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Block copolymer nanotemplating of tobacco mosaic and tobacco necrosis viruses ☆

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Abstract

This paper examines the interaction between a block copolymer and a virus. A poly(styrene-*b*-4-vinylpyridine) block copolymer was loaded with nickel, and cast from a selective solvent mixture to form a cylindrical microstructure (PS/P4VP–Ni). The nickel ions were confined within the P4VP block of the copolymer. The binding of tobacco mosaic virus (TMV) and tobacco necrosis virus on microphase-separated PS/P4VP–Ni was examined. A staining technique was developed to simultaneously visualize virus and block copolymer structure by transmission electron microscopy. Electron microscopy revealed virus particles associated with block copolymer microphase-separated domains, even after extensive washes with Tween. In contrast, virus associated with PS/P4VP block copolymers lacking Ni were readily removed by Tween. The cylinder long axis of the microstructure was oriented using a hot press and a cooled channel die for quenching, resulting in PS/P4VP cylinders that had a strong anisotropic directional preference. When exposed to flowing solutions of TMV, the PS/P4VP–Ni surface exhibited an ability to retain TMV in a partially aligned state, when the direction of flow coincided with the long axis of the PS/P4VP–Ni cylinders. These results suggest that Coulombic interactions provide a robust means for the binding of virus particles to block copolymer surfaces.

Keywords: Block copolymer; Nanopatterning; Virus

1. Introduction

This paper investigates the interaction between a block copolymer surface and viruses. A polystyrene-*b*-poly(4-vinylpyridine) copolymer surface was investigated for its ability to bind and pattern two different plant viruses, tobacco mosaic virus (TMV) and tobacco necrosis virus (TNV). This study is unique in its use of a self-assembled block copolymer template for the creation of regularly shaped metal-loaded domains for the purpose of interacting with biological species such as viruses.

Research has shown that nanotextured surfaces can influence the behavior of viruses and cells [1,2]. Because much of the chemistry involved in signaling and recognition in biology involves surface interactions, having a tunable surface that can be altered to study different organisms or to elicit different reactions could be a valuable research tool [3–5,7,8,6]. A nanopatterned block copolymer surface seems a natural fit for a microfluidic system, as its ease in processing should allow it to be integrated, especially in the capacity of binding studies, species detection or structural characterization. A number of methods on the use of polymers to assist in the patterning of viruses have been reported. Niu et al. [9] have fabricated one-dimensional nanofibers with high aspect ratio, narrow dispersity and high processibility. The self-assembly of TMV is assisted by aniline polymerization. The electrostatic attraction of the amine group on the monomer to the negative charge on the virus causes the monomer to accumulate on the TMV surface, inducing head-to-tail assembly. Clark et al. [2] have utilized the electrostatic interactions of self-assembled monolayers to order viruses on the surface of thin films.

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Much current research on this topic of biological-abiotic surface interfaces involves patterning that exists on the microscale. Lithographic techniques can produce a wide variety of structures, such as arrays of lines and dots [10], that have been proven to affect the direction of the growth of cells, the adhesion of cells to a substrate, and the shape and health of cells attached to the substrate [11]. Lithographic techniques have also been used to pattern viruses and direct their assembly onto surfaces [12,13]. Rather than using lithography to pattern an inorganic surface, block copolymers were employed in this study to create a surface decorated with nanometer-sized periodic structures. Block copolymers microphase separate into periodic domains of nanometer dimensions. Control over domain morphology can be exerted through simple choice of block length and composition, allowing a wide range of available morphologies and functional groups. In a block copolymer, single blocks can be chosen so that they have the chemical ability to take up metal ions. Metal loading occurs through metal ion sequestration in the receptive block. Previous work [6] has shown that nickel-templated block copolymers preferentially interact with recombinant proteins. Such block copolymers have been used for the directed self-assembly of metal nanoparticles [14]. Divalent metal cations have been found to promote TMV precipitation from aqueous solutions [15]. The block copolymer of this study, polystyrene-*b*-poly(4-vinylpyridine), was exposed to nickel salts while in solution, resulting in high specificity in targeting the nickel ion to the receptive block of each block copolymer. Transmission electron micrographs showed that nickel metal nanoparticle formation is not observed. Rather, the receptive poly(4-vinylpyridine) block complexes the nickel ion and confines it with apparently minimal leakage between blocks. Tween detergent was used to examine the strength of the binding of the virus to the copolymer surface by disrupting weak functional group interactions between the virus and the copolymer.

The purpose of experiments presented here was to provide a better understanding of the nature of the virus–copolymer interaction. It was necessary to resolve the tendency of rod-like virus particles to lie randomly on the copolymer surface rather than self-orienting to maximize contact with the attracting block of the copolymer surface. To have any chance of large-scale ordering of the virus, the surface of the block copolymer itself must have a degree of long-range microstructural order. This was accomplished through the use of applied stress above the glass transition temperature of the polymer. Tween detergent was again used to disrupt weak bonding between the copolymer and the virus.

2. Materials and methods

2.1. Poly(styrene-b-4-vinylpyridine)

The PS/P4VP block copolymer purchased from Polymer Source had a molecular weight ratio of 20.0 K/19.0 K for the polystyrene and poly(4-vinylpyridine) blocks, respectively. The polystyrene block had a degree of polymerization of 192, and the poly(4-vinylpyridine) had a degree of polymerization of 180. The total block copolymer had a polydispersity of 1.09.

