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Printing and Aligning Mesoscale Patterns of *Tobacco mosaic virus* on Surfaces

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In recent years, patterning surfaces with chemically-active moieties on the micrometer and nanometer scale has attracted intense interest due to its great potential for basic research, for the construction of electronic and photonic devices, and for possible diagnostic applications. Because large rod- or tube-like biological molecules such as DNA,[1] plant viruses,[2–4] protein tubes[5] and protein fibers[6] are often some tens of nanometers in size and can be produced with atomic precision, they are ideal building blocks for the study of molecularly-directed assembly of biomolecular arrays. There is growing interest in using such entities for nanoscale structuring, as functional units[7] or as templates.[1–6,8–11] One-dimensional structures are of particular interest due to potential quantum electronic-transport behavior[12] and also for wiring of nanodevices.[13] Possible applications such as in nanoscale biologically-active devices and biotemplated sub-10 nm structures require a transfer to a substrate surface on a selected area in a defined orientation.[14] An attractive biomolecule for templating one-dimensional nanostructures is the *Tobacco mosaic virus* (TMV), a tube-like supramolecular unit, made up from ~2130 identical protein subunits assembled in a right-handed helix around a single strand of RNA. The virion (virus particle) is a rigid rod, 18 nm in diameter and 300 nm in length.[15] Virions are physically and chemically very stable; they can be suspended in several polar solvents (for example, acetone and ethanol) without substantial changes of their structural integrity.

We have used TMV as molecular building block for mesoscale chemical structuring of surfaces. While for small molecules (such as alkane thiols) a molecule-covered scanning probe tip can be used for selective placement of molecules on a substrate,[14] to our knowledge this has not been demonstrated for TMV or other very-large supramolecular structures. However, TMV nanoarrays at the single-particle level have been fabricated by initially generating chemical templates of 16-thiohexadecanoic acid on a gold thin film with dip-pen nanolithography (DPN), followed by spatially-selective adsorption of plant-derived virions, including TMV.[16] An alternative, very-simple, and effective approach for mesoscopic structuring of surfaces with molecules is microcontact printing (μCP).[17–19] With this method, a chemically-structured surface can be obtained, for example by printing a self-assembled monolayer. One can then employ the chemical difference between the printed and bare substrate, for example for the spatially-selective adsorption of biomolecules.[17,20] More attractive and even simpler is the direct transfer (direct printing) of biomolecules in the desired pattern,[21] as shown for proteins and also for DNA.[20,22] An important question is up to what sizes large molecules or supramolecular assemblies can be printed without loss of structural integrity. Here we demonstrate that very-large supramolecular units (more than ten linearly-aggregated TMVs, each containing more than 2000 protein subunits) can be printed by a simple and elegant direct transfer. The processes of inking a hydrophilized siloxane relief structure (stamp) with a suspension of TMV particles, drying and contacting the stamp with a flat hydrophilic surface transfers patterns of particles with great precision. The particles are randomly placed inside the patterns. However, when the inking of the contact stamp is carefully controlled, nano-objects can assemble (''bottom-up'') by discontinuous de-wetting on the stamp. In this work, we show that selective adsorption and alignment of TMV on siloxane stamps can result in extended, printable nanostructures. This edge-printing process works for objects as large as TMV, which is 300 nm long and 18 nm in diameter, and for several micrometer-long lines of end-to-end assembled virions of a single virion width, which are similar to naturally-occurring TMV aggregates. The printing process consists of simply contacting the stamp with a flat substrate.

When a TMV suspension is dried on poly(dimethyl siloxane) (PDMS) stamps, atomic force microscopy (AFM) images show disordered, microscale spots with multilayer aggregates of TMVs. This is due to de-wetting on the strongly-hydrophobic stamp surface (water droplet contact angle ~108°) during drying. Successful printing requires a well-defined wettable surface. For this, we oxidized the PDMS surface with an oxygen plasma, rendering it hydrophilic (''Ox-PDMS'', contact

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ADVANCED MATERIALS

angle ~10°). In contrast to hydrophobic stamps, Ox-PDMS can be covered with well-dispersed TMV, simply by contacting the stamp with an aqueous virus suspension (concentration range 0.01 to 1 mg ml^{-1}). As in conventional microcontact printing, the stamp is dried with a gas stream, thereby removing surplus suspension and leaving a thin, well-dispersed layer of randomly-adsorbed virus particles. Such stamps can easily transfer the virions from their protruding features onto Mg^{2+}-pre-treated mica surfaces, as shown in Figure 1a and 1b. Although we have printed complete virions, the procedure is analogous to standard contact printing of small molecules. Due to the random distribution and orientation of the particles, the patterns were not homogeneously covered with virions; hence, some of the square-shaped areas in Figure 1a show very few virions, and some areas in Figure 1b show virions adsorbed on a virion layer (white spots between the two squares on the left).

