Electronic Supplementary Material (ESI) for Chemical Communications

Detection of viral nucleoprotein binding to anti-influenza aptamers via SERS

Pierre Negri, Andreas Kage, Andreas Nitsche, Dieter Naumann and Richard A. Dluhy

EXPERIMENTAL METHODS

Material and Reagents. (1-Mercaptoundec-11-yl)tetra(ethyleneglycol) (95%) was purchased from Sigma-Aldrich (St. Louis, MO). Molecular biology grade water was purchase from Fisher Scientific. All other chemicals were of analytical grade and used without any further purification.

Preparation of SERS-Active Ag Nanorod Arrays. Ag nanorod array substrates were prepared by an oblique-angle vapor deposition (OAD) technique using a custom-designed electron-beam/sputtering evaporation system.[1, 2] Standard 2.54 × 7.62 cm glass microscope slides (Gold Seal®, Becton Dickinson Company, Franklin Lakes, NJ) were cleaned using Piranha solution (4:1 conc. H_2SO_4: 30% H_2O_2) for 15 minutes, rinsed several times with copious amounts of deionized water and dried under a gentle stream of nitrogen gas. The glass slides were placed into a vapor deposition chamber with background pressure maintained at less than 5×10^{-6} Torr. A 20 nm layer of Ti (99.99%, Kurt J. Lesker Company, Clairton, PA) was deposited onto the glass slide substrate at a rate no greater than 2.0 Å/s. The Ti underlayer serves as an adhesion layer on the glass slide to stabilize the subsequent Ag layers. Next, a 500 nm layer of Ag (>99.99%, Kurt J. Lesker Company) was deposited onto the substrate at a rate of 3.0-4.0 Å/s. Finally, the substrate was rotated to 86° relative to the incident vapor source. Ag nanorods were then deposited at a constant rate of 2.0-3.0 Å/s until a quartz crystal microbalance (QCM) registered a final nominal thickness of 2000 nm. The QCM was placed inside the deposition chamber and positioned at normal incidence to the vapor source. We have established a calibration relationship between nanorod length and the mass of Ag deposited on the quartz crystal where 2000 nm nominal thickness results in tilted nanorods of ~900 nm length. After deposition, the SERS-active substrates were allowed to cool down to room temperature in vacuum inside the deposition chamber for 2 hours. The SERS-active substrates were then removed from the deposition chamber and stored in a nitrogen purge type glove box

inside a Petri dish to avoid any surface contamination or deterioration of the surface condition due to temperature or atmospheric humidity.

These vapor deposition conditions result in optimal SERS substrates with high aspect ratio silver nanorods with overall nanorod lengths of ~900 nm, diameters of ~80-90 nm, densities of ~13 nanorods/μm^2, and a tilt angle of 71° with respect to the substrate normal.[1, 2] The Ag nanorod substrates result in a SERS enhancement factor of $>10^8$ with less than 15% variation in SERS intensity from batch-to-batch.[2] Prior to their use, the SERS-active Ag nanorods were cleaned for 4 minutes in an Ar^+ plasma using a plasma cleaner (Model PDC-32G, Harrick Plasma, Ithaca, NY) to remove any surface contamination.[3]

SERS Micro-Well Array Fabrication. Following nanofabrication of the Ag nanorod substrates, a patterned microwell array was fabricated onto the SERS nanorods for multiplexed sample analysis. This multi-well array was constructed using a polymer molding process in which liquid polydimethylsiloxane (PDMS, Sylgard® 184 Silicone Elastomer Kit, Dow Corning, Midland, MI) is added to a mold and cured by low temperature heating. The patterned substrate was produced according to previously published procedures[4, 5] with slight modifications as follows. A stainless-steel mold/substrate assembly was pre-heated in a bench top oven (Fisher Scientific) at 55°C for 10 minutes prior to the addition of a mixture of PDMS base, curing agent, and accelerator (20:2:1, w/w) through the opening of the substrate/mold assembly. The mixture was allowed to cure on the SERS-active substrate at 55°C for approximately 45 minutes, and cool down to room temperature for about 10 minutes. Finally, the well patterning plate was de-attached gently from the patterned substrate, creating a uniform, 4 × 10 well PDMS-pattern SERS-active micro well array substrate.

