Glutaraldehyde Modified Mica: A New Surface for Atomic Force Microscopy of Chromatin

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ABSTRACT We have found that mica surfaces functionalized with aminopropyltriethoxysilane and aldehydes bind chromatin strongly enough to permit stable and reliable solution imaging by atomic force microscopy. The method is highly reproducible, uses very small amounts of material, and is successful even with very light degrees of surface modification. This surface is far superior to the widely used aminopropyltriethoxysilane-derivatized mica surface and permits resolution of structure on the nanometer-scale in an aqueous environment, conditions that are particularly important for chromatin studies. For example, bound nucleosomal arrays demonstrate major structural changes in response to changes in solution conditions, despite their prior fixation (to maintain nucleosome loading) and tethering to the surface with glutaraldehyde. By following individual molecules through a salt titration in a flow-through cell, one can observe significant changes in apparent nucleosome size at lower [salt] and complete loss of DNA from the polynucleosomal array at high salt. The latter result demonstrates that the DNA component in these arrays is not constrained by the tethering. The former result is consistent with the salt-induced loss of histones observed in bulk solution studies of chromatin and demonstrates that even histone components of the nucleosome are somewhat labile in these fixed and tethered arrays. We foresee many important applications for this surface in future atomic force microscopy studies of chromatin.

INTRODUCTION

Atomic force microscopy (AFM) is proving to be an extremely useful analysis technique for the study of chromatin. Novel structural properties of individual nucleosomes and of nucleosomal arrays as well as their interaction with nucleosome-remodeling machinery have been analyzed recently by scanning probe microscopy (SPM) techniques (Bash et al., 2001; Schnitzler et al., 2001; Yodh et al., 2002). These recent studies extend earlier SPM investigations of chromatin (Allen et al., 1993; Fritzsche and Henderson, 1997; Leuba et al., 1994; Martin et al., 1995; Sato et al., 1999; Yodh et al., 1999). Despite this progress, SPM studies are still hindered by the unreliable nature of the deposition process, a major handicap when only small amounts of sample are available.

To date, most surface modification techniques have been based on electrostatic attachment of biopolymers to an oxide (usually mica) surface. One simple approach is direct attachment to clean glass, spontaneously activated with hydroxyl groups on exposure to water (Leuba et al., 2002). Another modification uses ion exchange to place positive charges on a mica surface (Hansma and Laney, 1996; Vesenka et al., 1992). Covalent attachment of charged groups using silanizing agents places amines on a mica surface by reaction with aminopropyltriethoxysilane (APTES) (Culler et al., 1985; Lindsay et al., 1992; Lyubchenko et al., 1993). However, even this method has yielded variable results in our hands. Recently, Facci et al. (2002) have described a new two-step surface modification in which an APTES-treated surface is subsequently exposed to glutaraldehyde to place reactive aldehydes on the modified mica surface. This reagent forms stable adducts with lysine residues (Richards and Knowles, 1968), and it has long been exploited both for studies of chromatin (Chalkley and Hunter, 1975) and as a protein coupling reagent in analytical applications (Ternynck and Avrameas, 1972). In this article, we discuss the application of glutaraldehyde-modified mica (GD-mica) to imaging of chromatin. The work describes an extremely useful new surface for solution analysis of chromatin, an area of great current research interest, and also suggests a reason for the variability of the APTES modified surfaces in chromatin studies. The fact that the new surface is more reliable than the APTES treatment alone suggests that the attachment of APTES to surface silanol groups is robust, but electrostatic attachment to the primary amine is less reliable than the covalent attachment used in the new process.

MATERIALS AND METHODS

APTES functionalization

A desiccator was purged with argon for 2 min and 30 μL of APTES (99%, Sigma-Aldrich, St. Louis, MO) placed into a small container at the bottom of the desiccator. Ten microliters of *N*,*N*-diisopropylethylamine (99%, distilled, Sigma-Aldrich) was placed into another small container, and the desiccator purged with argon for a further 2 min. Mica sheets were stripped on one side until smooth and immediately placed into the desiccator. The