As in conventional contact printing, successful pattern transfer requires a careful balance of adsorption energies – the virions have to adhere to the Ox-PDMS, but should be able to detach by interacting more strongly with the substrate (here Mg^{2+}-mica). The adhesion to the stamp is most likely due to interactions of the hydroxyl groups on the Ox-PDMS with various hydrophilic groups on the viral surface. In contrast to Ox-PDMS, a freshly-cleaved mica surface is negatively charged, as are virus particles suspended in water. We found that pretreatment with aqueous $MgCl_2$[23] improves pattern transfer: TMV interacts with Mg^{2+}-mica electrostatically, or through bridges formed by Mg^{2+}, interacting simultaneously with both the negative charges of the mica and the functional groups on TMV such as carboxylates.[24]

Figure 1c and 1d prove that the same procedure works on oxidized silicon wafer substrates, which are terminated with hydroxyl groups, even with higher coverage. As in the case of mica, we observed 100% selectivity, that is to say, the virions did not cover any non-contacted area. Note that the stripe pattern in Figure 1d, produced by virions randomly adsorbed on the printing stamp, is decorated by virions on its entire width. Such patterns can be formed by using high concentrations of TMV in the ink, for example 10 mg ml^{-1}, followed by drying the Ox-PDMS stamp with argon, or by allowing a droplet of a suspension of low concentration (0.2 mg ml^{-1}) to dry completely (in air for ~30 min). The corresponding adsorbed virions on a stamp are shown in Figure 2a. Here the stamp structure comprised 4 μm wide stripes with alternating void regions of approximately 1 μm width.

Obviously the interaction strength between TMV and the various substrate surfaces plays an essential role. We recently found substantial differences between TMV adsorption on hydrophilic and hydrophobic surfaces,[24] which are confirmed by the μCP results presented here. Because the outer surface of the TMV is covered with functional groups such as hydroxyl and carboxylate groups, the affinity to hydrophilic surfaces (Ox-PDMS, Mg^{2+}-mica, oxidized silicon) is very high. Water should be present as a thin film on TMV and on Ox-PDMS, and support this process. The strong adsorption prevents any mobility on Ox-PDMS and on mica or oxidized silicon: even contact AFM imaging with its intrinsically-high lateral forces cannot move TMV. When the Ox-PDMS surface is scanned at high forces, adsorbed TMV is dissected by the lateral movement of the scanning tip rather than shifted (see Supporting Information). Consequently, TMV particles are transferred in the same (random) distribution and orientation that they occupy on the Ox-PDMS surface.

The patterns (randomly-distributed TMV particles in predefined areas) shown in Figure 1 might be valuable for biological applications such as biomolecule detector arrays; however, ordered assemblies of virus particles are more desirable in order to expand the scope of applications to photonic or nanoelectronic devices, where metallized TMV can build up functional units like interconnects or channels.[8,25] For a better control of the printing process in μCP, we varied several experimental parameters. For example, a droplet of 0.2 mg ml^{-1} TMV suspension was contacted with an Ox-PDMS surface for 30 min, followed by its removal with a stream of inert gas, which removes in fact most of the virions (instead of allowing it to dry in air). In this case, TMV particles appear to bind with enhanced end-to-end aggregation at the base of all of the protruding regions of the stamp (Fig. 2b-d), similar to the aggregation on flat surfaces in the presence of divalent metal ions reported by Nedoluzhko et al.[26] In other words, while the protein-protein interactions operate towards helical arrangement in a single virion during virus assembly in vivo, the same interactions can cause end-to-end contact of two virions, which ''interlock'', even in vitro. In both cases, the preference for linear aggregates is based on the specific propensity of the coat protein to arrange helically, even in the absence of the helix-stabilizing RNA.[27] In fact, the virion assemblies (Fig. 2d, grey) are longer than those observed in standard adsorption experiments (up to about hexamers, Fig. 2d, black). The same result was found when a low-concentration (0.01 mg ml^{-1}) virion suspension was allowed to slowly dry in air on Ox-PDMS (without removal of TMV by a gas stream, see Supporting Information).

