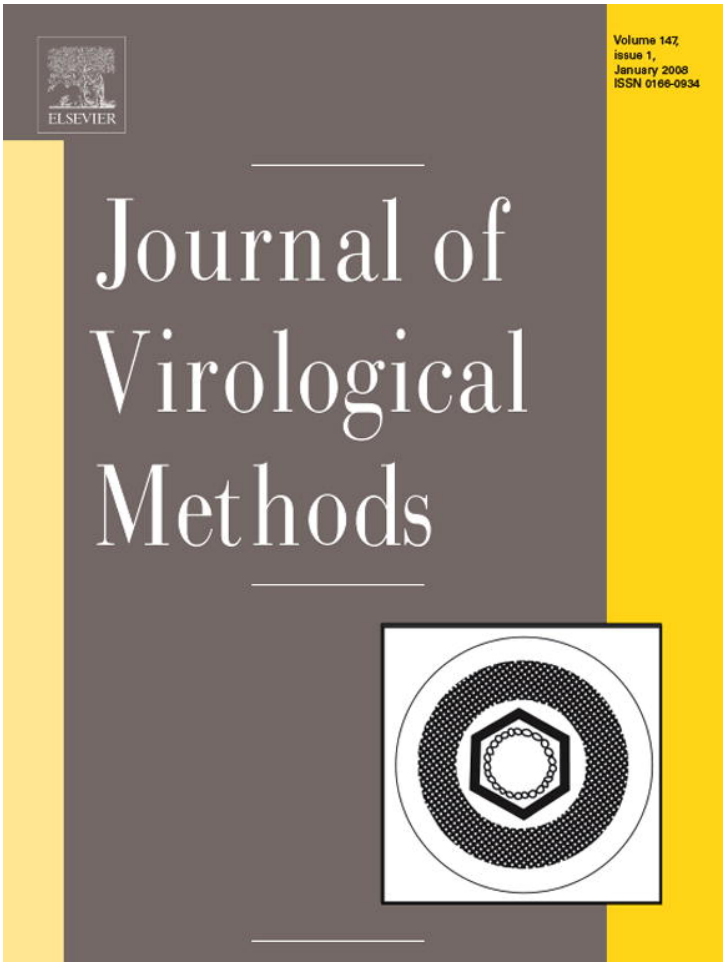

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Use of surface plasmon resonance imaging to study viral RNA:protein interactions

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Abstract

Surface plasmon resonance imaging (SPRi) is an emerging microarray technology that is label-free, rapid and extremely flexible. Here the capabilities of SPRi are demonstrated in results of proof-of-concept experiments detailing a method for studying viral genomic RNA:protein interactions in array format. The principal RNA is the well-characterized origin of assembly (OAS) containing region of Tobacco mosaic virus (TMV) RNA, whereas the principal protein is the primary subunit for TMV virion assembly, the 20S capsid protein aggregate. DNA probes complementary to TMV and non-TMV RNA fragments were covalently attached to a thin gold layer deposited on glass. These DNA probes were used to discreetly capture *in vitro* transcribed TMV and Red clover necrotic mosaic virus (RCNMV) RNA2 (used as a negative control for the subsequent protein binding). The 4S TMV capsid protein monomers were isolated from TMV particles purified from infected plants of *Nicotiana tabacum* L. and were induced to form 20S stacked disc aggregates. These 20S stacked disc aggregates were then injected onto the array containing the RNA fragments captured by the DNA probes immobilized on the microarray surface. The discrete and preferential binding of the 20S stacked disc aggregates to the array locations containing the TMV OAS RNA sequence was observed. The results demonstrate that SPRi can be used to monitor binding of large RNA molecules to immobilized DNA capture probes which can then be used to monitor the subsequent binding of complex protein structures to the RNA molecules in a single real-time, label-free microarray experiment. The results further demonstrate that SPRi can distinguish between RNA species that have or do not have an origin of assembly sequence specific for a particular viral capsid protein or protein complex. The broader implications of these results in virology research are found in other systems where the research goals include characterizing the specificity and kinetics of viral or host protein or protein complex interactions with viral nucleic acids.

Keywords: Surface plasmon resonance imaging (SPRi); Microarray; Viral assembly; Tobacco mosaic virus (TMV)

1. Introduction

The interactions between viral genomes and certain viral and host proteins are central to the replication and transcription strategy of all known viruses. Tools enabling researchers to study the interactions between viral nucleic acid and proteins more efficiently can accelerate basic research as well as the development of effective management and treatment strategies against viruses.

