Increased density and coverage uniformity of viruses on a sensor surface by using U-type, T-type, and W-type microfluidic devices

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Microorganisms, molecules, or viruses in the fluidic environment are usually at considerably low Reynolds numbers because of small diameters. The viscous forces of molecules and viruses dominate at considerably low Reynolds numbers. This study developed three microfluidic devices, that is, T type, U type, and W type devices, to control the flow movement, which can increase the adhesion density of viruses on the surface of the sensor. The linker 11-mercaptopundecanoic acid (11-MUA) and Turnip yellow mosaic virus (TYMV) were used in this study and measured by a confocal microscope. Fluorescent intensity and coverage of 11-MUA and TYMV were used to identify the adhesion density quantitatively. Results indicate that 11-MUA layers and TYMV disperse randomly by the dipping method. Attachment tests for T-, U-, and W-type devices demonstrated average fluorescence intensities of 1.56, 2.18, and 2.67, respectively, and average fluorescence coverage of 1.31, 1.87, and 2.55 times those of dipping techniques, respectively. The T-type device produced the lowest fluorescence coverage uniformity (10%–80%), whereas the W-type device produced the highest fluorescence coverage uniformity (80%–90%). Fluorescence intensity correlates positively with flow within a specified flow range; however, the exact relationship between fluorescence intensity and flow requires further study. Attachment tests for TYMV virus samples indicated that the W-type device produced an average fluorescence intensity of 3.59 and average fluorescence coverage of 19.13 times greater than those achieved through dipping techniques. Traditional immersion methods achieved fluorescence coverage of 0%–10%, whereas that of the W-type device reached 70%–90%. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4722294>]

I. INTRODUCTION

The study of viruses is conducted because viruses cause numerous infectious diseases, such as common cold, influenza, measles, severe acute respiratory syndrome (SARS), hepatitis, yellow fever, polio, smallpox, and acquired immune deficiency syndrome (AIDS). A number of viruses known as oncoviruses contribute to specific forms of cancer, for example, infection with hepatitis B and hepatitis C viruses, which are associated with liver cancer. Although more than 5000 viruses have been described, millions of various types of viruses are under investigation. Enzyme-linked immunosorbent assay (ELISA) (Staley *et al.*, 2007; Lequin, 2005) is one

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of the most widely used methods in immunology to detect specific viruses. Each antibody can be distinguished and bound by its matched antigen, and the function is commonly used to detect the presence of viruses in a sample. A known quantity of captured antibodies is first bounded on the surface, and antigen-containing viruses are subsequently washed over the surface to bind the viruses to the surface. After the viruses are affixed on the surface, primary antibodies and enzyme-linked secondary antibodies are applied to bind specifically to the viruses. The secondary antibodies are linked to enzymes that can convert to detectable signals. For fluorescence ELISA, antigen/antibody complexes fluoresce when light of the appropriate wavelength is shown on the sample. The amount of antigens in the sample can be inferred through the magnitude of the fluorescence.

Western blotting is the other commonly used method in the study of viruses. In Western blotting (Burnette *et al.*, 1981; Towbin *et al.*, 1979), the virus samples are separated by using gel electrophoresis, such as SDS-PAGE (sodium dodecyl sulfate—polyacrylamide gel electrophoresis) and subsequently transferred to a membrane (typically nitrocellulose or polyvinylidene fluoride, PVDF). The membrane is dipped into a buffer liquid with primary antibodies to target viruses. The membrane is exposed to secondary antibodies after it is rinsed to remove unbound primary antibodies. The secondary antibodies are linked to biotins or to reporter enzymes, such as alkaline phosphatase or horseradish peroxidase. In the last step, a sensitive sheet of photographic film is placed against the membrane. The sheet is exposed to the light to create images of viruses. However, ELISA and Western blotting require several experimental steps, longer processing times, expensive detection equipment and well-trained expertise. The sensitivity is occasionally not satisfied for real-time detection. Researchers must duplicate large numbers of viruses to satisfy the sensitivity of the sensor. However, these steps substantially increase the total processing times and may risk the health of researchers.

Recently, optical and electrical pathogen diagnostic tests in biochips were developed to detect specific viruses because of their excellent selectivity and sensitivity (Varshneya and Li, 2009; Lee *et al.*, 2009a, 2009b; Rosi and Mirkin, 2005). Another advantage of these methods is the ease with which they can be integrated with microchannels, and thus, form a pathogen diagnosis and analysis system (Lee *et al.*, 2009a, 2009b; Julich *et al.*, 2011). Li and Su (2006) applied traditional ELISA theory with the microchannels formed by silicon and glass to detect *Escherichia coli* 0157:H7. Gao *et al.* (2005) used electrokinetically driven actuators combined with the indirect ELISA theory to detect various antigens from *Helicobacter pylori* and *Lactobacillus rhamnosus* (control group). The lowest detected concentration was 1 ng/ml, and the higher the concentration of *H. pylori*, the stronger the detected fluorescence signal. Eteshol and Leckband (2001) applied the PDMS microchannel to the theory of heterogeneous sandwich ELISA to detect the Sheep IgM antigen in the test solution; and the detected concentration of the antigen was as high as 15 ng/ml. Niemeyer and Ceyhan (2001) used the scanometric approach to detect proteins. Specific antibodies for target proteins were immobilized on a surface. After the target proteins were bound to the capture antibodies, antibody-labeled gold nanoparticles were bound to the proteins to generate a sandwich system. The use of either spectrophotometrically or a scanner resulted in a detection limit of approximately 200 pM, which is comparable with ELISA.

