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DIRECT VISUALIZATION OF UNSTAINED NUCLEIC ACIDS ON A METAL SUBSTRATE

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Unstained DNA has been directly imaged for the first time on a metallic substrate. Reproducible, three-dimensional images of molecular morphology following aqueous deposition onto tungsten have been obtained. The images confirm the importance of reducing surface tension forces during drying. Preadsorption of poly-l-lysine seems to improve image quality although optimum conditions are quite different than those reported in the TEM literature for insulating substrates. Our results suggest that poly-l-lysine should be preadsorbed as a neutral molecule at high pH. Following adsorption, the pH should be reduced in order to establish a net positive surface charge density. The presence of positive charges on a tungsten surface helps to preserve DNA morphology following adsorption from aqueous solution.

1. Introduction

The electron microscope has provided valuable insight into the morphology of DNA by imaging isolated DNA molecules on thin, non-metallic substrates after heavy metal staining. The metal stain improves image contrast and preserves essential features of molecular morphology during electron bombardment. In practice, the presence of a stain limits the resolution of the image to one or two nanometers [1]. Since the stain may coat an adsorbed molecule unevenly, its image may be subtly distorted.

Unstained images of isolated DNA molecules have also appeared in the electron microscope literature [2]. Since electron beam induced damage is difficult to avoid, such images can be misleading. Image processing can be used to extract recognizable features from noisy, low-dose micrographs [2]. However, the reliability of such procedures has been questioned when one knows, a priori, the expected shape of the molecule [3].

In this paper we present images of unstained DNA obtained by Field-Ion Tomography, a novel point-projection imaging technique [4–6]. Non-destructive, three-dimensional pictures of molecular morphology on tungsten have been obtained at a resolution of ~ 2 nm. In addition to demonstrating the feasibility of imaging DNA by Field-Ion Tomography, we will show that deposition difficulties encountered with insulating substrates are also encountered when metallic substrates are employed. Our observations may have direct bearing on the interface problem associated with hybrid devices which employ biological and material science technologies [7].

2. Substrate considerations

Fig. 1 shows a Field-Ion Tomographic image of a clean tungsten substrate. A clean substrate is prepared by routine electrochemical polishing in NaOH [8] followed by thermal annealing at ~ 2000 K in a vacuum of ~ 10^{-9} Torr. Tomographic imaging was performed in the usual way [4–6] at 20 K and 10^{-9} Torr following a brief (~ 60 s) transfer from laboratory ambient.

Fig. 2 shows the result of a control experiment in which a clean tungsten substrate was first imaged in a commercial transmission electron microscope (TEM) [9] immersed into 0.15M NaCl (for 150 s), rinsed in 18 MΩ water and then removed into air and dried. Our substrates are typically imaged in

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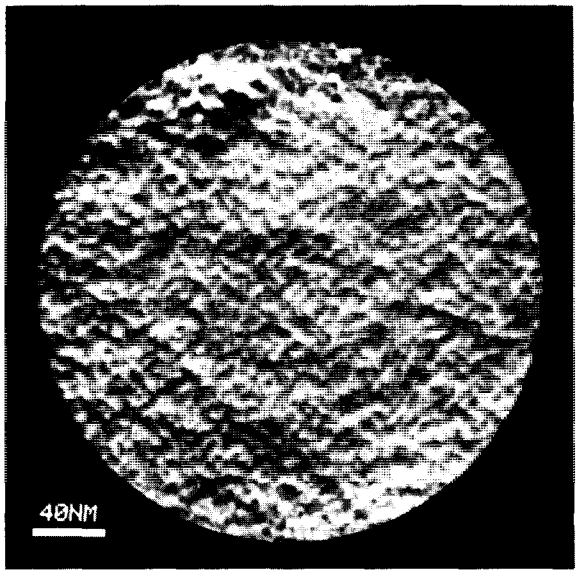

Fig. 1. A Field-Ion Tomographic image of a clean tungsten substrate imaged at ~ 20 K and 10^{-9} Torr. The substrate was cleaned by thermal annealing at ~ 2000 K in 10^{-9} Torr, and then transferred through laboratory ambient into the tomographic microscope for imaging [P151.5].

