

# Preparation of DNA films for studies under vacuum conditions

## The influence of cations in buffer solutions

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**Abstract.** Experiments were carried out to determine the optimum conditions required for the preparation of uniform films of supercoiled plasmid DNA to be used in irradiation experiments under high vacuum conditions. Investigations reveal that significant damage to the DNA molecules occurs due to the evacuation process when films were formed from DNA samples in ultra high purity water only. A variety of bases were tested for their possible protective capabilities and sodium hydroxide solution was found to be the most effective in maintaining the supercoiled structure of plasmid DNA during the preparation process. Using a transmission electron microscope we also examined the structure of the DNA films which are formed upon evacuation and how the proposed adducts influence the preparation process. It was found that the addition of bases cause the DNA to aggregate, noting that a base is required for the stability of the DNA molecules. The experimental results presented in this paper show that it may not be possible to perform experiments on so-called pure DNA under vacuum with no stabilizers being added to the sample before the evacuation process.

## 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 to DNA and its complexes by low energy electrons [1], ions [2] and VUV [3]. For the experimental results to be quantitative it is of great importance for the biological material to remain as intact as possible during the preparation process. One of the most important issues to be dealt with is the removal of constituent water from DNA molecules during sample evacuation. It has been shown that the subtraction of such water initially leads to damage of the molecules in the form of single (SSB) and double strand breaks (DSB), and eventually complete decomposition of the DNA molecule to its basic building blocks, the nucleotides and nucleosides [4].

To avoid DNA decomposition the solution containing the molecules that are to be investigated must contain other species that can stabilize the structure during the drying process and prevent the removal of constituent water molecules. The main problem with studies of DNA

films containing such adducts is their possible influence both on the damage formation and its mechanism, and there is also the issue of the effect they have on the way in which the film is formed. The most commonly used compounds for DNA stabilisation are trizma base [5] and ethylenediaminetetraacetic acid (EDTA) [6]. Both molecules are much more complex than simple hydroxides, possessing three (trizma base) and four (EDTA) hydroxyl groups, which may, depending on the experimental conditions, interfere with projectile–DNA molecule interactions while acting like protectants. Therefore, simpler stabilizers must be sought.

The temperature and environment in which films are made are also important factors in the preparation process. In order to control the substrate temperature a simple system, similar to the one described previously [7], was assembled. The temperature of the system was controlled using a set of Peltier units, which allowed stable and reproducible temperatures to be applied to the samples during the DNA film evacuation. The apparatus was designed so that all film preparations were performed in anaerobic conditions, in a dry nitrogen environment. We have established the optimal conditions for the preparation of DNA films to be used in DNA irradiation studies

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under high vacuum conditions and will show that it is not possible to perform experiments on so-called pure DNA under vacuum with no stabilizers being added to the sample before the evacuation process.

## 2 Experimental details

### 2.1 Materials

Plasmid DNA pBR322 in UHP H<sub>2</sub>O was purchased from Inspiralis Limited (Norwich, UK). NaOH, KOH, Mg(OH)<sub>2</sub>, Ca(OH)<sub>2</sub>, boric acid, EDTA and trizma base were purchased from Sigma-Aldrich (DK). Agarose was purchased from Cambrex (UK). Tantalum foil was purchased from Johnson Technica A/S, Frederikssund, Denmark.

### 2.2 Sample and solutions preparation

All bases 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 pH of 10.0 and measured after filtration. For NaOH an additional solution of pH 8.0 was made. Such high pH was necessary to obtain lower base content when mixed with aqueous DNA solution. When base solutions were mixed with DNA solution, the pH was 8.0 or 7.5, depending on the incident base and water content.

All DNA samples used to make films were prepared by diluting stock DNA solutions either with UHP H<sub>2</sub>O or with appropriate base solutions to obtain the final DNA concentration and pH of interest.

### 2.3 Substrate preparation

Tantalum foil (0.5 mm thick) was used as a support for DNA samples stability investigations. The reasons for this are the high *Z*-number of Ta and the layer of tantalum oxide which is formed on the metal surface. The high *Z*-number reduces secondary electron emission upon low electron irradiation, and the oxide layer creates a stable, chemically inert surface, ensuring no DNA-substrate interaction. In addition, tantalum has already been shown to be a good choice for DNA-low energy electrons experiments [8].