Measurements of the electric properties, such as variation of impedance and the amount of biological agents, can also be estimated. Several recognized layers (for example, self-assembled monolayers, specific linkers, and antibodies) were first deposited on the surface of the biosensor, and impedance of the biosensor was measured before and after adhesion of biological agents. Edwards *et al.* (2001) applied a microchannel with two electrodes within a fixed pitch. The presence of pathogens in the test solution can be detected by measuring electrical changes when pathogens pass the pitch between the electrodes. Chang *et al.* (2005) first modified an RGD-C peptide on an electrode to increase the amount of Madin-Darby canine kidney (MDCK) cells adhering to the electrodes to facilitate electrical measurement. In other studies on electrical measurement, the link action between antibody and antigen was also used to fix the pathogens in the test solution in the target area (Suehiro *et al.*, 2006; Javanmard *et al.*, 2007). Yun *et al.* (2007) used highly aligned multiwalled carbon nanotubes as electrodes to

develop a label-free immunosensor. Anti-mouse IgG was subsequently covalently immobilized on the nanotube electrodes. Cyclic voltammetry and electrochemical impedance spectroscopy were used to measure the binding of mouse IgG, and a detection limit of 200 ng/ml was obtained.

Regardless of whether optical or electrical measurement methods were used, the measurement signals were occasionally too weak and difficult to judge when the volume of microorganisms in the sensing area was considerably low. Traditional biological detection methods are generally based on the principle of soaking the antigen-antibody and fixing the microbial molecules to the sensor area. Because of the influence of Brownian motion, for microbial molecules in static liquid, relying on diffusion movement to attach the biological molecules to the sensing area requires considerable time, and the likelihood of successful dispersion on the sensing area is low. Tabeling (2005) argued that when a fluid flows through a square-shaped downward concave slot, the fluid causes a degree of swirling flow in the corner of the slot, and the side length ratio of the square-shaped slot influences the flow state of the fluid in the slot. Bruus (2008) also observed that the fluid created a swirling flow in the corner of the square-shaped slot when the Reynolds number of the microchannel increased. The effect turn on vortices was also investigated in the literature recently. Tsai and Wu (2011) developed a planar micromixer based on multidirectional vortices in a curved channel. They demonstrated that the C-shaped microchannel with several inner micro-barriers could enhance the efficiency on mixing two different fluorescent solutions. Buchegger *et al.* (2012) presented an enzymatic bioreactor using a millisecond microfluidic flow mixer. A novel passive T-mixer was developed to reduce mixing time at low Reynolds number. The formation of fluid layers enabled fast mixing and enhanced the reaction of enzymatic bioreactors. Jiang *et al.* (2011) used an optical detection technique using two-photon fluorescence imaging microscopy to depict two-dimensional maps of mixing dynamics by chaotic advection in microdroplet. The chaotic advection inside microdroplets introduced by the periodic flow in terms of two symmetric or asymmetric vortices inside microdroplets moving through a serpentine channel was measured experimentally and matched well with theoretical and computational results. Static mixers are also an alternative way to produce high vortical shear. Cheng and Chang (2011) designed a mixer with a special pattern to mix two types liquid (H^+ and OH^-) to adjust pH value in the microchannel. They demonstrated electrokinetic control of fluid pH and generation of stable pH gradients across flow chambers.

The effect of shear on a single nanoparticle and on a surface assay has also attracted considerable attentions recently. Kardous *et al.* (2010) used an acoustic mixer to improve performances of their immunosensors. Therefore, increasing the microbial pathogens to a fixed number considerably improves the accurate measurement of the microbial pathogens. Cheng *et al.* (2010) developed an open flow-based microfluidic device to distinguish between target and non-target DNA. They found that shear discrimination of non-specifically bound target DNA molecules on the nanocolloid surface at high flow rate and thus offer high detection specificity between end mismatch probe sequence and target DNA sequence. However, there is still lack of study to increase the adhesion density of viral particles on the sensor surface.

To improve the low efficiency of the traditional immersion method, this study used the fluidic flow in the microenvironment through bio-molecular motion to effectively solve the traditional problems encountered in the soaking method. Three major research objectives are included in this paper. First, we designed T-type, U-type, and W-type folding flow channel chips to increase the binding of 11-mercaptopundecanoic acid (11-MUA) and Turnip yellow mosaic virus (TYMV) on the sensor surface. Second, this study used NCD-4 fluorescent molecules to verify that the design of the microchannel structure can efficiently bind 11-MUA and capture TYMV. Finally, three types of microchannel environments were compared to determine the fluorescence intensity of fluorescent coverage differences.