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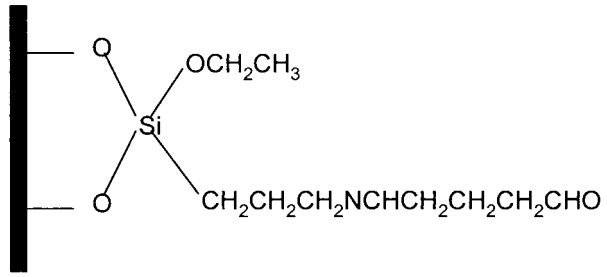

FIGURE 1 Schematic showing the modified surface that results from APTES treatment of the surface followed by glutaraldehyde exposure. The exposed aldehyde group reacts with lysine residues.

desiccator was purged for another 3 min and then sealed off, leaving the mica exposed to APTES vapor for times that were varied between 30 min and 2 h (there appeared to be no consistent effect of exposure time within this range). After this exposure, the APTES was removed, the desiccator purged, and the treated mica (AP-mica) stored in the sealed desiccator until needed. APTES was used both as received and as redistilled. Distillation was found to have no effect unless the as-received material was older than ~2 months or had been exposed to ambient air for some hours.

Procedure for glutaraldehyde functionalization and chromatin binding

Two hundred microliters of a 1 mM glutaradehyde (grade I, Sigma-Aldrich) solution in water was pipetted onto AP-mica immediately on removal from the storage desiccator and incubated for 10 min. A structure for the functional group attached to the mica surface (GD-mica) is shown in Fig. 1. The surface was rinsed with water from a Nanopure ultrapure water system, and 60 μL chromatin solution (0.3 μg DNA per milliliter in water) was pipetted onto the treated surface and allowed to incubate for 30 min. The surface was then rinsed again with water. The prepared sample was mounted into the SPM liquid flow cell (Molecular Imaging, Phoenix, AZ) and imaged immediately.

Imaging conditions

In situ imaging was carried out with a Macmode PicoSPM (Molecular Imaging) equipped with triangular Si_3N_4 Cantilevers (Molecular Imaging) with a spring constant of 0.1 N/m. Measurements were performed at ~8 kHz driving frequency and 5 nm of amplitude with 8% amplitude reduction. The scanning rate was 1.78 Hz. Salt titration studies were carried out by injecting NaCl solutions of increasing concentration into the flow cell in situ. Imaging in air was carried out with a Nanoscope III AFM (Digital Instruments, Santa Barbara, CA) using NCH silicon cantilevers (Nanosensors, Wetzlar, Germany) with a spring constant of 42 N/m. Drive amplitude was ~20 nm with a 30% reduction set-point. Chromatin was spread as described by Bash et al. (2001) using solutions corresponding to DNA concentrations that ranged from 1.5 μg/mL to 0.75 μg/mL as needed to achieve a reasonable surface coverage (the activity of the AP-mica being highly variable).

Nucleosome arrays

The 172-12 DNA template used in this work was originally constructed by Simpson et al. (1985). The plasmid containing this DNA template was a generous gift from Prof J. Hansen. The mouse mammary tumor virus promoter (MMTV) DNA template was constructed as described elsewhere (Fragoso et al., 1995). DNA isolation, nucleosome reconstitution, and glutaraldehyde fixation were all done as previously described (Bash et al., 2001). Chromatin sample concentrations are expressed in terms of [DNA] with the nucleosome loading (at a given histone concentration in the reconstitution) determined empirically as described by Bash et al. (2001).

RESULTS AND DISCUSSION

Comparison of AP- and GD-mica

AP-mica has been used in our laboratories for chromatin structural studies for some time (Bash et al., 2001; Yodh et al., 1999). Thus, to characterize the properties of the GD-mica surface, we compared the abilities of the two surfaces to image the same chromatin sample, 172-12 nucleosomal arrays. The 172-12 DNA template, developed for chromatin studies by Simpson and coworkers (Simpson et al., 1985), can be reconstituted in vitro with histones and provides a well-behaved model chromatin system whose properties have been extensively analyzed, including detailed AFM studies (Bash et al., 2001). We typically reconstitute these arrays to subsaturating levels with nucleosomes because studies of subsaturated arrays can yield more information on template loading issues than studies of saturated ones, and the properties of these arrays (numbers of nucleosomes, distances, etc.) are easier to analyze by AFM if the templates are not fully saturated (Yodh et al., 1999).

