

The influence of the substrate temperature on the preparation of DNA films for studies under vacuum conditions

M.A. Śmiałek^{1,a}, N.C. Jones², R. Balog³, N.J. Mason⁴, and D. Field³

¹ Atomic Physics Division, Department of Atomic Physics and Luminescence, Faculty of Applied Physics and Mathematics, Gdańsk University of Technology, Gabriela Narutowicza 11/12, 80 952 Gdańsk, Poland

² Institute for Storage Ring Facilities, Aarhus University, Ny Munkegade, Building 1520, 8000 Aarhus C, Denmark

³ Department of Physics and Astronomy, Aarhus University, Ny Munkegade, Building 1520, 8000 Aarhus C, Denmark

⁴ Department of Physics and Astronomy, The Open University, Walton Hall, Milton Keynes, MK7 6AA, UK

Received 7 September 2010 / Received in final form 6 December 2010

Published online 18 March 2011 – © EDP Sciences, Società Italiana di Fisica, Springer-Verlag 2011

Abstract. Experiments were carried out to determine the dependence of the physical form of supercoiled DNA films on the initial temperature of the substrate. Such films are often used in irradiation experiments involving low energy particles, like electrons or photons. In order to obtain absolute values for cross sections for such experiments, the spatial distribution of the sample in the film has to be well estimated. These investigations aim to correlate the size and form of a DNA film with the initial temperature of the substrate, on which the liquid sample is deposited prior to evacuation. From our previous studies we concluded that the presence of agents preventing DNA structural water evaporation is required in order to preserve the supercoiled plasmid DNA form under vacuum conditions. Therefore, we examined the temperature dependence on films prepared from plasmid suspended in solutions containing magnesium hydroxide, Tris-Cl buffer and various concentrations of sodium hydroxide. To visualise the films we used a conventional light microscope with a CCD camera and a scanning electron microscope. The results revealed a significant influence of the temperature of the substrate on both the area of the substrate covered by the film as well as on the spatial distribution of DNA molecules. An increase in the amount of sodium hydroxide that stabilises supercoiled DNA under vacuum increases DNA aggregation. After these investigations we conclude that the best temperature of the substrate to produce uniform and thin films should be between $-5\text{ }^{\circ}\text{C}$ and $-10\text{ }^{\circ}\text{C}$ for substrates which are not atomically flat and above $0\text{ }^{\circ}\text{C}$ for atomically flat substrates.

1 Introduction

The study of the properties of DNA, both chemical and physical, under high vacuum conditions has been highlighted recently through a series of experiments aimed at elucidating the damage induced in DNA and its complexes by low energy electrons [1], ions [2] and VUV [3]. For such model studies plasmid DNA is frequently used as it is easy to handle and, due to its three conformers (supercoiled, relaxed and linear), provides information on supercoiled molecule relaxation and double strand break (DSB) formation, that can be easily seen by agarose gel electrophoresis (AGE). To study particular interactions with biological material under vacuum conditions, such as DNA, suitable preparation of the sample is required in order that films are reproducibly formed and are stable under the experimental conditions required. Our recent results showed that without adding cations, which stabilise plasmid DNA under vacuum conditions, it is difficult to sustain plasmid DNA in its supercoiled form [4].

Freeze-drying is commonly used in the preparation of samples intended for use in high vacuum conditions. This technique causes lyophilization of liquid samples and is used in most cases to prevent DNA aggregation during solvent removal [5]. Nonetheless, this procedure may influence the formation of the film causing DNA network development [6]. Although commonly used, no studies were performed to assess how the freeze-drying technique influences the DNA film formation in the presence of external factors, like stabilisers or the temperature of the substrate. In this work we focus on the influence of the substrate temperature during the freeze-drying process on film formation. Previous studies have reported that films have been formed with the substrate kept at temperatures varying from RT [7] to liquid nitrogen temperatures [1] while depositing the liquid sample. Our previous study reported the influence of stabilisers, such as sodium, potassium, magnesium and calcium hydroxides, required to maintain the supercoiled form of DNA on the formation of films [4], and here we now present details of investigations of how films are affected by these stabilisers when varying the substrate temperature.

^a e-mail: smialek@mifgate.mif.pg.gda.pl

2 Experimental details

2.1 Materials

Plasmid DNA pBR322 in UHP H₂O was purchased from Inspiralis Limited (Norwich, UK). NaOH, Mg(OH)₂, boric acid, EDTA and trizma base were purchased from Sigma-Aldrich (DK). Agarose was obtained from Cambrex (UK). Tantalum foil was purchased from Johnson Technica A/S, Frederikssund, Denmark. 10 mM Tris-Cl buffer was purchased from Qiagen (UK) and used with no further purification.

2.2 Sample and solutions preparation

Sodium hydroxide and magnesium hydroxide pellets were dissolved in ultra-high purity (UHP) water and filtered through a 0.2 micron syringe filter to remove possible impurities. Solutions of each of the bases were made up to a concentration of 0.1 mM (pH 10.0). For NaOH an additional solution of concentration 1 μ M (pH 8.0) was made. When base solutions were mixed with DNA solution, the pH was 7.7 or 9.9, depending on the base and water content. Samples suspended in Tris-Cl buffer had a final concentration of 5 mM of the buffering agent.