II. SENSING PRINCIPLE

The virus TYMV (Bransom *et al.*, 1995) is a tymovirus of the family Tymoviridae and causes a bright yellow mosaic disease that exhibits vein clearing and molting of plant tissues.

The TYMV were propagated in cabbage leaves and stored at -20°C . In this research, TYMV was used for the following reasons: first, the virus is a plant virus that does not threaten the health of researchers; second, TYMV is easy to duplicate; and third, the properties of TYMV are commonly known and the diameter of a single TYMV is approximately 28 nm. Therefore, we believe that TYMV is a suitable candidate to study the mechanism of antibody and antigen binding.

The structure of TYMV was simulated by RASMOL, which is a computer program written for molecular graphics. The amino groups and thiol groups were discovered on the TYMV surface. The succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) could conjugate with the linker which has the amino group derived from lysine. After the reaction, the maleimide-activated linker can link to the thiol group of TYMV particle. Quantum dots with modified amino group on the surface can be linked with the same function. These amino groups can be easily linked with antibodies and quantum dots. Alexa 594 was applied to the TYMV with the amino groups to examine the virus distribution under a confocal microscope. The other type of fluorescent was NCD-4 (N-Cyclohexyl-N-4-Dimethylamino naphthyl carbodiimide), and it can also be linked with TYMV by the carboxy groups on the TYMV. The self-assembled monolayers (SAMs) and the linker were used to affix the virus to the sensing surface in the microfluidics channel. The type of SAMs used in this paper was 11-MUA. At one end of the chemical structure of 11-MUA are thiol groups, which can be attached on the gold film of the sensing surface. At the other end of the 11-MUA is a carboxyl functional group. This functional group can form a connection between the linker and 11-MUA. The linker layer was EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide Hydrochloride) and NHS (N-hydroxysuccinimide). The TYMV were captured by the designed SAMs after EDC interacted with 11-MUA. The compound NHS was used to prevent EDC layer hydrolysis. Therefore, we achieved a connection between the virus and the sensing surface. After the virus with quantum dots was anchored to the sensing surface of the microfluidics channel, a confocal microscope was used to monitor the surface of the silicon membrane. A laser beam with a wavelength of 615 nm was passed through an aperture and was focused by an objective lens onto a small focal volume on the sensor surface. A mixture of emitted fluorescent lights (594 nm) and reflected laser lights from the quantum dots were subsequently re-obtained by the objective lens. A photodetection device was used to record the reflected light signal.

The soaking/dipping method is commonly used in the biological experiment to bind viruses because of easy operation. This paper developed three microfluidic devices and compared to the performance of viral sensing of dipping device when other conditions remained the same. The substrates of dipping and microfluidic devices were all glass. The amount of samples were all 20 ml and viral concentrations were 10 ng/ml. The incubation process all underwent 10 h at 25°C . The dipping and microfluidic devices were all placed on static plate to make sure devices which would not be moved.

III. MICROFLUIDIC DESIGN, SIMULATION, AND EXPERIMENT

A. Design of folded microfluidic device

Functional groups, antibodies, and viruses are typically used in the soaking method to deposit self-assembled monolayers and often result in uneven immersion. For example, when using immersion deposition of self-assembled monolayers with a molecular self-assembly diameter of only a few Å, this type of molecule in the solution is mainly affected by Brownian motion and diffusion. Random molecular motion results in an uneven response. High soaking concentrations result in molecular aggregation. When small molecules become large molecules, the diameters increase in conjunction with the Reynolds numbers of particles. When the inertia force (gravity) is greater than the viscous force of the fluid, the random movements result in macromolecules depositing on the wafer surface, leading to a reaction of local aggregation. Low soaking concentrations result in only sporadic responses.

This study used folding microfluidic chips to address these disadvantages. Folding channels can be used to extend the distance and time of the molecular reaction on the chip area, thereby

increasing the efficiency of the diffusion. In addition, the elements within the flow are mainly affected by fluid traction forces. Controlling the fluid flow rate can increase the probability of a collision reaction zone, increasing the connection with the density and coverage uniformity. By using internal partitions to extend the flow path, the microchannel points were divided into T-type, U-type, and W-type structures, as shown in Fig. 1.

B. Analysis of flow field in microfluidic device

No partitions were set up in the T-type microchannel, as shown in Fig. 1(a). Through a reaction area of the microchannel measuring $3\text{ mm} \times 3\text{ mm} \times 100\text{ }\mu\text{m}$, most of the fluid molecules followed a 3 mm basic flow path through the reaction zone. Finite element software, COMSOL MULTIPHYSICS, was used to analyze the microchannel flow field to design favorable structural parameters. Simulation results were used to predict the experimental parameters, such as flow velocity and vorticity. The simulations demonstrated a larger difference in the flow section at the central axis of the entrance, and this difference diminishes farther from the central axis. A considerably small flow rate increased the potential for “aggregation” and “sporadic response.” The fluid in the sensing zone is nearly stationary in the two lower corners of the T device. In the reaction zone, the highest flow rate value is 0.01 m/s, with an average flow rate of 0.00185 m/s, and a residence time of 1.6 s. In addition, the residence times represent the times of viruses in the microfluidic channels and also might indicated the times of viruses to react with self assembly monolayers on the surface of device. As the distance from the central axis of the entrance increases, the stream in this zone becomes thinner, indicating that the number of fluid molecules passing through the zone also decreased.

