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Transfer-printing of single DNA molecule arrays on graphene for high resolution electron imaging and analysis

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Abstract

Graphene represents the ultimate substrate for high-resolution transmission electron microscopy, but the deposition of biological samples on this highly hydrophobic material has until now been a challenge. We present a reliable method for depositing ordered arrays of individual elongated DNA molecules on single-layer graphene substrates for high resolution electron beam imaging and electron energy loss spectroscopy analysis. This method is a necessary step towards the observation of single elongated DNA molecules with single base spatial resolution to directly read genetic and epigenetic information.

Keywords

Graphene; DNA; transmission electron microscopy; electron energy loss spectroscopy; nanopatterning; genetics/epigenetics

Direct imaging and identification of the identity of bases in individual DNA molecules has been a long standing goal. Three things are required to do this. The first is a method with sufficient spatial resolution to resolve closely-spaced monomer units in the macromolecule. The second is a contrast mechanism to differentiate the base or monomer identity. The third is controlling the conformation of the long biopolymer so the order of the sequence monomer identities can be unambiguously determined. While various combinations of these requirements have been demonstrated, until now the three have not been simultaneously satisfied in one method. For example, numerous methods have been demonstrated for elongating DNA molecules on surfaces or in fluid channels^{1–9} and these have been interrogated by optical imaging techniques with fluorescent labels for molecular identification. While the requirements on molecular conformation and contrast are satisfied, the optical techniques do not have the required practical spatial resolution. Tanaka et al. recently reported single-molecule DNA sequencing capabilities with a scanning tunneling microscope (STM) by using an oblique pulse-injection method to deposit the molecules onto a copper surface. They show they can identify the electronic fingerprint of guanine bases in long-chain DNA molecules with atomic resolution¹⁰. While scanning probe techniques have the required spatial resolution, the method is somewhat limited as it is based upon local differential density of state. Transmission electron microscopy (TEM) also possesses the required spatial resolution¹¹, with the advantage of contrast based on elemental composition using electron energy loss to identify natural or labeled elemental differences. Conventional TEM and STM are primarily imaging techniques, not designed for rapid chemical analysis. Conventional instrumentation may not be compatible with industrial sequencing. We believe the ultimate resolution and elemental composition analysis possibilities of electron beam

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analysis suggests that appropriately designed instrumentation could obtain both genetic and epigenetic information from single molecules. In this approach having ordered and elongated molecules greatly simplifies these analyses. Elongation of molecules for direct electron imaging has been an issue that we have been addressing. We showed, for example, that electrospinning of DNA in nanofibers can present individual elongated DNA molecules in a thin support medium for electron beam analysis,¹⁻² but the thickness of the supporting medium limits the possibilities for elemental and spatial analysis. The ideal case would be to have no supporting medium, but this is of course not possible. The best possible case would be to have a high-strength, electrically-conducting, single element, low atomic number support on which one could reliably elongate and place molecules. The best candidate for this is single-atom-thick graphene.¹²⁻¹⁵ At a single layer thickness of 0.34 nm, the minimal scattering cross-section of graphene also minimizes background (noise) contributed by inelastic and multiple scattering within the substrate. In this work we demonstrate a reliable technique for elongating individual DNA molecules and transferring them in ordered arrays to a single-atom-thick mechanical support layer for high resolution scanning transmission electron microscopy and elemental analysis by electron energy loss spectroscopy. This approach satisfies the three requirements above and adds the advantage of being able to pre-treat the DNA and create spatial order that can simplify the analysis. While the complexity of the STEM systems limits the throughput and practicality of this approach it demonstrates the possibilities for electron beam analysis of individual biopolymers.