2.3 Substrate preparation

In order to control the substrate temperature while depositing the liquid containing the plasmid DNA a simple system was assembled, similar to one described previously [5]. The temperature of the substrates was controlled using a set of Peltier units, which allowed stable (± 1 °C) and reproducible temperatures between -25 °C and $+20$ °C to be applied to the samples during the DNA film evaporation. To prevent any damage resulting from air condensation, the preparation process was carried out in anaerobic conditions, under a dry nitrogen atmosphere.

DNA films were prepared on 0.5 mm thick tantalum foil, attached to small copper blocks. Tantalum foil was chosen as a substrate due to the high atomic number of Ta and the layer of tantalum oxide which is formed on the metal surface. The high atomic number reduces secondary electron emission upon low energy electron irradiation, and the oxide layer creates a stable, chemically inert surface, minimising DNA-substrate interaction. In addition, tantalum has already been shown to be a good choice for DNA – low energy electrons experiments [8]. To ensure an even Ta surface, the foil was polished on fine sand paper wheels (P2500, 8.4 μ m grain size) and was cleaned in an ultrasonic bath in pure ethanol (EtOH) and blow-dried with dry N₂. To re-use the substrates, samples were washed off with UHP H₂O, followed by EtOH wash and dried.

2.4 DNA film preparation and analysis

To prepare the films, DNA in an aqueous stock solution was diluted with either UHP H₂O, Tris-Cl buffer or with

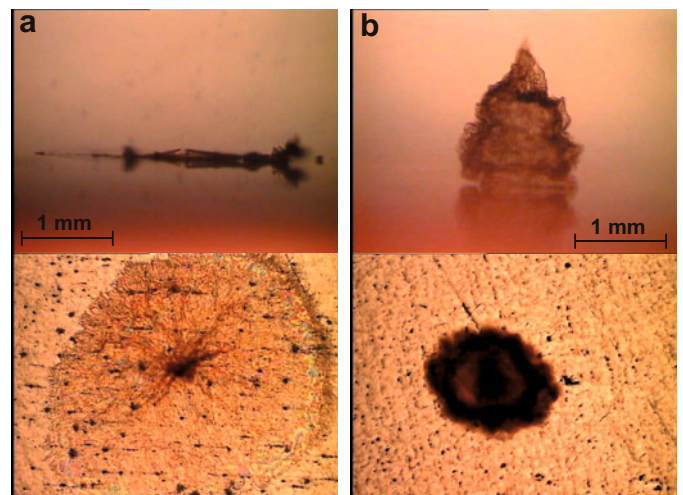


Fig. 1. (Color online) Images from light microscope of side view (top line) and top view (bottom line) of DNA films prepared from 2 μ L UHP H₂O containing 100 ng plasmid DNA, placed on the substrate at (a) -10 °C and (b) -20 °C.

base solutions to obtain 100 ng DNA per either 2 or 6 μ L of solution. The DNA solution was then placed on the tantalum support at $+4$ °C, -5 °C, -10 °C or -20 °C, under a dry N₂ atmosphere in the freeze-dryer, which was assembled in a glove box. Under these conditions it was possible to observe if any water from the glove box condensed on the tantalum supports and thus to see if the environment was as dry as assumed prior to sample deposition. Samples were then subjected to medium vacuum (10^{-5} mbar) for one hour. After evacuation the chamber was brought back to atmospheric pressure with dry nitrogen.

After preparation, the DNA films were recovered from the tantalum supports with 7 μ L of tris buffer. To quantify damage levels, agarose gel electrophoresis (AGE) was employed. Samples were run on 1.4% agarose gels at 0.5 V/cm for 16 h in the presence of 1 \times TBE buffer. Gels were later stained in a 1 \times SYBR Green I/1 \times TBE solution for 40 min and de-stained in 1 \times TBE for another 20 min. Fluorescent images of the gels were recorded using the SYNGENE U:Genius gel documentation system. To quantify the proportions of various DNA forms present in the gels, densitometric analysis was performed using ImageJ software (available from: <http://rsb.info.nih.gov/ij/>). Since SYBR Green I binds equally to all plasmid conformers [9], no correction factors were used. The total surface area of the substrate covered by the films was also estimated using the ImageJ software.

In order to explore the morphology of the DNA film surface, a traditional light microscope with CCD camera and a scanning electron microscope (SEM) were employed. No additional modification, such as metallisation, was required prior to imaging with SEM. The advantage of such analysis being that we were able to image samples that would be produced in the same way as for irradiation studies. Using this method it was possible to see the film structure as well as the level of substrate area coverage.

