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14. ABSTRACT

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RPPR Final Report
as of 28-Nov-2017

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Proposal Number: 65392LS **Agreement Number: W911NF-14-1-0286**
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Final Report for Period Beginning 15-Jun-2014 and Ending 14-Jun-2017
Title: Controlled Microfluidic Assembly and Functionalization of Complex Biomolecules
Begin Performance Period: 15-Jun-2014 **End Performance Period:** 14-Jun-2017
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STEM Degrees: 1 **STEM Participants:** 3

Major Goals: Biological molecules can confer unique recognition features for a range of targets including pathogens, toxin and explosives. However, this recognition generally requires the assembly of nano- and meso-scale macromolecules derived from individual subunits. Unfortunately, assembly of such systems usually requires a cellular environment. This greatly limits the ability to integrate the unique functionality of these molecules into devices that are readily useable by soldiers in the field. Thus, there is a need to develop systems for the synthesis of complex macromolecules in a cell-free environment. There is also a need to engineer novel biological components with designer functions that can modify, enhance or extend the recognition capabilities of macromolecules. The goals of this study are to develop unique microfluidic systems for the control and assembly of designer virus-like particles (VLP) with engineered recognition and response capabilities.
Specific aims use programmable microfluidic systems for the assembly of multifunctional receptor-based VLPs onto transducer surfaces. These studies are directed at developing novel sensing systems for the detection of target analytes in real-time and under label-free conditions.

Accomplishments: A summary of major milestones are listed below: See the attached progress report for accomplishment details.
- A series of TMV coat protein variants for use in the assembly of multiplexed TMV-VLPs have been developed and tested for expression and VLP nano-rod assembly. ?
- Macromolecular systems for end specific assembly of multifunctional TMV VLPs were developed. ?
- Demonstration of potential reusability of the capillary-microfluidics integrated impedimetric sensors for label-free antibody sensing using a pH-dependent sensor regeneration method. ?
- Application of the open-channel capillary microfluidic platform for rapid prototyping of nanostructured microdevices. ?
- Development and characterization of VLP coat proteins with selective positive end charges that confer pH-dependent disassembly/reassembly for the directional end-to-end assembly of multi-modal nanorods.
- A novel electrowetting biofabrication technology has been developed allowing programmable functionalization of bio-nanoreceptors onto 3D device substrates. ?
- This fabrication system allows the precise positioning and controlled deposition of ~1?l samples onto 3D micropillar substrates. ?

- The electrowetting system produces a 7-fold increase in device surface functionalization, as measured by VLP fluorescence, over a control system. ?
- The developed VLP functionalized impedance biosensor produced a 4.12% impedance change per log10 (ng/ml) antibody concentration with a detection limit of 55 pM anti-FLAG IgG. This detection limit represents a 100-fold improvement over our previous VLP-based optical sensors.

Training Opportunities: Both graduate and undergraduate students benefited from the cross-disciplinary training and professional development activities provided through this project. Specifically students from both biology and engineering tracks that participate in this project have presented their work at conferences and meetings as listed in the products section of this report.

Results Dissemination: See Products for a list of papers and conferences in which the results of this project were disseminated to the scientific community. In addition, results from this study were also incorporated into undergraduate lectures for courses in Virology and an Honors Seminar. These project lectures were used to demonstrate the potential of cross-disciplinary studies (Virology and Engineering) to produce novel solutions to complex problems.

Honors and Awards: Awardee: Sangwook Chu
Award: Outstanding Graduate Assistant Award
Source: University of Maryland
Date awarded: May 11, 2017
This award recognizes the outstanding research and teaching contributions Sangwook Chu has made to the University of Maryland. This award is given to the top 2% of University Graduate Assistants in a given year.

Protocol Activity Status:

Technology Transfer: Nothing to Report

PARTICIPANTS:

Participant Type: Graduate Student (research assistant)
Participant: Adam Degan Brown
Person Months Worked: 12.00 **Funding Support:**
Project Contribution:
International Collaboration:
International Travel:
National Academy Member: N
Other Collaborators:

Participant Type: Graduate Student (research assistant)
Participant: Sangwook Chu
Person Months Worked: 12.00 **Funding Support:**
Project Contribution:
International Collaboration:
International Travel:
National Academy Member: N
Other Collaborators:

Participant Type: Graduate Student (research assistant)
Participant: Faheng Zang
Person Months Worked: 12.00 **Funding Support:**
Project Contribution:
International Collaboration:
International Travel:
National Academy Member: N
Other Collaborators:

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Participant Type: Undergraduate Student
Participant: Emily Larkin
Person Months Worked: 2.00 **Funding Support:**
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National Academy Member: N
Other Collaborators:

CONFERENCE PAPERS:

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Conference Name: American Society for Virology: 35th Annual Meeting
Date Received: 24-Aug-2016 Conference Date: 18-Jun-2016 Date Published: 24-Aug-2016
Conference Location: Virginia Tech, Blacksburg, Virginia.
Paper Title: Engineering Capsid Protein Carboxylate Groups to Control Viral Rod Assembly
Authors: Adam Brown, James Culver
Acknowledged Federal Support: **Y**

Publication Type: Conference Paper or Presentation **Publication Status:** 1-Published
Conference Name: The 15th International Conference on Micro and Nanotechnology for Power Generation and Energy Conversion Applications
Date Received: 24-Aug-2016 Conference Date: 01-Dec-2015 Date Published: 01-Dec-2015
Conference Location: Boston, MA
Paper Title: Bio-nanotextured high aspect ratio micropillar arrays for high surface area energy storage devices
Authors: Sangwook Chu, Kosta Gerasopoulos, Reza Ghodssi
Acknowledged Federal Support: **Y**

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Conference Name: Hilton Head Workshop 2016: A Solid-State Sensors, Actuators and Microsystems Workshop
Date Received: 24-Aug-2016 Conference Date: 05-Jun-2016 Date Published: 05-Jun-2016
Conference Location: Hilton Head, SC
Paper Title: CAPILLARY MICROFLUIDICS-INTEGRATED IMPEDANCE SENSOR FOR RAPID LABEL-FREE ANTIBODY SENSING
Authors: Faheng Zang, Sangwook Chu, James Culver, Reza Ghodssi
Acknowledged Federal Support: **Y**

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Conference Name: The 15th International Conference on Micro and Nanotechnology for Power Generation and Energy Conversion Applications
Date Received: 24-Aug-2016 Conference Date: 01-Dec-2015 Date Published: 01-Dec-2015
Conference Location: Boston, MA
Paper Title: Rapid fabrication of supercapacitor electrodes using bio- nanoscaffolds in capillary microfluidics
Authors: Faheng Zang, Sangwook Chu, Kostas Gerasopoulos, James Culver, Reza Ghodssi
Acknowledged Federal Support: **Y**

