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PII: S0167-9317(17)30396-9
DOI: doi:10.1016/j.mee.2017.11.020
Reference: MEE 10671

To appear in: *Microelectronic Engineering*

Received date: 12 October 2017
Revised date: 28 November 2017
Accepted date: 29 November 2017

Please cite this article as: Monica Marini, Marco Allione, Sergei Lopatin, Manola Moretti, Andrea Giugni, Bruno Torre, Enzo di Fabrizio , Suspended DNA structural characterization by TEM diffraction. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Mee(2017), doi:10.1016/j.mee.2017.11.020

# Suspended DNA structural characterization by TEM diffraction.

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**Keywords**: DNA, super-hydrophobic devices, diffraction, interbases distance.

**Abstract**

In this work, micro-fabrication, super-hydrophobic properties and a physiologically compatible preparation step are combined and tailored to obtain background free biological samples to be investigated by Transmission Electron Microscopy (TEM) diffraction technique. The validation was performed evaluating a well-known parameter such as the DNA interbases value. The diffraction spacing measured is in good agreement with those obtained by HRTEM direct metrology and by traditional X-Ray diffraction. This approach addresses single molecule studies in a simplified and reproducible straightforward way with respect to more conventional and widely used techniques. In addition, it overcomes the need of long and elaborated samples preparations: the sample is in its physiological environment and the HRTEM data acquisition occurs without any background interference, coating, staining or additional manipulation. The congruence in the results reported in this paper makes the application of this approach extremely promising towards those molecules for which crystallization remains a hurdle, such as cell membrane proteins and fibrillar proteins.

## 1. Introduction

The diffraction pattern taken by Raymond Gosling under Rosalind Franklin [1] supervision, shown in the so-called “Photo 51”, has been a milestone for biology. The information contained in the image was crucial for the correct determination of the helical structure of DNA by Watson and Crick [2]. That moment changed the approach to biological sciences, highlighting the needed for new techniques capable to investigate biological matter down to the single molecule scale and below.

X-Ray diffraction on crystallized biological systems is the state of the art, worldwide known technique for structural investigation, and has been used to resolve the double helix main features since the 50s, but the high-quality crystals needed still remains a major limitation. In addition, only a fraction of the biomolecules can be reduced in fibers or crystallized and, even when possible, the most flexible parts of the species cannot be resolved. A number of proteins cannot satisfy the X-Ray diffraction requirements. For example, proteins embedded in the membrane phospholipid bilayers have to be separated from their native environment, fibrillar proteins (*e.g.* amyloid proteins) cannot be studied as single molecules [3,4] and not all the biologically interesting systems do crystallize. The above mentioned issues highlight the necessity to develop new methods to reveal macromolecules structures and their relation to other compounds [5].

In the last years, we reported a novel approach for structural characterization of biomolecules by HRTEM and spectroscopy, overcoming the limitations displayed by the conventional techniques [5–10]. The first attempt to directly image DNA allowed researchers to distinguish the double helix period of 27 Å. The fine-tuning of the droplet dimension and deposition technique, micro-pillars arrangement, buffer composition and samples preparation dramatically increased the resolution down to the single base. Besides the nucleic acids helices, repair proteins and membrane proteins [7–9] direct imaging was performed by conventional Transmission Electron Microscopy (TEM) and High Resolution TEM (HRTEM) to achieve a resolution to 3.3 and 1.5 Å, respectively.

Briefly, the method proposed by our group involves the use of biological molecules dissolved in physiologically compatible buffers and super-hydrophobic devices. The super-hydrophobic surfaces are composed by a regular pattern of cylindrical micro-pillars and holes between them. A few microliters of the solution containing the analyte of interest is deposited on the micro-structured device under controlled temperature conditions, obtaining a droplet suspended on the micro-pillars with a quasi-spherical shape and a contact angle >150°. The evaporation of the buffer causes the droplet volume reduction while maintaining its shape and shrinking towards the center. In this process, the DNA molecules contained in the droplet and attached to the top of a pillar are stretched to the top of the consecutive one, accordingly to the dehydration movement. With this approach double strand (ds) DNA, single strand (ss) DNA, neuronal cell membranes and related ion channels, proteins such as Rad51 and lysozyme [8,11] have been suspended over the devices and characterized, ensuring no background signal and no need of staining techniques prior the visualization. The versatility of the device and the wide range of samples that can be tested on it, make this system a suitable platform not only for HRTEM direct imaging and diffraction but also for detection. Among the the recent literature available related to biosensing [12–14], the combination of

high resolution imaging, EM diffraction and Raman spectroscopy techniques applied to biomolecules, highlights the strong potential of this approach in the biodetection field.