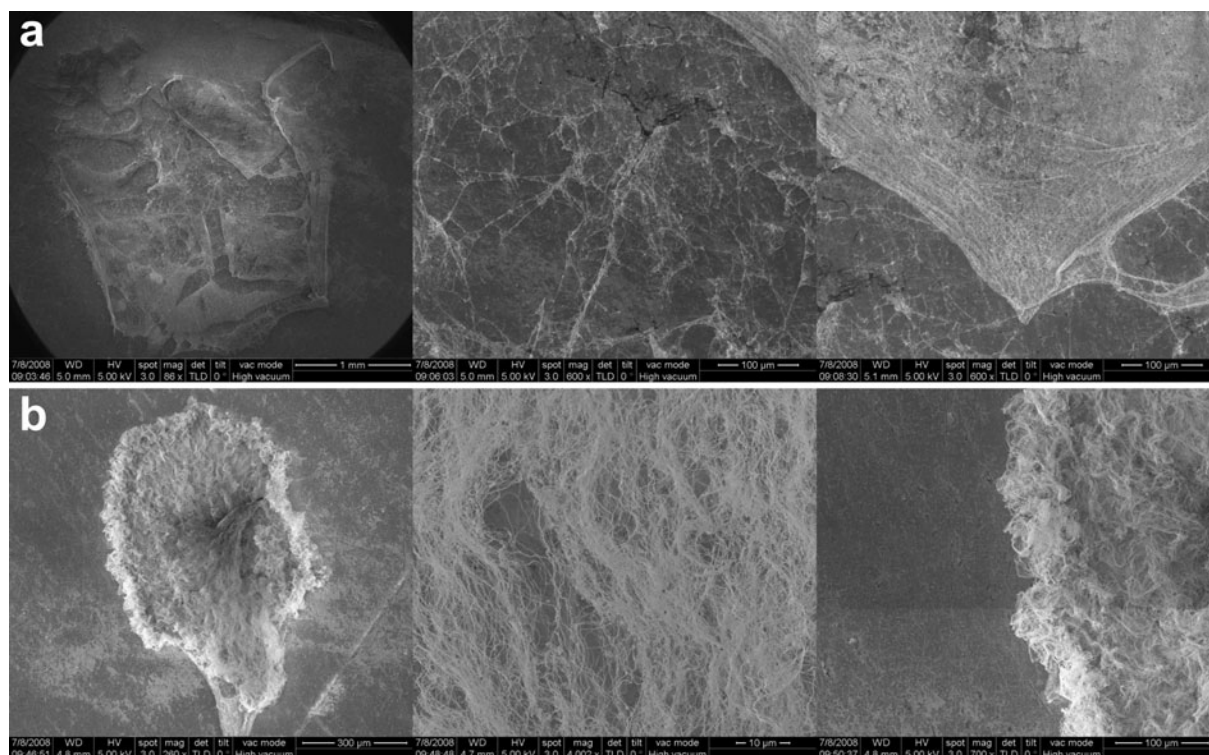


Fig. 2. Images of DNA films prepared from 2 μL UHP H_2O containing 100 ng plasmid DNA, placed on the substrate at (a) +4 $^\circ\text{C}$ and (b) -20 $^\circ\text{C}$, together with close-ups of the central area and edges of the samples.

3 Results and discussion

3.1 Light microscope analysis

When samples were prepared using plasmid DNA suspended in UHP H_2O and placed on the tantalum foil at -20 $^\circ\text{C}$, the resulting DNA film was clearly visible by eye as a very compact, high structure. In contrast, the DNA films prepared at a substrate temperature of -10 $^\circ\text{C}$ are broader and flat. These films were observed under a light microscope to determine their shape and dimensions. Figure 1 shows images obtained for DNA films from solutions containing 100 ng DNA in 2 μL of UHP H_2O , deposited at (a) -10 $^\circ\text{C}$ and (b) -20 $^\circ\text{C}$. The top images are side views of the prepared films, and bottom show the top view of both films. It can be seen that the film prepared at -10 $^\circ\text{C}$ forms a large, flat circle of approximately 2.5 mm in diameter and with a height of about 0.1 mm. The film prepared at -20 $^\circ\text{C}$ formed a compact structure of 1 mm in diameter and a height of approximately 1.5 mm. From these observations it can be concluded that the shape of the DNA film, which is formed using the freeze-drying technique is strongly dependent on the temperature at which the liquid sample is added to the substrate. For samples deposited at substrate temperatures higher than -20 $^\circ\text{C}$, the droplet can spill over the surface of the substrate before cooling to the freezing point. At lower temperatures, the small volumes of liquid sample used in the experiment freeze immediately once touching the cooled surface. After the water has been removed in the evacuation process,

the dried DNA film retains the shape of the initial frozen sample droplet.

3.2 AGE and SEM analysis of DNA film formation

After examining the samples with a traditional microscope, samples were prepared and then examined with a SEM to determine the properties of DNA films formed at various substrate temperatures. First, samples prepared from a solution containing just UHP H_2O were examined. Figure 2 shows the DNA films formed at a substrate temperature of (a) +4 $^\circ\text{C}$ and (b) -20 $^\circ\text{C}$, together with close-ups of the centre of the sample area and of sample edges.