Tantalum foil substrates were supported on copper blocks to ensure maximum thermal contact with the cooling plate, which allowed the substrate temperature to be controlled during sample placement and evaporation. The substrates were held at +4 °C while the DNA was added, and it was possible to observe if any water from the glove box condensed on the tantalum supports and thus to monitor if the environment was as dry and clean as assumed. To ensure an even Ta surface the foil was polished on fine sand paper wheels and was cleaned in an ultrasonic bath in pure EtOH and blow-dried with dry N<sub>2</sub>. To re-use the substrates, samples were washed off with UHP H<sub>2</sub>O, followed by EtOH wash and dried.

### 2.4 DNA film preparation and analysis

To prepare DNA films, DNA in aqueous stock solution was diluted further with either UHP H<sub>2</sub>O or with base solutions to obtain 100 ng DNA per 6 μL of solution. A volume of 6 μL of DNA solution was then placed on the tantalum support at +4 °C under dry N<sub>2</sub> atmosphere, in a small evacuation chamber, which was assembled in a glove box. Samples were then subjected to medium vacuum (10<sup>-5</sup> mbar) for time periods varying from one to sixteen hours. After evacuation the chamber was brought back to atmospheric pressure with dry nitrogen. It was found that the optimum time required for a stable DNA film preparation was about 1 h of evacuation.

After preparation, DNA films were recovered from the tantalum supports with 7 μL 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 × TBE buffer. Gels were later stained in 1 × SYBRGreen I/1 × TBE solution for 40 min and de-stained in 1 × TBE for another 20 min. Fluorescent images of the gels were recorded using SYNGENE U:genius gel documentation system. To quantify various DNA forms present in the gels, densitometric analysis was performed using ImageJ software (available from: <http://rsb.info.nih.gov/ij/>). As SYBR Green I binds equally to all plasmid conformers [9], no correction factors were used.

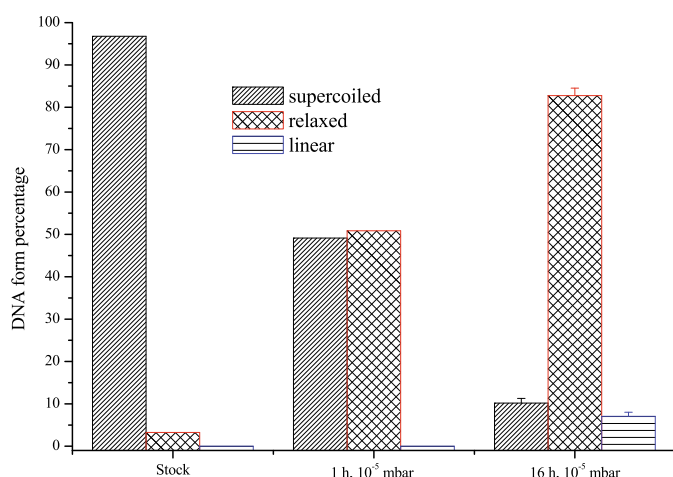
In order to explore the morphology of the DNA film surface a transmission electron microscope (TEM) was employed. The advantage of such analysis was that no modifications to the substrate or the sample were required and hence we were able to image samples that would be produced in the same way as for the irradiation studies. Using this method it is possible to see the film structure as well as the level of area coverage.

## 3 Results and discussion

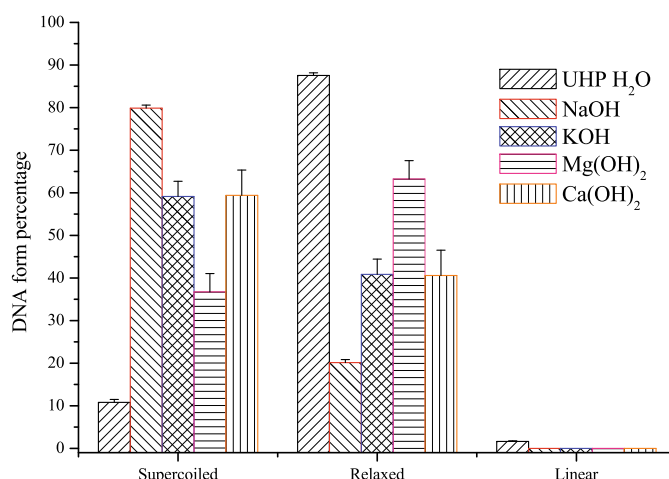
### 3.1 AGE analysis of plasmid survivability under vacuum conditions