2.2. Tested tobacco virus species

Two different virus species, TMV and TNV, were used in order to examine the interaction between viruses and the block copolymer surface. The TMV virion is a rigid rod 300 nm long with a diameter of 18 nm. The isoelectric point of TMV is 3.5; therefore at pH 7, TMV has an overall negative surface charge, with a linear charge density of 0.5–2 electrons/Å [16]. TMV forms head-to-tail oligomeric string aggregates that are much longer than the length of a single TMV virion. Unlike the TMV rod, TNV has a regular icosahedral shape with an average diameter of 26 nm [17]. TNV has an isoelectric point of 4.5, thus, like TMV, TNV has a negative charge at pH 7.

2.3. Ultramicrotoming and electron microscopy

A Leica EM UC6 ultramicrotome was used to section samples for electron microscopy. PS/P4VP–Ni and PS/P4VP specimens were fixed in Spurr's fast-curing resin. Embedded specimens were microtomed at room temperature. Specimens were cut to 100 nm thickness so that they would be visible in the transmission electron microscope and able to endure the planned virus exposure and Tween detergent wash procedures. Microtomed films were mounted on 600 mesh copper grids, model number 600TT, purchased from Ted Pella. Electron microscopy was performed on a Hitachi H600AB electron microscope with 100 kV accelerating voltage.

2.4. Nickel metal complexation and film preparation

PS/P4VP was dissolved in chloroform, a good solvent for both polystyrene and poly(4-vinylpyridine) blocks. A polymer/chloroform concentration of 1 mg/ml was used. In order to dissolve $Ni(NO_3)_2$ in the PS/P4VP/chloroform system, the metal salt was added to the solution at a concentration of 100 μg/ml, and the mixture was titrated with tetrahydrofuran until a clear solution was formed. At the point of solution formation, the amount of tetrahydrofuran added was 8.5% of the total solution volume. The amount of nickel ion provided for complexation was in excess to the number of available surface pyridines. However, it is believed that the ratio of nickel ions complexed in the vinly pyridine block should be close to 1:6, as nickel normally has hexavalent complexation behavior with nitrogen. While we did not explicitly examine the amount of nickel uptake, the transmission electron microscopy (TEM) images suggest that there is not an excess of nickel remaining in the film. Bulk films of the copolymer and nickel were cast in flat-bottomed basins formed from Bytac film in a chloroform-saturated desiccator to slow the evaporation process. Film formation via evaporation occurred

over the course of a week. These static cast films were further processed using stress applied in a heated channel die in order to give a preferential orientation in the direction of the long axis of the PS/P4VP cylindrical microstructure. Binding analysis of the virus–copolymer system consisted of visualization through electron microscopy. Binding experiments were performed with the PS/P4VP films cut and mounted on 600 mesh copper TEM grids.

2.5. Stress-induced orientation of PS/P4VP–Ni block copolymer morphology

A custom-made aluminum mold with integrated cooling water galleries was used to heat the copolymer past its glass transition temperature, transmit applied stress and quench the copolymer once it experienced flow. A schematic of this die is shown in Fig. 1. PS/P4VP–Ni films loaded into the channel die were translucent with a light blue color. Samples were heated to 160 °C in a standard Carver 2400 digitally controlled heat press. A small preloaded force of 100 lb was initially applied to the cold sample prior to flow to ensure proper mechanical and thermal contact of the channel die with the copolymer. Flow initiated when the preload pressure was observed to drop to zero, indicating the copolymer could no longer support a mechanical load. This occurred shortly after reaching the target temperature of 160 °C. Pressure was immediately raised to 2000 lb and the copolymer was allowed to flow until it reached the ends of the mold. At this point, the heaters were turned off and cooling water was flowed through the mold, dropping the system temperature to room temperature at a rate of approximately 280 °C /min. The applied pressure was maintained during the quenching process. Once cool, the copolymers were removed from the mold after being carefully marked to show the direction of flow [18,19].

2.6. Virus staining technique

Electron microscopy was the analytical tool used to examine the morphology of the block copolymer and virus species. In order to properly image the virus, a stain was applied to provide contrast in the electron beam. Phosphotungstic acid (PTA) is commonly used when staining TMV and TNV. It was found, however, that PTA caused significant interference with the existing block copolymer contrast, as observed in Fig. 2. The tendency was for the virus to be weakly stained, accompanied by a loss in contrast from the metal-containing microstructure of the copolymer. A successful alternative was to stain the block copolymer/virus system with a 1% solution of uranyl acetate (UA) [20]. When applied to the block copolymer/virus surface, UA was able to clearly show virus particles while not interfering with the visualization of the block copolymer microstructure. The staining procedure was always a 30 s exposure to a 1 mg/ml solution of UA. Staining was performed by holding the grid of interest containing the copolymer film in self-closing tweezers and allowing a 10 μl drop of UA staining solution to rest on the surface for 30 s. At the end of the stain time, the drop was rapidly removed by gently contacting the grid bottom and sides with filter paper. Once the stained film was fully dry, it was allowed to air-dry for at least 30 min before insertion into the electron microscope.

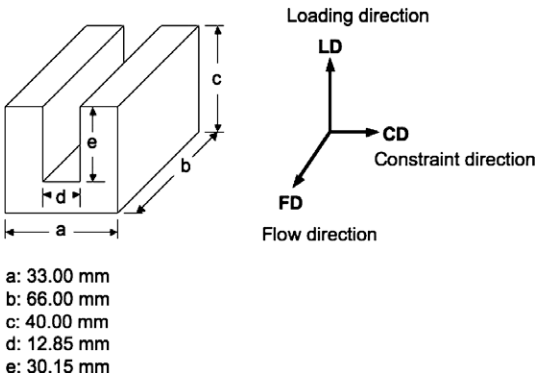

Fig. 1. Dimensions of the channel die used to orient the PS/P4VP block copolymer. Also shown are the three major axes of deformation.