However, end-to-end aggregation of virions cannot be the only driving force for the selective placement of virus particles at the base of the relief structure since single virus particles can also be found. We believe that the selective placement of virus particles is due to discontinuous de-wetting (Fig. 2c), caused by selective wetting of the recesses by solvents that de-wet on the stamp surface, a process that has previously been used for selective filling of microwell arrays in PDMS.[19,28] This process should not depend much on the shape of the suspended particles. Indeed, we found that using carboxylate-coated fluorescent polymer beads (in the place of TMV), coat only the stamp recesses, with preferred aggregation at the bases of the protruding features (see Supporting Information). If de-wetting indeed plays a role, it should be possible to switch from discontinuous to continuous de-wetting by changing the solid/liquid interaction. We prepared mixed aqueous/ethanolic suspensions of TMV particles, since ethanol does not only wet Ox-PDMS, but even hydrophobic PDMS to a certain

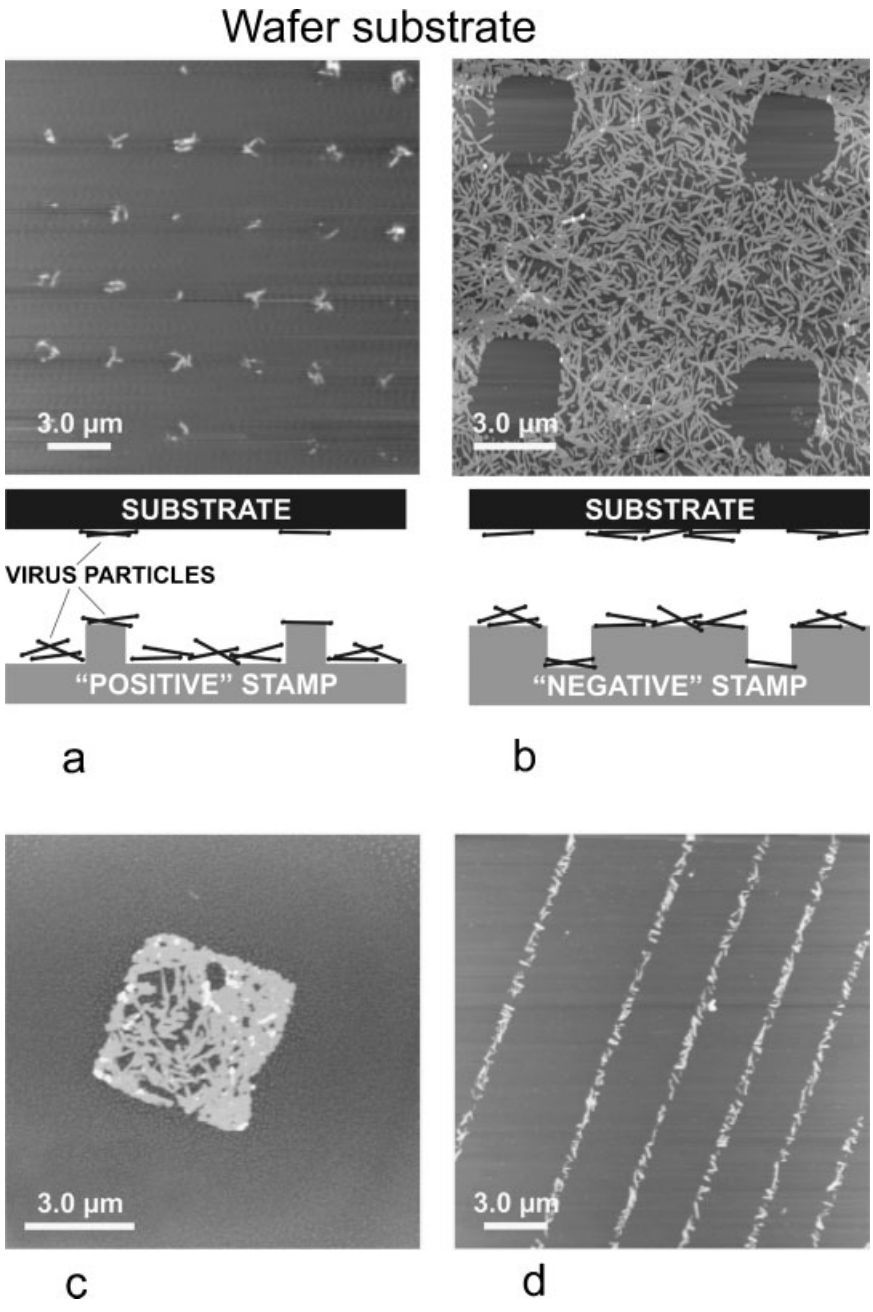

Figure 1. Conventional microcontact printing of TMV particles: Ox-PDMS (surface-oxidized printing stamps) were contacted with a concentrated (10 mg ml^{-1}), aqueous virus suspension for 30 min, blow-dried with argon (removal of surplus suspension), and printed on flat mica (pre-treated with aqueous $MgCl_2$) or on flat