Herein we show the successful switch from the real space to the reciprocal space using HRTEM and their application to biological materials suspended over dedicated super-hydrophobic devices. The information obtained from the images acquired in the direct space strongly support the HRTEM diffraction pattern of the DNA bundles studied, confirming the versatility and the wide applicability of the newly developed approach. The combined method herein presented can be applied to a wide range of biological molecules. This property gives to researchers the possibility to perform HRTEM diffraction and high resolution imaging also on the samples that cannot undergo to analysis with conventional techniques, overcoming a significant hurdle of structural biology.

## 2. Materials

### 2.1. Super-hydrophobic device realization

The devices used in this work were fabricated as previously reported [7,8]. Briefly, the super-hydrophobic samples were realized by using commercial Si <100> wafers, characterized by a thickness of 50 μm. The device with holes is mostly defined with two steps of optical lithography performed on the same side of the wafer. The first lithography step defines the pattern of pillars and is followed by the deposition of thin metal layers: Titanium (10 nm), gold (50 nm) and Chromium (50 nm) were evaporated in this order. The second lithography step is needed in order to obtain the holes through the device. Etching with Deep Reactive Ion Etching (DRIE) of silicon precedes the oxygen plasma treatment done to remove the resists excesses. A second DRIE process is performed to create the pillars, using the previously deposited metal as a mask. The samples obtained are then coated with a thin layer of Teflon-like fluorocarbon polymer (FDTS) of a final thickness of approximately 2 nm.

### 2.2. Sample preparation

The DNA of the lambda phage was used as a reference molecule for this work due to its commercial availability (New England Biolabs, NEB UK) and due to its verified self-organization into reproducible bundles of suitable length. An aliquot of the DNA sample was diluted in saline buffer containing 6.5 mM NaCl and 10 mM Tris-HCl, pH 9.3, to reach the DNA concentration of 150 ng/μl. A droplet of 5 μl volume was pipetted on the super-hydrophobic device placed on a hot plate

at 25 ˚C and 50% of relative humidity until the complete evaporation of the buffer solution was achieved.

### 2.2 Microscopy characterization of the DNA samples

After complete dehydration, samples were imaged by SEM (FEI, Quanta 200) working at an acceleration voltage of 3 kV. No coating or additional treatment was performed, to avoid any interference with the micrographs analysis. HRTEM imaging was performed working at 80 keV with a Titan Cs Image (FEI).

## 3. Results and Discussion

In this work, we applied TEM diffraction to the study of nucleic acids helix features in a single bundle of suspended dsDNA. The dsDNA form of the Lambda phage was chosen as a test system due to its length, covering the pillar-pillar distance of 12 μm. Lambda DNA was dissolved in a physiological compatible saline buffer to reach a concentration of approximately 150 ng/μl and a few microliters droplet of the freshly prepared solution was deposited on a super-hydrophobic device. Recent literature shows several examples of devices able to repel water; the arrangement and the shape of the micro-structures on the device are chosen on the basis of the sample and of the final application, spanning from detection to visualization [15]. In our case, the device is designed to have a circular pattern of cylindrical micro-pillar (diameter 6 μm, distance 12 μm, height 10 μm). Among the purposes of the super-hydrophobic device herein proposed, the concentration of a diluted solution of macromolecules and their precise localization are two of the main objectives in this work. The droplet starting dimensions of nearly 3 mm diameter decrease down to 400 μm while the dehydration occurs. Accordingly to the droplet shrinkage, three main concurrent effects take place: the non-suspended material (which includes nucleic acids and salts) become more concentrated; the buffering salts excess is sieved from the biomolecules; the DNA helices self-arrange themselves in bundles self-aligned between micro-pillars. The ordered network of DNA bundles obtained was imaged by Scanning Electron Microscopy (SEM, **Figure 1a-c**). As reported in **Figure 1a** (top right) and in **Figure 1b** (on the right) a saline residual corresponds to the final point of the evaporation where the concentrated droplet collapses. Straight bundles of diameters ranging between 200 nm and 5 nm were homogenously dispersed all around the salt residual. No crystals due to salts presence were observed on the bundles, confirming the self-sieving effect and ensuring a DNA bundle free from any additional contribution potentially interfering with the HRTEM electron beam.