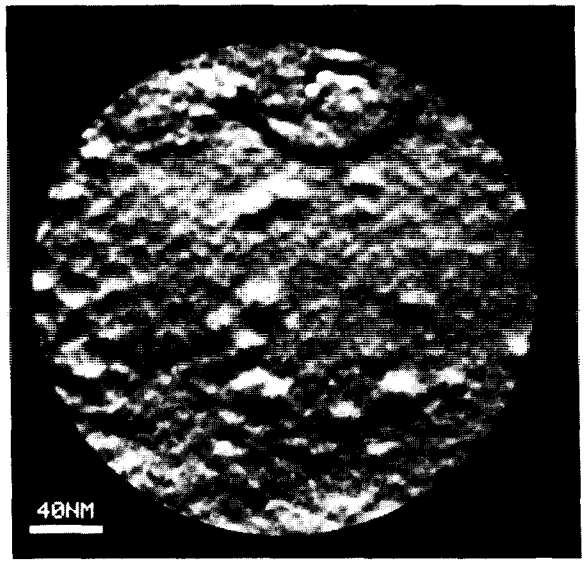

Fig. 2. A Field-Ion Tomographic image of a clean tungsten substrate imaged at ~ 20 K and 10^{-9} Torr after imaging in a commercial TEM. This image displays features which are characteristic of TEM contamination [P149.7].

the TEM in order to determine their radius of curvature, from which our image magnification is deduced [4]. In this way, a magnification accurate to within 20% can be obtained.

The features seen in the image of fig. 2 have been associated with contamination introduced by TEM imaging. If a clean substrate is immersed into 0.15M NaCl and then rinsed in pure water, an image essentially identical to the clean substrate image (fig. 1) is obtained. We have not been able to control the contamination introduced by TEM imaging. At times no contamination is seen; at other times, image features similar to those seen in fig. 2 are obtained. Occasionally, more contamination is observed in the image. In all cases a cold sleeve cooled to liquid nitrogen temperature was used during TEM imaging. In the future, we intend to avoid TEM imaging until after tomographic imaging is completed. The problem with TEM imaging after DNA deposition is the inherent radiation damage which may make subsequent tomographic images difficult to interpret.

3. Deposition considerations

Two lyophilized, synthetic nucleotide copolymers were obtained [10]. In general, these were dissolved in 0.15M NaCl whose pH was not controlled. Tris (0.01M) was occasionally used as a buffer at pH ~ 7. The use of Tris did not seem to affect image quality.

Deposition was accomplished in the usual way from solution [11], followed by a rinse in pure (18 MΩ) water without breaking the water–air interface. For some experiments, an additional rinse in 200 proof (absolute) alcohol was used in an attempt to reduce surface tension at the liquid–air interface during substrate withdrawal into air. The alcohol was not distilled prior to use. A DNA concentration of ~ 63 μg/ml deposited for 150 s was judged to give a submonolayer coverage from an independent visual test of protein adsorption [12]. If 0.01M Tris–0.01M EDTA buffer was used (at pH ~ 7), deposition conditions could not be visually assessed. The indium substrate used in the visual test appeared to be eroded away, probably as a result of the known chelating properties of

EDTA. Since there is a possibility that EDTA could affect other metals substrates during deposition, its use was discontinued.

4. Discussion

Fig. 3 shows the result of depositing poly(AT) onto tungsten. Despite its relative insolubility in alcohol, lyophilized poly(AT) was dissolved in absolute alcohol. After deposition, the specimen was then rinsed in absolute alcohol and dried in air. The exact deposition conditions were not recorded, and the large feature seen in the image was not reproduced (during ten separate imaging attempts using ten different tungsten substrates). However, the feature seen in fig. 3 could be reproducibly reimaged on the same substrate without noticeable change.

Fig. 4 shows the variety of reproducible image features seen if poly(GC) is deposited onto a tungsten substrate from water in the usual way and then moved, into air, through absolute alcohol. Image features are characteristically large and relatively formless. Surface coverage is nearly complete.

Fig. 5 shows the result of moving a poly(GC) covered substrate into air through 18 MΩ water. In this case, pure water was used as the final rinse (rather than alcohol). Image features appear smaller and more globular in appearance than those seen in fig. 4. Surface coverage is less, and even a few strand-like features can be observed. The absolute alcohol used to obtain the images shown in figs. 3 and 4 was not distilled. It was found to leave a submonolayer coverage of an (unknown) organic impurity when dried on an indium coated glass substrate [12]. Differences in the size, shape and coverage of the image features seen in figs. 4 and 5 reflect the use of alcohol in the former case. The more globular features seen in fig. 5 are consistent with the type of feature expected if surface tension forces dominate during drying. TEM images show that DNA (which is nominally a long, stranded molecule in solution) is notorious for tangling into globular "bush-like" structures when deposited onto carbon substrates