oxidized silicon wafers for 10 to 20 s. The virion-covered protrusions transfer a pattern composed of randomly-adsorbed virus particles. a) AFM image (contact mode) of a mica surface, patterned with TMV in discrete squares with a ''positive'' stamp (stamp features: 1 µm squares). The first spot in the second row carries a single virus particle, all of the others at least three. b) AFM image (noncontact mode) of a mica surface, patterned with TMV particles in discrete areas with a ''negative'' stamp (stamp feature: grid (inverse squares)). c) AFM image (noncontact) of an oxidized silicon surface, patterned with TMV particles with a ''positive'' stamp (stamp features: 4 µm squares; only a single square was imaged). d) AFM image (noncontact) of an oxidized silicon surface, patterned with TMV in discrete stripes with a ''positive'' stamp (stamp features: stripes).

extent[28] – in fact, this is the prerequisite for the standard method of inking with long-chain alkane thiols. In TMV suspensions with increasing amounts of ethanol we still observed selective placement of virions (see Supporting Information). At around 75% ethanol, the discontinuous de-wetting is broken, the entire stamp is coated, and randomly-adsorbed TMV particles are detected (see Fig. 3a). Obviously, the surface chemistry of the stamp is decisive: an amine coating of the Ox-PDMS (by

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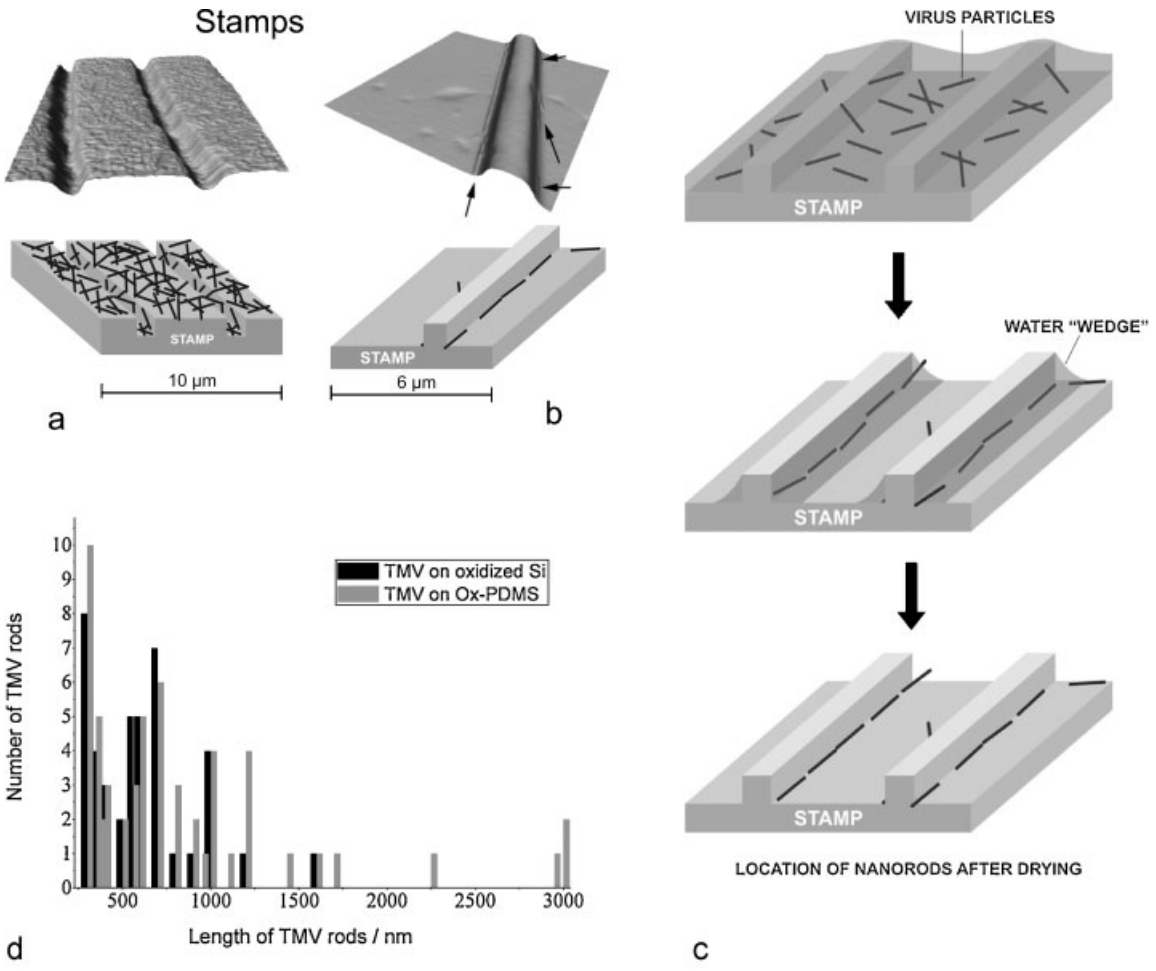