2.7. Binding of TMV on PS/P4VP–Ni and PS/P4VP

The TMV binding ability of the PS/P4VP–Ni surface was tested by directly exposing TEM grid-mounted samples to an aqueous TMV solution. In order to test the binding strength of TMV to the metal-loaded copolymer surface, detergent washes were performed to analyze the amount of virus held by the copolymer. Initial TEM pictures were taken to establish the appearance of the TMV on the copolymer surface. Wild-type TMV was prepared according to literature procedures [16]. Samples of PS/

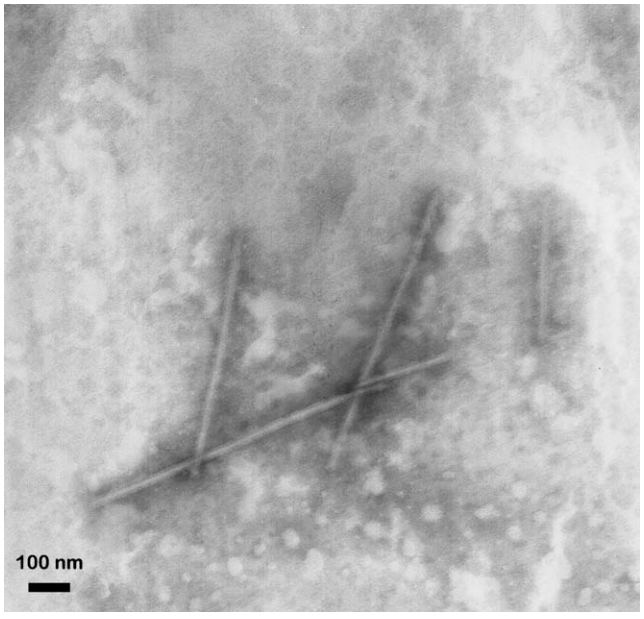

Fig. 2. Image of TMV stained with phosphotungstic acid. The underlying PS/P4VP copolymer with nickel-containing microstructure is obscured by overstaining with phosphotungstic acid.

P4VP–Ni and non-nickel PS/P4VP were mounted on copper TEM grids as per the stated sample preparation method. A 10 μl droplet of the TMV solution of approximately 0.1% mg/ml was placed on the grid and allowed to remain there for 1 min. After 1 min, the droplet was wicked away with filter paper. The sample was then immediately exposed to a 10 μl droplet of uranyl acetate stain for 30 s, and then wicked away with filter paper. Once dry, the specimen was ready for electron microscopy. Tween 20 detergent washes (product no. P5927, available from Sigma-Aldrich) were performed after virus exposure and before UA staining. Primary washes consisted of a Tween-only solution. For Tween washes of 2 min or less, the TEM grid containing microtomed copolymer was held in tweezers and gently circulated in a basin of 1% Tween for the required time. After exposure, the grid was wicked dry, rinsed with 10 μl of water, and wicked dry again. Upon drying, the washed sample was stained by exposing the grid to 10 μl of UA for 30 s. For Tween washes lasting longer than 2 mi, the grids were washed in autoclaved 1.5 ml centrifuge tubes. The tube was filled with 1 ml of the Tween wash solution. The grid containing copolymer and virus was dropped into the centrifuge tube, being sure that it did not float on the surface, but rather sank to the bottom. This centrifuge tube was then gently tumbled for the desired wash time. The sample was then rinsed with 10 μl of water, wicked dry and then stained for 30 s with 10 μl of UA. Tween washes were also performed with a mixed solution of 1% Tween and 1 M NaCl. Washing procedures for the Tween/NaCl solution were identical to those used for Tween-only washes.

2.8. Flow-induced exposure of TMV and TNV to PS/P4VP–Ni

PS/P4VP–Ni films were applied on the surface of a copper TEM grid. An apparatus was built that held the edges of the TEM grid and allowed the surface of the grid to remain unclamped, lying at a 45° angle to the horizontal countertop. A 1 ml sterile syringe was loaded with 1 mg/ml TMV solution. A flat-tipped Luer-locking needle was attached to the syringe. This syringe and tip were inserted into a syringe pump set to 3 ml/min pump rate. A schematic of this setup is shown in Fig. 3. The TMV was flowed onto the surface of the TEM grid at 3 ml/min. When the TMV solution was spent, the grid was wicked dry, gripped in tweezers, and then exposed to UA for 30 s, as in previous experiments. TEM analysis of the flow experiment followed after a 30 min drying period. TMV, because of its 300 nm length, often lays across several cylinders, and therefore it is unable to help determine the effect of a single cylinder on the binding activity of the surface. Wild-type TNV was dissolved in a solution of approximately 1 mg ml. Microtomed samples of channel-die oriented nickel-PS/P4VP were mounted on copper TEM grids and held in tweezers. As before, a 10 μl drop was allowed to remain on the surface for exactly 1 min and then wicked away with a filter paper. UA was used to stain TNV in an identical procedure to TMV. The sample was exposed to a 10 μl droplet of uranyl acetate stain for 30 s following TNV exposure, and then wicked away with filter paper. Once dry, the specimen was ready for electron microscopy.

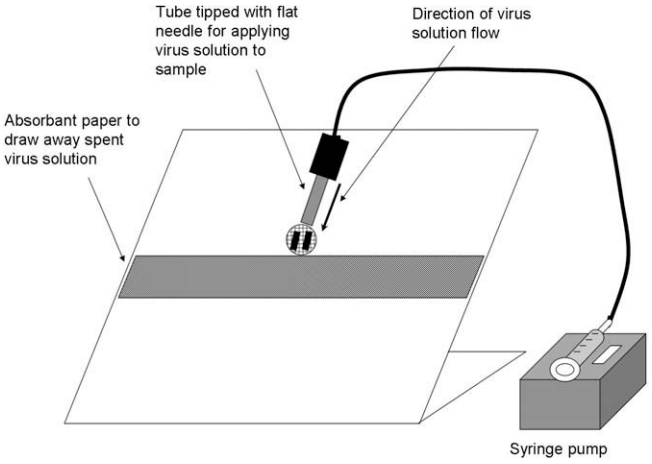

Fig. 3. Schematic of the dynamic exposure apparatus built for exposing PS/P4VP–Ni films to flowing TMV solution. A syringe pump is connected to a tube which ends in a flat-tipped needle. At the barrel of the needle, a TEM grid with affixed microtomed sections of PS/P4VP–Ni is held stationary. The TEM grid is oriented so that the long axis of the copolymer points in the direction of virus solution flow.