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Conference Name: A Solid-State Sensors, Actuators and Microsystems Workshop
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Conference Location: Hilton Head, SC
Paper Title: LOCALIZED BIONANORECEPTOR 3D-ASSEMBLY VIA ELECTROWETTING: AN INTEGRATED MICRO/NANO/BIO FABRICATION TECHNOLOGY
Authors: Sangwook Chu, Faheng Zang, Adam Brown, James Culver, Reza Ghodssi
Acknowledged Federal Support: **Y**

Publication Type: Conference Paper or Presentation **Publication Status:** 1-Published
Conference Name: American Chemical Society
Date Received: 28-Aug-2017 Conference Date: 20-Aug-2017 Date Published: 20-Aug-2017
Conference Location: Washington D.C.
Paper Title: Hierarchical self-assembly of novel tubular nanoparticles and surface-attached nanoscaffolds from modified Tobacco mosaic virus capsid protein
Authors: Adam Brown, Sangwook Chu, Reza Ghodssi, James N. Culver
Acknowledged Federal Support: **Y**

Publication Type: Conference Paper or Presentation **Publication Status:** 1-Published
Conference Name: 30th IEEE International Conference on Microelectromechanical Systems (MEMS 2017)
Date Received: 28-Aug-2017 Conference Date: 22-Jan-2017 Date Published: 22-Jan-2017
Conference Location: Las Vegas, NV
Paper Title: 3D-EBP: A PROGRAMMABLE 3D BIONANORECEPTOR ASSEMBLY
Authors: Sangwook Chu, Ben Hurwitz, Thomas E. Winkler, Adam D. Brown, James N. Culver, Reza Ghodssi
Acknowledged Federal Support: **Y**

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Authors: Faheng Zang
Acknowledged Federal Support: **Y**

Final Report: W911NF1410286

Controlled Microfluidic Assembly and Functionalization of Complex Biomolecules

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University of Maryland, College Park, MD 20742

Introduction:

Biomaterials hold the potential to revolutionize a broad range of energy, manufacturing, sensor, and medical processes. Furthermore, bio-enhanced processes are increasingly being looked to as potentially cost effective methods to produce or enhance a diverse array of micro devices. However, knowledge required for the controlled assembly of protein-based macromolecules is limited. In addition, the integration of these biomaterials into functional devices is often difficult to control, leading to their inactivation or functional loss. In this project we have used the well-defined plant virus *Tobacco mosaic virus* (TMV) to develop novel strategies for the assembly, patterning and functionalization of nanoscale surface features for specific sensor applications. Key features of this project include the development of macromolecular systems for the end specific assembly of multifunctional TMV-VLPs, an open-channel microfluidic platform with integrated impedance sensing electrodes, a rapid microfluidic VLP assembly and functionalization system and the demonstration of label-free antibody sensing at detection limits of 55 pM, representing a 100-fold improvement over previous VLP-based sensors.

Aim 1. Programmable systems for the surface assembly of multiplexed TMV-VLPs. Efforts have focused on the development of TMV coat protein (CP) seed assemblies for the directional and layered assembly of VLP nanorods on a substrate surface.

Accomplishments:

The self-assembly of virus particles from identical protein subunits is directed by specific intermolecular forces that control the conformation of individual subunits and their ability to self-associate with like subunits or nucleic acid. The genetic manipulations of these forces through the introduction of specific amino acid additions or substitutions within the encoding CP open reading frame can be used to manipulate both the size and flexibility of the subunits as well as their ability to self-associate. These capabilities make it possible to engineer new self-assembling particles of unique sizes and shapes. TMV is a particularly well-suited biotemplate for these efforts due to its well-defined and robust structure, economically scalable production methods, well-understood molecular interactions, available molecular genetic engineering tools and virus-like particle (VLP) system.

As outlined in Table 1 a number of TMV-VLP mutants have been developed and investigated for their directional assembly from either the 5’ or 3’ nanorod ends. For these studies an array of TMV-VLPs with modified electrostatic charge distribution profiles at either end of the VLP nanorod were tested for and confirmed to bind to TMV nanorod ends having complimentary electrostatic charge distributions to produce multiplexed TMV-VLPs. Specifically, VLPs composed of TMV CP expressed in bacteria were created by replacing a negatively charged carboxylate-containing amino acid residue with either a neutrally charged

Table 1: List of modified VLP CP constructs for directional assembly indicating negative (–), neutral (Ø), or positive (+) additions and targeted end for attachment.

Name	Axial E50	Axial D77	Lateral E95/E97/D109	Lateral E106	Tag	End for Attachment	Assembly
E50Q/D77N	Ø	Ø	–	–		both	Yes
E50Q Flag	Ø	–	–	–	FLAG	5’	Yes
R46G/E50K	+	–	–	–	HA	5’	Yes
D77N HA	–	Ø	–	–	HA	3’	Yes
D77H Flag	–	+	–	–	Flag	3’	Yes
E50K His	+	–	–	–	His	5’	No
D77K Flag	–	+	–	–	FLAG	3’	Yes
Lat95/97/106/109	–	–	Ø	Ø		both	Yes
Ax50-Lat95/97/109	Ø	–	Ø	–	FLAG	5’	Yes
Ax77-Lat106	–	Ø	–	Ø	HA	3’	Yes

carboxamide-containing residue or a positively charged residue. The substitution of these negatively charged residues eliminates electrostatically repulsive interactions between adjacent subunits within a VLP rod. This drives CP self-assembly as well as creates a VLP rod with a unique combination of surface charges at either end. The modified rod end charges of these subunits, positive or neutralized, can be used to direct their assembly onto a specific end of the negatively charged TMV or TMV-VLP rod (Figure 1A & B). Studies confirm that this approach allows the two distinct CPs / rods to self-assemble into a single continuous chimeric rod of