It can be seen that for the sample prepared at +4 $^\circ\text{C}$ the film covers a large area of approximately 2.5 mm in diameter. The film area exhibits cracks, which are due to the evacuation of the film and reduces the area coverage. For the film prepared at -20 $^\circ\text{C}$ it can be seen that the film is much more compact and covers a smaller area than that prepared at +4 $^\circ\text{C}$. The film was also flattened by partial denaturation of DNA induced by the incident electron beam in the microscope. The density of the film is lower due to the physical form of sample material. The edge of the sample is better defined at -20 $^\circ\text{C}$ than in the case of the film prepared at +4 $^\circ\text{C}$ and there is a higher concentration of sample at the edge than in the centre of the film.

As shown previously [4], plasmid DNA sustains the supercoiled form under vacuum conditions only if ionic stabilisers are added. With this in mind, the temperature

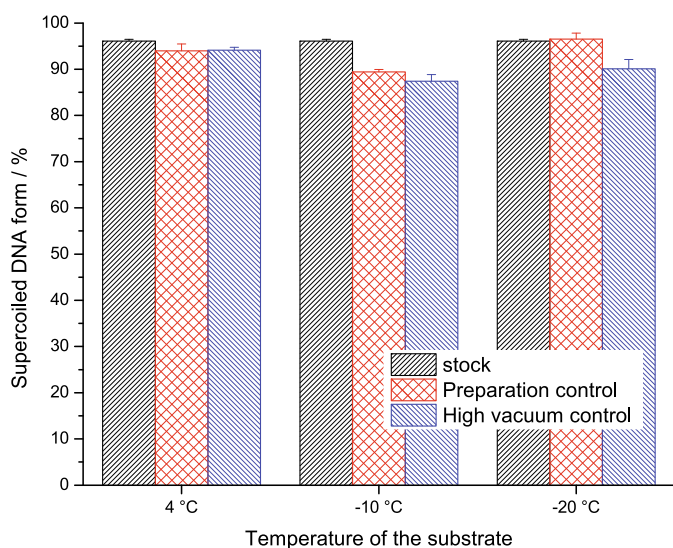


Fig. 3. (Color online) Loss of supercoiled plasmid DNA in a form of films under medium (10^{-5} mbar) and high (10^{-7} mbar) vacuum conditions, samples contained $1 \mu\text{L}$ of 100 ng DNA in UHP H_2O and $5 \mu\text{L}$ of NaOH, pH 10.0, deposited on Ta substrate at $+4$, -10 and -20 °C, assessed from AGE.

dependence of the formation of films containing low and high concentrations of NaOH was examined using AGE. It can be seen in Figure 3 that samples containing $1 \mu\text{L}$ of 100 ng DNA in UHP H_2O and $5 \mu\text{L}$ of NaOH, pH 10.0, survived the evacuation process (10^{-5} mbar) and experimental conditions (10^{-7} mbar) with less than 10% damage to supercoiled plasmid DNA molecules.

DNA films, prepared by mixing $1 \mu\text{L}$ DNA solution with $1 \mu\text{L}$ of NaOH, pH 8.0, were also examined with a SEM and are shown in Figure 4. The images were taken for films prepared from a solution containing 100 ng DNA and deposited on the substrate at (a) $+4$, (b) -5 and (c) -20 °C. Close-ups of the sample area (middle line) and the sample edges (bottom line) are also shown in the figure. It can be clearly seen that in the case of the sample prepared at $+4$ °C the DNA film spreads over a large area of approximately 2.5 mm in diameter, marked with a characteristic “rim”, although the sample edges are rough and thus the effective coverage is about 40% lower than in case of films deposited at -10 °C and -20 °C. Also, a close-up of the sample edge reveals an increased sample concentration at the edge as compared to the central region.