A partition ($200\text{ }\mu\text{m} \times 1.5\text{ mm} \times 100\text{ }\mu\text{m}$) was set up in the U-type microchannel, as shown in Fig. 1(b), dividing the microchannel into two sections of $1.4\text{ mm} \times 100\text{ }\mu\text{m}$. The partition impeded the flow in the original path, forcing the fluid to flow around the obstacle. Most of the fluid molecules followed a 6 mm basic flow path through the reaction zone. Simulations indicate that the forced diversion resulted in a more even flow distribution through the reaction zone, with a maximal flow of approximately 0.01 m/s, an average flow of 0.00397 m/s, and residence time of approximately 1.5 s. The partition impedes the flow from its original path, forcing the fluid to flow around the obstruction. If viruses or antibodies are distributed uniformly in the flow, the denser flow distribution indicates that more viruses or antibodies will be carried in the cross-sectional area. This result assists in increasing the probability of antigen and antibody collision.

Three partitions ($200\text{ }\mu\text{m} \times 2.4\text{ mm} \times 100\text{ }\mu\text{m}$) were set up in the W-type microchannel, as shown in Fig. 1(c), dividing the channel into four sections of $600\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$. The design was based on extending the flow path as an extension of the U-type microchannel. The reduction of the cross-sectional microflow area increased the flow resistance. For the respective input flows, the fluid pressure for a specific channel wall is greater than in other microchannel structures. Most of the fluid molecules in the reaction zone followed the 12 mm basic flow path. The W-type devices have more uniform flow rates and denser flow line in the channel in the results of finite element simulation. If viruses or antibodies are distributed uniformly in the flow, more uniform flow rates, and the denser flow distribution indicates that more viruses or antibodies will be carried in the reaction zone. We assume that W-type microchannel differs from other structures because it exhibits superior flow distribution in the reaction zone. In the rest paragraph of the article, we also find the W-type microchannels exhibited regional fluorescence coverage of 80% to 90% which is the best results among dipping method, U-type device and T-type device. Although the deviation from the central axis of the entrance is specified, the flow rate can be maintained at a certain level. The flow rate rapidly reaches a steady state because of the microchannel length and the effect of the cross-sectional area. In the reaction zone, the highest value of the flow is 0.018 m/s, the average flow rate is 0.00926 m/s, and the residence time is approximately 1.3 s. The microchannel cross-sectional area of the maximum number of flow line results in the greatest concentration, through which the highest number of fluid molecules passed. Although the retention time was the shortest of all the structures

(a) T-type microfluidic device

(b) U- type microfluidic device

(c) W- type microfluidic device

FIG. 1. Schematic diagram of the folded microfluidic devices. There are inlet channels from the right and outlet channels to the left for three folded microfluidic devices. The reaction area of three devices is all $3\text{ mm} \times 3\text{ mm} \times 100\text{ }\mu\text{m}$ in the central part of channels. (a) T- type device. No partitions were set up in the T-type microchannel. (b) U- type device. A partition ($200\text{ }\mu\text{m} \times 1.5\text{ mm} \times 100\text{ }\mu\text{m}$) was set up in the U-type microchannel dividing the microchannel into two sections of $1.4\text{ mm} \times 100\text{ }\mu\text{m}$. (c) W- type device. Three partitions ($200\text{ }\mu\text{m} \times 2.4\text{ mm} \times 100\text{ }\mu\text{m}$) were set up in the W-type microchannel dividing the channel into four sections of $600\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$.

considered, the internal flow can be adjusted by the inlet flow. The advantageous flow distribution of this structure can be used to increase the feasibility of future experiments.

C. Fabrication of microfluidic devices

The traditional mold processes by photolithography allow miniaturization of dimensions and exhibit higher precision. Photolithography usually uses a spin-coater to deposit the positive or negative photoresist on silicon substrate. Subsequently, a mask is used to cover the layer of photoresist, and a UV light is used to expose the photoresist. Finally, a complete structure of the mold is fabricated by the developing process. The advantage of photolithography is its high precision; however, the disadvantages are the manufacturing complexity and low reusability of the mold. Based on the requirements of this study, we used micromachining systems to simplify the process and created a reusable master pattern. First, stainless steel or brass was processed by a computer numerical control (CNC) milling machine to produce the inserts designed in the study. After the processed metal inserts were cleaned, pre-polymer (Sylguard-184 A) and cross-linker (Sylguard-184B) were fully mixed in a ratio of 10:1. Polydimethylsiloxane (PDMS) was subsequently poured into the channel and substrate inserts. The PDMS was heated in a vacuum chamber at 50 °C for 5 h to complete solidification. After solidification, the PDMS can be removed from the inserts. Additionally, the atmospheric pressure plasma can modify the surface energy of the PDMS channel and glass substrate. The parameters were 0.5 Torr, 29.6 W, 15–20 min represent pressure, plasma efficiency, and surface modification time, respectively. The microchannel was subsequently bonded with the substrate after the PDMS was used to implement the surface modification procedure.