Aptamer. The polyvalent 5'-C6-disulfide anti-influenza aptamer was provided by AptaRes AG, Mittenwalde, Germany. The AptaRes polyvalent anti-influenza aptamer used in this study was isolated against the nucleoprotein constituents of a commercially available split-virion inactivated influenza

vaccine using a cell-free, MonoLex™ combinatorial process, as previously described.[6] The single-stranded DNA aptamer consisted of 63 base pairs with a molecular weight of 25,987 Daltons. The as-received lyophilized aptamer was dissolved in 77 µL of molecular biology grade water to make a 100 µM solution.

Aptamer Immobilization on the SERS-Active Ag Nanorod Substrate. The 5' C6 disulfide anti-influenza ssDNA aptamer was immobilized onto the Ag nanorod substrate by addition of 20 µL of 1000 nM of the aptamer solution to a patterned microwell overnight at room temperature to yield a self-assembled monolayer (SAM) of the aptamer. After this time, the aptamer solution was removed from the substrate surface and the microwell was rinsed three times with 20 µL aliquots of molecular biology grade water to remove any unbound material. To minimize non-specific binding of DNA or proteins to the Ag surface, 20 µL of 100 nM of the spacer molecule (1-mercaptoundec-11-yl)tetra(ethyleneglycol), $HS(CH_2)_{11}(OCH_2CH_2)_4OH$, was added to the microwell overnight at room temperature. After incubation, the solution of the spacer molecule was removed from the substrate and the microwell was rinsed three times with 20 µL aliquots of molecular biology grade water to remove any unbound spacer molecules.

Influenza Virus Nucleoprotein Samples. The AptaRes polyvalent influenza aptamer used in this study was isolated against the nucleoprotein constituents of the viruses contained in the commercially available Fluarix™ Influsplit SSW® 2009/2010 split-virion inactivated influenza vaccine (GlaxoSmithKline, GmbH & Co., KG, Munich, Germany). The three monovalent split viruses used in this vaccine are the i) A/Uruguay/716/2007 NYMC X-175C, ii) B/Brisbane/60/2008, and iii) A/Brisbane/59/2007 IVR-148, influenza strains. The Robert Koch Institut (Berlin, Germany) supplied the influenza nucleoproteins from these viruses in the form of purified, whole virus cell lysates. These samples consisted of cell lysates from the three isolated monovalent influenza virus strains contained in the Influsplit SSW® vaccine, and characterized as to their hemagglutinin (HA) content. The sample provided were i) 100 µl of A/Uruguay/716/2007 NYMC X-175C, HA concentration of 172 µg/ml, ii) 100 µl of B/Brisbane/60/2008,

HA concentration of 207 μg/ml, and iii) 100 μl of A/Brisbane/59/2007 IVR-148, HA concentration of 144 μg/ml.

Binding Buffer. The buffer used in the binding experiments was prepared by dissolving 20 mM Tris HCl, 15 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ in molecular biology grade water at pH 7.3 and stored at 4°C. The buffer and working tools were DNase free.

Procedure for the Binding of Influenza Viral Nucleoproteins to the Aptamer-Modified SERS-Active Substrate. Binding of the nucleoproteins of the virus samples to the aptamer-functionalized SERS-sensing surface was accomplished by adding 20 μL of 1 μg/mL HA content of the virus lysate diluted in the binding buffer to the aptamer-modified, Ag nanorod microwell. The substrate was then incubated at 37°C for 8 hours in a humid environment to prevent dehydration of the samples on the substrate surface. This was achieved by depositing small droplets (20 μL) of molecular grade water around the SERS-active substrate in the Petri dish. The humidity was maintained by covering the Petri dish with a Parafilm® sheet and sealing it with the cover of the dish. After incubation, the virus solution was removed from the substrate, and any non-specifically adsorbed virus components were removed by two washes with 20 μL aliquots of binding buffer followed by one final rinsing step with 20 μL aliquot of molecular biology grade water to remove the salts and chaotropic agents present in the buffer and virus samples. The substrate was then allowed to dry for one hour in a clean and dry environment prior to analysis.

