFM Imaging

A dimension 3100 AFM from Veeco-Digital Instruments (Santa Barbara, CA) was utilized for all topography measurements. The ViriChips were imaged in TappingTM mode using silicon Ultralevers (Veeco) under ambient conditions. Scan size was set to $25 \,\mu m^2$. The scan size was determined based on the virus dimensions and the pixel density of data capture. We found that the maximal surface area of a single scan that would provide adequate morphological information on the viruses at the captured pixel density of 512×512 was $25 \,\mu m^2$.

2.7. Experimental samples

Grab samples of primary sludge were obtained from the Des Moines Wastewater treatment plant. Sputum and urine were obtained from human volunteers. CB3 was inoculated into the crude samples (10⁸ TCID50 ml⁻¹) and incubated at room temperature with continuous mixing for 30 min. Virus was separated from particulates and other components of samples using a previously described extraction protocol [22]. Briefly, each sample and uninoculated control was adjusted to 100 mM Tris HCl, pH 7.5, 0.2 M NaCl, 5% (vol/vol) glycerol and 10% foetal bovine serum, mixed using a Vortex Jr. for 2 min and centrifuged ($10\,000 \times g$; 5 min, 4 °C). Samples of the supernatants were prepared in Viriblock and applied to a ViriChip using the standard assay protocol.

2.8. Data analysis

Each experiment was repeated at least three times and five fields of 25 μ m² (a dataset) were imaged on each chip for each data point. The numbers of virus particles in each of the fields were counted and the mean and standard deviations were calculated utilizing the data from all respective determinations. Virus detection was defined as one or more virus particles observed in each image of a dataset. The limit of detection was defined as the concentration at which at least one virus particle was observed in every image of a dataset. Points derived from a dataset were plotted using Sigmaplot (SPSS Inc.).

3. Results

ViriChips were constructed with 600 μ m diameter antibody domains against a single virus type on each chip. These ViriChips were individually exposed to each of the six group B coxsackieviruses as described in the protocol of the standard assay. Each of the six group B coxsackievirus types bound extensively to its specific ViriChip (figure 1) with little or no binding observed on the non-specific chips (figure 2). Although the same concentration of virus was applied to each of the ViriChips, the number of virus particles binding on the specific homologous chips varied greatly. Under the conditions used in this assay, 100 (CB1) to 2500 (CB4) particles bound to the specific homologous antibody surface while fewer than ten particles were observed attached to the chips with nonhomologous antibody. The viral particles could be readily identified by their distinct morphology and the uniformity of the particle shapes and sizes. Coxsackievirus particles could also be easily differentiated by this method from other particulates occasionally seen on surfaces. No virus binding was observed in the antibody-free regions of the chip.

CB1, CB4 and CB6 were used to optimize the assay conditions and determine the limits of detection. In concentration dependent kinetic studies, 1 μ l of each serial dilution of each of these viruses was exposed to the corresponding specific ViriChip. The binding kinetics as a function of concentration were linear, with a tenfold increment in virus concentration resulting in a tenfold increase in the number of virus particles captured within a range of 10^3 TCID₅₀– 10^7 TCID₅₀ applied to the assay surface (figure 3). At higher concentrations of virus, coverage of antibodies and spatial limitations on the capture surface limited the number of particles captured. The neutralization titre of the antibody also seemed to correlate with the capture profile of the antibodies. The antibody against CB4 had a higher neutralization titre than anti-CB1 and anti-CB6, which could be responsible for the larger number of viruses being captured by the anti-CB4 surface. Also, saturation binding on anti-CB4 chips was reached at a lower virus concentration than was observed on anti-CB1 chips or anti-CB6 chips. Under the conditions used, the AFMIA for CB4 had a detection limit of



Figure 1. AFM images of group B coxsackieviruses (CB) on ViriChips with homologous antibody. Six groups of ViriChips (seven chips in each group) were constructed. Each group had a specific group B coxackievirus antibody immobilized. Individual ViriChips within each group were incubated under standard conditions with one of the CB preparations at 10^6 TCID50 μ l⁻¹ in Viriblock. The seventh chip in each group was a virus negative, Viriblock control. Representative images of the CB viruses binding to their homologous antibodies are presented. Top (left), CB1 on anti-CB1; top (middle) CB2 on anti-CB2; top (right) CB3 on anti-CB3; bottom (left) CB4 on anti-CB4; bottom (middle) CB5 on anti-CB5; bottom (right) CB6 on anti-CB6. Virus particles were identified by their height and relative dimensions. Images of non-homologous pairs and controls are not presented.



Figure 2. Specificity of group B coxsackievirus capture on ViriChips. The 6×6 matrix of homologous and non-homologous chips was analysed and the number of virus particles bound to each $25 \ \mu\text{m}^2$ field was obtained. The mean virus particle count was calculated and plotted. A display scale was chosen to optimize graphic representation of relative particle counts for all samples, and resulted in truncation of the CB4 data (~2500 particles) at the 500-particle maximum for the graph.