Figure 2. Drying TMV suspensions on stamps: AFM images (noncontact mode) of TMV particles adsorbed on Ox-PDMS stamps from 0.2 mg ml^{-1} aqueous TMV suspension. a) The suspension was dried in air, leaving a thick, but continuous layer of virions, adsorbed in a random distribution on the "negatively"-structured stamp surface. The raised regions are ~4 μm wide and 300 nm high. b) The suspension was removed with an argon stream after 2 min contact. End-to-end assemblies of TMV particles (arrows) formed at the base of the protruding ~1 μm wide and 300 nm high stripe. c) Model of the discontinuous de-wetting operating in (b): Formation of water wells in recesses, and of water wedges and finally aligned particles upon further drying. d) Length distribution of TMV (number versus length of the rods in nm) of TMV randomly adsorbed on flat oxidized silicon wafers (black, as in (a)) compared with TMV adsorbed and aligned at the bases of protruding features of Ox-PDMS structures (grey, as in (b)).

applying 3-aminopropyltriethoxysilane (APTES)) can break the discontinuous de-wetting already for a pure aqueous TMV suspension (Fig. 3b). Note that in both cases the suspensions were dried with an argon stream (as in Fig. 2b), but still the virus suspensions tended to coat the entire surface (raised and recessed regions of the stamp), rather than selectively covering the bases.

We found that the duration of the oxygen plasma treatment of the PDMS surface has little influence; end-to-end assemblies of TMV similar to those in Figure 2b were observed when the plasma treatment time was extended from 2 to 10 s. This also correlates with contact-angle measurements, which show that exposure from 2 to 30 s does not change the water-droplet contact angle of the stamp surface significantly from ~10°, which translates into a nearly completely hydrophilic character.