Use of Respiratory Syncytial Virus (RSV) As A Negative Control. Respiratory syncytial virus (RSV) was used as an additional negative virus control for the aptamer-covered substrates. RSV strain A2 was propagated using Vero cells maintained in Dulbecco's Modified Eagles Medium (DMEM; GIBCO BRL Laboratories, Grand Island, NY) supplemented with 2% heat-inactivated (56°C) FBS (Hyclone Laboratories, Salt Lake City, UT). Upon detectable cytopathic effect (three days post-infection), RSC/A2 was harvested in serum-free DMEM followed by freeze-thaw cycles (-70°C/4°C), after which the contents were collected and centrifuged at 4,000 × g for 15 minutes at 4°C. The virus titer was

approximately 10^7 PFU/mL as determined by immunostaining plaque assay as previously described.[7] Lysis of RSV/A2 was performed by mixing 35 µL of the virus sample with 140 µL AVL chaotropic lysis buffer (Qiagen, Valencia, CA). The lysate was then subjected to buffer exchange on a zebra spin desalting column (Thermo Scientific, Rockford, IL). Briefly, binding buffer was accomplished by loading the column bed gel with 50µL aliquots of the binding buffer and centrifuging at 1,000 × g for one minute at room temperature to pass the binding buffer through the column resin bed. The procedure was repeated four times to ensure complete pre-equilibration of the resin bed with the binding buffer. To complete the buffer exchange procedure, 140 µL of the RSV lysate was applied to the pre-equilibrated resin bed and the column was centrifuged at 1,000 × g for two minutes at room temperature to collect the sample. The sample was then diluted to a concentration of about 10^5 PFU/mL in the binding buffer for the SERS experiment.

Incubation of the RSV control sample with the aptamer-functionalized SERS-sensing surface was accomplished by adding 20 µL of the RSV virus solution in binding buffer to the aptamer-modified, Ag nanorod microwell. The other binding conditions were kept identical to those of the influenza nucleoprotein samples, described above.

Use of ss-DNA Sequence As a Negative Control. A random 22-mer DNA sequence HS-C_6H_{12}-$^{5'}$ACT CCA TCA TCT AAC ATA TCA A$^{3'}$ was purchased from Integrated DNA Technologies (IDT, Coralville, IA) and used without further purification. This 22-mer DNA sequence was used as a negative control in the influenza binding experiments. The immobilization of the 22-mer DNA control sequence was accomplished using the same experimental protocols that were employed for the immobilization of the aptamer, as described above. Also, experiments to assess the binding of the influenza nucleoproteins on this self-assembled, 22-mer DNA functionalized surface used the same experimental protocols that were employed for the capture and binding of the influenza nucleoproteins on the aptamer-coated substrate, as described above.

Raman Spectroscopy. Measurements were performed using a confocal Raman microscope (InVia, Renishaw, Inc., Gloucestershire, United Kingdom). Laser excitation was provided by a 785 nm near-IR diode laser. The incident laser beam was delivered to the sample by epi-illumination through a 20× (N.A. = 0.40) objective onto an automated sample stage. The laser illumination spot produced by this system through this objective has a rectangle pattern that is approximately 4.8 × 27.8 μm. The laser power used was 0.01% (~0.36 mW), as measured at the sample. SERS spectra were collected from ten different spots in a given well using a 30 second acquisition time with one accumulation. Spectra were collected between 2000 and 500 cm^{-1}.

Experimental Design and Reproducibility. In order to ensure reproducibility of results, the following experimental design was used. Twenty (20) individual Ag nanorod substrates were used in this study. Each substrate was patterned in a 40-well format, as previously described;[4, 5] at least 25-30 of those wells were used during the course of each experiment. The anti-influenza aptamer was applied to the wells at concentrations of between 1 – 1000 nM; each concentration of aptamer was added to a minimum of two wells.