D. Experiment of fluorescence streamline

We used fluorescence microscopy and high-speed camera observations of fluorescent microparticles in the flow channel to verify that the microflow design and analysis was consistent with the experiment. Microbial molecules and fluorescent particles exhibited similar patterns of movement in the fluid, and the Reynolds number of the fluorescent particles was small; thus, the viscous effect was considerably larger than the inertial effect. Because of the small Reynolds number, the fluorescent particles and the viruses exhibited difficulty in moving freely in the fluid flow lines, which is similar to a person trying to move through syrup. In the fluorescent flow line verification of the T-type microchannel, the closer to the inlet section of the central flow axis, the denser the flow line. The flow in the area below the flow lines was thinner and slower. We discovered a problem with bubbles. Increasing the flow can resolve the bubble problem; however, this substantially increases the fluid pressure of the microchannel structure, and damages the structure by causing leakage, as shown in Fig. 2(a).

In the fluorescent flow line verification of the U-type microchannel, most of the fluorescent particles were obstructed by the partition; however, a small number of fluorescent particles passed through the gap below the partition. The PDMS microchannels were fabricated first and bonded with glass substrates. The gaps appeared because of manufacturing errors of processes such as the surface roughness of the PDMS channels and glasses. The parallelism between PDMS channels and glasses also can be one of the factors to create the gap. If the gap is larger than the size of fluorescent particles around 10 μm , the particles will pass through the gap below the partition. This phenomenon does not impact future related experiments because the reaction zone is positioned directly below the partition and the particles passing through can increase the attachment efficiency, as shown in Fig. 2(b).

In the fluorescent flow line verification of the W-type microchannel, the structure of the microchannel produced characteristics of a high-intensity flow line and motion path, as shown in Fig. 2(c). In addition, the flow rate can be calculated by the simultaneous exposure of fluorescent particles and the length of the motion path. The longer the motion path, the faster the flow rate, and vice versa. Most of the particles moved uniformly through the channel, reducing particle clustering.

(a) T- type streamlines of fluorescence

(b) U- type streamlines of fluorescence

(c) W- type streamlines of fluorescence

FIG. 2. Experimental streamlines in (a) T-type device. In the fluorescent flow line verification of the T-type microchannel, the closer to the inlet section of the central flow axis, the denser the flow line. The flow in the area below the flow lines was thinner and slower. We discovered a problem with bubbles. (b) U-type device. In the fluorescent flow line verification of the U-type microchannel, most of the fluorescent particles were obstructed by the partition; (c) W-type device. In the fluorescent flow line verification of the W-type microchannel, the structure of the microchannel produced characteristics of a high-intensity flow line and motion path. (Image from videos.) (enhanced online). [URL: <http://dx.doi.org/10.1063/1.4722294.1>] [URL: <http://dx.doi.org/10.1063/1.4722294.2>] [URL: <http://dx.doi.org/10.1063/1.4722294.3>]

FIG. 3. The carbodiimide group ($N=C=N$) in NCD-4 can be captured by carboxyl group in 11-MUA. The attachment process is illustrated in Figs. 3(I)–3(IV). (I) A glass substrate was cleaned by ethanol and acetone. (II) Gold was evaporated on a glass substrate. (III) The dipping method or microchannel circulation system was applied to the substrate at room temperature. A layer of 11-MUA self-assembled monolayers was deposited on the substrate over 10 h with a Moore volume concentration of 18.32 mM. (IV) In a low-temperature environment (-20°C), NCD-4 solution was soaked onto the substrate for 20 h with a Moore volume concentration of 854.99 nM.

E. Attachment process of 11-MUA layer and NCD-4 fluorescent particles

This study used a gold-plated glass substrate as test specimens for “self-assembled monolayers” and “microchannel circulation systems.” The carbodiimide group ($N=C=N$) in NCD-4 can be captured by carboxyl group in 11-MUA. The attachment process is illustrated in Figs. 3(I) to 3(IV), and described as follows:

Step I. A glass substrate was cleaned by ethanol and acetone.

Step II. Gold was evaporated on a glass substrate.

Step III. The dipping method or microchannel circulation system was applied to the substrate at room temperature. A layer of 11-MUA self-assembled monolayers was deposited on the substrate over 10 h with a Moore volume concentration of 18.32 mM.

Step IV. In a low-temperature environment (-20°C), NCD-4 solution was soaked onto the substrate for 20 h with a Moore volume concentration of 854.99 nM.