Surface plasmon resonance imaging (SPRi) is a label-free microarray technology that yields results in real-time (Brockman et al., 2000). SPRi microarrays, or "biochips", have been used to measure interactions between DNA and DNA (Frutos et al., 1997), DNA and protein (Maillart et al., 2004; Wegner et al., 2003), DNA and RNA (Nelson et al., 2001, 2002), protein and protein (Wegner et al., 2003), peptide and protein (Wegner et al., 2002), protein and carbohydrate (Smith et al., 2003) and provided the instrument contains the necessary modifications, binding kinetics (Wegner et al., 2004). These experiments have used custom-fabricated microarrays containing DNA, protein, or peptides as the surface-immobilized capture probes. Coupling such a broad range of capabilities with the ability to custom-fabricate arrays that meet the specific needs of the researcher make SPRi a flexible technology and an ideal tool for the virology research laboratory.

Surface plasmon resonance (SPR) reflectivity measurements are surface-sensitive, spectroscopic methods that detect changes in the thickness and/or index of refraction of ultrathin organic

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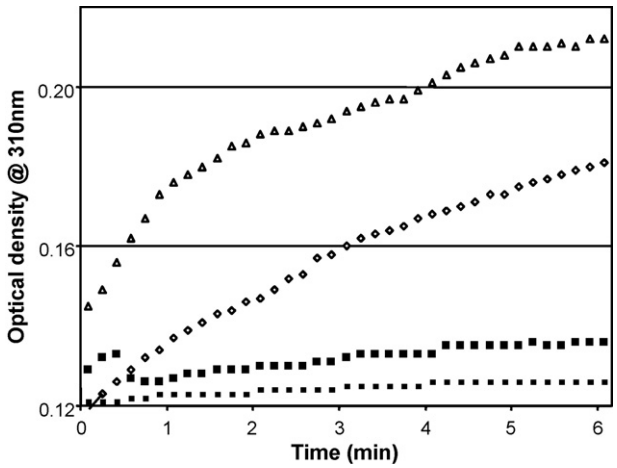

Fig. 1. *In vitro* reassembly of TMV and non-TMV RNA fragments with TMV 20S stacked disc aggregate as measured by monitoring the increase in turbidity (light scattering) at 310 nm. Ten micrograms of each RNA fragment were mixed with 800 μg of the protein solution and measurements were taken every 10 s for 6 min. TMV RNA full length (◊), TMV RNA OAS+ (△), TMV RNA OAS− (■) and RCNMV RNA2 (▬).

and biopolymer films on metal (Au, Ag, Cu) surfaces (Brockman et al., 2000). The SPRi instrument used to collect measurements was manufactured by GWC Technologies (Madison, WI, www.gwctechnologies.com) (see Fig. 1). For a detailed description of the underlying theory, instrument configuration and how the biochip is fabricated and SPR imaging measurements are taken from them, please see Brockman et al. (2000).

Here, the utility of SPRi was demonstrated by testing its ability to distinguish between RNA species that have or do not have the TMV OAS sequence (Zimmern and Butler, 1977). The immobilized DNA probes were used to array TMV and non-TMV RNA fragments that were then probed with a solution of 20S stacked disc aggregates of the TMV capsid protein, prepared as described (Durham, 1972).

2. Materials and methods

2.1. DNA capture probe preparation

In designing the DNA capture probes, the free energy (ΔG) minimization program contained in the RNAstructure™ program version 3.7, was used to predict the secondary structure of the RNA targets that were synthesized *in vitro* (see Section 2.4). The RNAstructure™ program is freeware designed by David H. Matthews, Michael Zuker and Douglas H. Turner and is available for download at http://rna.chem.rochester.edu/RNAstructure.html. Once the predicted secondary structures of the target RNA molecules were determined, the "oligo walk" feature of the program enabled the selection of DNA capture probe sequences with the most favorable ΔG for target RNA binding. Using this overall ΔG as the selection criterion, the entire length of the target RNA molecules were scanned and the sequences having the most favorable theoretical binding capacity for the target RNAs were selected as potential DNA capture probes to be screened.

The best DNA capture probe for each target RNA was synthesized (Integrated DNA Technologies, Coralville, IA) and modified to incorporate a 15-thymine or adenine spacer arm amended with a 5′-thiol group at the 5′-end of end of the probe sequence (Brockman et al., 1999). The sequence of the DNA capture probe specific for the TMV OAS(+) RNA fragment is 5′-/5Thio/AAA AAA AAA AAA AAA CGT ATG TTG TGT GGA ATT GT-3′ whereas the sequence specific for the TMV OAS(−) fragment is 5′-/5Thio/AAA AAA AAA AAA AAA TTG TTA ATG GTT GTT GTT GTT TGT TGT TTG-3′. The DNA capture probe used to capture RCNMV RNA2 was 5′-/5Thio/TTT TTT TTT TTT TTT ATT ATA GTT TTT CTC CGT GC-3′ The spacer arms were designed to reduce steric hindrance when the target binds to the capture probes attached to the array, while the thiol group enabled covalent attachment of the DNA probes to the array (Guo et al., 1994).