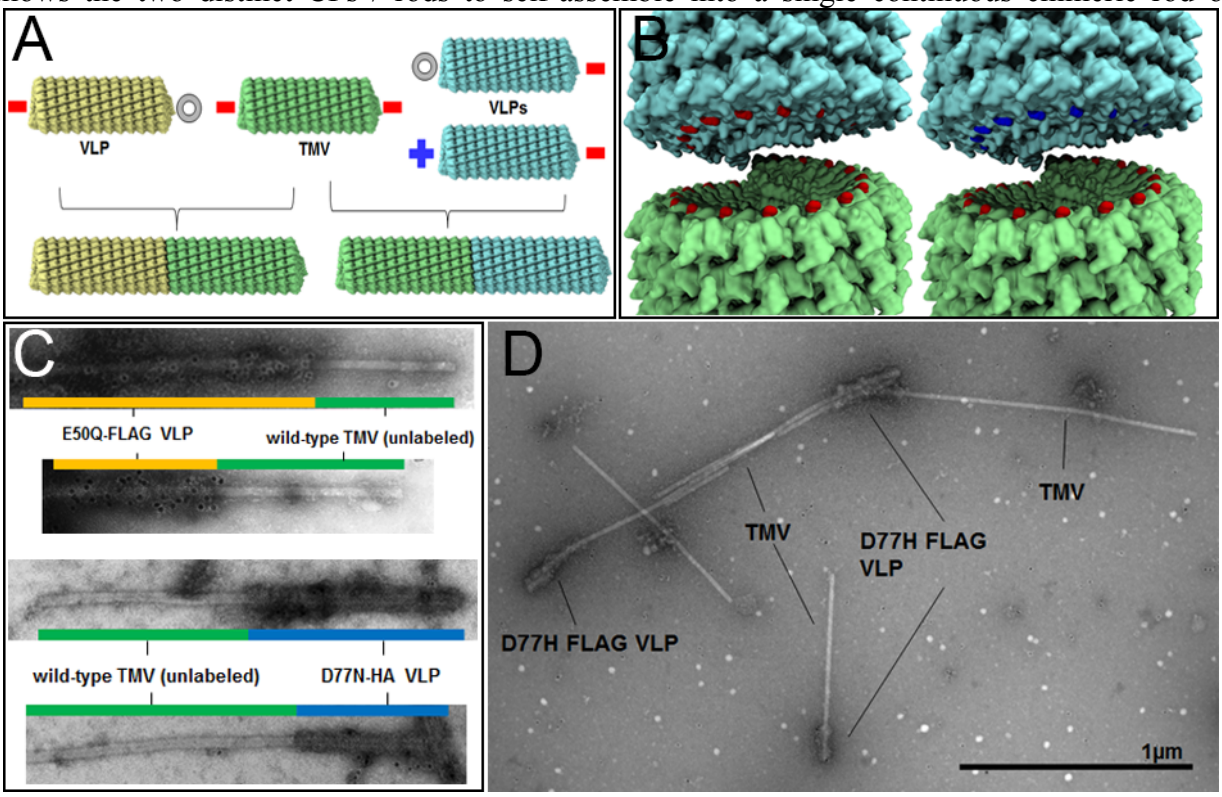

Figure 1. (A) Schematic of charge-directed end-to-end assembly of TMV (green, center) and VLPs modified by replacement of exposed negatively charged residues with either neutral or positively charged residues at the 3’ end (yellow, left) or 5’ end (blue, right) (B) Detail of negative electrostatic charges, in red, responsible for repulsive interactions between the 3’ end (top left) and 5’ end (bottom left) of TMV rods with wild-type amino-acid distribution and of an attractive end-to-end interaction between the 5’ end of wild-type TMV (bottom right) and a VLP with neutral or positively charged 3’ end residues, in dark blue (top right) (C) TEM images of gold-nanoparticle-immunolabeled VLP with E50Q (neutralization of 3’ end charges, yellow) or D77N (neutralization of 5’ end charges, blue) attached to unlabeled TMV (green) (D) immunolabelled VLP with positive 5’ end charge modification D77H showing end-to-end attachment to unlabeled TMV retaining negative charges at either end.

predetermined order (Figure 1C & D). To date several designed charge modifications have been developed that direct VLP assembly from either the 3' or 5' end of a TMV nanorod (Table 1).

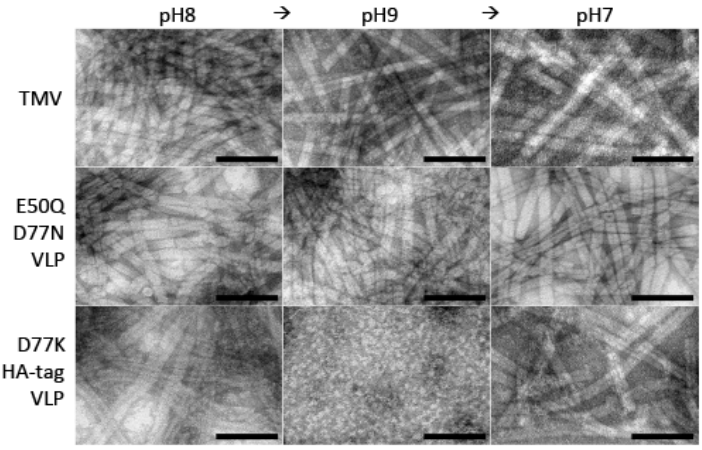

Figure 2. pH-dependent disassembly and reassembly of D77K TMV VLP figure A. Transmission electron micrographs of wild-type TMV (top row), E50Q D77N VLP (center row), and D77K HA-tag VLP (middle row) sequentially dialyzed (left to right) to pH8, to pH9 and then back to pH7 showing disassembly of D77K HA-tag VLP at pH9 (bottom center) and its subsequent reassembly upon return to pH7 (bottom right).

Results from these studies confirmed that the neutralization of these repulsive intersubunit sites could be used to direct the assembly of TMV CPs to a specific end of the virus nanorod. As shown in Figure 1C and D amino acid substitutions that neutralize (E50Q) or produce a unique + to – interaction (D77H) between subunits can be used to direct CP assembly to one end of the TMV nanorod. However, among the CP mutations investigated in this study two, R46G/E50K and D77K, displayed unique assembly and disassembly profiles (Table 1). Both of these mutations were designed to introduce novel positive surface charges to either the 5' (D77K) or 3' (R46G/E50K) CP interfaces. Both of these mutations were found to generate VLP nanorods that disassemble under unique environmental conditions. Specifically, as shown in Figure 2 the D77K mutation induces rod disassembly at pH9 while the wild-type TMV and VLP E50Q/D77N rods remain stable at pH 9. Additionally, shifting the disassembled D77K CP back to pH 7 results in the reassembly of the nanorods. Based on current structural predictions the larger side chain volume of the substituted K residue induces a significant instability at the CP interface when deprotonated, resulting in the rapid destabilization of the TMV nanorod. Similarly, as shown in Figure 3 R46G/E50K displays a unique assembly – disassembly – reassembly profile that is dependent upon ionic strength. Structural modeling of this mutant suggests that the predicted salt-bridge between E50K and D77 could be ionically stabilized. Again the larger side chain of the E50K substitution may provide a stabilizing force when interacting with D77 on the opposing subunit. However, the E50K substitution is also positioned to interact with R71 of the opposing subunit, conferring a repulsive interaction. This repulsive interaction may be mitigated by negative ions, allowing the ionic strength of the solution to control the assembly of this nanorod. Protein modeling experiments are planned to further define the mechanisms behind these different assembly traits. Interestingly, current data indicates that D77K nanorods are