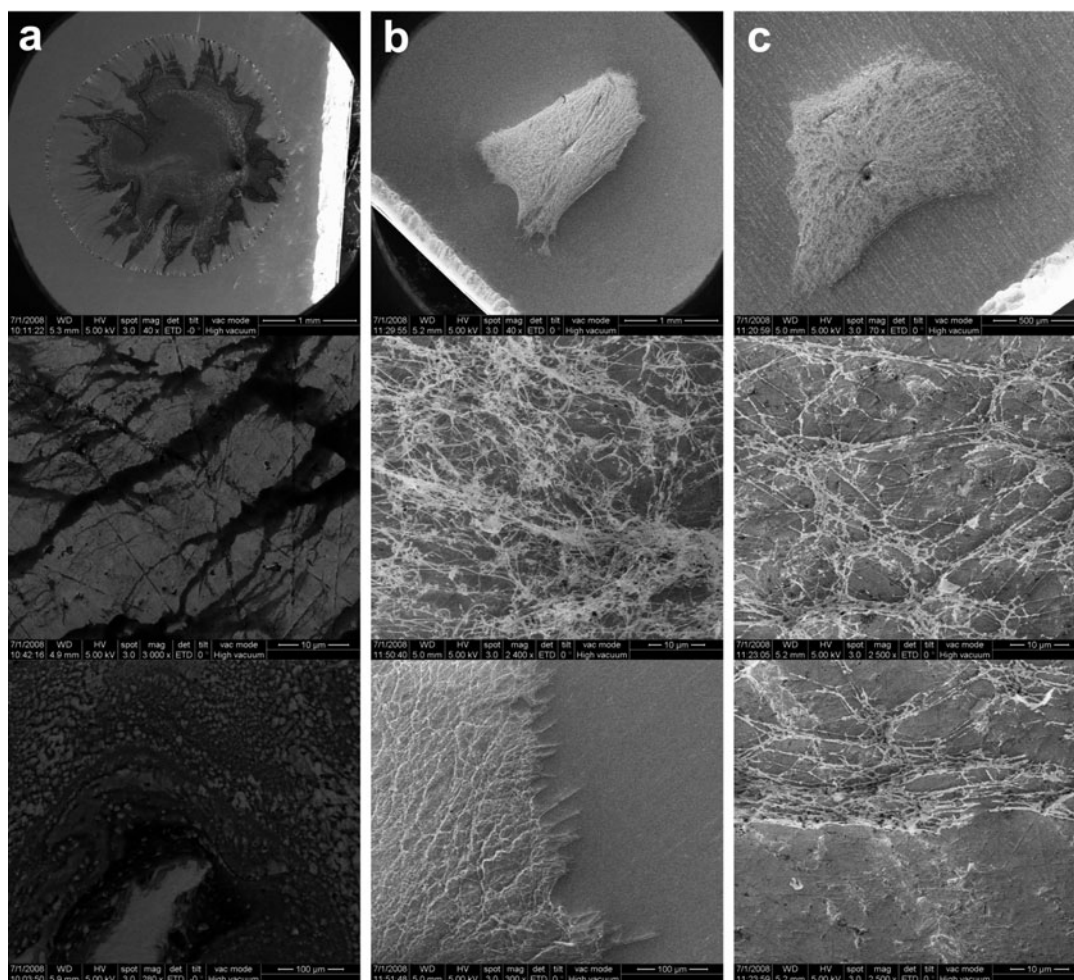


Fig. 4. Images of DNA films prepared from a solution of $1 \mu\text{L}$ UHP H_2O containing 100 ng plasmid DNA and $1 \mu\text{L}$ NaOH pH 8.0, placed on the substrate at $+4$ °C (a), -5 °C (b) and -20 °C (c), together with close-ups of the central area and edges of the samples.

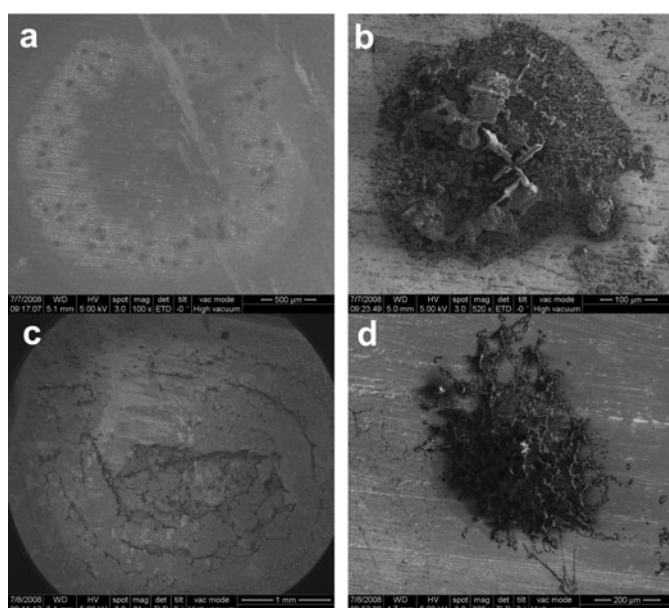


Fig. 5. Samples containing 5 μL NaOH, pH 10.0, placed on the substrate at (a) +4 $^{\circ}\text{C}$ and (b) -20 $^{\circ}\text{C}$ and 100 ng DNA in 1 μL UHP H₂O mixed with 5 μL NaOH, pH 10.0, placed on the substrate at (c) +4 $^{\circ}\text{C}$ and (d) -20 $^{\circ}\text{C}$.

For samples prepared at lower temperatures the area coverage decreases. Thus films prepared at -5 $^{\circ}\text{C}$ have a diameter half that of those prepared at +4 $^{\circ}\text{C}$, and films formed at -20 $^{\circ}\text{C}$ are 2.5 times smaller compared to those prepared at +4 $^{\circ}\text{C}$. It can also be seen that the sample is more compact with a decrease in temperature. A similar situation was also observed with the light microscope (Figs. 1a and 1b). It can be clearly seen from the tilted images of the samples prepared at different temperatures, that the height of the films varies more than the substrate area covered by the sample. Thus, the density of the film prepared at -20 $^{\circ}\text{C}$ is much lower than the density of the film prepared at -10 $^{\circ}\text{C}$.