The reactive thiol concentration of each probe was determined using 5′,5′-dithiobis(2-nitrobenzoic) acid (Ellman's reagent, Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer's protocol. Probes were ready to be attached to the surface when the molar concentrations of the DNA and reactive thiol were equivalent

2.2. Gold chip preparation

GenTel BioSurfaces, Inc. (Madison WI, www.gentelbio.com) kindly provided the gold substrates, constructed as briefly described here. First, an ultrathin chromium layer (∼1 nm) was vapor-deposited onto one side of high index glass (refractive index = 1.728) substrate to serve as an anchor for the gold. Then, a thin layer (47.5 nm) of pure gold (99.9999% pure) was vapor-deposited onto the chromium layer.

2.3. Array preparation

To prepare multi-component DNA arrays, a seven-step procedure was adapted from a protocol described previously (Brockman et al., 1999). The method aims to maximize the control over the array surface and to enable the sequential construction of a complete surface consisting entirely of robust covalent bonds. The robustness of the entire surface serves to maximize the density of capture probes, minimize non-specific adsorption upon the background regions of the array and to render the array reusable.

2.3.1. Step 1: Creation of an amine-terminated self-assembled monolayer (SAM)

The gold-coated chip (as prepared in Section 2.2) was reacted with a 1 mM solution of 11-mercaptoundecylamine (MUAM), an amine-terminated alkanethiol (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) in absolute ethyl alcohol (EtOH) USP (AAPER Alcohol and Chemical Co., Shelbyville, KY) at room temperature overnight. The surface was then rinsed with EtOH to remove any unreacted MUAM and dried under a N_2 stream. The gold is now uniformly terminated in amine groups which will be differentially targeted to covalently construct the probe-containing and non-specific adsorption resistant background regions of the array.

2.3.2. Step 2: Creation of a reversibly protected hydrophobic surface

The gold-coated chip was then incubated with 3 mM *N*-(9-fluorenylmethyloxycarbonyloxy)succinimide (Fmoc-OSu) (EMD Biosciences, Inc., La Jolla, CA) for 30 min at room temperature to form a reversibly protected hydrophobic surface). The Fmoc-OSu was first dissolved in 300 μL dimethyl sulfoxide (DMSO) (certified ACS) (Fisher Chemicals, Fairlawn, NJ) and then combined with 200 μL of 100 mM triethanolamine (TEA) buffer (pH 7.0) (Sigma–Aldrich, St. Louis, MO) prior to addition to the surface. After the 30-min incubation, the modified surface was rinsed with DMSO to remove any unreacted Fmoc and dried under a N_2 stream. This step results in a uniformly protected, chemically resistant surface. In subsequent steps, the selective removal of the protective groups from precisely defined and controlled regions of the array (hence the term reversible) restores the reactive nature of the surface terminating amine group. This enables layers of discreet surface chemistries to be deposited upon the array in order to covalently attach the DNA capture probes in an ordered array, while the background regions of the array remain inert.

2.3.3. Step 3: Creation of a patterned surface using photolithography

Next, a quartz mask containing a checkerboard pattern of 750 μm × 750 μm square openings was placed over the Fmoc-protected surface and exposed to UV light (mercury/xenon lamp, 400 W for 1 h) (Oriel Instruments, Stratford, CT) to create a photopatterned surface. The UV light passed through the openings in the quartz mask and oxidized the gold–sulfur bonds that anchored the MUAM to the surface of the array. The chip was then rinsed with EtOH to remove the released MUAM-Fmoc from the exposed features and dried under a N_2 stream. The re-exposed bare gold "pads" (now surrounded by the Fmoc-protected hydrophobic background) were reacted with MUAM as described in Section 2.3.1 (except that the reaction time was no more than 4 h). This resulted in an array of reactive hydrophilic MUAM pads surrounded by a hydrophobic, Fmoc-terminated background. The step serves two purposes, first by creating the checkerboard of hydrophilic squares surrounded by a hydrophobic background, the solutions containing the crosslinker and DNA probes attached in subsequent steps will be confined to the hydrophillic regions defined by the hydrophobic borders. Second, the hydrophilic regions are now receptive to surface chemistry modification leading towards a surface receptive to receiving the thiol-modified DNA capture probes in the next two steps.