3. Results and discussion

PS/P4VP was employed in these experiments because it is known to take up metal ions [14,21,22]. Metal chelation occurs through the lone electron pair on the pendant pyridine moiety. At room temperature, both polystyrene (T_g

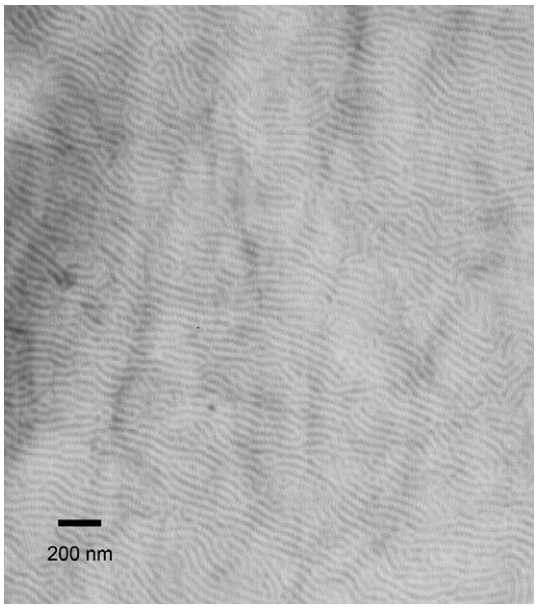

Fig. 4. TEM micrograph showing the lamellar microstructure of PS/P4VP when static cast from chloroform, a non-selective solvent. Contrast between polystyrene and poly(4-vinylpyridine) is provided from iodine vapor staining.

97.06 °C) and poly(4-vinylpyridine) (T_g 143.65 °C) blocks are in a glassy state, limiting the diffusion of metal ions from poly(4-vinylpyridine) to polystyrene. Metal ion localization in the poly(4-vinylpyridine) is important to the nanopatterning process, because it insures that the tested viruses will be templated by the metal-loaded poly(4-vinylpyridine) block and not feel competing attraction to polystyrene due to unintended metal diffusion from block to block.

A lamellar block copolymer microstructure is expected when a film is cast from a non-specific solvent for both polystyrene and poly(4-vinylpyridine) blocks, which in this case was chloroform, as seen in Fig. 4. Ni^{2+} does not, however, dissolve in chloroform. In order to dissolve both Ni^{2+} and PS/P4VP block copolymer, a solvent mixture of 8.5% tetrahydrofuran and 91.5% chloroform was used. The copolymer microstructure resulting from casting of this solvent mixture was cylindrical, as seen in Fig. 5. Ni^{2+} binds to the P4VP block, causing the observed darkened cylinders in Fig. 5. In this micrograph, both the cylinder ends and sides are visible. The long axis of the P4VP cylinders are approximately 20 nm in diameter, with an intercylindrical distance of approximately 20 nm and cylinder lengths greater than 1 μm.

Preferential orientation of the copolymer microstructure was achieved by exerting pressure on the block copolymer above its glass transition temperature. Heat and flow of the block copolymer within the channel die had the effect of giving a preferential direction to the long axis of the cylinders formed through microphase separation of the PS/P4VP–Ni copolymer. The partial orientation of the block copolymer microstructure was visualized through electron microscopy. Samples microtomed parallel to the flow axis, seen in Fig. 6, showed only the sides of the cylinders, preferentially aligned in the flow direction. Samples microtomed parallel to the constraint direction, as in Fig. 7 showed only the hexagonally close-packed ends of the cylinders. For virus testing, it was desired to use the cylinder long axis. Therefore, samples were microtomed to expose the cylindrical long axis of the block copolymer, and these surfaces were used in virus-binding tests.

TMV was bound by the nickel-loaded PS/P4VP–Ni block copolymer preferentially over the non-metal-loaded PS/P4VP. As observed in the TEM micrograph in Fig. 8, TMV remained visible on the block copolymer microstructure after 1% Tween washes of up to 5 min, as seen in

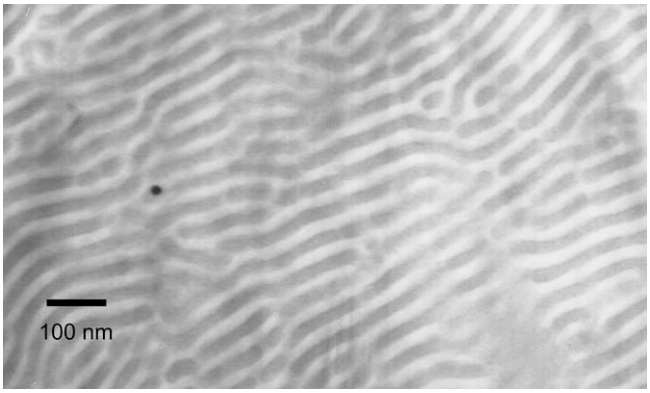

Fig. 6. PS/P4VP–Ni microtomed along the flow direction of the channel die, showing preferentially oriented cylinders. Contrast comes from nickel confined in the P4VP block.