DNA is often characterized using fluorescence microscopy or atomic force microscopy, but few studies¹⁶⁻¹⁹ have characterized or analyzed it by electron microscopy. In fact, light elements (with low atomic number) are essentially transparent to electron microscopes which generate contrast from the charges on atomic nuclei. DNA is mostly made of carbon, nitrogen, and oxygen, with small amounts of hydrogen and phosphorus giving an average atomic number of 5.5. DNA is, as a result, inherently low contrast for electron imaging. Previous studies have reported the deposition of coiled double-stranded DNA or single-stranded oligonucleotides by incubation and drying of the molecules on graphene.²⁰⁻²¹ But the characterization of DNA in its coiled state hinders obtaining information that can be more easily read from elongated molecules. In fact, chromatin, also contains valuable information in epigenetic or genetic disorders such as cancer. Thus analyzing and identifying the placement of these epigenetic marks with single nucleotide resolution across the entire genome would represent a significant step forward. In the present paper, we present a technique to transfer regular arrays of individual elongated DNA molecules onto single-layer graphene substrates. Our method relies on assembling DNA on a microstructured PDMS stamp by capillary assembly. We previously reported this experimental procedure²²⁻²³ to transfer arrays of single phage lambda DNA molecules from a PDMS stamp to a hydrophilic and positively-charged surface by simple contact in dry conditions. Here, graphene being highly hydrophobic, the transfer is performed with solvent mediation. We obtain regular arrays of single phage lambda DNA molecules adsorbed on graphene following a Poissonian distribution and with a 91% success rate.²⁴ We prove that subsequent imaging of the assembled molecules is possible using a transmission electron microscope without any prior metallization or labeling of the DNA molecules.

To prepare phage lambda DNA solution, 100 μ l phage lambda DNA solution (Sigma, 48 502 bp, 329 μ g/ml diluted to 50 μ g/ml in 10 mM Tris-HCl / 1 mM EDTA, pH 8) was heated at 65°C for 5 min and dipped into ice water to avoid molecular concatenation. For fluorescence imaging, the solution was then fluorescently labeled with YOYO-1 intercalator (Invitrogen) by adding 1.5 μ l of YOYO-1 (100 μ M); incubation was conducted in the dark at room temperature for a minimum of 2 hours. Following the labeling reaction, samples were protected from light and stored at 4°C. Phage lambda DNA solution was further diluted to a final concentration of 10 μ g/ml in the same buffer with 0.1% v/v Triton X-100. Note that for

electron beam imaging, the DNA molecules were used unlabeled. To direct the capillary assembly of phage lambda DNA, we used PDMS stamps with topographical cavities obtained from the replication of a positive silicon master. The silicon micropatterned master was achieved by ultraviolet photolithography and the pattern transfer by deep reactive ion etching. The PDMS prepolymer solution containing a mixture of 10:1 mass ratio of PDMS oligomers and a reticular agent from Sylgard 184 Kit (Dow Corning, Wilmington, DE) was then poured onto the silicon master and cured at a temperature of 80°C during 2 hours. The cured PDMS was peeled off and cut into 1.8 cm × 1.4 cm stamps. In a general manner, the design of the topographic patterns requires a prior reflexion in terms of distribution, dimension, depth, and orientation. In fact, the size of the patterns determines the number and the positioning of the objects to be assembled for assemblies of controlled geometry. Given the dispersion in size of the objects in solution, the patterns are usually intentionally enlarged to facilitate the assembly by compensating the fluctuations in size among the objects. The depth of the patterns is also a key geometrical parameter to take into consideration as it determines the number of layers to be deposited inside the patterns and ensures the subsequent transfer of the assembled objects. When the stamp is used as support for capillary assembly, the design rule to keep in mind is that the deformation of the liquid contact line has to be minimized in order to avoid its premature disruption, and allow the forces involved to direct and gather the objects at the liquid front line to fill the cavities appropriately. So additionally, the periodicity of the patterns has to be large enough so the contact line can get pinned on each row of patterns without missing one. In the case of DNA molecules' assembly, the silicon master was designed with protruding microfeatures 5 μm and 8 μm in diameter, 5 μm high and with different periodicities (20 μm, and 25 μm). Therefore, the corresponding PDMS stamps are the negatives of the master and consist of microcavities with the same sizes. The directed assembly is carried out using a dedicated setup. The microstructured PDMS stamp where we want the DNA molecules to be assembled is placed on a motorized translation stage below a fixed glass spreader at a distance of about 1 mm. A 15 μl droplet of DNA molecules in solution at a concentration of 10 μg/ml is injected between the glass and the substrate. The liquid contact line is therefore moved over the substrate at a constant velocity of 0.5 mm/sec for the trapped DNA molecules to be stretched. The experiment is conducted at ambient temperature. The experimental parameters (speed, concentration) are adjusted to enable the directed assembly and combing of single DNA molecules with high placement accuracy. The assembly is performed throughout the entire surface of the PDMS stamp, so approximately over an area of more than 1 cm², allowing the analysis of approximately ~250'000 molecules over an entire substrate.