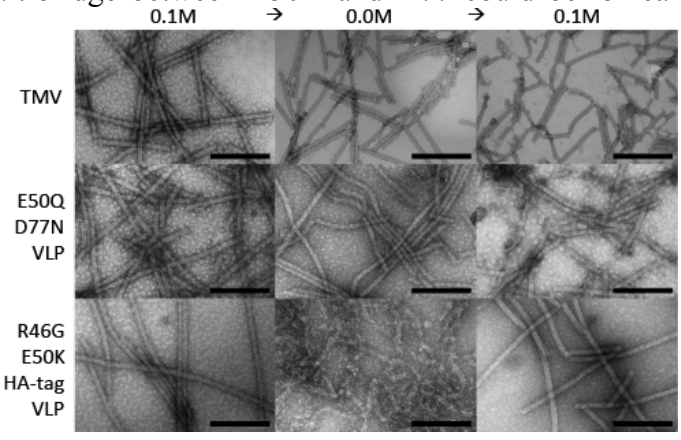

Figure 3. Ionic strength dependent disassembly and reassembly of R46G/E50K TMV VLP. Transmission electron micrographs of wild-type TMV (top row), E50Q/D77N VLP (center row), and R46G/E50K HA-tag VLP (middle row) sequentially dialyzed (left to right) from 0.1M Na-phosphate buffer to 0.0 M water and back to 0.1M Na-phosphate buffer. The bottom panels show R46G/E50K disassembly (bottom center) and its subsequent reassembly upon restoration of ionic strength (bottom right).

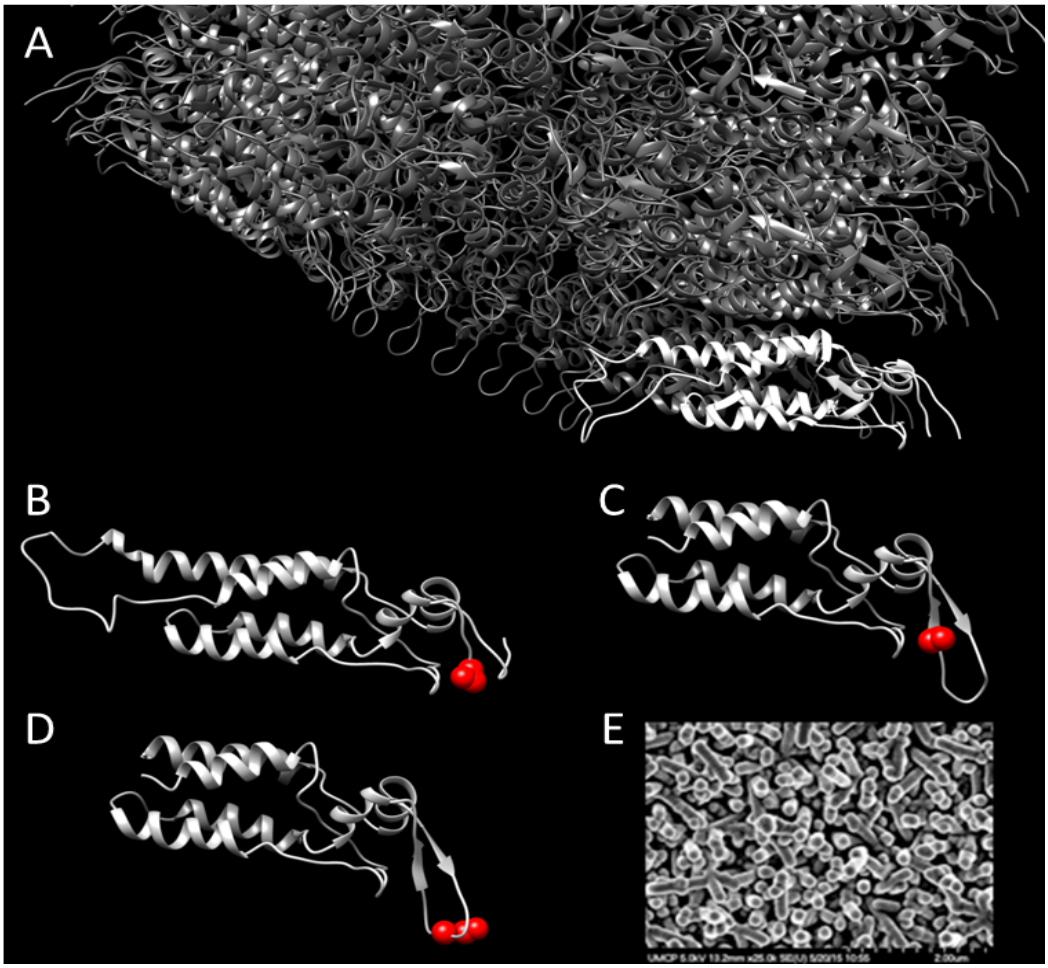

Figure 4. Model comparison of TMV-VLP CP with circular permutants with and without relocated cysteine residue and scanning electron microscope image of surface attached VLP permutant. A, 3' end of TMV nanorod B, unmodified TMV coat protein showing location of cysteine residue for surface attachment (red) C, Permutant VLP coat protein showing original location of cysteine residue for surface attachment (red). Note the inaccessibility of the cysteine residue to the outer subunit surface. D, Modified permutant coat protein with cysteine residue (red) positioned for surface binding. E, TEM micrograph of modified permutant coated with Ni metal to show surface binding.

primarily affected by pH and not by ionic strength while the R46G/E50K nanorods disassemble only at low ionic strength with pH having little effect. Thus, these two mutations allow us to utilize either pH or ionic strength as drivers in nanorod assembly and disassembly, making them ideal for controlling the assembly of chimeric VLPs in solution and within microfluidic devices (See Figure 6B).

Additional studies in this Aim were directed and the development of a modified CP permutant with restored capability for vertical attachment to gold-plated substrates so as to provide a basis for the sequential deposition of VLP constructs with desired functionalities such as the piezoelectric VLP discussed in Aim 2. The permutant moves the N and C termini of the TMV CP from the outer rod surface to the inner channel surface. Combined with the stabilizing E50Q/D77N mutations, the permutant produces a nanorod with a wider, 8-10nm, inner channel that can accommodate larger modifications. Unfortunately, initial attempts to attach these permutant nanorods to surfaces using the cysteine residue at CP residue position 2 failed due to

steric hindrances associated with the cyclization of the CP N and C termini. Based on structural assessments the relocation of the cysteine residue to a more distal position on the connecting loop restored surface attachment as shown in Figure 4E.

Aim 2. Assembly and functionalization of macromolecular VLP structures. Studies have focused on the structural design and assembly of bi-functional VLPs that bind metals or generate piezoelectric currents within the inner channel of the TMV-VLP while displaying analyte specific receptor peptides on their outer surfaces for label free detection.