 $10^3 \text{ TCID}_{50} \mu l^{-1}$; the other viruses were detected at a minimum level of $10^4 \text{ TCID}_{50} \mu l^{-1}$.

The capacity of the AFMIA to capture and identify coxsackieviruses in body fluids and environmental samples



Figure 3. Effect of virus concentration on binding to ViriChips. ViriChips with antibodies to CB1, CB4 and CB6 were prepared as in section 2. The reported neutralization titres were less than 1:16, 1:524 000 and less than 1:16, respectively. Twofold dilutions of each of the virus stocks were prepared in Viriblock and applied to ViriChips under standard conditions. The number of virus particles bound to each 25 μ m² field was counted and the means calculated.

was examined. CB3 in urine, sputum and primary sludge were exposed to anti-CB3 ViriChips as described in materials and methods. The ViriChip captured the specific virus type from each of the different samples (figure 4). In a single extraction protocol, we could detect 32%, 26% and 108% of the virus particles from primary sludge, sputum and urine, respectively, relative to the control samples in PBS. The level of contaminants and particulates in these samples did not significantly interfere with the capture of virus onto the surface.



Figure 4. ViriChip immunosensor assay for CB3 in primary sludge, sputum and urine. A CB3 stock was added to grab samples of primary sludge, sputum, urine and control phosphate-buffered saline (0.1 ml) each to a titre of 10^5 TCID50 μ l⁻¹. The mixtures were incubated for 30 min at room temperature following thorough mixing. The mixtures were brought to 1× with respect to virus extraction buffer, mixed for 2 min on a vortex mixer and centrifuged at $10000 \times g$ for 5 min at 4°C. The supernatant fractions were collected and samples were diluted twofold into Viriblock and applied to anti-CB3 ViriChips under standard assay conditions. Following capture the ViriChips were imaged and virus particles were counted. Left-hand image—CB3 extract from primary sludge; middle left-hand image—CB3 extract from sputum; middle right-hand image—CB3 extract from urine; right-hand image—CB3 extract from phosphate-buffered saline.

Very little debris was observed by AFM on these surfaces and such debris was easily distinguished from the virus particles.

4. Discussion

4.1. Coxsackievirus B ViriChip

We report the development of an immunocapture assay, the AFMIA, using type-specific antibodies for each of the group B coxsackieviruses and label-free readout by AFM. With this assay we have demonstrated the detection and identification of the group B coxsackievirus particles from environmental samples and medically relevant materials. This AFM immunocapture assay uses a solid phase affinity substrate, termed the 'ViriChip', containing a virus-specific antibody capture domain. ViriChips were exposed to samples and read by AFM without molecular or biological amplification. The nature and proper orientation of the antibodies used permitted the capture of specific whole viral particles. Conditions of the assay were optimized with respect to construction of the chip and protocol for the assay. The AFMIA was found to be sensitive (10³ TCID₅₀ μ l⁻¹), specific in that viruses were captured only by homologous antibodies on ViriChips and, in its current format, had a dynamic range of three logs.

The AFMIA for the group B coxsackieviruses uses typespecific monoclonal antibodies that have been produced using strains of viruses recognized as being prototypical for each type. Whereas these antibodies have little or no crossreactivity with heterologous coxsackieviruses, they cannot be expected to capture all of the strains within a particular type and group. Thus, using these antibodies for group B coxsackieviruses may limit the AFMIA's utility with respect to strains that are closely related to the prototypical strains. Group-specific capture antibodies for enteroviruses have not been reported but several studies have described neutralization monoclonals with group specificity [23-25]. Peptide mapping suggests that sequences near the amino terminus of VP1 are responsible for this group specificity [25]. Peptides constructed from these sequences may lead to hybridomas secreting group capture monoclonals. Nonetheless, at present, the primary limitation to expanding the assay to capture all the strains is the availability of capture

antibodies with either group or type specificity. The creation of arrays consisting of multiple type-specific capture antibodies on a ViriChip is another approach to capture multiple strains on a single chip. The construction of arrays of type-specific capture antibodies for a group of viruses or the viruses known to cause a particular syndrome or disease may lead to the rapid identification of disease aetiologies or exclusion of agents from further consideration.

4.2. AFMIA performance

The importance of the AFMIA stems from its ability to detect whole virus particles and identify them using two integrated sets of criteria without damaging the virus or its genome. First, the dimensional properties of the captured particles as determined by AFM should be in agreement with those for the *family* of viruses suspected, and second, the capture by a typespecific antibody indicates positive type-specific interaction. The combination of these properties should permit placement of a virus within a *family*, sub-family and a serotype depending on the nature of the epitope recognized by the capture antibody.