Graphene was grown using chemical vapor deposition on copper foils.²⁵ It was verified to be predominantly single-layer by Raman spectroscopy.²⁶ The graphene sheets were then transferred on silicon dioxide substrates or molybdenum TEM grids with lacey carbon support films (Pacific Grid Tech, San Francisco, CA) following the graphene transfer technique developed in ²⁷. After chemical vapor deposition, a 50 nm-thick poly(methyl-methacrylate) (PMMA) film was spin-coated on the copper foil. Copper was etched in a ferric chloride solution; the graphene/PMMA was then transferred on TEM grids and PMMA was dissolved in a dichloromethane bath for 6 hours.^{25, 28}

Figure 1 displays the scanning electron microscope image of single layer graphene suspended over the lacey carbon TEM grids. For AFM imaging, graphene was prepared by mechanical exfoliation²⁹ and deposited on a freshly cleaned silicon dioxide substrate. To transfer the formed DNA arrays, a droplet of solvent (absolute ethanol) is placed on the graphene substrate (graphene on SiO₂ or on TEM grids). Ethanol having a low surface tension, it spreads easily creating a thin film of liquid all over the substrate. Ethanol is then left to evaporate, but not fully, and the PDMS stamp with the assembled DNA molecules is

then brought into contact with the wet graphene substrate for 2–3 min for the solvent to fully evaporate. The PDMS stamp is then peeled away (Figure 2).

The molecules' transfer on graphene was controlled under an upright epifluorescence microscope ($\times 20$ and $\times 50$ objectives) from Olympus equipped with a 512×512 camera (Photometrics). In the directed assembly technique, patterning is used to create a well-defined spatial distribution of forces that direct the motion of molecules in solution towards specific areas of a substrate. In our case, we use a PDMS stamp with topographical features to direct that assembly process. The experimental parameters, namely, the concentration and the displacement speed are chosen to trap and stretch individual molecules. After capillary assembly, the resulting DNA array is transferred onto the graphene substrate. In practice, we deposit a droplet of ethanol not fully allowed to dry on the graphene substrate and the PDMS stamp is brought into contact with that wet surface for 2 min, without exerting any additional pressure at the back of the stamp. The solvent placed between the PDMS stamp and the substrate during contact mediates the transfer. The PDMS stamp is finally removed, leaving the DNA array on the graphene surface (Figure 2). In general, for the transfer to occur, the molecules need to have more affinity for the target surface than for the PDMS stamp's surface. In the present case, the two surfaces are highly hydrophobic (PDMS vs graphene) with a measured contact angle of $108^\circ \pm 2^\circ$ and $92^\circ \pm 2^\circ$ respectively, so when the contact is made in dry conditions, the molecules are not naturally transferred from the stamp to the graphene surface.