2.3.4. Step 4: Immobilization of the heterobifunctional linker used for DNA attachment

Next, a pneumatic picopump (model PV830, World Precision Instruments, Sarasota, FL) was used to place a 1-mM solution of the heterobifunctional linker sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SSMCC) onto the hydrophilic MUAM-reactive pads. The SSMCC contains both an *N*-hydroxy-sulfosuccinimide (NHS) ester and a maleimide functionality. The NHS-ester end of the SSMCC molecule reacts with the free amine groups on the MUAM surface, creating pads terminated in maleimide groups, which are reactive towards thiols (Brockman et al., 1999).

2.3.5. Step 5: Immobilization of the DNA capture probes

To covalently bind the capture probes to the array, the picopump was used to spot a 1-mM solution of each DNA capture probe onto the appropriate SSMCC-modified pad, following the pattern shown in Fig. 2. The spotted array was then incubated in a 28 °C water bath (to prevent evaporation) for 2 h. During the reaction, the thiol on the capture probes formed a covalent maleimide bond with the SSMCC-modified surface. After incubation, the array was rinsed with RNAse-free, diethylpyrocarbonate (DEPC)-treated water and then soaked the chip in 2× SSPE (360 mM NaCl, 20 mM sodium phosphate (pH 7.7)/0.2% SDS) for 1 h to remove excess DNA. The array was again rinsed with RNAse-free, DEPC-treated water and dried under a N_2 stream.

2.3.6. Step 6: Preparation of array background for blocking non-specific adsorption

Next, the Fmoc groups were chemically removed from the background regions of the array during a 15-min exposure to a 1 M solution of Tris(2-aminoethyl)amine (TAEA) (Sigma–Aldrich), in *N*,*N*-dimethyl formamide (DMF) (Fisher Chemicals). After the 15-min incubation, the array was rinsed with RNase-free, DEPC-treated water to remove the released Fmoc groups, thus re-exposing the underlying MUAM monolayer and dried the array under a N_2 stream.

2.3.7. Step 7: Creation of background that resists non-specific adsorption

In the final step of the array fabrication, the MUAM background was reacted with 4 mM methoxypoly(ethylene glycol) propionic acid (mPEG-SPA, MW 2000) (Nektar Therapeutics (formerly Shearwater Polymers), Huntsville, AL) (in 100 mM TEA, pH 8.0), which served to resist the non-specific adsorption of analyte proteins to the array background.

2.4. TMV and RCNMV RNA production

The RNA used in these experiments was transcribed *in vitro* from cDNA clones of TMV and Red clover necrotic mosaic virus (RCNMV) RNA2. Two TMV RNA fragments were generated. The fragment containing the OAS (OAS+, 1422 nucleotides (nts)) included nucleotides 4944–6366 of the viral genome and the fragment not containing the OAS sequence (OAS−, 1527 nts) included nucleotides 80–1607 of the viral genome. These fragments were amplified from the full-length infectious clone pTMV004 (Dawson et al., 1986) using the polymerase chain reaction (PCR) and each PCR product was cloned into pGEM-T using a TA Cloning kit (Promega, Madison, WI) according to the manufacturer's instructions. The TMV OAS+ PCR products were cloned into the SacII/SpeI sites of pGEM-5Zf(+) (Promega). The two subclones, pOAS(+) and pOAS(−), were then linearized with Spe I (New England Biolabs, Beverly, MA) and run-off *in vitro* transcription was performed using the