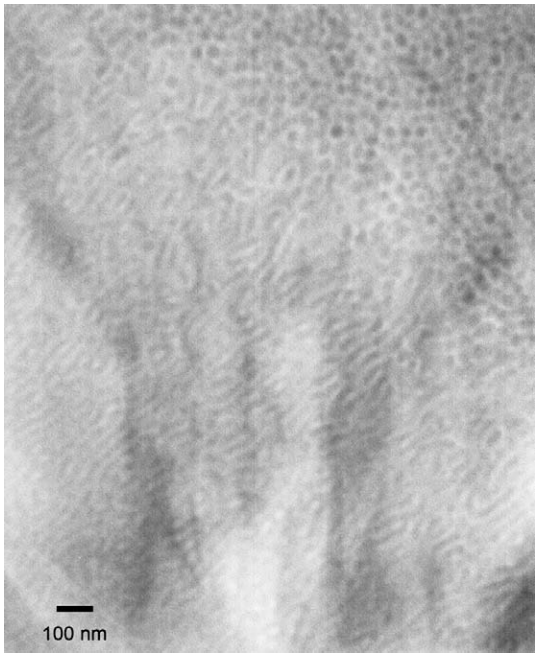

Fig. 5. TEM micrograph showing both the sides and the ends of the cylindrical microstructure resulting from PS/P4VP–Ni film formation from static casting from a 8.5% tetrahydrofuran–91.5% chloroform solvent mixture. The Ni^{2+} ion in the P4VP block provides contrast.

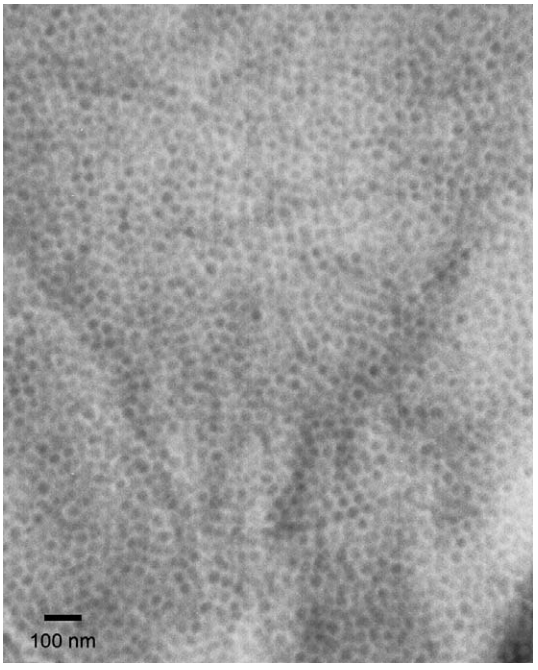

Fig. 7. PS/P4VP microtomed along the constraint direction, showing the cylinder ends. Contrast comes from nickel confined in the P4VP block.

Fig. 8. After 6 h, the surface still resembles that seen after 5 min, with some virus still visible. PS/P4VP that was prepared without nickel was seen to lose almost all visible TMV within 2 min. In order to view the microstructure of the block copolymer, the polymer was stained after microtoming with a 2 h exposure to iodine vapor, preferentially contrasting the P4VP block. TMV is observed on the non-metal PS/P4VP surface (Fig. 9a). After 1 min of Tween wash, approximately 55% of the TMV is removed, and after 2 min in Tween, virtually no virus is seen on this surface (Fig. 9c). Although there should be significant non-specific binding, owing to the large number of the virus surface functional groups interacting with the surface, the dominant binding force holding the virus on the surface is either Coulombic binding of the negatively charged virus to the metal-loaded microstructure of the copolymer surface, or a result of chelation between the virus and nickel ions on the copolymer surface.

TNV was tested for its binding on PS/P4VP–Ni. While it is a much smaller virus than TMV, TNV is similar in that it carries a negative charge at pH 7. Because of its size, approximately 26 nm, it is possible for the TNV particles to fit within the confines of the metal-loaded P4VP block portion of the surface microstructure. As seen in Fig. 10, single TNV virions were seen to fit within both the PS and P4VP portions of the copolymer surface microstructure. When exposed to detergent, TNV associated with PS was washed away. There was a distinct correlation of the binding ability of single nickel-loaded P4VP cylinders, and individual TNV virions. This is important because it suggests that unlike charge binding occurs between the metal-loaded copolymer and the virus without the need for collective action between different P4VP–Ni cylinders. Again, as in the TMV tests, TNV was seen to bind to the nickel-containing PS/P4VP block copolymer surface following Tween 20 washes.