Figure 3 shows a fluorescence micrograph of individual DNA molecules transferred to CVD graphene on silicon dioxide with solvent mediation. We observe that the transfer is performed reliably over large areas. All the molecules present at the surface of the PDMS stamp are transferred. Furthermore, we observe the presence of periodic fluorescent spots that correspond to the material initially contained in the PDMS wells during capillary assembly. The transfer method is so effective that even the material trapped and not directly in contact with the surface is transferred. However, not all liquids or solvents are proper to transfer the assemblies from a PDMS stamp onto a substrate. In our case, the category of good solvents such as trichloroethylene, hexane, toluene are to be excluded as they irreversibly deform and damage the PDMS stamp, and are not compatible with biology in a more general manner. In this regard, water could have been a candidate to consider, but its surface tension in the presence of a hydrophobic surface prevents the creation of a thin layer of liquid and prevents the PDMS stamp from contacting the surface in a conformal manner as well. Consequently, we suggest different hypotheses concerning the influence of ethanol. On the one hand, compared to ethyl acetate for example, ethanol only exerts a swelling extent of 6.3% on a weight basis of bulk PDMS.³⁰ But this swelling may modify in some manner the PDMS features and facilitate the release of the assembled molecules. On the other hand, during contact of the stamp with the substrate, in liquid, the wetting of the surface is locally and temporarily modified while the evaporation process may lead to the creation of capillary forces that direct the elongated molecules towards the wetting target surface. Thus, the natural evaporation process may facilitate the release of the trapped and elongated molecules. However, it is still difficult to know which one of these candidates is responsible for molecule transfer. We also notice that the fluorescence intensity of YOYO-1 intercalated DNA molecules on graphene is somewhat lower than the fluorescence intensity one could obtain with molecules transferred onto glass. This suggests that there is a certain degree of quenching provoked by graphene, in agreement with previous results.³¹ This, however, does not prevent the characterization of molecules using fluorescence microscopy. To further demonstrate that the DNA array is composed of individual molecules, we performed the transfer on exfoliated graphene. In fact, the presence of residual iron particles ~ 10 nm in diameter on the CVD graphene after its transfer to silicon dioxide inhibits the proper characterization of individual molecules with such a technique. Thus we chose

exfoliated graphene, well-known for its atomically-flat surface as our substrate for AFM imaging purposes. For AFM imaging and measurements we used a NanoScope IIIa from Digital Instruments. All imaging was done in tapping mode in air, with a resolution of 512×512 using NC silicon AFM probes (Bruker Company).

Figure 4 shows a bright field, fluorescence and AFM image of the same area of a silicon dioxide substrate with exfoliated graphene after transfer of a DNA array. In the bright field image we observe the presence of the PDMS stamp feature imprints. In the fluorescence image, figure 4B, the elongated DNA molecule which is part of the array and positioned on the exfoliated graphene is not visible possibly due to quenching. However, its presence can be detected by AFM. Figure 4C1 shows an enlarged image of a DNA molecule on silicon dioxide. From the corresponding cross-section we observe that it is a single molecule measuring 1.57 nm in height. Figure 4C2 shows a magnified image of a DNA molecule on exfoliated graphene. In contrast to the measurement on silicon dioxide, the roughness is comparable to the height of the molecule (2 nm on average). We attribute this roughness to impurities attracted to exfoliated graphene during the transfer process that we do not observe on silicon dioxide. The measurements from the different AFM and fluorescence images show that the DNA molecules measure in average $16.3 \mu\text{m} \pm 4.4 \mu\text{m}$ long, which is approximately equal to the theoretical length of individual phage lambda DNA molecules.³²

By extension, the transfer process can be performed on any type of graphene substrates such as TEM grids with lacey carbon support films. In this case, commercial molybdenum TEM grids pre-coated with a web of amorphous carbon fibers (lacey carbon) are used as a support to suspend atomically-thick graphene films.