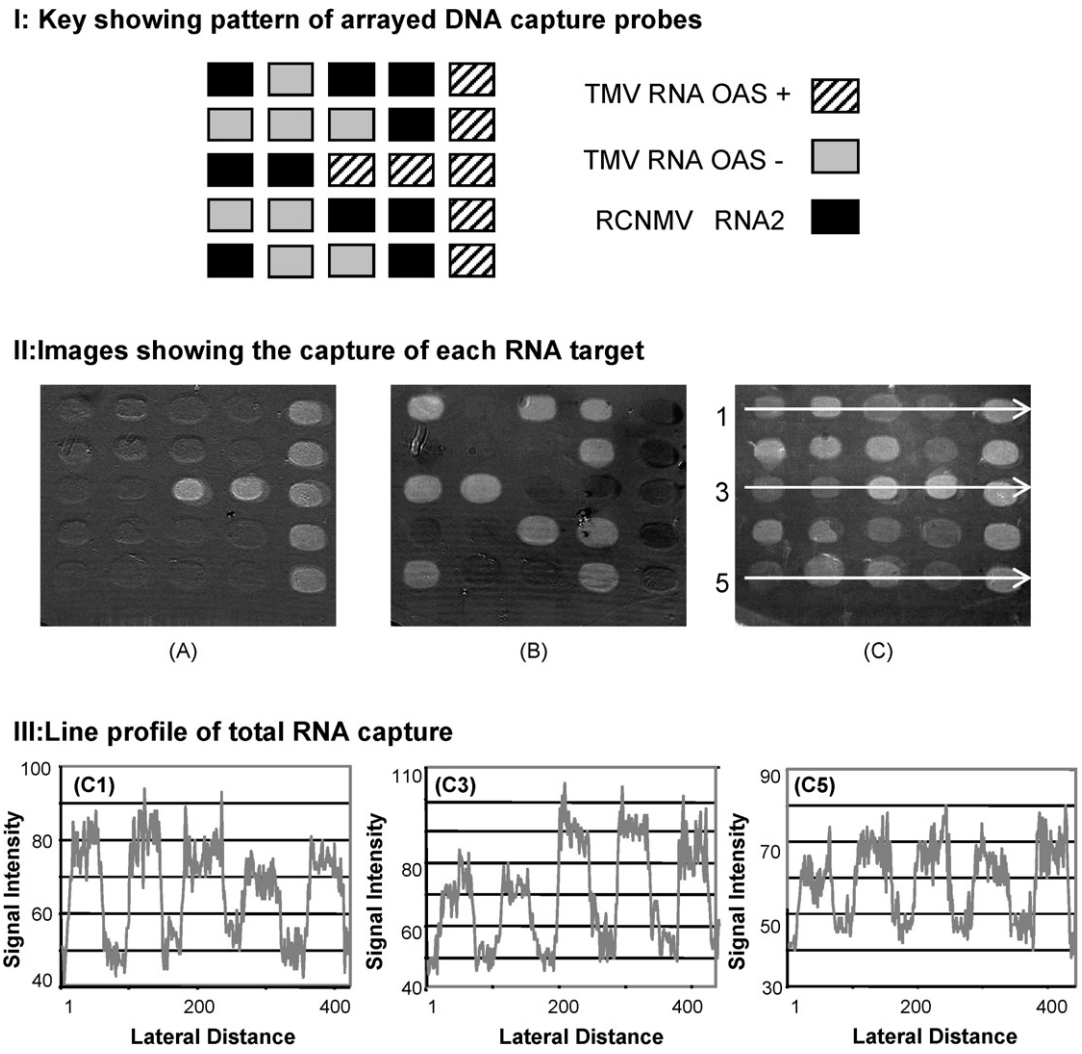

Fig. 2. (I) Pattern of arrayed DNA capture probes, which are complementary to unique regions within each RNA target. (II) SPR difference images showing the capture of (A) TMV RNA OAS+, (B) RCNMV RNA2 and (C) composite image showing capture of all three RNAs prior to protein injection. III. Line profiles across rows 1(C1), 3(C3) and 5(C5) of image (C) in panel II.

Ribomax^{TM} T7 Large Scale RNA Production System (Promega) according to the manufacturer's instructions.

The RCNMV RNA 2 was obtained using pRC2IG (Xiong and Lommel, 1991), a full-length cDNA clone of RNA2 (1402 nts), as the template for run-off *in vitro* transcription. The *in vitro* transcription was performed using the Ribomax^{TM} T7 Large Scale RNA Production System (Promega) after it had been linearized with Sma I (New England Biolabs).

The RNAs were extracted with phenol:chloroform (pH 4.5) and precipitated with 2.5 volumes of EtOH at −20 °C overnight. The RNAs were analyzed by formaldehyde-reducing agarose gel electrophoresis (1%) in 1× MOPS buffer and visualized with ethidium bromide to verify fragment size.

2.5. TMV capsid protein preparation

The protein used in these experiments was derived directly from TMV purified from plants of *N. tabacum* L. cv. Bottom Special inoculated with run-off *in vitro* transcripts of full-length TMV RNA derived from pTMV004. Infected tissue (100 g) was harvested 2 weeks after inoculation and virus was purified following a previously described method (Valverde et al., 1991). The amount of virus was quantified by spectrophotometry at 265 nm, using $\varepsilon_{265}^{0.1\%} = 3.06$ (Boedtker and Simmons, 1958). The virus preparation was tested and shown to be TMV by analysis using ELISA with a commercially available anti-TMV antibody (Agdia Incorporated, Elkhart, IN) and also by its ability to produce classic TMV symptoms upon subsequent re-infection of *N. tabacum* L. plants.