A comparison of the same 172-12 reconstituted chromatin sample visualized by tapping mode in air on an AP-mica surface or by Macmode (GD-mica in solution) is shown in Fig. 2, *a* and *b*. The qualitative appearance of the chromatin molecules on the two surfaces is the same. On both surfaces, the molecules are well spread out, and the nucleosomes are clearly visualized. Table 1 makes a quantitative comparison of the features of 172-12 chromatin molecules deposited on the two surfaces. The average number of nucleosomes per molecule is similar in the images from both surfaces. The slight differences may be a consequence of the small sample size, or they may reflect a small amount of nucleosome loss from molecules imaged in solution (R. Bash and H. Wang, unpublished observations). Nucleosome widths and heights on the two surfaces are quite similar. The significant differences in naked DNA heights are expected as a consequence of the gentler imaging in solution. The difference in naked DNA widths may be accounted for by a difference in the sharpness of the two types of AFM probes used for the different imaging methods. Taken together, these results indicate that chromatin molecules deposited on the two surfaces behave quite similarly.

It was noted in previous studies that nucleosomes are lost from unfixed chromatin deposited on AP-mica surfaces (Yodh et al., 1999). Fig. 2 *c* shows that nucleosomes are also lost from unfixed molecules deposited on GD-mica surfaces. Thus, when using GD-mica for chromatin imaging, prior fixation is apparently still necessary to maintain

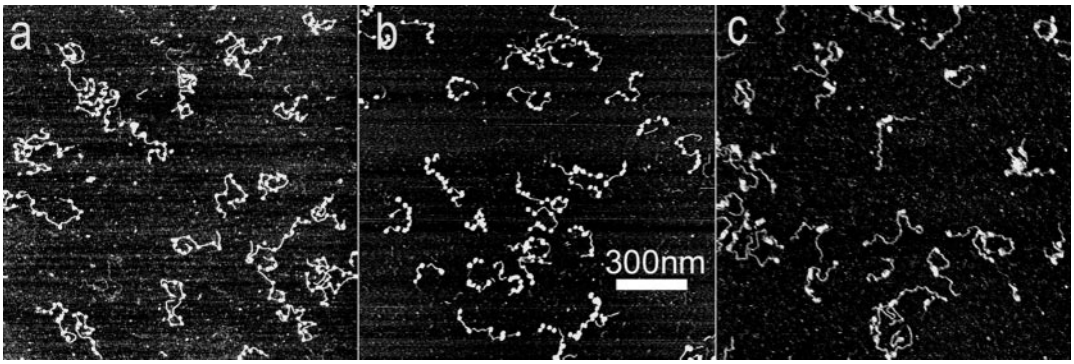

FIGURE 2 AFM images of fixed, 172-12 polynucleosomal arrays (*a*) imaged on APTES-mica by tapping mode in air or (*b*) imaged on the GD-mica surface using Macmode and (*c*) unfixed 172-12 arrays imaged as in *b*. The samples used with the glutaraldehyde-treated surfaces have a DNA concentration of 300 μg/L. The AP-mica required several trials with sample DNA concentrations that ranged from 15 mg/L to 750 μg/L to establish the concentration needed to achieve a coverage like that shown in *a* in a given run. The vertical scale of the images (*black* to *white*) is 3 nm.

samples that accurately reflect the nucleosome loadings originally present on the reconstituted arrays.

Solution biochemical studies of in vitro reconstituted nucleosomal arrays have largely been limited to templates derived from the sea urchin 5S rDNA sequence, apparently for technical reasons, although AFM analysis using AP-mica does not seem to suffer from the same limitations (R. Bash, H. Wang, D. Lohr, S. Lindsay, in preparation). Thus, we wanted to test whether the GD-mica surface will allow imaging of nucleosomal arrays made with other DNA sequences. For that purpose, an ~2-kb DNA fragment from the MMTV promoter region was reconstituted in vitro with HeLa histones. This chromatin region was chosen because it undergoes nucleosome remodeling in response to hormone induction in vivo (Hager, 2001; Hager et al., 1995) and in vitro (Fletcher et al., 2000) and has become an extremely important model system for studying the nucleosome changes associated with gene activation in eukaryotes. The images of these arrays on GD-mica are as clear as those from the 172-12 arrays. Some images of these MMTV nucleosomal arrays will be shown below (see Fig. 4).