The results of the preliminary studies conducted using plasmid DNA samples suspended in UHP H<sub>2</sub>O are shown in Figure 1. The loss of supercoiled DNA and appearance of SSBs and DSBs can be clearly seen, with 40% of the supercoiled DNA lost after only 1 h of evacuation and a 80% loss after 16 h as compared to the stock solution. After such a long evacuation period multiple DSBs (MDSBs) were also observed on the gel in the form of smeared, broad, fast progressing band (data not shown). Such a rapid loss of the primary DNA form would make it impossible to conduct irradiation experiments under vacuum conditions regardless of incident particles type and energy. Therefore, a simple idea of introducing mono- and dications as DNA stabilizers was tested.

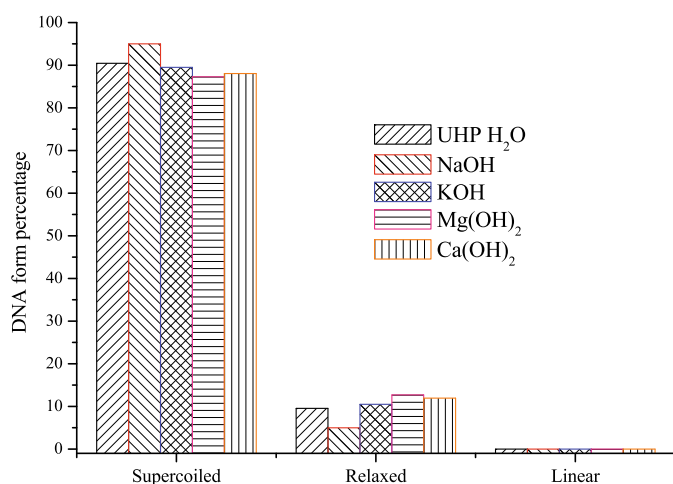
Figure 2 shows the influence of pH 10.0 base solutions on plasmid DNA stability when mixed in a ratio of 5:1 with the stock plasmid in UHP H<sub>2</sub>O. It can be seen that



**Fig. 1.** (Color online) Loss of supercoiled plasmid DNA and the appearance of linear and relaxed forms in samples evacuated from a UHP H<sub>2</sub>O solution, when subjected to 10<sup>-5</sup> mbar vacuum after 1 h and 16 h of evacuation with respect to untreated stock solution.



**Fig. 3.** (Color online) Loss of the supercoiled form of plasmid DNA arising from different basic solutions after evacuation of the samples at 10<sup>-5</sup> mbar for 16.5 h at +4 °C substrate temperature.



**Fig. 2.** (Color online) Assessment of base influence on plasmid DNA stock solutions; the smallest loss of supercoiled DNA form is seen with NaOH solution, which even resulted in the DNA molecules being more stable than DNA suspended in only UHP H<sub>2</sub>O.

the addition of base to DNA solutions in itself does not cause damage to the DNA. The lowest levels of damage were observed for the samples mixed with NaOH solution, with respect to the samples in UHP H<sub>2</sub>O solution. In addition, the number of damaged molecules in solution containing NaOH was lower than in the case of DNA in UHP H<sub>2</sub>O stock solution. This suggests that the absence of ions stabilizing DNA may cause sample deterioration even at the storage stage.