F. TYMV viral attachment process

The fabrication processes of recognition layers are shown in Fig. 5. First, the substrate and the microfluidic channel were fabricated by PDMS casting. A layer of Au film was deposited on the substrate by sputtering, as shown in Fig. 4(I). Subsequently, 20 mM 11-MUA solution

FIG. 4. The succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate could conjugate with the linker which has the amino group derived from lysine. After the reaction, the maleimide-activated linker can link to the thiol group of TYMV particle. The viral attachment process is illustrated in Figs. 4(I)–4(IV). (I) A layer of Au film was deposited on the substrate by sputtering. (II) 20 mM 11-MUA solution was injected on the sensor surface by the microfluidics system. This linked the 11-MUA with gold films. (III) EDC and NHS solutions (molar ratio 2:1) were also injected on the sensor surface by the microfluidics system. (IV) TYMV particles with fluorescent particles (NCD-4) in the buffer were introduced over the sensor surface.

was injected on the sensor surface by the microfluidics system. This linked the 11-MUA with gold films. Figure 4(II) shows the self-assembled monolayers as linkers. In Fig. 4(III), EDC and NHS solutions (molar ratio 2:1) were also injected on the sensor surface by the microfluidics system. In the final step in Fig. 4(IV), TYMV particles with fluorescent particles (NCD-4) in the buffer were introduced over the sensor surface at a different flow rates. The sensor surface was rinsed and dried in fast protein liquid chromatography (FPLC) to avoid nonspecific adhesion. A confocal microscope was subsequently used to monitor the sensor surface.

Self assembly monolayer was employed for enhancing the capture of virus. However, there is always some unspecific adsorption/binding that can also contribute to the result. In this paper, we just provide a model to test the possibility and efficiency of the system to catch the viral particle we have used. Therefore, we design the SAMs to sense the efficiency of viruses caught and optimize the best condition for the reaction. This reaction can be visualized directly by observing the specific fluorescent viral particles. Unspecific adsorption/binding would not be counted in the experiment. Once the condition optimized, the applications also can use the specific antibody to detect the specific virus caught on the surface of the device.

There is a strong connection between the viral concentration and the detection performance. The concentration in the experiment was chosen at 10 ng/ml. In this concentration, the average fluorescence coverage and the average fluorescence intensity of TYMV detection in soaking device were only 4.23% and 342 A.U., respectively. It showed that the numbers of viruses might not be underestimated in this concentration. The paper mainly focuses on developing the different types of devices to improve the fluorescence coverage and the average fluorescence intensity at the same viral concentration. In the future research, we will study the relation between the fluorescent intensity and the viral concentration.

IV. EXPERIMENTAL RESULTS

A. Experimental results of NCD-4 test

This study used the traditional immersion method as the control group. The experimental results indicate that the average fluorescence is approximately 996.34 A.U. and the average fluorescence coverage is approximately 33.37%. Without a considerable concentration gradient in the liquid or random movement patterns, molecular diffusion and Brownian motion lead to intermolecular aggregation, as shown in Fig. 5(a).

The liquid was nearly stationary in the two lower corners of the T device, and the attached results indicated sporadic reaction, as shown in Fig. 5(b). At a fluorescence strength of $x = 1.2$ mm, the intensity indicated that fluorescence intensity and simulated flow were mostly positively correlated (Fig. 6(a)), and may be related to the number of particles in the unit cross-sectional area. Experimental results indicate that the average fluorescence intensity is approximately 1558.08 A.U., and the average fluorescence coverage is approximately 43.73%. Relative to the immersion method, the attachment efficiency of the T-type microchannel increased considerably, mainly because of the lack of uniformity in flow distribution. To resolve this problem, we used internal partitions to increase flow uniformity and doubled the length of the basic flow path to increase the probability of collision.

The U-type microchannel was used to implement 11-MUA and NCD-4 attachment experiments; and the attachment results shown in Fig. 5(c). The optical measurements indicate that the gap below the partition is approximately $16.67 \mu\text{m}$. Only a small amount of liquid passed through this area, and the area below the partition exhibited relatively weak fluorescence. At a fluorescence strength of $x = 1.2$ mm, the intensity is mostly positively correlated to the simulated flow from $y = 0$ to $y = 3$ mm, as shown in Fig. 6(b). The negatively correlated zone is near $1500 \mu\text{m}$ because this zone was positioned at a point where the fluid turned. Experimental results indicate that the average fluorescence intensity is approximately 2 171.43 A.U., and the average fluorescence coverage is approximately 62.34%.

The experimental results for the T-type and U-type microchannels were compared by using internal partitions to ensure increased attachment efficiency and coverage uniformity. Three internal partitions were added to create a W-type microchannel, for which the 11-MUA and

FIG. 5. Confocal fluorescence 2D (left) and 3D (right) micrographs of MUA/NCD4 (a) soaking method. The average fluorescence was 996.34 A.U. and the average fluorescence coverage is 33.37%. Without a considerable concentration gradient in the liquid or random movement patterns, molecular diffusion and Brownian motion lead to intermolecular aggregation. (b) T-type devices. The average fluorescence intensity was 1558.08 A.U., and the average fluorescence coverage was 43.73%. The liquid was nearly stationary in the two lower corners of the T device, and the attached results indicated sporadic reaction. (c) U-type devices. Compared to T-device, the attached results showed more uniform fluorescence distribution in the U-device. The average fluorescence intensity was 2171.43 A.U., and the average fluorescence coverage was 62.34%. (d) W-tape devices. The average fluorescence intensity was 2658.33 A.U. and average fluorescence coverage was 85.13%. Compared to the T-type and U-type microchannels using the traditional immersion method, these results exhibit a substantial improvement in 11-MUA and NCD-4 attachment efficiency.