2.5.1. 20S stacked disc aggregate formation

The TMV 20S stacked disc aggregate was produced following a protocol based on that described by Durham in 1972 (Durham, 1972). Briefly, a 1% solution of virus was dialyzed overnight in a 3-mL 10,000 NMWCO Slide-A-Lyzer^{TM} cassette (Pierce) against 100 mM ethanolamine/5 mM HCl buffer (pH 11.0) and then for an additional 4 h against 12 mM Tris/10 mM HCl buffer (pH 7.9 at 25 °C), all at 4 °C. The now-degraded

virus was then subjected to ultracentrifugation at 150,000 × *g* (39,500 rpm) for 1 h (model L8-80M, SW60 rotor, Beckman Coulter, Fullerton, CA). After decanting, the supernatant liquid was loaded onto a 1-mL DEAE cellulose column equilibrated with 120 mM Tris/100 mM HCl buffer (pH 8.3 at 25 °C) at 4 °C. Under these conditions, the RNA was retained on the column, while the protein was recovered in the flow-through. The flow-through (1 mL) was collected, equilibrated to pH 5.0 by dialysis in a 3-mL 10,000 NMWCO Slide-A-Lyzer™ cassette for 2 h against 1 L of 50 mM NaOAc (pH 5.0, ionic strength 100 mM) at 4 °C and stored at 4 °C as a solution of primarily 4S capsid protein monomers. Finally, the method of Turner et al. (1989) was used to catalyze the formation of the 20S stacked disc aggregates. The 4S capsid protein solution was dialyzed in a 3-mL 10,000 NMWCO Slide-A-Lyzer™ cassette (Pierce) against 25 mM sodium phosphate buffer (pH 7.0, ionic strength 100 mM) for 2 h at 4 °C. The protein was transferred to a 2-mL microfuge tube and incubated overnight at 20 °C. The protein concentration was determined by spectrophotometry at 282 nm ($\varepsilon_{282}^{0.1\%} = 1.27$) (Fraenkel-Conrat, 1957) and the maximum/minimum ratio was determined at 282/251 nm to be 2.54, indicating that this procedure yielded a high-quality 4:1 mixture of 20S:4S capsid protein subunits, as described by Durham (1972).

2.6. RNA and 20S stacked disc aggregate validation

The ability of the 20S:4S capsid protein subunits prepared as described in Section 2.5 to encapsidate the RNA fragments produced as described in Section 2.4 were tested by incubating 10 μg of each *in vitro* transcript RNA with 800 μg of the protein complex and monitored for an increase in light scattering at 310 nm (Butler and Klug, 1971). The results of this experiment are shown in Fig. 1.

2.7. SPR imaging of fragment reassembly

2.7.1. Step 1: Flow cell assembly and fitting to the SPR imager

After the capture probes were covalently attached, the functionalized biochip was assembled in the SPR imager (GWC Technologies). A single small drop (~100 μl) of index matching fluid (Series M, Index of Refraction = 1.720) (Cargille Laboratories Inc., Cedar Grove, NJ) was placed on the back of the biochip. The index matching fluid served to couple the biochip optically to the equilateral prism, which was carefully placed onto the drop of index matching fluid to not introduce air bubbles. The assembly was then secured using a clamp. The flow cell was then placed onto the assembly and secured (Fig. 1). After the inlet and outlet hoses were connected to the flow cell, PBS was pumped through the chamber (at 100 μL/min) to facilitate the removal of air from the system. Once the system was primed and all air bubbles removed, the array was allowed to equilibrate in PBS for 5 min. Then, a baseline reference image was collected to be used for subtractive imaging once the target molecules have been adsorbed. GWC Technologies' software was used to manually define the regions of interest (ROI) on the array to be monitored throughout the experiment. Through subtractive imaging, each subsequent image was processed to generate a final image showing hybridization over time as the experiments proceeded. Intensity images were collected and processed using the software package V++ (Digital Optics Ltd., Auckland, New Zealand) provided with the SPR imager and software macros provided by GWC Technologies. The macros automate the subtractive imaging, image collection, frame averaging and numerical output transfer from the previously defined ROIs' into an Excel (Microsoft Corporation, Redmond, WA) spreadsheet format.

2.7.2. Step 2: RNA target treatment and application

Individual RNA targets were sequentially injected onto the array after being treated as follows. Each target RNA was heated (95 °C, 5 min) to partially denature the RNA, cooled on ice for 1 min and then re-equilibrated to room temperature to mitigate temperature effects on imaging. Each RNA target (1 mM, 500 μL in PBS) was then injected onto the biochip at 250 μL/min (see Fig. 2).

2.7.3. Step 3: Application of 20S stacked disc aggregate

Once the RNA molecules were attached to the array, 250 μL of the 20S stacked disc aggregate preparation (2 μg/μL, in PBS) was injected and binding was monitored by collecting images every 10 s for 6 min (see Figs. 3 and 4).

3. Results

3.1. Conventional TMV reassembly assay

The results of the experiment indicated that the neither the TMV OAS− sequence nor the RCNMV RNA became encapsidated by the TMV protein complex, but the TMV OAS+ sequence and the full-length TMV were encapsidated as measured by their increased light scattering (Fig. 1). These results confirmed that the RNA fragments and the 20S stacked disc aggregate solution created for this study functioned as expected according to well-established methods. Because the experiment was carried out using constant mass of each RNA (10 μg), the effective concentration of OAS elements is higher in the smaller TMV OAS+ fragment than the larger full-length TMV RNA. This results in the higher initial rate and total signal for the TMV OAS+ fragment seen in Fig. 1.