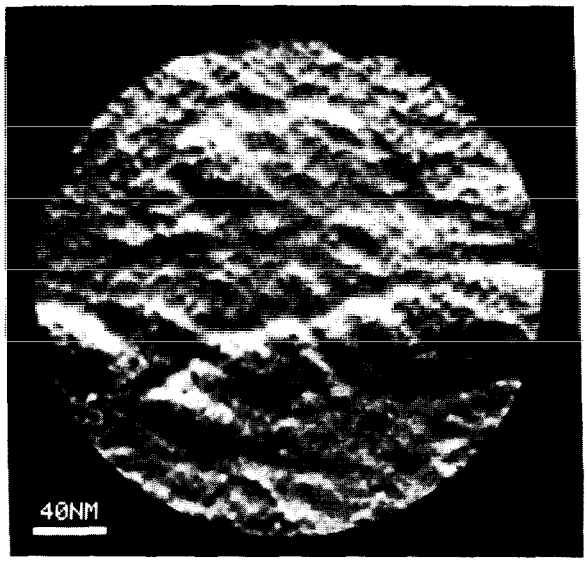

Fig. 3. A Field-Ion Tomographic image of Poly(AT) DNA on a tungsten substrate imaged at ~ 20 K and 10^{-9} Torr. The features seen in this image have not been reproduced on other specimens (see text) [PNEG153.4].

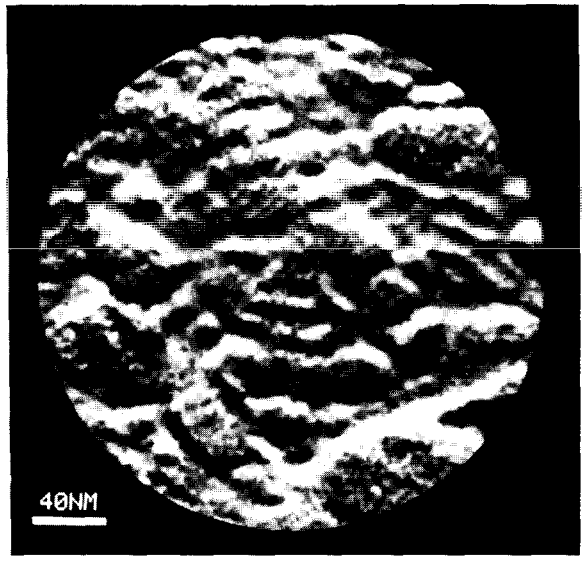

Fig. 4. A Field-Ion Tomographic image of Poly(GC) DNA on a tungsten substrate imaged at ~ 20 K and 10^{-9} Torr. The substrate was rinsed in pure (18 MΩ) water after deposition without crossing the air–liquid interface. A final rinse in absolute alcohol was followed by removal through the air–alcohol interface. The specimen was dried in air (P118.5].

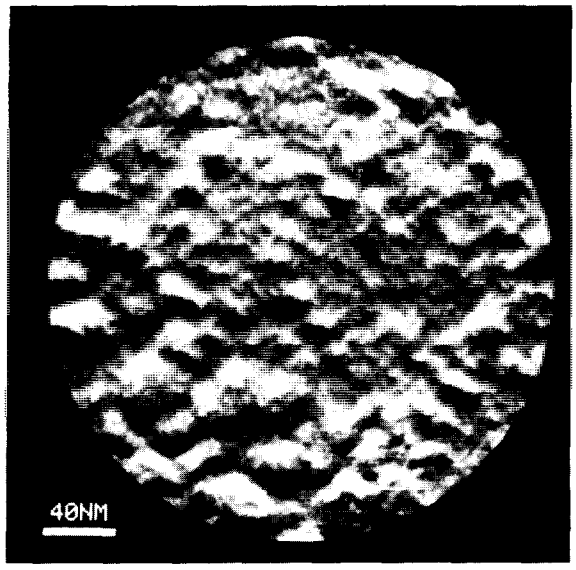

Fig. 5. A Field-Ion Tomographic image of Poly(GC) DNA on a tungsten substrate imaged at ~ 20 K and 10^{-9} Torr. Deposition conditions were identical to those which produced the image shown in fig. 4 except the final rinse in pure (18 MΩ) water was followed by removal through an air–water interface. The specimen was air dried [P148.5].