Accomplishments:

The structure and assembly characteristics of the TMV VLP represent a potentially useful scaffold in which to incorporate peptides with novel functionalities. Efforts in this aim focused on the design of a piezoelectric peptide for display within the inner channel of the TMV-VLP and its surface attachment. The addition of a piezoelectric response to the VLP is aimed at using the VLP itself to generate a detectable change in electric potential. When fused with an analyte binding peptide we anticipate that the strength of the piezoelectric response will change upon the binding of the target analyte, allowing the VLP macromolecule to both directly sense and report binding events. To date we have designed and produced a TMV-VLP construct that stably self-assembles and displays a piezoelectric peptide within its inner channel. We have also demonstrated the ability of the TMV-VLP construct to display piezoelectric activity (Figure 5). Piezoelectric activity was consistently observed as a voltage change in response to pressure applied to a tube filled with purified TMV-VLPs. Controls with an agar gel or an unmodified virus did not yield a similar charge change. Reversal of polarity resulted in the inverse charge responses, consistent with piezoelectric activity.

Subsequent efforts in this aim were directed at attaching the piezoelectric permutant VLP to an electrode surface for further studies. Attempts to use cysteine modifications to attach permutants, either the original (Figure 4C) or repositioned (Figure 4D) cysteine VLP constructs were unsuccessful. This may be due to the length and overall negative charge of the attached

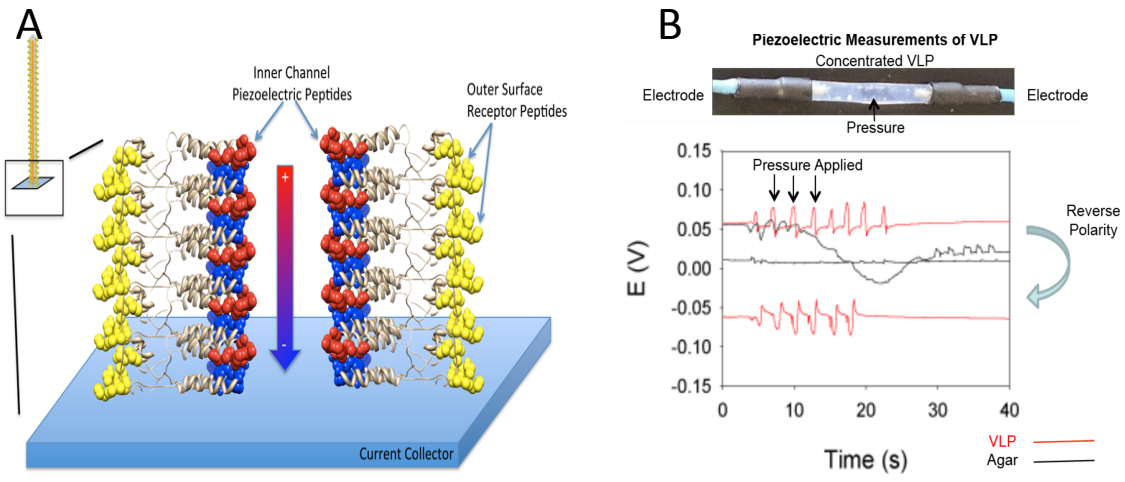

Figure 5. Design of piezoelectric TMV-VLP assemblies. Model shows a CP assembly configuration with stacked positive - negative inner loops. The outer surface is free for the display of analyte binding receptor peptides. A, Diagram of surface assembled piezoelectric VLP design with internal piezoelectric peptides (red-blue) and external analyte binding peptide (yellow). B, Potentiostat testing of piezoelectric TMV-VLP concentrated in a tube format.

piezoelectric peptide directly interfering with surface attachment. Efforts continue to investigate these scaffolds for their conductive and energy applications as well as their ability to enhance sensor activity. Our current goal is to use surface assembly-competent VLP subunits from the end directed assembly systems developed in Aim 1 to connect device surfaces to inner channel functionalized VLPs. As shown in Figure 6A we plan to use a seed layer of unmodified CP permutant subunits/disks that readily attach to the electrode via the repositioned cysteine residue. Piezoelectric modified subunits carrying either D77K or R46G/E50K substitutions will then be used for assembly onto the surface attached seed subunits (See Figure 1A). Either pH for the control of D77K or ionic strength for the control of R46G/E50K assembly will be used to control this process (Figures 2 and 3). Preliminary studies have shown that it is possible to use ionic strength to control the end assembly of R46G/E50K onto the target rod ends in a solution system (Figure 6B).

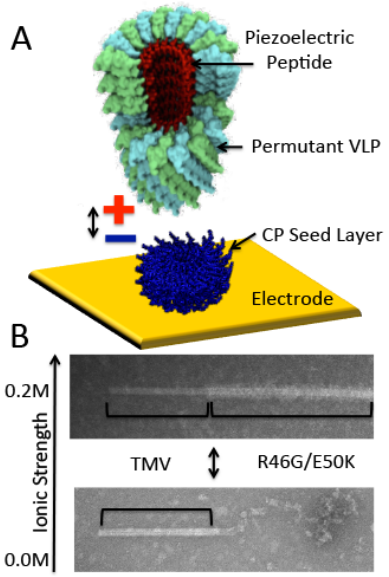

Figure 6. Controlled nanorod end assembly. A, Diagram indicating how a surface attached CP seed layer, either disk or small aggregates, can be used drive the surface attachment of functional unique VLP nanorods such as the piezoelectric permutant. B, Solution demonstration of how ionic strength can be used to drive the assembly of VLP nanorods using the R46G/E50K mutation.

Aim 3. System-based microfluidic investigations for monitoring and controlling the assembly of macromolecules. Efforts have investigated the reusability of integrated microsystems for monitoring TMV-VLP self-assembly and biosensing, and the potential use of capillary microfluidics for the rapid fabrication of nanostructured devices. Additional efforts have focused on the integrated assembly of TMV-VLPs onto three-dimensional microelectrodes as a means to enhance sensing performances.

Accomplishments:

We have developed an integrated microsystem comprising capillary microfluidics and impedimetric sensors for rapid on-chip TMV-VLP assembly and label-free immuno-sensing. Using open-channel microfluidics, TMV-VLP sensing probes displaying FLAG epitopes are autonomously delivered onto an impedance sensor surface, forming a dense biorecognition layer within 6 minutes due to enhanced surface evaporation-assisted assembly. The TMV-VLP functionalized impedance sensor is able to perform label-free sensing with detection limit down to 55 pM target antibody (anti-FLAG) concentration. These results highlight the significant potential of the integrated system for rapid transducer functionalization and biosensing. Efforts also focused on investigating the reusability of the microfluidics-integrated impedimetric system for biosensing and implementing capillary-microfluidics as a temporary tool for the rapid fabrication of nanostructured microdevices.