Loading characteristics of the GD-mica surface

Imaging on dried surfaces usually requires less concentrated samples than solution imaging because the molecules are concentrated on the surface during the drying process. However, we found that to produce fields that were not excessively covered (i.e., to avoid crowding), samples deposited onto GD-mica for solution imaging had to be diluted relative to the solutions used for AP-mica imaging in air. Thus, the GD-mica is quite efficient in capturing molecules. All samples used for these GD-mica studies were diluted to 0.3 μg/mL (DNA concentration). In contrast, samples for AP-mica imaging required DNA concentrations that varied from 15 μg/mL to 0.75 μg/mL, depending upon the (uncontrolled) activity of the surface. Thus, the GD-mica surfaces required samples that were 2.5 times to 50 times more dilute than those required for AP-mica. Furthermore, the GD-mica surfaces yielded clearly and easily visualized molecules in all (100%) of the depositions. The AP-mica only yielded interpretable data in approximately one-half of the depositions, even though the samples were more concentrated.

For insights on the modification level required for chromatin binding, we carried out a series of depositions on surfaces modified to various extents with GD. This was achieved by varying the GD concentration. As can be seen in Fig. 3, there is only a gradual (20%–30%) decrease in the ability of the surface to tether chromatin as the [GD] drops into the micromolar and nanomolar range. This small variation in sample molecule density as the glutaraldehyde concentration is changed by many orders of magnitude implies that adsorption is more sensitive to the sample concentration and less sensitive to the degree of glutaraldehyde treatment, at least in this [GD] range. The GD-mica surface can even bind chromatin fairly efficiently at subnanomolar levels. The glutaraldehyde surface is therefore effective at very low levels of modification. The dashed line in the plot indicates the upper

TABLE 1 Comparison of chromatin imaged in air on APTES treated mica with chromatin imaged in water on APTES-GD mica

Preparation	Nucleosomes (per molecule)	DNA width (nm)	Nucleosome width (nm)	DNA height (nm)	Nucleosome height (nm)
APTES mica, imaged in air	9.25 ± 0.4	7.8 ± 0.2	17.6 ± 2.2	0.52 ± 0.1	2.88 ± 0.3
APTES-GD mica imaged in water	8 ± 0.5	13 ± 2	16.4 ± 0.2	0.87 ± 0.1	3.22 ± 0.3

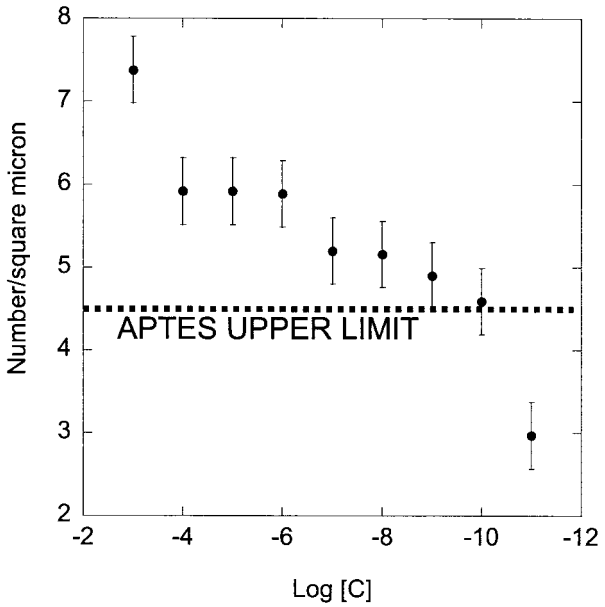

FIGURE 3 Plot of the coverage (molecules per square micron) versus gluaraldehyde concentration used for surface modification. All data were acquired with an equivalent DNA concentration of 300 μg/L. The dashed-line indicates the upper limit of coverage for this concentration on the AP-mica. The glutaraldehyde treatment provides reliable tethering down to the nanomolar level. Error bars are estimated uncertainty based on variance in counts of different images.

range of molecule densities obtained with the same concentration of sample on AP-mica alone (the full range extends to zero as no molecules are found tethered in many experiments on AP-mica at this low sample concentration). Thus, by approximately nanomolar glutaraldehyde, the upper limit of the bare APTES tethering is reached. A 200-μL drop of a nanomolar solution contains $\sim 10^{11}$ molecules and covers $\sim 10^{14}$ mica unit cells, so only $\sim$1 in 1000 sites can be modified. At the lowest levels of modification, the images appear to be separated by bare regions, so the modification is probably not homogeneous.