In addition to a series of aptamer concentrations, we also studied a series of influenza samples with HA concentrations ranging from 0.1 μg/ml to 10.0 μg/ml. Each combination of aptamer and influenza sample, as well as the blanks and controls, was studied on each patterned substrate, resulting in at least 40 replicates for each combination of concentrations. A minimum of 10 SERS spectra were acquired in each well. Therefore, we acquired a minimum of 400 SERS spectra for each specific combination of aptamer concentration with influenza nucleoprotein sample, including blanks and controls. The spectra presented in Figures 1 and 2 are an average of 10 individual spectra for each particular type of sample.

Assay Sensitivity. The sensitivity of this SERS-based influenza assay was assessed against the standard ELISA method of viral nucleoprotein detection using the anti-influenza aptamer as outlined in the manufacturer's literature supplied with the aptamer (www.aptares.net). The standard ELISA method for detection of the

influenza viral nucleoproteins is specified for a concentration range of 6.0 – 0.06 μg/ml with respect to influenza HA. All the spectra shown in the manuscript were acquired using influenza nucleoprotein samples adjusted to a concentration of 1.0 μg/ml, relative to influenza HA. We also have obtained SERS spectra at 0.1 μg/ml HA concentration with equivalent results. Therefore, the SERS-based influenza assay described here is at least as sensitive as the standard ELISA assay used for aptamer-based detection of influenza.

Data Analysis. Off-line spectral manipulation and analysis, *i.e*., baseline correction, band height/area and peak wavenumber determination, was performed using GRAMS 32/AI Version 6.0 (Galactic Industries Corporation, Nashua, NH). Mulivariate statistical analysis and classification of the samples was achieved using principal components analysis (PCA).[8, 9] This data analysis was performed with PLS Toolbox version 6.2 (Eigenvector Research Inc., Wenatchee, WA), operating in a MATLAB environment (R2011a, The Mathworks Inc., Natick, MA). SERS spectra used to generate the classification models were first processed by taking the first derivative of each spectrum (1st derivative, 2nd-order polynomial, 15-point Savitzky-Golay) and then normalizing the spectrum to unit vector length.[9] The normalized first derivative spectra were mean-centered and auto-scaled prior to PCA.[10, 11]

References

1. S. B. Chaney, S. Shanmukh, Y.-P. Zhao and R. A. Dluhy, *Appl. Phys. Lett.*, 2005, **87**, 31908-31910.
2. J. D. Driskell, S. Shanmukh, Y. Liu, S. B. Chaney, X. J. Tang, Y. P. Zhao and R. A. Dluhy, *J. Phys. Chem. C*, 2008, **112**, 895-901.
3. P. Negri, N. Marotta, L. A. Bottomley and R. A. Dluhy, *Appl. Spectros.*, 2011, **65**, 66-74.
4. J. L. Abell, J. D. Driskel, R. A. Dluhy, R. A. Tripp and Y.-P. Zhao, *Proceedings of the SPIE*, 2009, **7321**, 732103-732111.
5. J. L. Abell, J. D. Driskell, R. A. Dluhy, R. A. Tripp and Y. P. Zhao, *Biosensors & Bioelectronics*, 2009, **24**, 3663-3670.
6. A. Nitsche, A. Kurth, A. Dunkhorst, O. Panke, H. Sielaff, W. Junge, D. Muth, F. Scheller, W. Stocklein, C. Dahmen, G. Pauli and A. Kage, *BMC Biotechnology*, 2007, **7**, 48-60.
7. R. A. Tripp, D. Moore, L. Jones, W. Sullender, J. Winter and L. J. Anderson, *J. Virol.*, 1999, **73**, 7099-7107.
8. M. J. Adams, *Chemometrics in Analytical Spectroscopy*, Royal Society of Chemistry, Cambridge, 2004.

9. K. R. Beebe, R. J. Pell and M. B. Seasholtz, *Chemometrics: A Practical Guide*, J. Wiley & Sons, Inc., New York, 1998.
10. M. Barker and W. Rayens, *J. Chemometr.*, 2003, **17**, 166-173.
11. G. Musumarra, V. Barresi, D. F. Condorelli, C. G. Fortuna and S. Scire, *J. Chemometr.*, 2004, **18**, 125-132.