Direct imaging and diffraction of the suspended DNA molecules by HRTEM is possible due to the particular design of the silicon device. A regular pattern of holes is fabricated between the micro-pillars allowing the electron beam to freely pass through the discontinuities of the device, resulting in a background-free imaging.

Diffraction by HRTEM was performed working at an acceleration voltage of 80 keV, previously reported as the best compromise between the radiation damage [16–18] occurring during suspended DNA imaging and a good resolution and contrast of the bundle [7]. The background-free images (**Figure 2a, b**) allowed directly analyzing sub-period details of DNA bundles without additional software processing. An interbase distance of ~2.7 Å was measured on the external edge of the bundles as reported in the related plot in **Figure 2c**. The data obtained are in good agreement with the expected A-DNA features, which differentiates from the B-DNA due to the lower hydration of the helices. As reported by the milestone paper from Franklin and Gosling, in presence of a relative humidity lower than 75% the DNA molecule assume an interbases distance of approximately 2.6 Å and a diameter of 23 Å [19]; in case of higher humidity rates (over the 75%), the rise per base pairs increases to 3.4 Å while the diameter changes to 20 Å. [2].

Afterwards, TEM diffraction was performed on the bundles. The d-spacing obtained by the diffraction patterns resulted to be ranging between 2.8 and 3.2 Å, following bundle direction (**Figure 3**). These values are coherent with the previously reported data both for the A- (2.7 Å < h < 3.0 Å) and the B-form (2.6 Å < h < 3.4 Å) of double strand DNA helices [20,21].

## 4. Conclusions

In this paper, we showed the preliminary data obtained on the DNA diffraction performed by HRTEM. DNA in its double strand form is one of the most characterized biomolecules, and it was chosen to perform this work as a test to evaluate the efficiency of the method. The data are extremely promising, however, a fine-tuning of the solution requirements, bundles dimension and HRTEM measurements are still in progress.

We intend to develop this technique as a central part of a combined approach to effectively and systematically address the study of the structural alterations to the pristine conformation of the nucleic acids, revealing the presence of other compounds causing helix disruption such as heavy metal ions or chemotherapeutic agents. In addition, the use of such a technique can be an innovative tool for the investigation of the macromolecules (such as flexible proteins and cell membrane proteins) that cannot be investigated with traditional methods.

In perspective of a systematic approach, a combination of this straightforward technique with more refined ones like HRTEM direct imaging, capable of submolecular resolution and spectroscopic studies to obtain valuable information on morphological and structural single point modifications and interactions between biological molecules and the environment.

## 5. Acknowledgments

The authors acknowledge financial support from the KAUST start-up funding and from Italian Ministry of Health under the projects: Project no.: GR-2010-2320665 and Project no.: GR-2010-2311677.