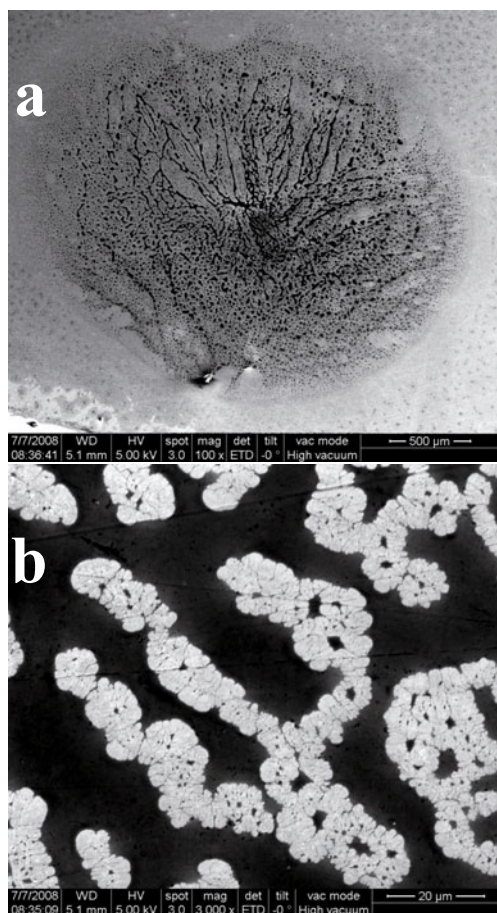
Solutions of plasmid DNA mixed with bases were used to prepare DNA films as described in Section 2.2. Samples were evacuated for 16.5 h and later recovered and analyzed with AGE. It can be seen in Figure 3 that after 16.5 h of evacuation again most of the supercoiled form from the samples in UHP H<sub>2</sub>O was lost (~80% loss), accompanied

by the appearance of DSBs. Of all the bases tested NaOH provided the best protection from the structural water evaporation during the evacuation process (~10% loss). The lowest protection was observed for Mg(OH)<sub>2</sub> solution (~50% loss). The remaining two base solutions provided moderate conditions for DNA protection under vacuum conditions (~25% loss of supercoiled DNA form in both cases).

### 3.2 TEM analysis of DNA film formation

An investigation of the film shape and material distribution over the surface was also conducted. The films of DNA supported on Ta foil, formed through evacuation of solutions for 1 h at 10<sup>-5</sup> mbar, were examined using TEM. Figure 4 shows micrographs for UHP H<sub>2</sub>O DNA film showing (a) the total sample area and (b) a close-up of the center of the sample. The film is circular and approximately 2 mm in diameter, with a higher concentration of DNA in the centre. It can also be seen that there is a large amount of uncovered tantalum surface within the sample area resulting in an uneven distribution of DNA molecules.

As a result of these observations, it was even more desirable to see if the presence of a base in the solution cause any changes to the DNA film itself. First, we examined the evaporated base solutions with no DNA molecules to see how base crystals redistribute over the Ta surface and for comparison with films containing DNA to see the base influence on the formation of the films. Figure 5 shows the results obtained for (a) KOH, (b) NaOH, (c) Ca(OH)<sub>2</sub> and (d) Mg(OH)<sub>2</sub>. It can be seen that KOH solution coverage is fairly uniform over the whole sample area, although some large crystalline structures are visible. Occurrence of such forms may cause DNA “clogging” and higher concentration within these crystalline centres. Also, the sample area itself seems to be greater than in case of the other



**Fig. 4.** Plasmid DNA in UHP H<sub>2</sub>O evacuated at  $10^{-5}$  mbar for 1 h, placed on the tantalum supports at +4 °C; (a) total sample area, (b) close-up of the sample centre. The dark places are covered with DNA, whereas bright ones are clean tantalum surface.

bases. For NaOH the sample edge seems to have higher concentration of the base, leaving the centre free from base crystals. Such a distribution would certainly lead to uneven distribution of the material over the surface during evaporation.

Samples prepared by evaporation of dication solutions (Ca(OH)<sub>2</sub> and Mg(OH)<sub>2</sub>) were characterized by rather large crystalline forms, concentrated in the middle of the sample area. This can especially be seen for Mg(OH)<sub>2</sub> solution in Figure 5d. These images, together with results obtained from AGE (see Sect. 3.1) led us to choose NaOH as the best candidate to stabilise DNA for the preparation of films, despite some issues with the uniformity of coverage with a NaOH solution.