Films with a higher base concentration formed in similar conditions to those shown in Figure 4 were also examined, as an increase in the concentration of the base prevents structural water evaporation and thus prevents damage to supercoiled DNA molecules. It was also interesting to see how the addition of the base to the sample influences the shape of the film formed during the evaporation process. We prepared films from samples containing 6 μL of pure sodium hydroxide, pH 10.0, placed on the substrate at +4 $^{\circ}\text{C}$ (Fig. 5a), and -20 $^{\circ}\text{C}$ (Fig. 5b), and from the base solution mixed with 100 ng DNA and placed on substrate at +4 $^{\circ}\text{C}$ (Fig. 5c) and -20 $^{\circ}\text{C}$ (Fig. 5d).

Again, it can be clearly seen that the films prepared at +4 $^{\circ}\text{C}$ are spread over a larger area than those formed at -20 $^{\circ}\text{C}$ and small crystalline features from the NaOH are spread over the edges of the sample. The sample also exhibits many small condensed fragments with large areas of clear substrate. In contrast, samples prepared at lower temperatures exhibit a high degree of crystal formation in both the pure base sample and the one containing DNA

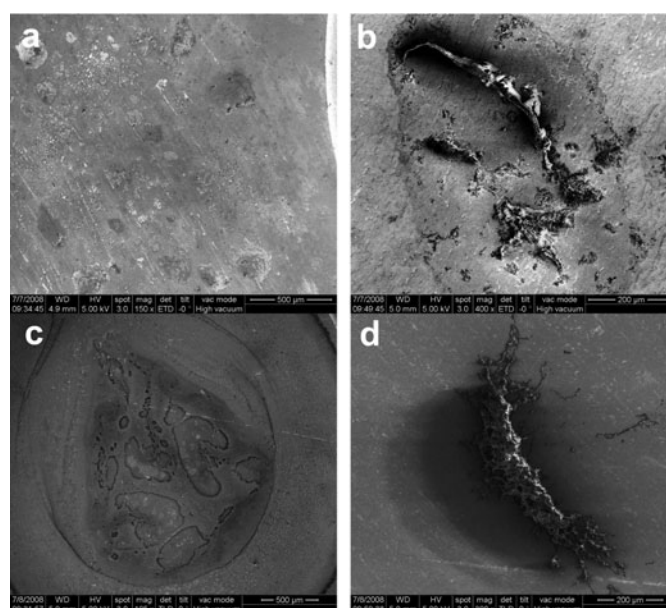


Fig. 6. Samples containing 5 μL Mg(OH)₂, pH 10.0, placed on the substrate at (a) +4 $^{\circ}\text{C}$ and (b) -20 $^{\circ}\text{C}$ and 100 ng DNA in 1 μL UHP H₂O mixed with 5 μL Mg(OH)₂, pH 10.0, placed on the substrate at (c) +4 $^{\circ}\text{C}$ and (d) -20 $^{\circ}\text{C}$.

molecules. The film prepared at -20 $^{\circ}\text{C}$ is much more condensed and thicker than the one prepared at +4 $^{\circ}\text{C}$, and spans a small and well defined area. This was also seen in Figures 2 and 4. Although the edge definition is better, a network system in a crystalline form of the sample is seen.

The influence of another base, magnesium hydroxide, on film formation under vacuum conditions was also tested and the SEM images are shown in Figure 6. This base gave the worst DNA survivability rate under vacuum conditions among all bases tested [4]. As for films containing NaOH (Fig. 5), the samples containing pure base show crystal formation at both temperatures tested. What is interesting to see with this base is that the films formed at a temperature of -20 $^{\circ}\text{C}$ (both with and without DNA) show a significantly different shape to other films, a dense, long structure is formed.

However, the most dramatic change in the shape of the film was seen for DNA samples mixed with Tris-Cl buffer (Fig. 7). We compared films prepared at +4 $^{\circ}\text{C}$ (Fig. 7a) and -5 $^{\circ}\text{C}$ (Fig. 7b) and examined the total area and the distribution of material within the central area and edge of the sample. The greatest difference can be seen at the edges. The sample prepared at +4 $^{\circ}\text{C}$ has a much higher concentration close to a well-defined edge than the sample prepared at -5 $^{\circ}\text{C}$. The material distribution within the central part of the sample is uniform for the +4 $^{\circ}\text{C}$ film, but the film prepared at a lower temperature formed small aggregates of various sizes, with the largest of these close to the centre of the sample. From all the bases tested as potential stabilisers, Tris-Cl buffer appears to give the most uniform film formation. However, as this buffer contains three hydroxyl groups and an amine group, which

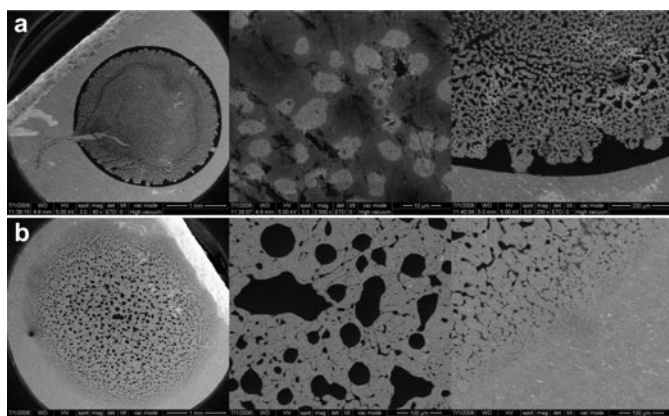


Fig. 7. 100 ng of plasmid DNA in 2 μL of 5 mM Tris-Cl buffer placed on the substrate at (a) +4 $^{\circ}\text{C}$ and (b) -5 $^{\circ}\text{C}$, together with close-ups of the central region and edges of the samples.

can bind to DNA, it is likely to interfere with the DNA destruction process by screening it from incident radiation of any kind and thus is not a useful DNA stabilizer.