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**Figures**

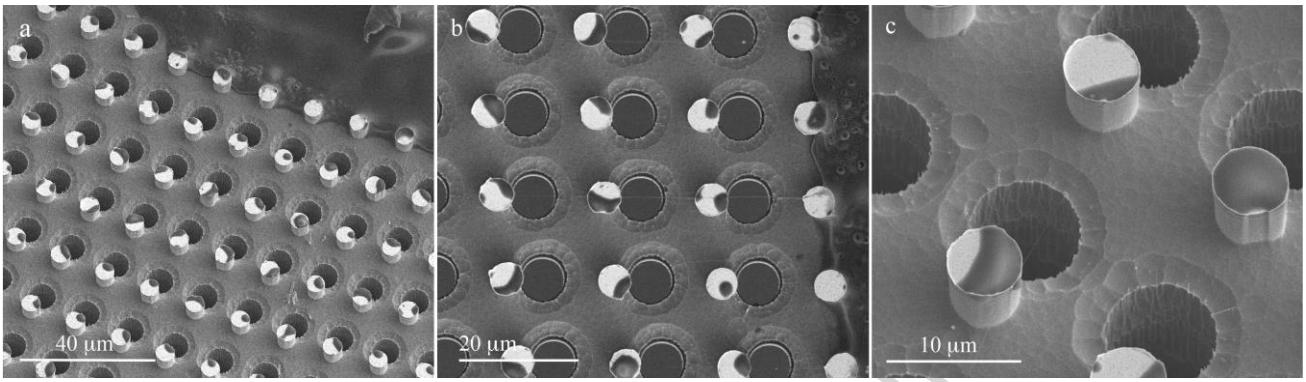



**Fig. 1. Suspended dsDNA bundles: SEM imaging.** a) As a result of the dehydration process, a salt residual is segregated and confined in a limited area (located by design at the center of the circular structure, not shown). DNA bundles are homogeneously distributed in the region of interest around the central residual area, in an oriented and regular manner. b) Higher magnification image showing a detail of the DNA bundle suspension. The DNA molecules are suspended over the holes of the micro-patterned device, allowing the downstream TEM direct imaging. c) The pillars height of 12 µm ensures the suspension of the macromolecules in a background-free environment. The sample in panels **a** and **b** was tilted of 25°.

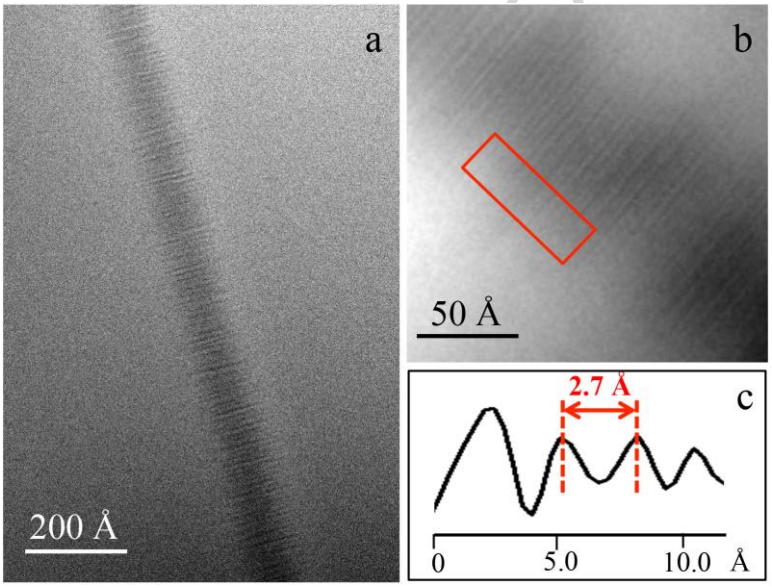



**Fig. 2. Suspended dsDNA bundles: HRTEM imaging. a,b**) HRTEM micrographs of a DNA bundle suspended over a super-hydrophobic device. Characteristics fringes related to sub-period features are clearly visible in both images. The red rectangle highlights the area where the metrology was performed for the plot in panel **c**). The distance between one fringe and the other is of 2.7 Å, in good agreement with previous works on the direct metrology of the DNA helix [7].