It is striking that the glutaraldehyde modification produces reliable tethering at levels below the theoretical limit in which all possible surface sites are modified. This implies that the prior APTES modification of the surface was, in fact, complete, even though AP-mica often does not yield interpretable images. Thus, we conclude that the failures of the APTES surface (to tether molecules) do not arise from a failure of the surface modification itself but rather from a failure of the modified surface to bind reliably the sample molecules. Because the activity of the APTES surface derives from an electrostatic interaction with the amines (charged at neutral pH), the variability must be associated with the surface charge. Trace amounts of contamination by very small ionic molecules could account for this effect. In contrast, the glutaraldehyde interaction with chromatin is highly reproducible and reliable.

Novel applications of APTES-GD surface

One of the most useful features of this new surface is that it allows easy imaging of chromatin samples in solution. To test these features and to gain insight on how the surface binds chromatin molecules, a salt titration was carried out on deposited chromatin arrays. For these studies, we reconstituted an $\sim$2-kb DNA template derived from the MMTV promoter region to subsaturated levels with HeLa histones, as described in Materials and Methods. The salt titration was carried out in a flow cell to allow the continuous monitoring of individual molecules. The results of one such titration are shown in Fig. 4. One of the several molecules that can be visualized over the entire course of the titration is marked by arrows.

It is known from solution studies (for review, see Van-Holde, 1988) that as ionic strength is raised from low levels (1 mM monovalent salt) to $\sim$0.2 M salt, chromatin first undergoes folding or compaction. In the range from 0.2 to 0.6 M, individual nucleosomes undergo a conformational change but remain intact except for a small percentage of DNA dissociation. Above 0.6 M NaCl, histones begin to dissociate from the nucleosome, first H2A-H2B and then, above 1 M NaCl, H3-H4.

Some of these transitions are observed in this in situ titration experiment. At low ionic strength, the MMTV nucleosomal array is clearly visualized. The resolution is comparable with that of 5S rDNA nucleosomal arrays, for example the 172-12 (Fig. 2). As the [NaCl] is raised from low levels to 0.4 M, there are subtle changes in the appearance of the arrays, such as reorientation of the DNA between nucleosomes, as well as shape and size changes in the nucleosomes themselves (see below). Also, particularly above 0.6 M, there is a steady increase in the level of background small material, perhaps reflecting the loss of histones H2A and H2B. Above 1 M [NaCl], there is virtually complete loss of DNA from the polynucleosomal array. This behavior is illustrated quantitatively in Fig. 5. From 0.6 to 1 M NaCl, there is a 5% to 20% loss of the DNA. This is followed by virtually complete loss at [salt] $>$ 1 M. The low levels of DNA loss at [salt] $<$ 1 M are in quantitative agreement with values from solution studies of mononucleosomes (van Holde, 1988). The loss process is also time dependent; longer time periods even at [salt] $<$ 1 M result in DNA loss (H. Wang, R. Bash, S. Lindsay, D. Lohr, unpublished observations). Time dependence has also been observed in the solution studies. Complete DNA loss seems to occur at lower salt concentrations on the GD-mica surface than in solution studies. This could be due to several factors: physical effects of the surface on nucleosome stability; higher local [salt] at the surface than in the bulk phase, and low nucleosome concentrations. In solution, DNA loss is also concentration dependent (van Holde, 1988). The ability of DNA to be lost from a fixed and surface-tethered chromatin template is consistent with the