Figure 7 shows the schematic of the integrated sensing microsystem (Figure 7A) with scanning electron microscopy (SEM) images of the open-channel capillary microfluidics (Figure 7B) and impedance sensor electrodes functionalized with TMV/VLPs. The system utilizes capillary micropumps, defined by KMPR 1050 negative photoresist featuring high-aspect-ratio geometry with smooth side-walls, for autonomous delivery of TMV-VLPs onto the transducer

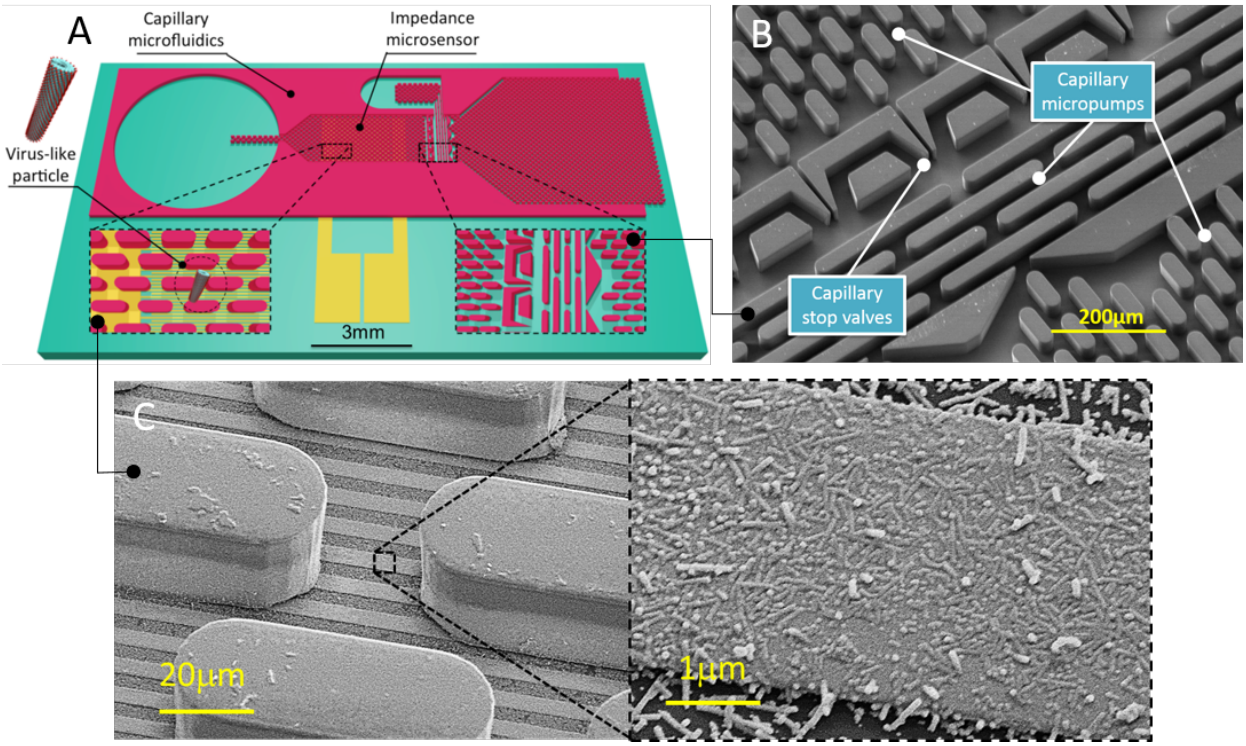

Figure 7. Integrated VLP-based sensing microsystem. A, schematic of the open-channel capillary microfluidics-integrated impedance sensor. B, SEM image of capillary pumps and stop valves for autonomous sample delivery and passive flow control. C. SEM image of impedance sensing electrodes functionalized with high-density TMV/VLPs.

surface. The capillary stop valves located at the end of sensing area allow confinement of the TMV/VLP samples during the surface functionalization step. A triggering fluid is introduced in perpendicular direction at the opposite side of the stop-valve (capillary channel shown in the center of the Figure 7B in diagonal direction) to open the valves for the successive rinsing and antibody introducing steps. As shown in SEM images in Figure 7C, dense arrangements of the VLP bionanoreceptors on surfaces was achieved using a novel evaporation-assisted assembly process; near confluent VLP coverage of the sensor surface (91%) in less than 6 minutes. This is a significant improvement compared to a regular self-assembly process which required 18-hours for functionalization.

Figure 8 shows real-time monitoring of electrical impedance during TMV-VLP assembly (Figure 8A), and label-free antibody sensing at different TMV-VLP sample concentrations (Figure 8B). The percentage impedance change (imaginary part of the complex impedance) at 100 Hz was monitored to analyse the VLP and antibody attachments on the electrode surface. When biological particles (relative permittivity of 4-13) attach to the electrode surface, they displace water (relative permittivity of ~80) on the electrode/liquid interface. This lowers the effective dielectric constant on the electrode surface and reduces the area (and thus the associated capacitance) of the ionic double layer at the electrode/electrolyte interface. Therefore, an increase in the amplitude of impedance (imaginary part) is expected as biomolecules attach to the electrodes. As shown in Figure 8A, using the open-channel capillary microfluidic device as a delivery platform, the impedance between the IDT electrodes increased by more than 120% and saturated within 6 minutes after introducing a 5µl drop of 0.2 mg/ml VLP-FLAG

nanoreceptors. In contrast, during the control experiment, the impedance changed less than 2% when only buffer solution was delivered.

The VLP-FLAG functionalized sensors were tested for sensing anti-FLAG antibodies and the results are shown in Figure 8B. First, the effect of VLP-FLAG concentration on sensor functionalization was studied for optimum sensor performances. Interestingly, the 0.2 mg/ml VLP-FLAG concentration resulted in highest signal transduction of antibody binding, with an impedance increase of 29.6% at 100 µg/ml antibody concentration while the other conditions (2mg/ml and 4mg/ml VLP-FLAG) showed around 20% change for the same target concentration. This is owing to reduced nanoreceptor layer thickness at lower VLP concentration that makes the captured antibodies closer to the sensor surface and improves the sensitivity. The selectivity of the sensor was verified using control non-specific bionanoreceptor-analyte combinations. These controls produced distinctively lower signal responses when compared to the anti-FLAG antibody responses, confirming the selectivity of the sensor for FLAG antibody detection. With a detection limit as low as 55 pM antibody concentration, the VLP functionalized impedance sensors produced in this study were shown to have great potential to perform rapid label-free biosensing in real-time.