4 Conclusions

In this work the influence of the initial substrate temperature on plasmid DNA film formation was examined. The influence of compounds stabilising DNA under vacuum was also tested. The preparation of films containing supercoiled plasmid DNA at various temperatures of the substrate (Fig. 3) has a very small influence (around 10%) on the survivability of supercoiled molecules. However, the results reveal a dramatic influence of both the temperature of the substrate and the stabilisers used on the size and shape of the film formed.

If we analyse the area of the substrate that was covered by the sample containing 1 μL of 100 ng DNA in UHP H_2O and 1 μL of NaOH, pH 8.0, with respect to the substrate temperature (Fig. 8), then an exponential increase with increasing temperature can be seen. Such a tendency was seen for all types of solution used in the experiments.

The total content of the base in the sample also has an influence on the shape and size of the film. The sample area of the film plotted against base content is shown in Figure 9. The diameter of the film formed decreases with increasing content of the base in the sample solution. Such behaviour was seen in all of the films prepared, regardless of the initial temperature of the substrate.

Results also show that the substrate temperature has an important impact on the shape of the edge of the sample. Apart from the sample prepared in Tris-Cl buffer, all samples prepared at temperatures below 0 $^{\circ}\text{C}$ showed more concise and well-defined sample edges than the ones prepared at above 0 $^{\circ}\text{C}$. The distribution of the material becomes more regular when decreasing the substrate temperature, which is in agreement with previous experiments [7]. Also, when base solutions were used as stabilisers, the surface of the films formed were full of cracks as a result of solvent evacuation. When comparing Figures 5–7

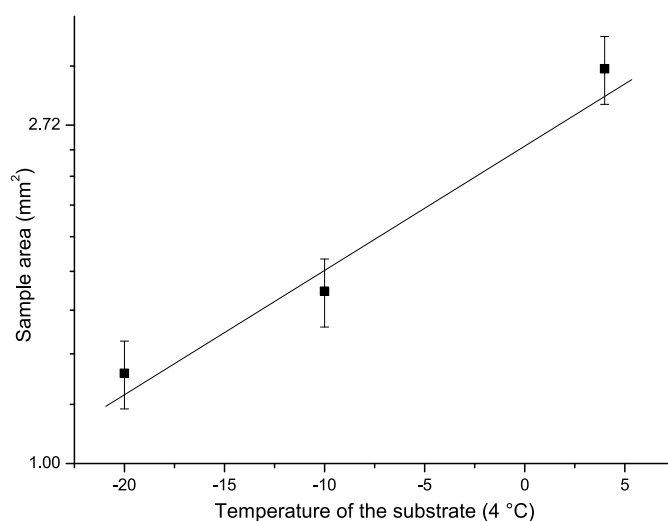


Fig. 8. An increase in sample area with increasing temperature of substrate during film preparation, calculated from samples containing 1 μL UHP H_2O with 100 ng plasmid DNA + 1 μL NaOH pH 8.0 placed on the tantalum support at +4 $^{\circ}\text{C}$, -5 $^{\circ}\text{C}$ and -20 $^{\circ}\text{C}$; the error bars shown on the graph represent uncertainty of the evaluation of the surface area of the sample; the solid line represents a guide to the eye.

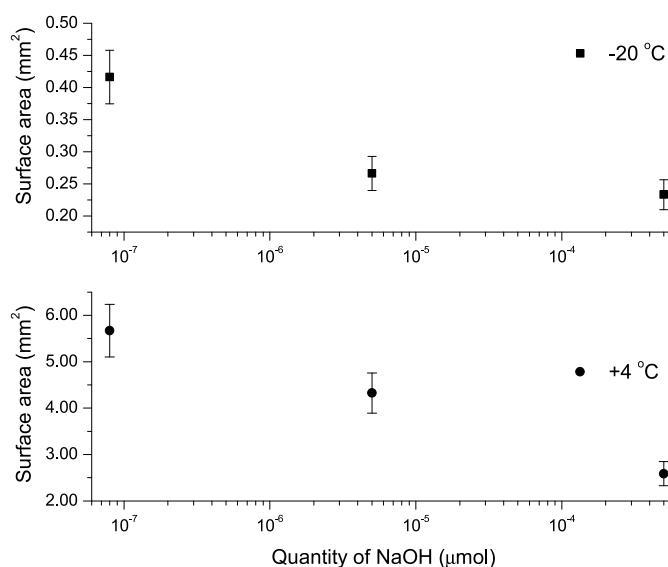


Fig. 9. A decrease in sample area with increasing base content in samples containing 100 ng DNA in: 6 μL UHP H_2O , 1 μL UHP H_2O + 5 μL NaOH pH 8.0 and 1 μL UHP H_2O + 5 μL NaOH pH 10.0, prepared at a substrate temperature of -20 $^{\circ}\text{C}$ (square symbol, top graph) and +4 $^{\circ}\text{C}$ (round symbol, bottom graph); the error bars shown on the graph represent uncertainty of the evaluation of the surface area of the sample.

the strong influence of the stabiliser on the morphology, shape and size of the film can be seen.