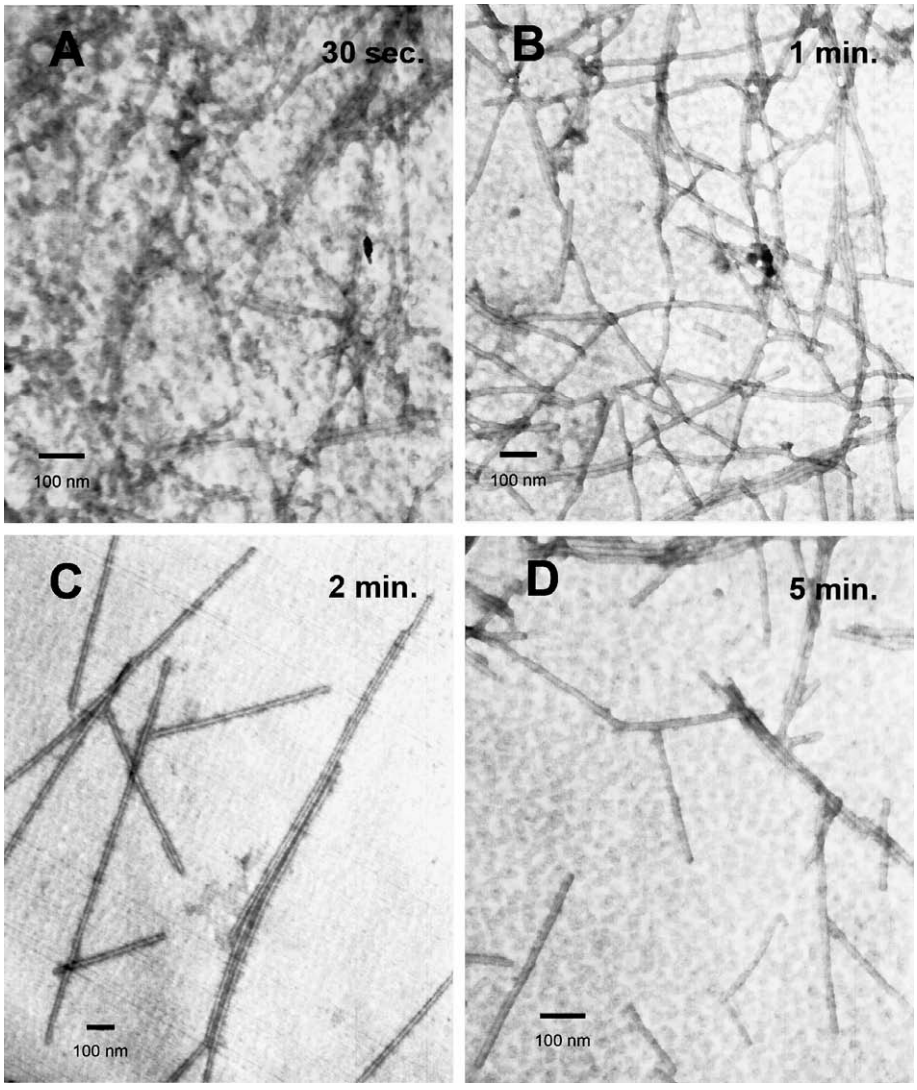

Fig. 8. TEM micrograph showing TMV binding to PS/P4VP–Ni. The pictures are taken after increasingly long Tween washes. In 8A, wash time is 30 s, 8B is 1 min, 8C is 2 min and 8D is 5 min.

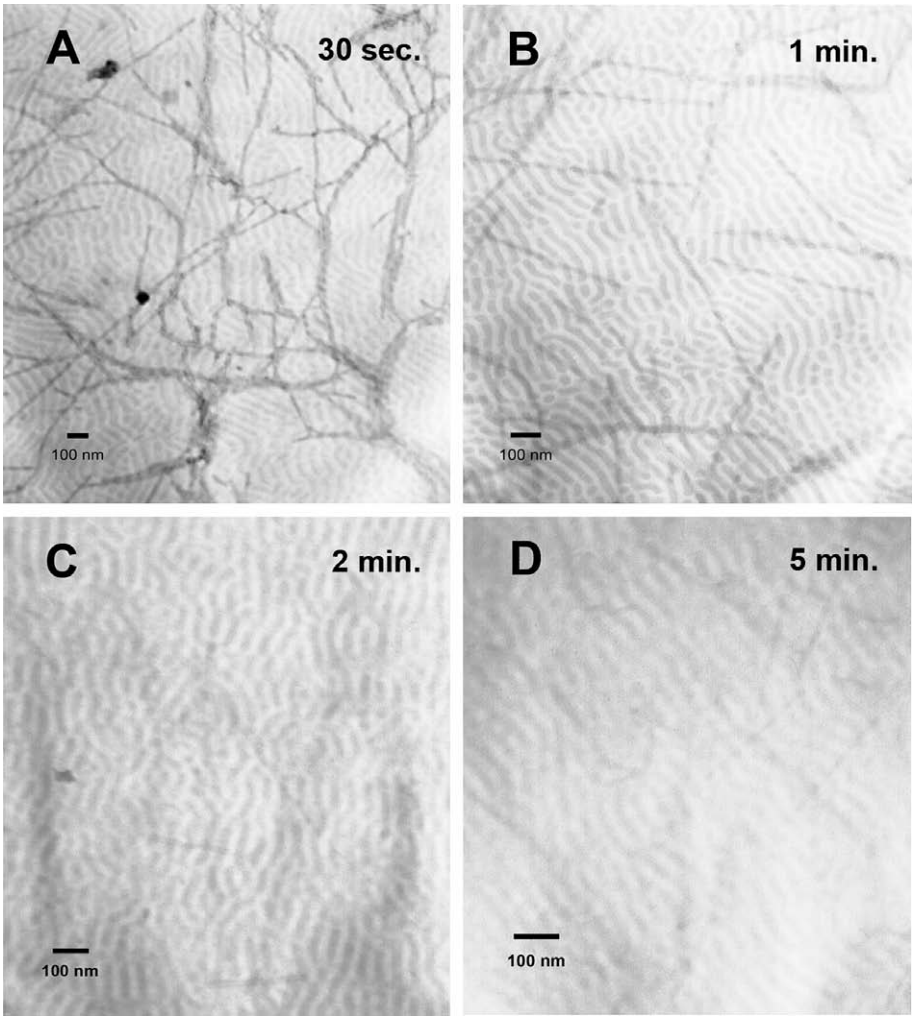

Fig. 9. Binding of TMV to PS/P4VP with no metal loading. Contrast in this TEM micrograph comes from post-virus exposure staining of the P4VP block with iodine vapor. As in Fig. 8, wash time in A is 30 s, B is 1 min, C is 2 min and D is 5 min. In D, after a 5 min exposure to Tween detergent, the virus is removed, leaving shadowy imprints but no visible virus particles.