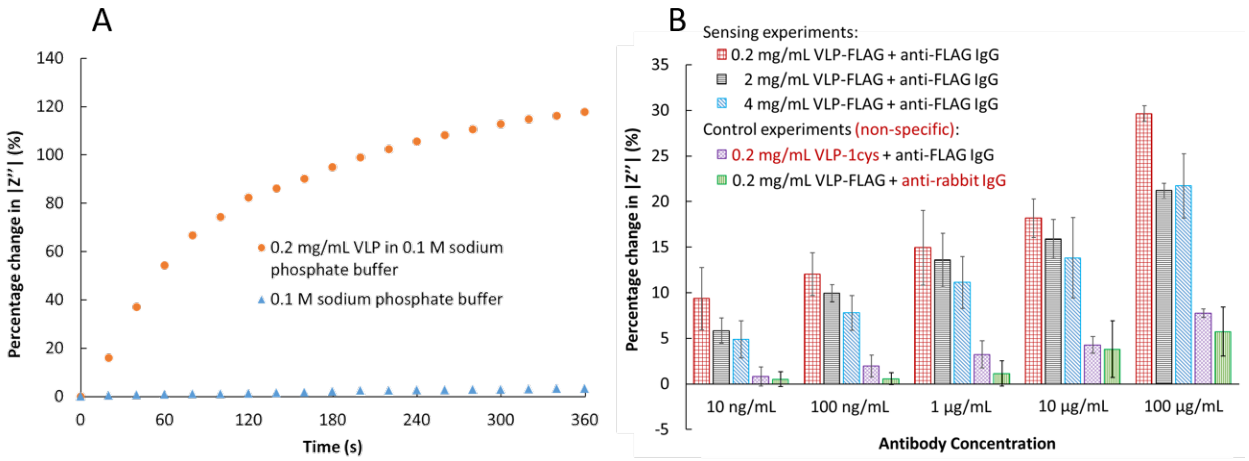

Figure 8. Evaporation-assisted VLP-FLAG assembly process and label-free antibody sensing. A, percentage changes of impedance in the presence or absence of VLPs. B, label-free antibody detection using the VLP-based impedance sensor.

For reusability studies it was determined that TMV-VLP particles self-assembled onto impedimetric sensing electrodes can be removed using various methods (e.g. temperature, pH control). Based on our previous work, which showed the disassembly of VLPs at pH > 9, we flushed the microfluidic devices with 5 µl of 2% NaOH solution for 5 min for sensor surface regeneration. Figure 9A compares the impedance responses from the same sensor before and after surface regeneration. The regenerated sensor shows similar responses compared to the new sensor with a similar increase in impedance following the target antibody concentration from 10 ng/ml to 1 µg/ml. However, the new sensor showed more stable and continuous impedance change compared with the regenerated sensor, with 15% and 10% change in impedance response at 1 µg/ml antibody concentration from the new and regenerated sensors, respectively. The morphology of the sensor surface after the NaOH washing step has been investigated using SEM

(Figure 9B and C). The characterization shows that over 95% of the gold surface previously assembled with TMV-VLP particles has been cleaned using NaOH solution, and ~ 5% of the area is still covered by TMV-VLP disk-like subunits (white round particles in Figure 9C). Though there are differences in the sensor characteristics due to the regeneration step, the VLP-functionalized regenerated sensor can still be utilized to perform label-free antibody sensing and quantify the concentration of the target molecule. In summary, the developed pH-based sensor regeneration method functions as a viable means to extend the life of sensor surfaces without sacrificing device integrity.

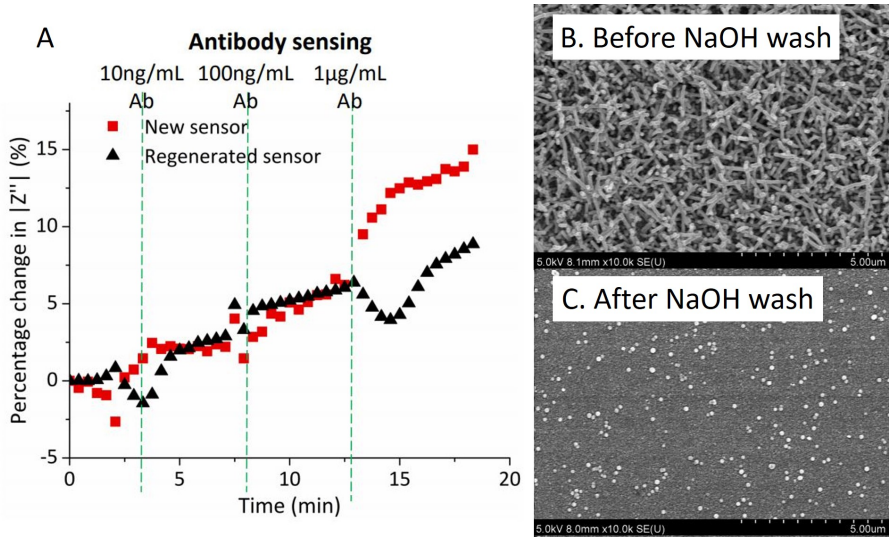

Figure 9. (A) Impedance responses to increasing target anti-FLAG concentration on a sensor before and after surface regeneration. (B, C) Comparison of SEM images showing the morphology of the metalized TMV-VLP particles on Au surface before and after cleaning with 2% NaOH solution at room temperature.

The capillary-microfluidic system we developed for the controlled assembly of TMV-VLP particles on sensor surfaces can also provide a novel platform for rapid prototyping of nanostructured devices. Based on our previous experience, developing electrochemical charge storage devices using TMV nanoscaffolds, our efforts have focused on investigating potential application of the open-channel capillary-microfluidics for the rapid fabrication of interdigitated nanostructured supercapacitor electrodes. As described in Figure 10A, the temporary capillary channels (AZ9260 photoresist) created through one-step photolithography allow the rapid introduction of TMV particles through the interdigitated electrode channels. This is followed by evaporation-assisted self-assembly and electroless metallization steps, resulting in the creation of high surface area nanostructured electrodes (Figure 10B). Compared to our previous methods, the open-channel capillary-microfluidics allowed use of minimal sample solution (~3 µl vs. 1 ml) and with a significant reduction in fabrication time (<1hr vs. 18hr). The fabricated electrodes were characterized as a NiO-based symmetric pseudocapacitor, and we were able to achieve 3.6-fold increase in areal capacitance with the capillary-microfluidics assembled TMV scaffolds (59 mF/cm^{-2}) compared with planar electrodes (16 mF/cm^{-2}). Combined, we believe that the open-channel capillary microfluidics developed in this work for controlled-assembly of macromolecules can bring a significant impact to a broader range of research contributing to rapid and simple assembly and fabrication of nanoparticles/nanostructures for a wide range of applications.