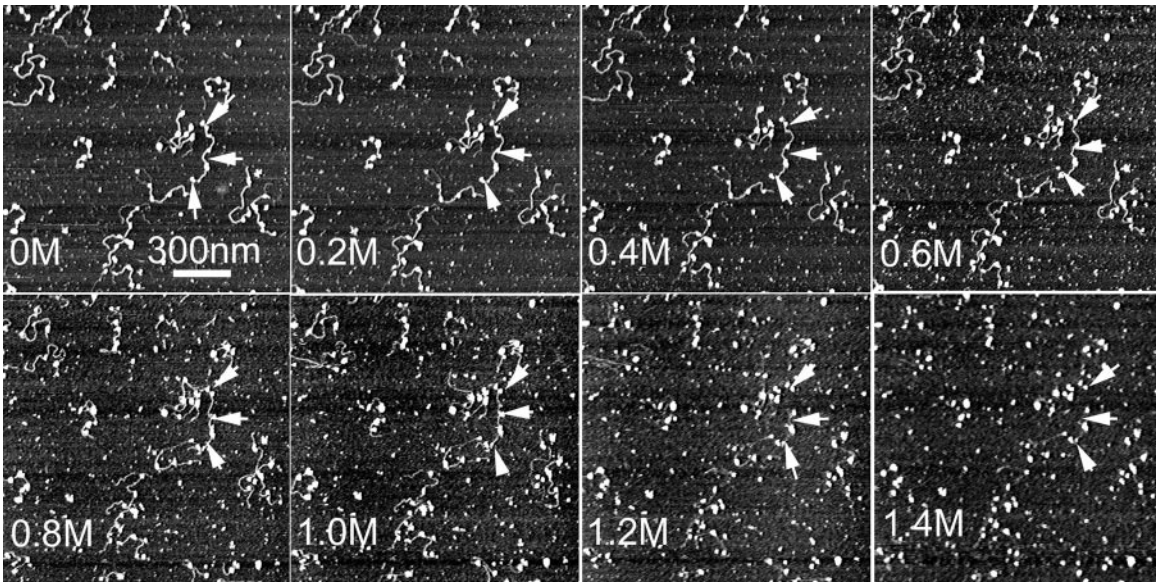

FIGURE 4 Series of images taken over the same region of the deposited sample as the salt concentration is increased in steps from 0 to 1.4 M (as labeled in the bottom left of each corner). The *x-y* scale for all images is marked by the bar. The vertical scale (*black* to *white*) is 3 nm. The sample is a reconstituted MMTV polynucleosomal promoter array (the same molecule is identified by *arrows* in each image). The DNA is completely lost from the array by 1.2 M NaCl. Note how the histone protein remains fixed in place on the substrate, whereas the DNA is free to move.

known cross-linking mechanism of glutaraldehyde; below 337 K, glutaraldehyde does not react with naked DNA, so glutaraldehyde-fixation (and, by implication, tethering) involves attachments to histones, thus, leaving the DNA free to move (Sewell et al., 1984).

Evidence for the more limited changes that occur below 1 M NaCl are shown in Fig. 6. These data quantify the measured height and width of the nucleosomes as a function of salt concentration. Note that the precise values of both of these quantities may be distorted in AFM measurements. Heights are generally smaller than crystallographic dimensions owing to compression of the molecules, surrounding adsorbates, and electrostatic effects (Muller and Engel, 1997), and widths are increased by the finite size of the scanning probe (Villarubia, 1994), i.e., tip broadening. However, the widths at the various [salt] reflect the same intrinsic tip broadening and thus, the trends in such plots are

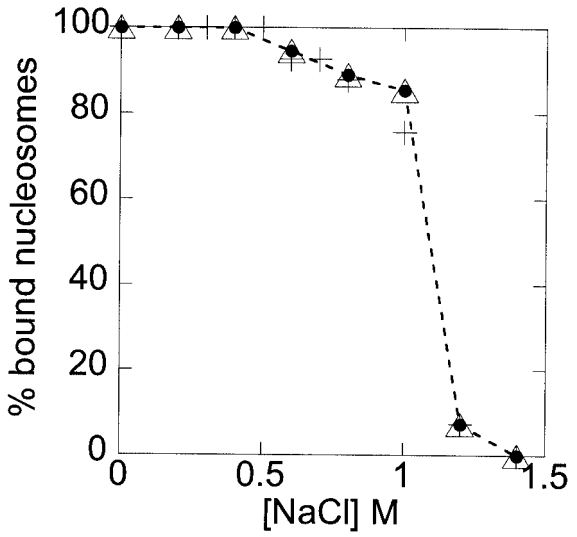

FIGURE 5 Plot of the fraction of DNA-bound nucleosomes as a function of salt concentration for reconstituted MMTV nucleosomal arrays from in situ salt titrations like the one shown in Fig. 4. Data for three runs are shown (*dots*, *crosses*, and *triangles*), showing that the transition point is remarkably reproducible.