Tween washes were performed on TNV as they were on TMV, and it was seen that results were consistent with results achieved by washing TMV with Tween. When exposed to PS/P4VP–Ni, the TNV was not removed completely after 5 min in a Tween wash, although there is continuously less and less virus over time. Fig. 11 shows TNV resisting Tween washes in a similar manner to TMV.

After 1 min in the Tween wash, it was observed that TNV had been mostly removed from the non-metal-containing polystyrene portion of the block copolymer microstructure. TNV virions were contained within the nickel-loaded 4-vinylpyridine cylinders. This is another positive indication that the virus does not bind through van der Waals or hydrogen bonding, but rather that the TNV specifically prefers the metal-loaded microstructure. The binding of TNV to the surface allowed observation of how individual viruses responded to individual parts of the block copolymer microstructure. Analysis of TNV micrographs showed that 87% of the TNV particles, after a 2 min Tween wash, remained adhered to the nickel-containing P4VP block of the copolymer. It thus seems likely

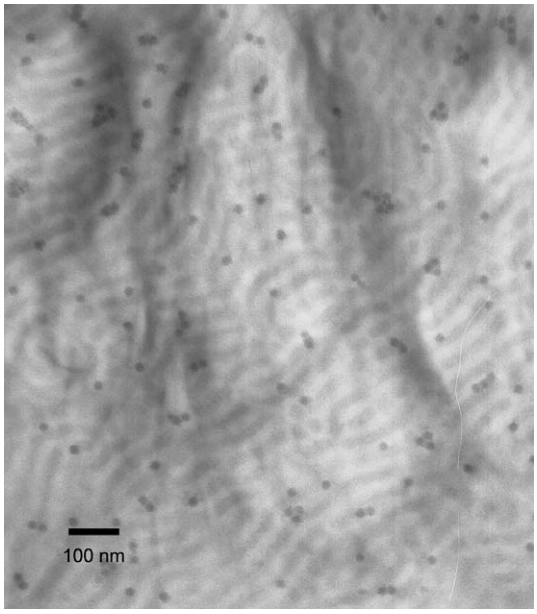

Fig. 10. TNV on the surface of the PS/P4VP–Ni block copolymer.

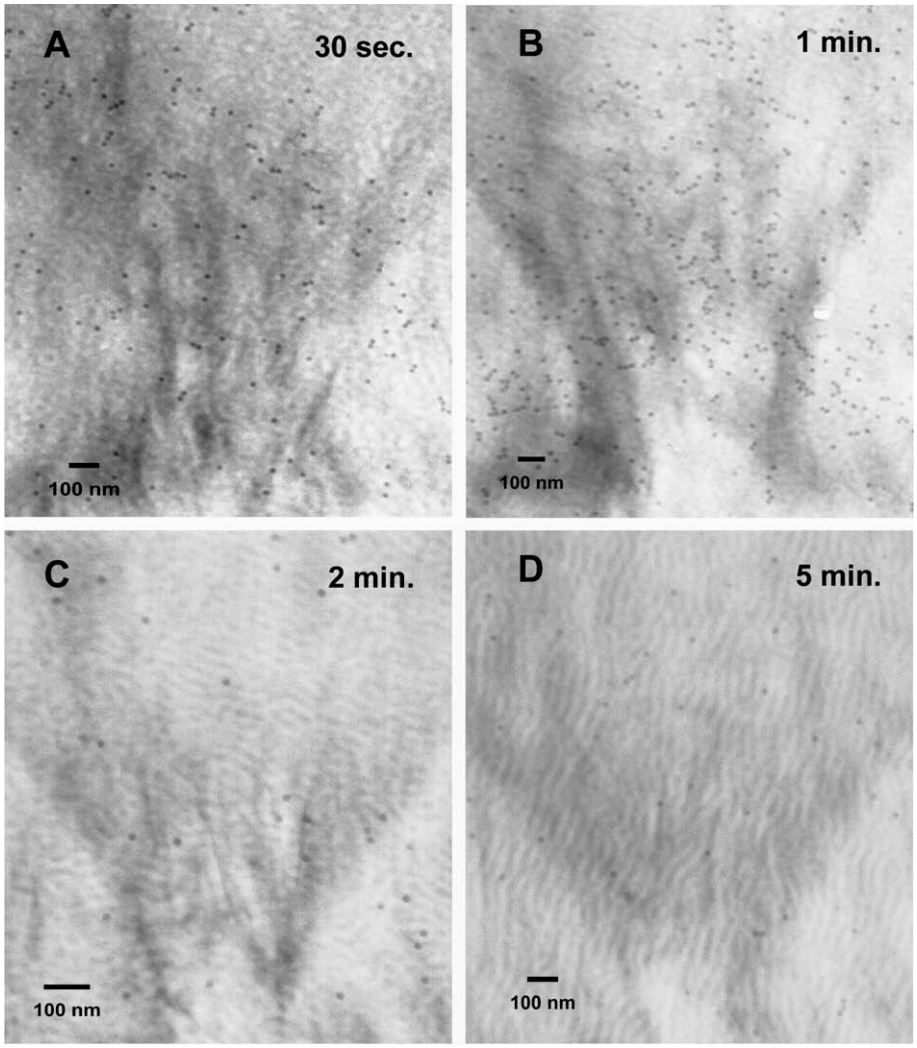

Fig. 11. Effect of Tween washes on the presence of TNV on the surface of PS/P4VP–Ni. As in TMV binding, 5 min of wash is not sufficient to remove all visible TNV from the surface.

that the virus particles are preferentially binding to the nickel-containing P4VP block. Non-nickel PS/P4VP was unable to show binding of TNV, as it did with TMV. Control experiments with PS and P4VP homopolymers only showed virus on the P4VP homopolymer after the Tween detergent wash procedure. Unoxidized nickel ions on the freshly microtomed PS/P4VP–Ni surface are assumed to carry the positive charge necessary for binding the TMV and TNV virions. PS/P4VP films, lacking the nickel ion and necessary positive charge, cannot strongly bind either TMV nor TNV.