FIG. 6. Fluorescent intensity and flow rate (a) T-type devices. At a fluorescence strength of $x = 1.2$ mm, the intensity indicated that fluorescence intensity and simulated flow were mostly positively correlated and may be related to the number of particles in the unit cross-sectional area. (b) U-type devices. At a fluorescence strength of $x = 1.2$ mm, the intensity is mostly positively correlated to the simulated flow from $y = 0$ to $y = 3$ mm, as shown in Fig. 6(b). The negatively correlated zone is near $1500 \mu\text{m}$ because this zone was positioned at a point where the fluid turned. (c) W-type devices. At a fluorescence strength of $x = 0.6$ m, fluorescence intensity and simulated flow are positively correlated at a flow velocity below 0.0108 m/s .

NCD-4 attachment experimental results, as shown in Fig. 5(d), demonstrated an average fluorescence intensity of approximately 2 658.33 A.U. and average fluorescence coverage of approximately 85.13%. Compared to the T-type and U-type microchannels using the traditional immersion method, these results exhibit a substantial improvement in 11-MUA and NCD-4 attachment efficiency. At a fluorescence strength of $x = 0.6$ mm, as shown in Fig. 5(d), the relationship between fluorescence intensity and simulated flow from $y = 0$ to $y = 3$ mm is shown in Fig. 6(c). Fluorescence intensity and simulated flow are positively correlated at a flow velocity below 0.0108 m/s; however, the exact relationship between fluorescence intensity and flow requires further study.

From the experimental results, a strong NCD-4 fluorescence reaction occurred under the partition area, and the optical measurement results indicate that no gap was present under the

FIG. 7. The overall average coverage rates at six distinct sections were subsequently used to analyze the attachment efficiency, and the average fluorescence coverage of the area was used to calculate attachment uniformity. The fluorescence image was processed in gray scale by using the 256 color scale method to calculate the average fluorescence coverage of the six sections in the figure. The calculations excluded the partition area. The average fluorescence coverage of the six areas was used to determine the uniformity of coverage, as shown. (a) In the traditional soaking method, the regional fluorescence coverage was approximately 20% to 40%. (b) For T-type microchannels, the regional fluorescence coverage ranged from 10% to 80% because the motion pattern of the microflow caused this structure to exhibit a coverage gap from 10% to 80%. (c) The U-type microchannels exhibited regional fluorescence coverage ranging from 50% to 90%, with the addition of partitions changing the original flow pattern. (d) The W-type microchannels exhibited regional fluorescence coverage of 80% to 90%. (e) Average fluorescent coverage of MUA at six sections for soaking device, T-device, U-device, and W-device are shown.

partition; thus, no liquid flowed through that area, and the area below the partition could not exhibit any fluorescence reaction. Experimental results are not consistent with the original speculation in that the area often exhibits anomalous fluorescent reactions and high uniformity of coverage. At the time that the microfluidic chip is packaged, the air plasma of the PDMS causes the PDMS surface to be modified as a layer of hydrophilic functional groups, including $-OH$ and $-COOH$, which is an important control mechanism for the fluorescent linkage of 11-MUA molecules and NCD-4. When the internal partitions and the gold substrate are sealed, it causes the PDMS surface molecules, $-COOH$, to transfer onto the gold substrate. The NCD-4 fluorescent particles were subsequently bonded onto the reaction area under the partitions and the device exhibited strong fluorescence reaction.

B. Experimental results of NCD-4 coverage uniformity

The overall average coverage rates at six distinct sections were subsequently used to analyze the attachment efficiency, as shown in Figs. 7(a)–7(d), and the average fluorescence coverage of the area was used to calculate the attachment uniformity. The fluorescence image was processed in gray scale, as shown in Figs. 7(a)–7(d), by using the 256 color scale method to calculate the average fluorescence coverage of the six sections in the figure. The calculations excluded the partition area. The average fluorescence coverage of the six areas was used to determine the uniformity of coverage, as shown in Fig. 7(e).

FIG. 8. Confocal fluorescence 2D (left) and 3D (right) micrographs of TYMV/A594 fluorescent particles. (a) soaking device. Few viruses were attached to the reaction zone through soaking methods. Average fluorescence intensity was 342.46 A.U., and average fluorescence coverage was 4.23%. (b) W-type device. Compared to traditional immersion methods, the experimental results in W-type device indicate that viruses are attached to the reaction zone in a uniform distribution. Average fluorescence intensity was 1229 A.U. and average fluorescence coverage was 85.13% in the W device.

In the traditional soaking method, the regional fluorescence coverage was approximately 20% to 40%, and the attachment efficiency was the lowest. For T-type microchannels, the regional fluorescence coverage ranged from 10% to 80% because the motion pattern of the microflow caused this structure to exhibit a coverage gap from 10% to 80%. The U-type microchannels exhibited regional fluorescence coverage ranging from 50% to 90%, with the addition of partitions changing the original flow pattern. The W-type microchannels exhibited regional fluorescence coverage of 80% to 90% because it had the largest number of particles flowing through the cross-section; thus, this structure offers optimal attachment efficiency and coverage uniformity.