In Figure 6 plasmid DNA films (top line) evaporated from solutions with different concentrations of NaOH can be seen, together with detailed images of the sample surface (bottom line). The images clearly show that there is a dramatic influence of the base content on the DNA film shape and surface coverage. The greater the NaOH concentration, the more condensed the film. As seen in Figure 5b, the crystalline forms are concentrated on the

edge of the sample, whereas the DNA film seems to be condensed more towards the centre of the film. Also, the total sample area appears to increase with base content. Unfortunately, the sample area coverage seem to worsen with increased stabilizing activity.

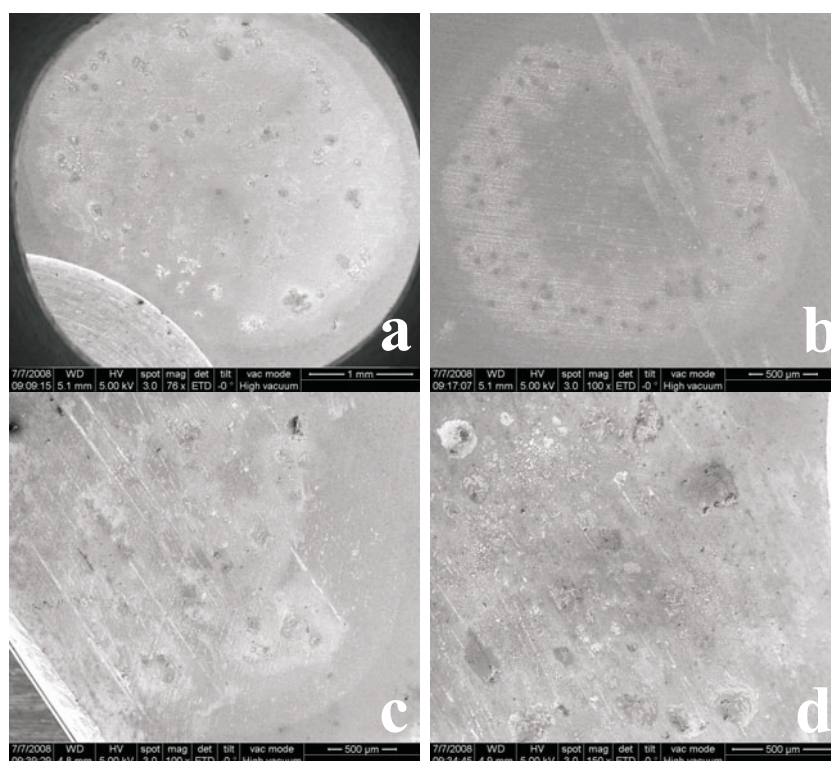
We also wanted to see how the DNA film would form when using a solution containing Mg(OH)<sub>2</sub> which gave, amongst all bases used in the experiment, the worst survivability rate from the AGE analysis (Fig. 7a). Moreover, we wanted to examine films formed from solutions containing trizma base, which is commonly used in vacuum experiments (Fig. 7b). It can be seen that in the case of Mg(OH)<sub>2</sub> the sample distribution is irregular and the sample area is surrounded by a “rim” (Fig. 7a, top line, marked with arrows). Also, as can be seen in Figure 7a, bottom line, there is a vast amount of small crystalline features distributed evenly across the sample area. Such features, which rise above the surrounding material, will affect the local flux of electrons through distortion of the potentials and cause uneven DNA irradiation by low energy electrons.

For films containing trizma base a much higher concentration of the sample can be seen in the edges of the area, accompanied by a quite regular distribution of remaining material within the central part of the film. Although the sample area seems to have the best definition amongst all compounds investigated, the complexity of trizma base and its possible involvement in the DNA destruction process in the presence of any kind of radiation, excludes trizma as a useful DNA stabilizer. In this connection trizma contains both three hydroxyl groups and an amine group. Trizma may therefore significantly distort the results of any DNA irradiation experiments through the generation of radicals and negative ions which may bind with or disrupt the target DNA.