Figure 5A shows a fluorescence image of nucleic acid-stained DNA molecules transferred on this type of grids. From this image we recognize the bright spots corresponding in periodicity to the patterns of the PDMS stamp. However, single molecules are not visible by fluorescence as the autofluorescence of the grid is much higher than that of silicon dioxide. Note that for the various graphene substrates (silicon dioxide or TEM grids), the layer of graphene was examined after transfer. No damage is perceived under optical microscopy or Raman spectroscopy. The single-layer sheet of graphene is preserved after the PDMS stamp is peeled off. Figures 5B, 5C, 5D, show the transmission electron micrographs obtained from DNA elongated and adsorbed on lacey carbon grids with suspended graphene. STEM imaging was performed using a field emission transmission electron microscope with monochromator (Tecnai F20) operated at 200 kV, with a 200 mm camera in dark field mode. Note that no plasma treatment or heating step was required prior to imaging. First, we observe that the images show little charging or contamination. This implies that our methodology as a whole is very clean and adapted to high resolution imaging purposes. Second, we observe that suspended single-layer graphene sheets remain on the grid after transfer and appear undamaged, so the forces exerted during PDMS peel-off are low enough to prevent graphene from rupturing. Third, while DNA could not be imaged using standard TEM grids without prior labeling, on single-layer graphene TEM grids DNA can be characterized with no difficulties and long exposure times. DNA appears to be undamaged after transfer, measuring 2–3 nm wide in the case of single molecules. If we compare the outside region of the lacey carbon and the inner area (Figure 5B), we can see that the contrast in the presence of suspended graphene is much higher. Figures 5C and 5D show higher magnification micrographs of the region shown in Figure 5B, where the molecule seems to present a B conformation with a 4.6 nm pitch. The periodicity of the double-helix is in this case 1.35 times greater than the theoretical pitch reported by Watson and Crick,³³ but these observations seem consistent with the ones reported in literature in similar conditions.^{17–18,34} Instead of considering the inelastic energy as a whole, the spectral distribution of the forward scattered electrons can be analyzed separately. A highly specific

signature lies in the characteristic core-loss edges: their energy position corresponds to a given atomic level and therefore identifies a given element within the irradiated volume. A way of exploiting electron energy loss spectroscopy (EELS) is to produce images representative of elemental distribution by scanning the probe, recording several energy-filtered images on both sides of the core-loss edge, and processing them pixel by pixel display maps of the resulting characteristic signal. We know that our support is constituted by a molybdenum lacey-carbon grid and graphene, both composed primarily of molybdenum, iron, and carbon elements.

We selected an area with elongated DNA molecules as shown by the bright field image (figure 6A), and we recorded a 20×20 EELS mapping within a 90 eV – 1140 eV window with a 4 seconds acquisition time per pixel and a 0.5 eV dispersion. Interested in obtaining a characteristic signature of DNA, we then filtered the resulting map, using the Cornell Spectrum Imager software, at an energy-loss of 130 eV corresponding to the edge of phosphorous. Although the total acquisition time was close to 1 hour, the specimen remained stable and no noticeable change of the phosphorous energy loss signal was observed. The presence of phosphorous is observed all along the molecule (Figure 6B). This suggests that phosphorous can be used as an indicator of individual bases. If the DNA molecule can be formed with non-native elemental labels on the different bases, the energy filtered images can reveal the base sequence. Figure 6C, we notice from the accumulated spectra extracted from the entire map without energy filtering, the K edges of carbon (285 eV), nitrogen (400 eV) and oxygen (532 eV) as expected. We see at around 130 eV the L₂₃ edge of phosphorous corresponding to the excitation of 2p electrons. Note that in that range, we also observe the L₂₃ edge of silicon (99 eV) more likely coming from PDMS residues during transfer.

By combining directed assembly on a PDMS stamp and microcontact printing with solvent mediation, we benefit from the advantages of both techniques at the same time: the control over the assembly process and the flexibility and simplicity of the printing technique. This printing method can handle single objects while preserving their intrinsic properties. It is adaptable to parallel processing over various hydrophilic/hydrophobic substrates and over large areas. In particular, the transfer on graphene opens a wide horizon of possible applications and could be an invaluable technique in MEMS/NEMS technologies but also to allow high resolution imaging of single DNA molecules using electron microscopy in a regular basis for applications in epigenetic and genetic analysis. By extension, we envision using this protocol to transfer base-labeled DNA and elongated chromatin with energy-loss resolved epigenetic labels. With this process, one could obtain a complete genetic and epigenetic map from individually selected chromosomes without the need for polymerase chain reaction. A STEM is not designed to be a cost-effective sequencing system, however, the possibility for directly reading single molecule information with the spatial resolution of electron beams is possible.