3.2. RNA capture on a biochip

The hybridization of the target RNAs to the biochip before capsid protein addition (see Fig. 2) revealed the DNA capture probes worked effectively with high specificity. Cross-hybridization between the probes was not discernable, each probe exhibited high specificity towards its targeted viral RNA. These results showed that SPRi can be used to study binding of large, unlabeled target RNAs (1401, 1422 and 1521 nts for RCNMV RNA2, TMV OAS+ and TMV OAS−, respectively) to covalently attached DNA capture probes without the use of

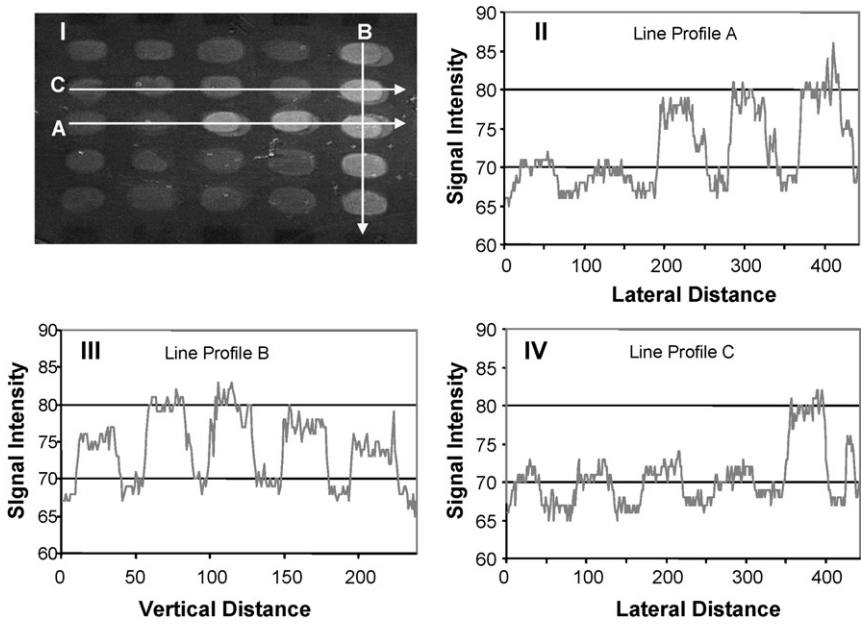

Fig. 3. (I) Final array image after 500 μg of TMV 20S stacked disc aggregate was injected onto the array containing the immobilized RNA and then incubated for 6 min. During the 6-min incubation, images were collected every 10 s to monitor binding. Panels II, III and IV are line profiles of image intensity along the lines A, B and C, respectively, drawn in panel I.

chemicals, such as formamide or high reaction temperatures in the flow cell.

3.3. 20S stacked disc aggregate capture

The binding of the 20S stacked disc aggregate to the appropriate target RNA (TMV RNA OAS+) was readily discernable in the final SPR image (Fig. 3). Line profiles across the image revealed that the features containing TMV RNA OAS+ consistently had higher signal than did the background regions of the array or those containing non-OAS features (Fig. 4). Although there was apparent binding to the non-OAS-containing RNA features of the microarray (Figs. 3 and 4), the TMV OAS+ features clearly exhibited higher signal intensity (Fig. 3) and rate of binding (Fig. 4), as expected. The signal intensities also apparently differed between the TMV RNA OAS+ features; several possible causes for this are discussed in Section 4.

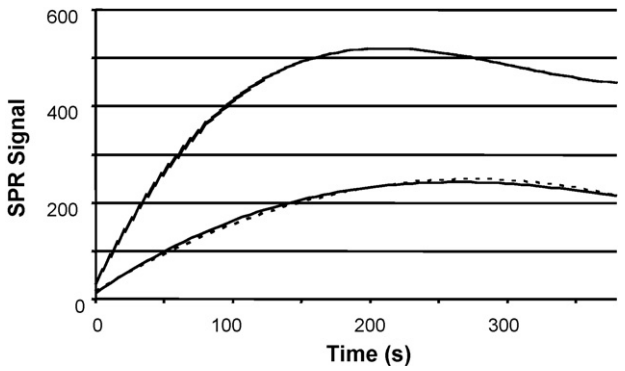

Fig. 4. The real-time tracing of the average signal intensity for TMV RNA OAS+ (▬), TMV RNA OAS− (—) and RCNMV RNA2 (· · ·) after the injection of the TMV 20S stacked disc aggregate onto the array. Data points were collected every 10 s for 6 min.