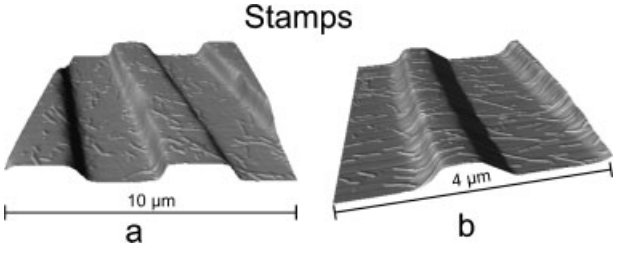
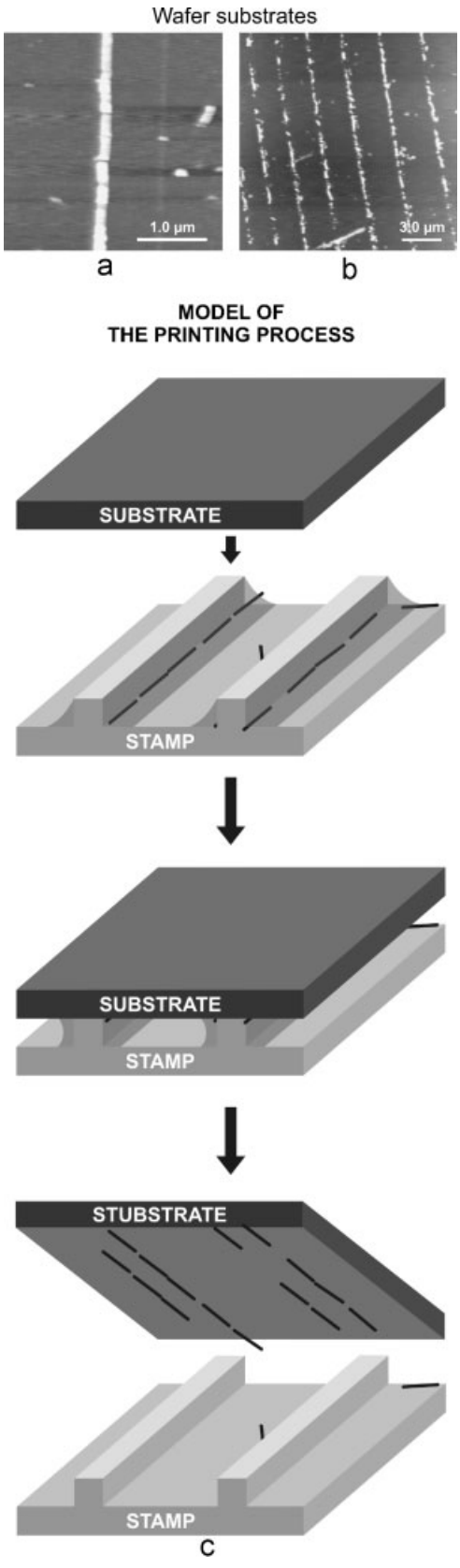


Figure 3. Wetting of virions on stamp surfaces: AFM images (noncontact mode) of randomly adsorbed TMV particles on Ox-PDMS surfaces after 30 min contact with 0.2 mg ml^{-1} TMV suspension, followed by removal of surplus suspension with an argon stream. a) Suspension in 75% ethanol +25% water. b) Aqueous suspension, but Ox-PDMS surface modified with 3-aminopropyltriethoxysilane (c.f. Fig. 2(b) for pure Ox-PDMS).



Most surprisingly, the patterns observed at the base of the protruding regions of the stamp structures (Fig. 2b) can easily be transferred by μCP to solid surfaces of interest. AFM observations revealed that end-to-end assembled TMV particles, selectively placed at the bases of the relief structures, were also found to be aligned and assembled when printed on oxidized silicon wafers (Fig. 4). The measured heights vary from 10 to 15 nm, which shows that the virions are not destroyed.[24] However, while the discontinuous de-wetting and selective placement of the TMV particles is highly reproducible, the transfer of the aligned particles to flat surfaces is successful only in about half of the experiments. Clearly, this could be improved by fine-tuning of solvent mixture, particle concentration in the ink, etc. In some cases, the transferred TMV lines consisted of multiple subunits with lengths of ~300 nm each, which matches the length of a single virion, separated by nanometer-sized gaps (Fig. 4a).

We have not been able to elucidate the printing mechanism completely; however, the presence of water in carefully-dried TMV samples shows that after drying in air or with a stream of argon, considerable amounts of water are still present. Indeed, infrared spectra of pristine and plasma-treated (oxidized) PDMS show adsorbed liquid water only in the latter. The mechanism of pattern transfer from the bases of the protruding features of the stamp structure to hydroxyl-terminated silicon wafers may involve transport of TMV particles via a meniscus of adsorbed water, similar to the mechanism of material transfer from the sides of the AFM tip in DPN, but occurring on a smaller scale, better comparable with edge-transfer lithography of molecules.[14,19] Discontinuous de-wetting would place larger amounts of water in the recesses than on the flat parts of the stamp. Upon contact with a hydrophilic flat surface, this water could form a wedge and transport TMV to the surface, while conformal contact with the protruding structures would hinder any adsorption inside the ''positive'' patterns (Fig. 4c). However, such a transfer cannot be perfect in terms of the amount of printed material. In fact, after a second and a third printing experiment without re-inking, we still found TMV particles at the bases of the protruding regions of the stamp structure, but in a lower density (see Supporting Information).