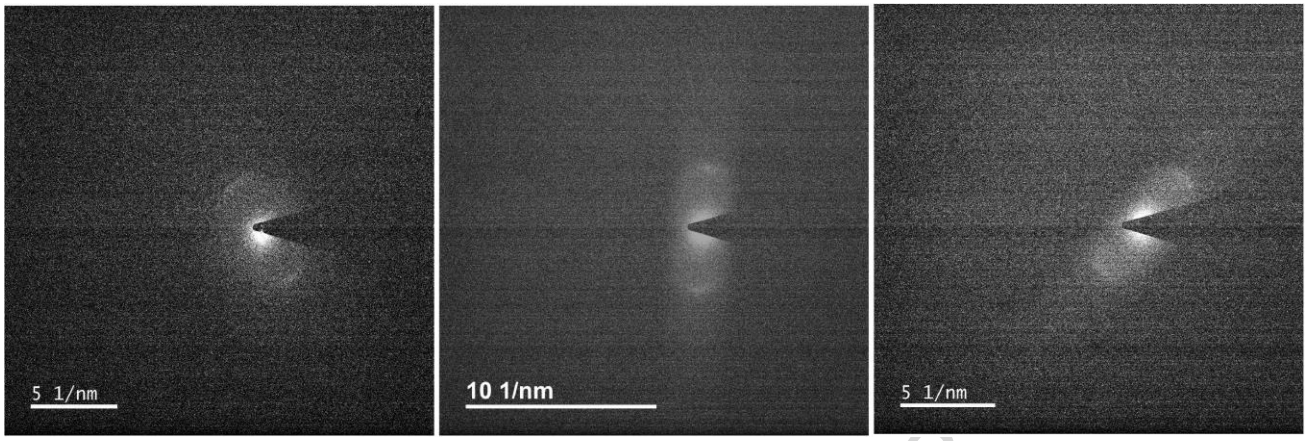
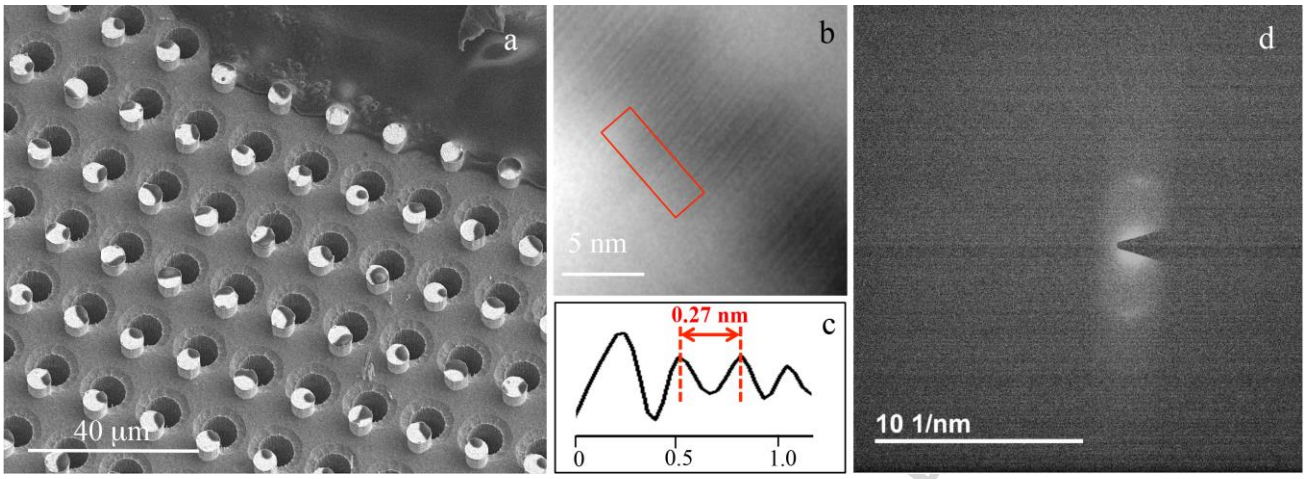



**Fig. 3. Suspended dsDNA bundles: TEM diffraction.** In the three panels the diffraction patterns obtained from three different suspended bundles of the same super-hydrophobic device are reported. The d-spacing measured ranges between 2.8 Å and 3.2 Å and corresponds to the interbase distance of DNA.




Graphical abstract

*Highlights*

**Suspended DNA structural characterization by TEM diffraction.**

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1. Micro-fabrication, super-hydrophobic devices and physiologically compatible preparation used to obtain suspended and backgropund free DNA samples;
2. TEM diffraction technique applied to the suspended sample to obtain diffractograms of DNA fibers;
3. Super-hydrophobic devices with a circular pattern of micro-pillars to sieve and suspended nucleic acids molecules;
4. TEM diffraction of suspended dsDNA show diffraction spacing in agreement with the previous bibliography.