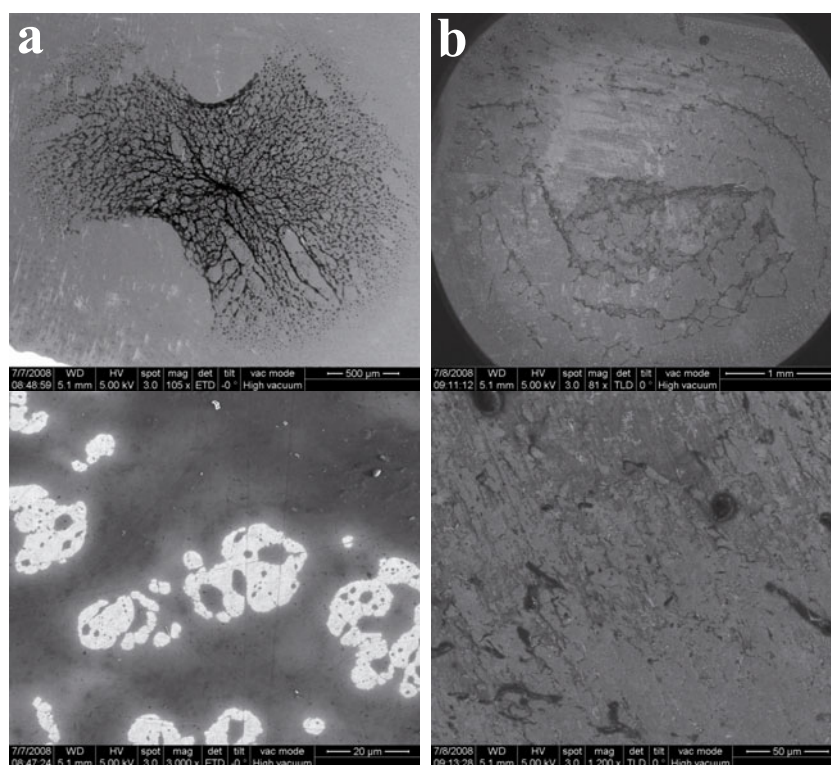
## 4 Conclusions

We have conducted an extensive series of experiments to determine the optimum conditions required to prepare thin films of plasmid DNA containing a high proportion of supercoiled molecules. The survivability of plasmid DNA under high vacuum environment was examined. It was found that it is not possible to sustain plasmid DNA in the supercoiled form under vacuum conditions, when samples are evaporated from UHP H<sub>2</sub>O alone. A set of simple bases containing mono- and dications were examined as potential DNA stabilizers. From data obtained from AGE analysis, NaOH was found to be the most efficient in preventing DNA structural water removal and thus material decomposition.