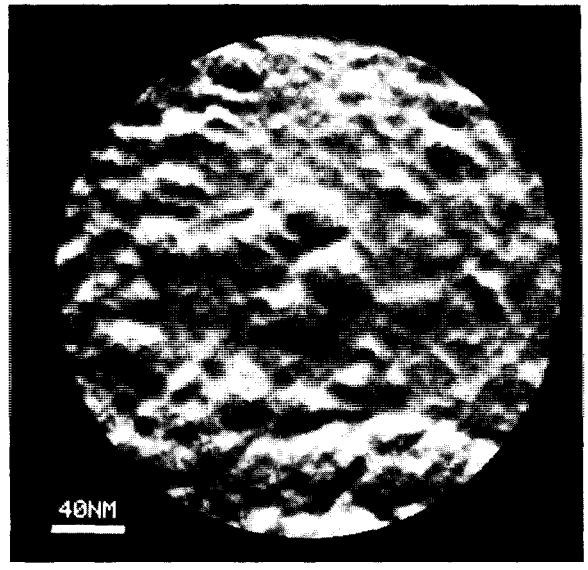

Fig. 6. A Field-Ion Tomographic image of Poly(GC) DNA on poly-l-lysine preadsorbed at neutral pH onto a clean tungsten substrate. Imaging at ~ 20 K and 10^{-9} Torr. DNA deposition conditions were identical to those which produced the image shown in fig. 5. Poly-l-lysine (mwt. ~ 2000) was preadsorbed from pure (18 MΩ) water at ~ 1 μg/ml for ~ 120 s. [P110.4].

from aqueous solution and dried in air [13].

Successful TEM images of DNA on carbon were first obtained after it was realized that the substrate had to be "conditioned" in a way which would minimize surface tension forces during drying. Initially, DNA was embedded within a layer of cytochrome *C*, ostensively to preserve the linear shape of the molecule [14]. However, the relatively large size of cytochrome *C* (compared to DNA) tended to distort the DNA structure. In order to avoid this problem, a procedure for surface conditioning was introduced which involved preadsorption of poly-l-lysine, a small (mwt. ~ 2000) synthetic polymer having a net positive charge [15]. DNA is known to possess a net negative charge at neutral pH. Therefore, adsorption onto a positively charged poly-l-lysine adlayer should encourage binding and even stabilize the molecule's nominal configuration. In fact, very good DNA images can be obtained using this procedure [13].

Fig. 6 shows the effect of adsorbing poly(GC) onto poly-l-lysine preadsorbed onto a clean tungsten substrate. The poly-l-lysine was dissolved in 18 MΩ water and deposited for ~ 20 s onto a clean tungsten substrate at a concentration of ~ 1 μg/ml. DNA was then adsorbed from 0.15M NaCl in the usual way without allowing the substrate to pass through the water–air interface. The substrate was removed into laboratory ambient and dried following several rinses in pure water (with the substrate kept below the surface of the liquid). This procedure is essentially identical to the procedure reported in the TEM literature [15] with one noticeable exception. In the TEM experiments, a carbon substrate was used. Typically, the carbon substrate is first exposed to a glow discharge, presumably to increase its "wetability". Since water will readily wet a clean metal surface, glow discharge conditioning was not deemed necessary in our experiments.

Contrary to expectation, the images which we obtained (e.g. fig. 6) did not contain features particularly suggestive of the long, rope-like strands characteristic of DNA in its normal configuration.

Recently, Feder and Giaever [16] addressed the general problem of protein adsorption from solu-

tion on to non-metallic (Lexan) substrates. Ferritin was used as a probe of adsorption conditions since its iron-rich core is readily seen in the TEM. Ferritin, like DNA, is a negatively charge molecule. Feder and Giaever found that the probability of binding ferritin to a Lexan surface was not encouraged by preadsorption of poly-l-lysine unless preadsorption occurred at high pH (~ 11), and subsequent ferritin deposition at neutral pH (~ 7). At high pH, poly-l-lysine is neutral. A neutral molecule can bind to a surface, presumably without regard for the nature and distribution of existing surface charge density. At neutral pH, poly-l-lysine is positively charged. Therefore, depositing ferritin at pH 7 encourages binding to the (positively charged) poly-l-lysine adlayer. In our experiments, a relatively poor probability of binding poly-l-lysine to tungsten would explain why the image features in figs. 5 and 6 are qualitatively similar. There appears to be not enough poly-l-lysine bound to the surface at neutral pH to affect the subsequent adsorption of DNA from solution.

Fig. 7 shows the result of adsorbing poly(GC) onto poly-l-lysine preadsorbed onto clean tungsten at pH 11. In this case we have essentially duplicated the condition which optimized ferritin binding to Lexan [16]. Our images under these conditions show more of the expected, stranded appearance of DNA. The results of adsorbing DNA at neutral pH onto poly-l-lysine (preadsorbed at high pH) are typically quite different than those obtained by deposition onto poly-l-lysine preadsorbed at neutral pH (fig. 6), or directly onto tungsten from saline (fig. 5). Since our poly-l-lysine (mwt. ~ 2000) is much smaller than DNA, it should not appreciably influence the type of image feature which we observe.