C. Experimental results of TYMV viral test

The experimental results indicate that few viruses were attached to the reaction zone through traditional immersion methods, as shown in Fig. 8(a). Average fluorescence intensity was approximately 342.46 A.U., and average fluorescence coverage was approximately 4.23%. The W-type microchannel was the optimal choice among the three designs. Compared to traditional immersion methods, the experimental results in W-type device indicate that viruses are attached to the reaction zone in a uniform distribution, as shown in Fig. 8(b). Attachment below the partition is similar to the experimental results for microchannel NCD-4 fluorescence attachment. The experimental results indicate that viruses are attached to the reaction zone in a uniform distribution, as shown in Fig. 8(b). When the fluorescence strength was at $x = 0.6$ mm, as shown in Fig. 8(b), the relationship between fluorescence intensity and simulated flow from $y = 0$ to $y = 3$ mm is shown in Fig. 9. Fluorescence intensity and simulated flow were mostly positively correlated, as shown in Fig. 9. Compared to an average fluorescence intensity of 342 A.U. and average fluorescence coverage of approximately 4.23% by the traditional soaking method, 1 229 A.U. and 85.13% were obtained in the W device. The overall average coverage rates at six distinct sections were subsequently used to analyze attachment efficiency, as shown in Figures 10(a) and 10(b), and the average fluorescence coverage of the area was used to calculate attachment uniformity. The fluorescence image was processed in gray-scale, as shown in Figures 10(a) and 10(b), by using the 256 color scale method to calculate the average fluorescence coverage of the six sections in these figures. The calculations excluded the partition area. The average fluorescence coverage of the six areas was used to determine the uniformity of coverage, as shown in Fig. 10(c). In the traditional soaking method, the regional fluorescence coverage was approximately 0% to 10%, and the attachment efficiency was the lowest. The W

FIG. 9. Fluorescent intensity of TYMV and flow rate in W-type device. Fluorescence intensity and simulated flow were mostly positively correlated in the experiment of TYMV attachment.

FIG. 10. The overall average coverage rates at six distinct sections were subsequently used to analyze viral attachment efficiency. The fluorescence image was processed in gray-scale using the 256 color scale method. The calculations excluded the partition area. (a) In soaking device, the regional fluorescence coverage was approximately 0% to 10%, and the attachment efficiency was the lowest. (b) The W device exhibited regional fluorescence coverage of 70% to 90%. (c) The average fluorescence coverage of the six areas is shown.

device exhibited regional fluorescence coverage of 70% to 90%. This structure offers optimal attachment efficiency and coverage uniformity. Attachment tests for TYMV virus samples demonstrated that the W-type microchannel produced an average fluorescence intensity of 3.59 times and average fluorescence coverage of 19.13 times greater than those achieved through traditional immersion techniques. Traditional immersion methods achieved fluorescence coverage of 0% to 10%, whereas that of the W-type microchannel reached 70% to 90%.

The real reaction between viral particles and SMA on the surface in the microfluidic devices is still under investigation. One possibility might be that concentration of viral particles increases when flow passes through and induces the shear on the surface. The viral particles move by Brownian motion in soaking device but they move by shear on the surface of microfluidic devices. According to Arrhenius equation (Carberry, 2001),

where $[A]$ is the concentration of linker on the sensor surface, $[B]$ is the concentration of viral particles near the sensor surface, and $[C]$ is the concentration of attached viruses on the surface.

If we assume $[A]$ is much larger than $[B]$ near the sensor surface, the rate of reaction v is

$$v = k[B], \quad (2)$$

where k is rate coefficient. To increase the concentration of viral particles near the sensor surface will increase the rate of reaction. However, this model just considers the binding reaction between the linker and viral particles but neglect the unbinding reaction by shear. Generally, the slow flow rate was used in our study and unbinding reaction seems to appear when flow rate was larger than 0.01 m/s. However, the exact surface reaction requires further study.

V. CONCLUSIONS

T-type, U-type, and W-type folding microfluidic chips were successfully designed based on flow movement in a microenvironment to enhance adhesion density and coverage uniformity of antibody-antigen binding on a sensor surface. Compared to traditional immersion methods, this approach considerably improved the attachment efficiency in the samples. Attachment tests for NCD-4 fluorescence for T-, U-, and W-type microchannels revealed average fluorescence intensities of 1.56, 2.18, and 2.67, and average fluorescence coverage of 1.31, 1.87, and 2.55 times that of traditional immersion techniques, respectively. The T-type microchannels produced the lowest fluorescence coverage uniformity (10–80%), whereas the W-type microchannels produced the highest fluorescence coverage uniformity (80%–90%). Within a specific flow range, fluorescence intensity correlated positively with flow; however, the exact relationship between fluorescence intensity and flow requires further study. Attachment tests for TYMV virus samples indicated that the W-type microchannel produced an average fluorescence intensity of 3.59 and average fluorescence coverage of 19.13 times greater than those achieved through traditional immersion techniques. Traditional immersion methods achieved fluorescence coverage of 0% to 10%, whereas that of the W-type microchannel reached 70% to 90%.

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