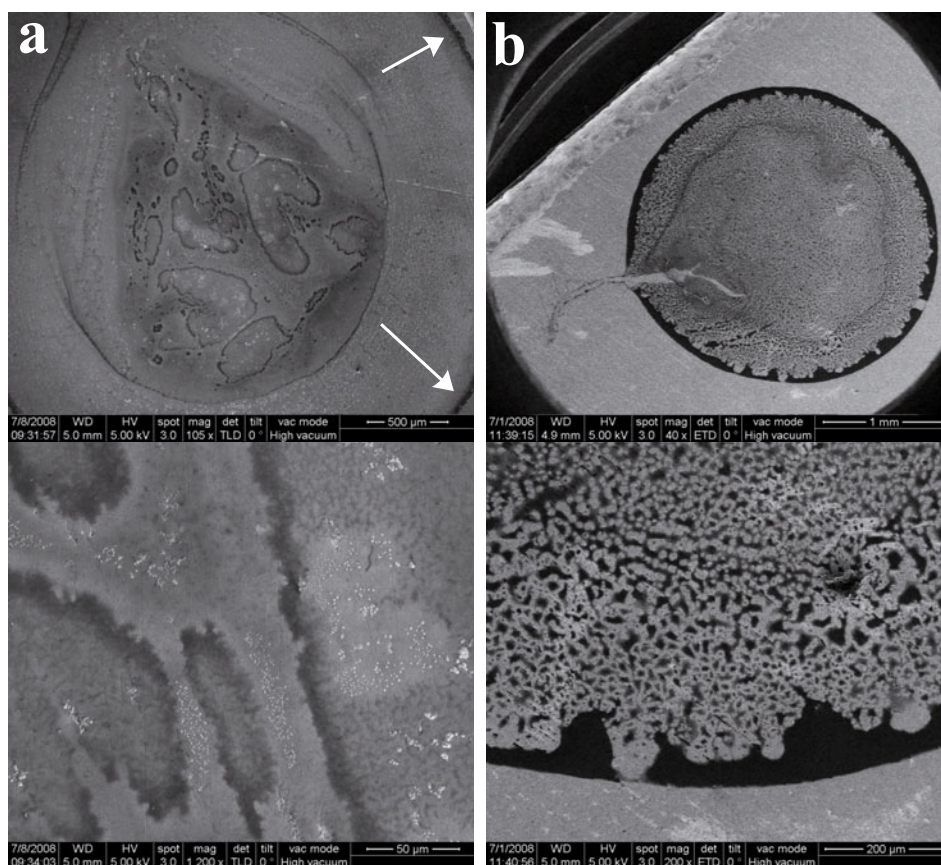
Images of DNA films prepared for vacuum studies were recorded using a TEM. This allowed both the area of the film formed from evaporated solutions and the morphology of the film to be examined. For NaOH, which was found to give the most stable samples after solvent evaporation, it was seen that the film surface is irregular and the area coverage is not uniform. This non-uniformity increases with the amount of stabilizers in solution, which



**Fig. 5.** Electron micrographs of 5  $\mu\text{L}$  (a) KOH, (b) NaOH, (c)  $\text{Ca}(\text{OH})_2$  and (d)  $\text{Mg}(\text{OH})_2$  evacuated at  $10^{-5}$  mbar for 1 h, placed on the tantalum supports at  $+4^\circ\text{C}$ .



**Fig. 6.** Plasmid DNA samples (top line) and their magnifications (bottom line) of 100 ng DNA in 6  $\mu\text{L}$  solutions, evacuated at  $10^{-5}$  mbar for 1h, placed on the tantalum supports at  $+4^\circ\text{C}$ ; (a) UHP  $\text{H}_2\text{O}/\text{pH } 8.0$  NaOH, ratio 1  $\mu\text{L}:5 \mu\text{L}$  (magnified 25 times), (b) UHP  $\text{H}_2\text{O}/\text{pH } 10.0$  NaOH, ratio 1  $\mu\text{L}:5 \mu\text{L}$  (magnified 20 times).



**Fig. 7.** Plasmid DNA samples (top line) and their magnifications (bottom line) of 100 ng DNA in 6  $\mu\text{L}$  solutions, evacuated at  $10^{-5}$  mbar for 1 h, placed on the tantalum supports at +4  $^{\circ}\text{C}$ ; (a) UHP  $\text{H}_2\text{O}/\text{pH } 10.0 \text{ Mg}(\text{OH})_2$ , ratio 1  $\mu\text{L}:5 \mu\text{L}$ , (b) plasmid DNA in tris buffer; the arrows mark a characteristic “rim” at the edge of sample containing  $\text{Mg}(\text{OH})_2$ .

seem to be causing the films to crack during evaporation. The most regular film was obtained for a DNA sample evaporated from trizma base-containing solution. Some distortion is seen at the edges of the sample, where a dense rim was observed. Although the desired area coverage was obtained, the idea of using this well-known and widely-used compound was abandoned due to its complexity and high reactivity, which might distort the direct interactions between projectiles and DNA target. On balance we feel that it is more important to give priority to the survivability of DNA under vacuum in irradiation experiments. Thus we would recommend that DNA uniformity is sacrificed and that DNA solutions in NaOH are used.

Another method of improving the film surface coverage would be to increase the initial amount of DNA molecules in samples. Unfortunately, this may cause DNA to aggregate or to form nets, as reported previously [10], instead of just uniformly increasing the surface coverage. Also, as shown in Figure 6, it is mesh formation from DNA molecules rather than a lack of material that is causing non-uniform film distribution.

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