4. Discussion

SPR imaging is an emerging, label-free microarray technique that offers real-time results for custom-designed microarrays. Here the application of this technique was demonstrated for not only monitoring the arraying of large target RNA molecules but also their subsequent probing in order to identify specific RNA fragments which contain a specific origin of assembly sequence recognized by a viral capsid protein. This demonstration adds DNA:RNA:protein interactions to those applications of SPRi previously described (Maillart et al., 2004; Nelson et al., 2001, 2002; Smith et al., 2003; Thiel et al., 1997; Wegner et al., 2002, 2003, 2004).

The two distinct events monitored chronologically in this experiment —the capture of large RNA fragments followed by the binding of the TMV 20S stacked disc aggregate protein to the appropriate RNA—each presented challenges that merit further discussion.

Three limitations affect using SPRi microarrays to monitor the capture of large RNA fragments. First, SPRi microarrays as constructed here are not resistant to the denaturant formamide, frequently used in northern blots to aid in RNA hybridization; formamide reacts with the amine-terminated monolayer upon which the entire microarray is constructed. Second, SPRi is a temperature-sensitive technique requiring that variations in temperature be held to a minimum for the duration of the experiment to obtain quality data. Thus, elevated temperature, another frequently used technique in northern blot hybridization, currently cannot easily be used in SPRi applications because of the diffi-

culty in precisely maintaining the temperature of the flow cell assembly and the fluid being delivered to it. Finally, although each DNA capture probe was selected to have the lowest free energy of hybridization for its RNA target, the theoretical free energy values differ for each probe-target pair and these differences may have contributed to the variability of RNA capture. This is especially relevant when the RNA is not thoroughly denatured by heat and/or formamide, because the secondary structure for each target can influence hybridization efficiency. These limitations most likely contributed to the different image intensities for the hybridization of each RNA target (Fig. 3), which presumably represent different amounts of RNA capture.

In spite of these limitations, strong RNA binding was obtained by heating the RNA (in the absence of formamide) to 95 °C and quickly cooling it back to room temperature before injecting the RNA onto the array. This result shows that a modified protocol for using elevated temperature can be employed with SPRi and that the restrictions placed on SPRi do not necessarily prevent the successful hybridization of large RNA molecules to the DNA capture probes immobilized on the array surface. Furthermore, it is anticipated that the hybridization of smaller RNA molecules will be less challenging due to their decreased secondary structure.

The capture of the TMV 20S stacked disc protein aggregate also presented several challenges that could affect the amount of protein that can bind to the immobilized RNA target. First, the TMV 20S stacked disc aggregate consists of 34 capsid protein monomers (two 17-monomer discs) and has a molecular weight approaching 600 kDa, making it a large target and potentially introducing steric hindrance that could prevent binding of other 20S stacked disc aggregates to neighboring RNA molecules. After the first 20S stacked disc aggregate binds to the TMV OAS sequence, encapsidation proceeds as the RNA is "pulled" through the elongating virus rod (Klug, 1999). Conceivably, tethering the RNA to the array surface as opposed to allowing it to move freely in solution, as in a conventional reassembly assay, restricts the movement of the RNA and subsequently, the amount of additional TMV 20S stacked disc aggregates binding to the target RNA. Additionally, identical RNAs within the same feature of the array may exert steric hindrance upon each other, resulting in the TMV 20S stacked disc aggregates not having access to all the TMV OAS sequence present. The steric hindrance exerted by the RNA molecules upon each other could be alleviated by reducing the density of RNA within each feature. This approach would increase the likelihood that the initial TMV 20S stacked disc aggregate can reach the target OAS sequence. The binding of one, or a few, TMV 20S stacked disc aggregates to the immobilized RNA would negatively impact SPRi signal intensity and might represent what was observed in these experiments.

In spite of these limitations, the OAS+ features show enhanced signal (Fig. 4) and rate (Fig. 4) for binding TMV 20S stacked disc protein aggregate over the negative control OAS− and RCNMV RNA2 features. Additionally, the final SPR image and line profiles (Fig. 3) exhibit excellent spot uniformity and uniform signal within each feature when considering the complex interactions taking place on the array surface.

5. Conclusions

The work presented here not only adds to the growing list of different interactions that SPRi can monitor, but also constitutes the proof-of-concept for a SPRi RNA microarray. To a virologist studying RNA viruses, SPRi offers a method to efficiently array, and then probe RNA fragments of virtually any size for interactions with proteins of interest. Although probing for encapsidation signals (packaging specificity) was demonstrated here, use of this method for testing hypotheses about the role of viral proteins in processes, such as RNA movement, RNA synthesis and gene silencing are directly implicated.

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