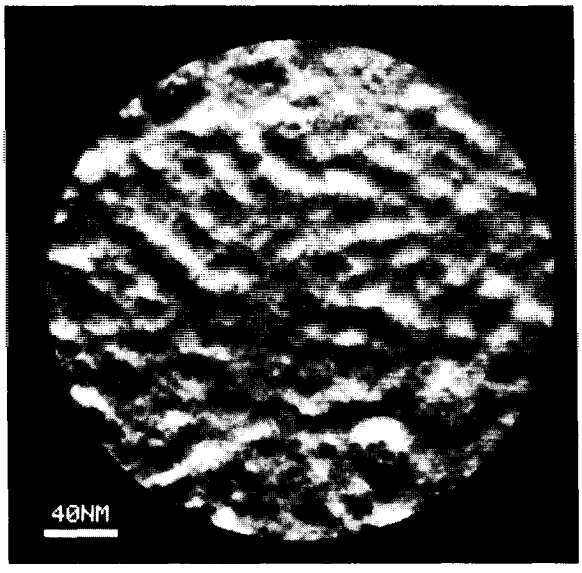

Fig. 7. A Field-Ion Tomographic image of Poly(GC) DNA on poly-l-lysine preadsorbed on a tungsten substrate at pH ~ 11. Imaging at ~ 20 K and 10^{-9} Torr. Deposition conditions were identical to those which produced the image shown in fig. 6 except the poly-l-lysine was preadsorbed at pH 11 and the DNA deposited at neutral pH [P120.4].

5. Conclusions

The image features presented in this paper (with the exception of fig. 3) are reproducible from specimen to specimen under identical deposition conditions. By this we mean that similar (but not necessarily identical) features have been obtained in about one out of every four imaging attempts. The remaining images generally display no consistent or recognizable detail. The statistical nature of our deposition procedure and the fixed, limited field-of-view of our microscope [4] are probably responsible for the specimen-to-specimen variation which we observe. Image reproducibility for the same specimen is extremely good, provided that the specimen is not physically damaged or subsequently imaged in the TEM. A specimen stored in laboratory ambient for months after initial tomographic imaging can be reimaged without noticeable change.

The trends observed for DNA under different deposition conditions suggest that for metal surfaces, the effect of surface tension forces during drying dominates the observed distribution of DNA on the surface. In general, we observe a greater number of strand-like features characteristic of DNA in solution if the DNA molecule is adsorbed onto poly-l-lysine preadsorbed onto our substructures at high pH. Our observations are consistent with previous results found for ferritin adsorption onto Lexan [16]. Similarly, we find that the binding of DNA to a metal surface is increased

if deposition occurs from a solution with a high salt concentration. However, under these conditions, the expected shape of the DNA molecule is not generally seen. Instead, aggregation and clumping is observed. Both our results and the previously published results for ferritin show that binding of negatively charged molecules is encouraged if adsorption occurs at neutral pH onto poly-l-lysine, preadsorbed at high pH. These results seem to contradict a previously published TEM procedure in which poly-l-lysine is adsorbed onto non-metallic substrates from pure water (neutral or low pH) in order to increase DNA binding [15].

Although we have been able to demonstrate that image features similar in appearance to those expected for DNA can be obtained, we have been unable to produce convincing images showing its characteristic double helix. However, the features which we see are consistent with an image resolution of ~ 2 nm, typical of our imaging conditions [4]. Our results indicate that it is essentially impossible to produce a well-defined coverage of a highly charged molecule (such as DNA) on a metal surface by simple adsorption from aqueous solution.

It is clear that the charge of the molecule in solution and the nature of the surface charge density will effect adsorption and binding. Surface tension forces during drying can rearrange surface species which are not tightly bound. Our results suggest that some form of surface conditioning will be necessary in order to provide a well-defined coverage of biological molecules at a metal surface. It appears that it will be quite difficult to completely cover a metal surface with biological molecules in a dense, close packed array. Complete coverages may be necessary in some technological applications in order to passivate a surface from further adsorption [7]. It is apparent that a more complete understanding of the attachment of biological molecules to metal and semiconductor surfaces will be needed if biologically active species are to be retained in a form suitable for the type of hybrid technologies which are being envisioned [7].

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