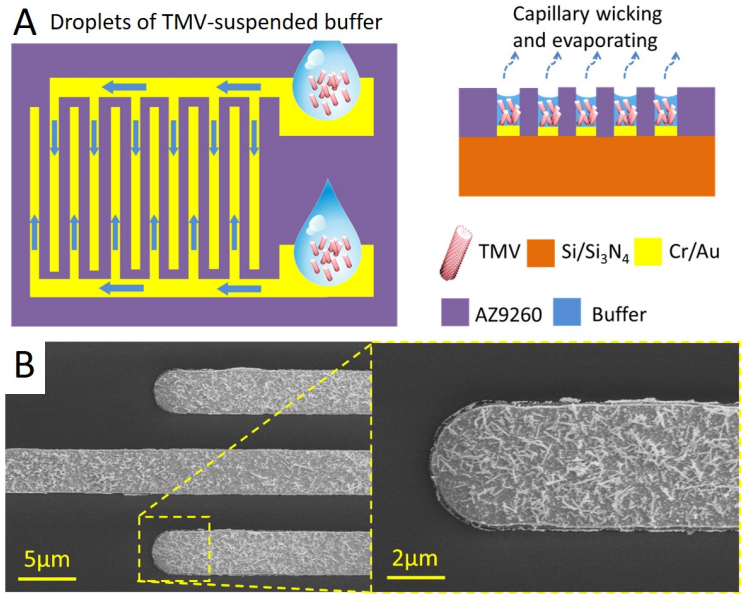

Figure 10. (A) Fabrication of TMV nanostructured electrodes through delivery and evaporation of TMV particles in temporary open-channel capillary-microfluidics defined by patterned photoresist. (B) Top-down SEM images of the nanostructured interdigitated electrodes fabricated in the capillary channels.

Additional efforts have focused on assembling TMV-VLP particles onto three-dimensional microelectrodes to increase surface-to-volume ratio and enhance TMV-VLP-based biosensing performance. For traditional capillary microfluidics systems, photoresist-based 3-D microposts have provided a simple means to increase transducer surface areas. However, this strategy is often compromised by wettability issues caused by surface tensions that tend to limit the access of receptor and sample solutions to the 3-D microcavities that occur between the microposts. We have investigated the assembly of TMV particles onto gold micropillar-array electrodes and have identified structural hydrophobicity – known as "Cassie-Baxter" wetting property – as a critical limiting factor for assembling TMV-VLP particles into the cavities of 3-D microelectrodes. To achieve the uniform assembly of TMV-VLP particles onto 3-D electrodes we developed a 3-D biopatterning technology by incorporating electrowetting principle (Figure 11A). This approach uses a small voltage differential to overcome the intrinsic hydrophobicity and drive TMV-VLP assembly into the cavities that occur between micropillars. The successful assembly of TMV1cys and VLPs onto 3-D electrodes is confirmed using SEM (Figure 11B) and fluorescence microscopy (Figure 11C) that show spatially controlled and uniform assemblies of TMV1cys within the micropillar array electrode. The increased density of TMV1cys per device foot-print produces a 7-fold increase in fluorescence intensity attributed to the µPAs when compared to similar assemblies on planar substrates. Combined, this work demonstrates the potential of electrowetting as a unique enabling solution for the development of controlled and efficient methods for the functionalization of macromolecules onto 3-D sensing electrodes.

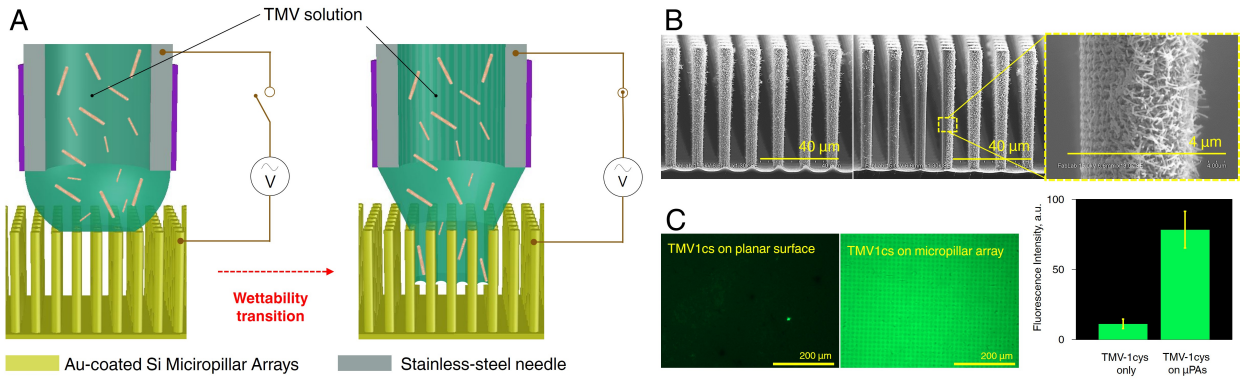

Figure 11. (A) Cross-sectional schematic of electrowetting-assisted 3-D biopatterning process. (B) Cross-sectional SEM images of TMV assembled micropillar arrays after electroless Ni metallization; (left) uniform coating of TMV across the pillar sidewalls, (center, right) formation of functionalization boundary on a single pillar sidewall located at the wetting edge. (C) Fluorescent microscopy characterization comparing TMV1cys functionalized on planar (left) and micropillar array (center) electrodes – (right) ~ 7-fold increase in fluorescence intensity is achieved with increase functionalization density on micropillar arrays.

Summary of Accomplishments:

- A series of TMV CP variants for use in the assembly of multiplexed TMV-VLPs have been developed and tested for expression and VLP nano-rod assembly.
- Developed and characterized VLP CPs with selective positive end charges that confer pH and ionic strength dependent disassembly/reassembly for the directional end-to-end assembly of multi-modal nanorods.
- A TMV-VLP with piezoelectric functionalized inner channel was developed and tested.
- Controlled VLP end assembly systems were developed using CP modifications responsive to ionic strength and pH.
- An open-channel microfluidic platform integrated with impedance sensing electrodes was developed for rapid bionanoreceptor functionalization using TMV functionalized VLPs.
- The developed VLP functionalized impedance biosensor produced a 4.12% impedance change per log10 (ng/ml) antibody concentration with a detection limit of 55 pM anti-FLAG IgG. This detection limit represents a 100-fold improvement over previous VLP-based optical sensors.
- Demonstrated the reusability of the capillary-microfluidics integrated impedimetric sensors for label-free antibody sensing using a pH-dependent sensor regeneration method.
- A novel electrowetting biofabrication technology has been developed allowing programmable functionalization of bio-nanoreceptors onto 3D device substrates with precise positioning and controlled deposition of ~1 µl samples onto 3D micropillar substrates.