A further observation is that the films prepared at temperatures above 0 $^{\circ}\text{C}$ are much lower than those prepared at temperatures below 0 $^{\circ}\text{C}$. Such low samples might cause DNA molecules to become stuck in an uneven surface of the substrate, when the substrate used is

not atomically flat, as in this work. This could prevent the biomolecules from being irradiated and make the recovery of the post-irradiation sample more difficult. On the other hand, the formation of thick DNA samples may shield molecules from the inner region of the film from the incident beam and thus have a strong impact on e.g. deriving cross sections for DNA damage levels resulting from low energy electron or photon irradiation. The preliminary results of irradiation experiments found this to be the case. As shown previously [10], a correction factor for penetration depth is therefore required. This problem is extremely important, especially when low energy electrons are to be used as incident particles as they will not penetrate the whole volume of the sample, charging only the outer surface.

Taking all the above conclusions into account it is believed that the best temperature of the substrate for DNA samples deposition during film preparation would be between $-5\text{ }^{\circ}\text{C}$ and $-10\text{ }^{\circ}\text{C}$, when a non-monocrystalline substrate is to be used. At these temperatures the liquid sample has some time to spread over the substrate before it freezes and the film will still remain flat and condensed, but the molecules will not be protected from the incident beam by features of the surface. In other cases, when atomically flat substrates can be used, such as mica or a metal mono-crystal, the best temperature for sample deposition and evacuation would range from $+4\text{ }^{\circ}\text{C}$ up to room temperature. Of course, using a different substrate, especially an atomically flat one, will change the properties of the film. There should not be a significant differences for DNA films prepared on a metal mono-crystal as the surface orientation of metallic substrates only influences the planar orientation of small molecules that will form a crystalline layer, and should therefore not have a great impact on large DNA molecules, which are more likely to form a network. Mica has been used for high energy particle irradiation studies, for example in hydrogen ion irradiation studies of DNA [6], however caution should be taken due to the surface charge of mica, which would make it unsuitable for LEE irradiation studies.

It should now be possible to control both the size and the shape of the sample, depending on the type of substrate used. Knowing the dependence of the physical form of the film on the initial temperature of the substrate, the uniformity, compactness and flatness of the film can to be estimated.

We would like to thank Dr J. Chevallier and Mr M.B. Sillassen from Department of Physics and Astronomy, Aarhus University, Denmark, for their assistance with the SEM. MAŚ would like to acknowledge the ESF-EIPAM action for supporting her visit to the Aarhus University.

References

1. B. Boudaïffa, P. Cloutier, D. Hunting, M.A. Huels, L. Sanche, *Science* **287**, 1658 (2000)
2. C.A. Hunniford, R.W. McCullough, R.J.H. Davies, D.J. Timson, *Biochem. Soc. Trans.* **37**, 893 (2009)
3. M.A. Śmiałek, S.V. Hoffmann, M. Folkard, K.M. Prise, D.E.G. Shuker, N.S. Braithwaite, N.J. Mason, *J. Phys. Conf. Ser.* **101**, 012020 (2008)
4. M.A. Śmiałek, R. Balog, N.C. Jones, D. Field, N.J. Mason, *Eur. Phys. J. D*, **60**, 3136 (2010)
5. M. Folkard, K.M. Prise, B. Vojnovic, B. Blocklehurst, B.D. Michael, *Int. J. Radiat. Biol.* **76**, 763 (2000)
6. J.A. Wyer, K.T. Butterworth, D.G. Hirst, C.J. Latimer, E.C. Montenegro, M.B. Shah, F.J. Currell, *Phys. Med. Biol.* **54**, 4705 (2009)
7. M. Folkard, K.M. Prise, B. Vojnovic, S. Davies, M.J. Roper, B.D. Michael, *Int. J. Radiat. Biol.* **64**, 651 (1993)
8. M.A. Huels, B. Boudaïffa, P. Cloutier, D. Hunting, L. Sanche, *J. Am. Chem. Soc.* **125**, 4467 (2003)
9. S. Even-Chen, Y. Barenholz, *Biochim. Biophys. Acta* **1509**, 176 (2000)
10. M.A. Śmiałek, S.A. Moore, N.J. Mason, D.E.G. Shuker, *Radiat. Res.* **172**, 529 (2009)
11. M. Folkard, K.M. Prise, B. Brocklehurst, B.D. Michael, *J. Phys. B At. Mol. Opt. Phys.* **32**, 2753 (1999)