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FIGURE 1.

Scanning electron microscope image of suspended graphene sheets on TEM grids with ultra-thin lacy amorphous carbon film. The inset graph displays the Raman spectra measured on a suspended graphene sheet. The 2D peak's full width at half maximum is 39 cm⁻¹.

FIGURE 2.

Schematic representation of the capillary assembly procedure. A. Capillary assembly process (1. The liquid meniscus is dragged over the microstructured PDMS stamp. 2. The meniscus encounters the topographical features, gets pinned during a given time, and during this pinning time the molecules are trapped inside the wells by the capillary forces exerted. 3. The meniscus finally disrupts and releases the molecules while stretching them. 4. Final assembly of individual DNA molecule arrays on the microstructured PDMS stamp). B. Transfer-printing with solvent mediation (1. A doplet of ethanol is placed on the graphene substrate. 2. The PDMS is put in contact with the wet surface for a few minutes and is finally peeled off).

FIGURE 3.

DNA transfer on CVD graphene. Fluorescence images of an array of single nucleic acid stained phage lambda DNA molecules transferred with solvent mediation onto a silicon dioxide surface with single-layer CVD graphene (excitation at 488 nm). B corresponds to a zoomed image of A.

FIGURE 4.

DNA transfer on exfoliated graphene. Bright field (A), fluorescence (B) and atomic force microscope (C) images of the same area of a substrate after transfer of nucleic acid stained DNA molecules onto a silicon dioxide wafer with exfoliated graphene. In A, the red dashed circle outlines a piece of exfoliated graphene. We also observe the imprints of the PDMS stamp's microfeatures. In B, the dashed area corresponds to the same piece of graphene outlined in A. We observe the array of the nucleic acid stained DNA molecule array (488 nm excitation). In C, the scanned area corresponds to the same area outlined in A and B, where we recognize the exfoliated graphene piece on the bottom right surrounded by silicon dioxide. C1 and C2 show magnified atomic force microscope images and the corresponding cross-sections of the delimited areas in C. C1 shows a single DNA molecule on silicon dioxide, C2 shows a single DNA molecule on exfoliated graphene.

FIGURE 5.

DNA transfer on TEM grids with suspended graphene. A. Fluorescence image of a lacey carbon TEM grid with suspended single layer graphene after transfer of the nucleic acid stained DNA array. The white arrows indicate the bright spots that are part of the periodic DNA molecule array. The DNA strands are not visible due to quenching effects. B. Transmission electron micrograph of elongated phage lambda DNA molecules on single layer graphene. The DNA molecules are not nucleic acid stained in this case. The distance between the molecules is short probably because we are looking at adjacent patterns where one molecule is stretched up to the next pattern, nearly meeting the next molecule. C and D are higher magnification micrographs of the molecule on the right in B. The inset in D shows a theoretical computer-simulated representation of B-form DNA. We observe that the pitch measured from the TEM micrograph is 1.35 times greater than the theoretical pitch of a B double helix.

FIGURE 6.

EELS analysis. On the left (A), bright field image of a single DNA molecule. The red frame corresponds to the scanned area for EELS. On the center (B), 20×20 EELS map from the insert in A, after energy filtering at 130 eV (PL_{23} edge of phosphorous). This map was acquired with a 4 s dwell time per pixel. On the right (C), accumulation of EELS spectra extracted from the 20×20 map without energy filtering (90 eV – 1140 eV window). The insert corresponds to the average of pixels along the molecule only, with a background subtraction (the energy window is reduced to 120 eV – 200 eV). We recognize the L_{23} edge of phosphorous.