In summary, we show that the TMV can be organized in mesoscale structures of a single virion width by simple and elegant printing. Microcontact printing with *micrometer*-sized stamp patterns can form *nanoscale* virus particle patterns. First, microcontact printing of TMV on a variety of surfaces is

Figure 4. Printing of aligned TMV: AFM images (noncontact model) of end-to-end assembled TMV lines (height ~10 nm) formed by printing stamps of the type in Figure 2b on flat oxidized silicon wafers. a) Gaps (3 to 8 nm high, 6 nm wide) between individual, 300 nm long virus particles are clearly discernible (the width of the particles is much exaggerated due to a blunt AFM tip). b) Large-area patterning; the distances between the lines correspond to the stripe widths on the stamp and vary from ~2.1 μm to ~2.8 μm from left to right. c) Model of the printing process: TMV diffusion in water wedges.

ADVANCED MATERIALS

possible if the surface of the siloxane printing stamp is rendered hydrophilic with an oxygen plasma. In this case, patterns can be filled with randomly-arranged virions. Secondly, careful control of the experimental parameters (solvent and concentration of the virus suspension, stamp surface chemistry, stamp drying) allows selective placing of aligned TMV at the relief features of the stamps, originating from discontinuous de-wetting. Thirdly, these virions can be transferred to flat, hydrophilic solid surfaces, presumably by transfer through a wedge of water, resulting in several-micrometer-long, end-to-end assembled TMV particles of a single virion width. Collectively, our results demonstrate that microcontact printing can produce nanoscale patterns of TMV particles, which could be used as functional units in future nano- and optoelectronics (for example, in combination with nanoscale metallization).[3,4,8] We suggest employing this powerful structuring method also for other large molecules, and for hard-matter nano-objects such as inorganic nanoparticles.

Experimental

Plasmid DNA containing a full-length copy of TMV cDNA was employed to mechanically infect *Nicotiana tabacum* cv. Samsun NN plants. For details about the isolation of the TMV particles from the plants and their storage see ref. [24]. AFM images were recorded with a Thermomicroscopes M5 Autoprobe operated in noncontact and intermittent contact modes, with MikroMasch NSC11 or NCHR Pointprobe Nanosensors cantilevers. For the μCP procedure, patterned PDMS (poly(dimethyl siloxane), Sylgard 184, Dow Corning) stamps were fabricated by casting an elastomeric polymer against silicon masters (Institut für Mikroelektronik Stuttgart, IMS), which were rendered hydrophobic with fluoroalkyl-trichlorosilane vapour before use.

A typical experiment required first rendering the stamps hydrophilic. The PDMS stamps were treated for >2 s with a 1 mbar oxygen plasma (nominal power 200 W, Technics Plasma 100-E, TePla), immersed for 30 min in up to 0.2 mg ml^{-1} TMV suspensions (in water or phosphate buffer), dried with an argon stream, and contacted for 10–20 s with Mg^{2+}-mica surfaces (mica pretreated with 100 mM aqueous $MgCl_2{\cdot}6H_2O$ (Aldrich) for 10 min). We also employed oxidized silicon wafers, terminated with hydroxyl groups by the so-called "RCA" procedure (for preparation see ref. [24]), which do not require pretreatment with salts.

3-Aminopropyltriethoxysilane (APTES) (Aldrich) was used for terminating an oxygen plasma-treated PDMS surface with amine functions. For such an experiment, an Ox-PDMS stamp was dipped in 10 ml of water, containing 6 μl APTES, for 2 min at room temperature and then washed with water. The contact angles of pure-water droplets were recorded with an OCAH 230 (Dataphysics) model contact-angle machine. Water was purified with a Millipore Gradient A10 apparatus. Pure ethanol (p.a. = 99.9%, Merck) was used for the ethanolic suspensions of TMV. Fluoresbrite™ carboxy NYO 0.05 micron microspheres (Polysciences, Inc.) were used in the confocal microscopy imaging (Leica TCS SP2-DM RE) of the stripe pattern of the stamp (Ox-PDMS).

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