Another interesting observation is the partial alignment of the TMV virions with the block copolymer microstructure, achieved with the help of shear-induced virus solution flow. The TMV virions were seen to lie at all angles randomly, with no preferred direction. The experiment involving dynamic exposure was performed with the flow direction coinciding with the long axis of the block copolymer. It was thought that this arrangement would make the TMV virions more likely to align with this long axis. As seen in Fig. 12, this situation is indeed possible, as single virions were seen to match up well with the block copolymer's microstructure.

4. Conclusions

Microphase separation of the PS/P4VP block copolymer cast from a mixed 91.5% $CHCl_3$ /8% THF selective solvent mixture resulted in a cylindrical microstructure with the nickel residing in the P4VP block. Nickel ion added in solution is confined within the block copolymer's P4VP microstructure by metal chelation to the pyridine nitrogen. A staining technique was developed to simultaneously visualize virus and block copolymer structure by TEM using a 1% solution of UA. It was shown that TMV and TNV viruses remain on the surface of nickel-loaded PS/P4VP block copolymers after detergent Tween washes.

The cylinder long axis of the microstructure was oriented using a hot press and a cooled channel die for

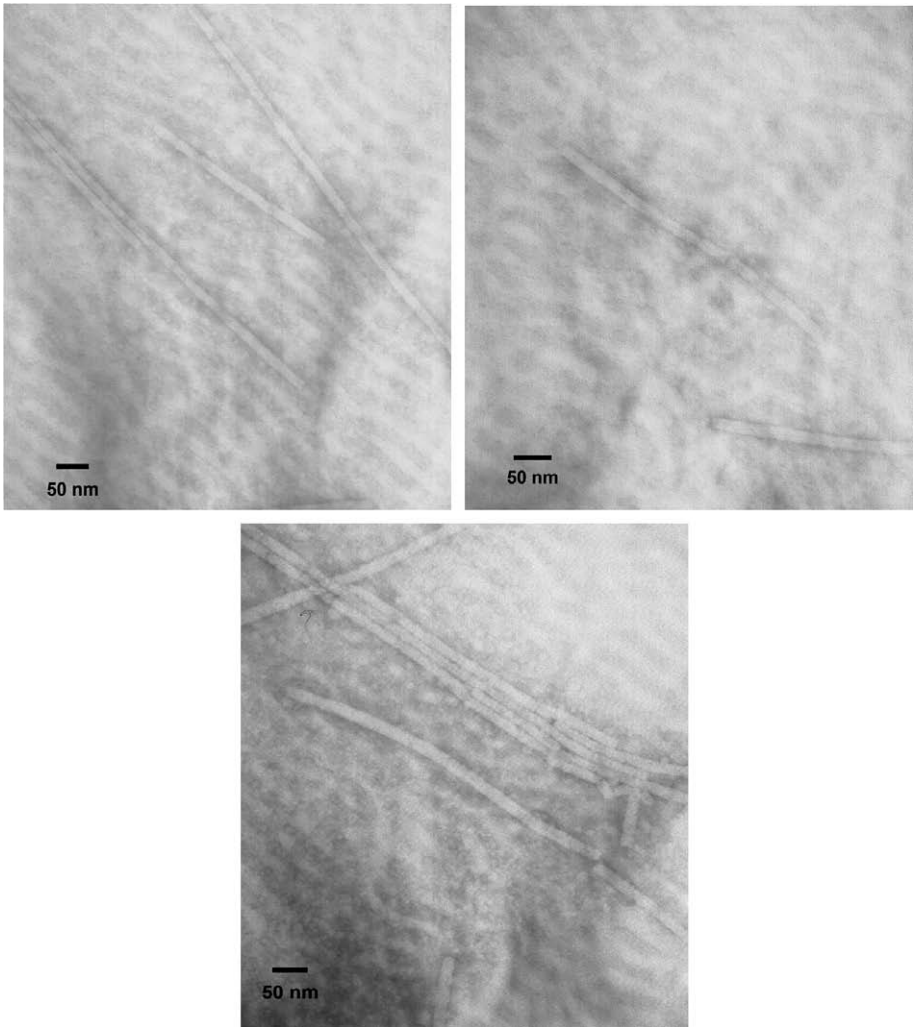

Fig. 12. TEM micrographs showing alignment of single TMV virions directly parallel to the flow axis of the PS/P4VP–Ni block copolymer microstructure.

quenching, resulting in PS/P4VP cylinders that had a strong anisotropic directional preference. This microstructure, when exposed by microtoming, was seen in the electron microscope to be able to bind both TMV and TNV virions when loaded with nickel.

Without nickel, the binding ability of the surface was no different than that of the Spurr's epoxy used to fix the PS/P4VP for microtoming. The biodetergent Tween was used to test binding because of its known ability to disrupt weak, non-specific binding in biological systems. In Tween washes of up to 2 h, TMV remained visible on the surface in increasingly diminished, but still significant, amounts. TMV, because of its size, was seen to cross several cylinders and often formed aggregates with other nearby TMV, although this did not seem to have an affect on the binding ability of the PS/P4VP surface. It was also observed that when exposed to flowing solutions of TMV, the PS/P4VP–Ni surface exhibited an ability to retain TMV in a partially aligned state, when the direction of flow coincided with the long axis of the PS/P4VP–Ni cylinders. It is therefore concluded that unlike-charge Coulombic interactions are the dominant mechanism in binding of the negatively charged TMV and TNV to the positively charged PS/P4VP–Ni block copolymer surface. The viability of virus nanopatterning by the PS/P4VP–Ni surface is validated by these results.

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