Currently, the AFMIA has a sensitivity of 10^3 TCID₅₀ μ l⁻¹. In contrast, ELISA-based detection using the same antibodies was reported by the manufacturer to have a detection limit of only 10⁷ plaque forming units. Furthermore, although RT-PCR is reportedly more sensitive (~0.1 TCID₅₀), RT-PCR measures only the presence of RNA and provides no information as to the bioinfectivity in the sample or whether there are even virus particles present [26, 27]. Also, the presence of enterovirus RNA in water samples infrequently correlates to the same endpoint with bioinfectivity studies and brings into question the relevancy of RT-PCR data from a public health standpoint [26, 28].

Electron microscopy (EM) is the only other technique that can detect viral particles. The sensitivity of EM to directly detect virus particles is 10^7-10^8 particles ml⁻¹, that is usually sufficient to allow for successful diagnosis of herpesvirus, poxvirus and some gastroenteric infections [29]. EM detection requires negative or positive staining or metal coatings of viruses that destroy the usefulness of virus particles for any further analyses. Direct immunoelectron microscopy, which is an improvement over EM, has a sensitivity of 10^6 particles ml⁻¹ [30]. Use of gold-labelled secondary antibodies in indirect IEM can increase the sensitivity but the procedure detects not only the complete virus particles but also the free viral proteins and fragments of virus particles. The most direct comparison of AFMIA is to IEM, both of which have similar sensitivities. The sensitivity of any immunoassay is highly dependent on the properties of the antibodies.

4.3. AFM in bioanalysis

The atomic force microscope [12] is a versatile analytical tool that is largely unexploited in the life sciences. It is well suited to analyses of structure and interactions of microorganisms and viruses. The Z-sensitivity of a typical AFM is in the ångström range, more than sufficient for virus morphology resolution and even protein-protein interaction screening. Thus, in its most fundamental operational mode, topographical imaging, the AFM offers a methodology for rapid detection and morphological characterization of micron and sub-micron scale pathogens. AFM has previously been used to obtain high-resolution images of viruses that have been immobilized on a variety of surfaces such as glass, mica, silicon and Langmuir-Blodgett films [31-33]. Atomic force microscopy permits discrimination among viruses based on their shape and size [31]. Moreover, from high-resolution AFM images, the viral capsomer packing patterns can be observed and triangulation numbers deduced [31]. This facilitates discrimination between viruses of similar sizes. However, this level of resolution is not required for the type of application described here.

Key advantages of the AFM approach include label-free detection, operation in biological liquids, potential retention of sample biological activity for subsequent bio-testing (e.g., infection of cell cultures) and extremely accurate height measurement as a corroborating diagnostic tool. The latter capability is useful because many pathogens, such as viruses, have well defined heights with little variation from particle to particle. With respect to these advantages, we have successfully eluted coxsackievirus particles from imaged ViriChip and infected green monkey kidney cells (data not shown). We have also used ViriChips, post AFM imaging, to deliver template coxackievirus RNA to RT-PCR reactions [34] and we have used the AFMIA in conjunction with infectivity analysis to determine the total to infectious particle ratio for preparations of these viruses.

4.4. Future directions

We believe that more extensive optimization of the AFMIA will include utilization of protein microarraying, microfluidics and newer AFM technology. Using protein micro- and/or nanoarraying [35] technology, it will be possible to create multiplexed ViriChips containing logical ensembles of tests. Also, based on the phenomenon of analyte harvesting [36–38], a reduction of the antibody domain size to 2–5 μ m should increase the sensitivity of the assay. Further, we have found that the efficiency of virus capture increases dramatically with decreased sample volume (data not shown). Thus, by incorporating a microfluidic sample delivery methodology to the ViriChip, it will be possible to precisely deliver the samples

to the capture domains and increase the efficiency of capture. The most recent generation of research grade AFMs have significantly improved scan rates, thereby reducing the data capture time for a ViriChip to about 30 s [39]. Moreover, commercial AFMs exist that are the size of a teacup and can be carried in a briefcase (e.g., EasyScan, NanoSurf, Switzerland). Applications for AFM in the life sciences such as AFMIA should motivate AFM manufacturers to re-think the AFM format and create portable and inexpensive AFMs for clinical and field-based biodetection and analysis.

5. Conclusions

We have developed a sensitive, solid phase immunoaffinity assay, AFMIA, by combining a nanotechnological tool, AFM, with a biotechnological method, immunoaffinity capture. This particular assay for coxsackievirus B may prove useful for diagnosis and identification of virus particles that are significant in the aetiology of viral myo/pericarditis, cardiomyopathies and pancreatitis. From a broader perspective this type of approach only begins to tap the potential of the AFM to become an integrated element of new, exciting and powerful bioanalytical and biodiagnostic systems.

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