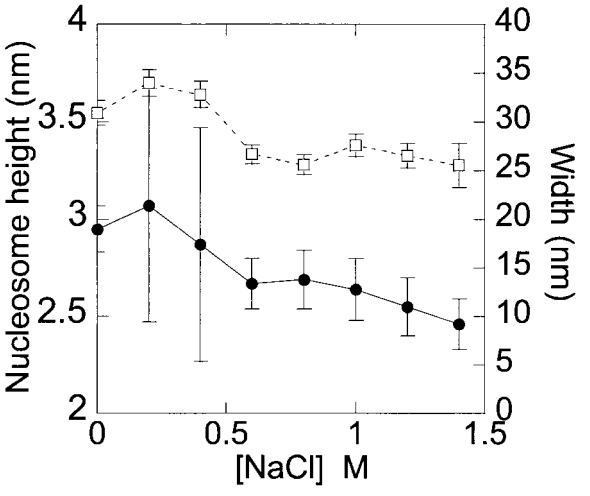

FIGURE 6 Plot of measured nucleosome height (*left scale*, ●) and measured nucleosome widths (*right scale*, □) as a function of NaCl concentration. Error bars are standard error derived from the measurements.

valid. For both the height and width measurements, the raw data show a clear trend toward smaller nucleosome dimensions as [NaCl] is increased from 0.2 to 0.6 M. That result is consistent with the loss of H2A-H2B noted in bulk solution studies. The changes again occur at lower [salt] than the bulk studies, probably for the same reasons noted above. This observation is especially remarkable because of the covalent tethering of the histones both to the GD-mica substrate, and to each other by the prior glutaraldehyde fixation. Examination of the data shows that some individual nucleosomes do not change size as salt is increased, but they are a small fraction of the total (2 of 25 followed in this way). This implies a surprising degree of lability in these fixed and covalently tethered samples. Interestingly, solution studies also detect a conformational change that has been interpreted as a "swelling" of the nucleosome in the low salt ranges (~0.3 M), perhaps corresponding to the slight width and height increases we observe in the 0 to 0.2 M NaCl range (Fig. 6).

What we do not see is also noteworthy. In the 0- to 0.2-M range, we do not see an increase in compaction, i.e., chromatin folding is not observed. This absence may be due to multiple attachment sites of the chromatin substrate to the GD groups on the surface, thus, preventing compaction. We have tried this experiment at lower GD modification levels but have been unsuccessful at inducing folding. However, salt treatment before deposition does result in compacted molecules (H. Wang, R. Bash, S. Lindsay, D. Lohr, unpublished results). The N-terminal tails of the histones extend out from the globular core of the nucleosome (Luger and Richmond, 1998) and are likely to be the targets of glutaraldehyde attachment, especially because they each contain several lysine residues, the presumed site of glutaraldehyde reaction. Because the tails also mediate chromatin folding (Fletcher and Hansen, 1996), the absence of folding could also reflect a loss of tail mobility.

CONCLUSIONS

A two-step modification of mica, APTES exposure, followed by glutaraldehyde treatment produces a surface that tethers chromatin strongly and reliably down to nanomolar levels of glutaraldehyde treatment, providing a vast improvement over the widely used APTES surface. The tethering on GD-mica occurs through linkages to the histones, leaving the DNA labile, as demonstrated by the salt-induced loss of the DNA in situ. Despite fixation and direct tethering of the histones, they also maintain some lability, as demonstrated by a decrease in nucleosome size with increasing [salt], in the concentration ranges lower than those producing DNA loss. On the other hand, the higher-order folding associated with polynucleosomal templates at physiological salt concentration is not observed, so this surface is not suitable for studying long-range rearrangements of chromatin in situ. The high reliability of the glutaraldehyde modification of the highly unreliable APTES-treated mica indicates that the APTES modification step itself is reproducible. The lack of reliability of the APTES surface alone must stem from variability associated with electrostatic tethering. The new surface is valuable for studies of small amounts of sample in solution, providing remarkably reliable imaging.

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