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Characterization of DNA–Hv1 histone interactions; discrimination of DNA size and shape

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ABSTRACT

We have studied the formation of histone Hv1–DNA complexes using an acoustic biosensor and AFM imaging. Our results show that DNA and histone molecules aggregate into amorphous accumulations which form a compact rigid layer on the sensor's surface. By measuring changes in the acoustic wave amplitude, it was possible to titrate surface bound DNA with Hv1 and discriminate between DNA molecules of different size and shape. From the kinetic analysis of real time data, K_{eq} was found equal to $3 \times 10^5\ M^{-1}$.

1. Introduction

Elucidation of the parameters governing the interactions between DNA and proteins is fundamental to our understanding and manipulation of genome packaging and gene regulation. Both processes require energetically unfavorable DNA deformation, since charge repulsions between phosphates along the double helix make DNA an exceptionally stiff polymer. A specific class of proteins, referred to as class 2 DNA bending proteins (e.g. histone octamer) [1], can induce DNA softening through electrostatic interactions between cationic amino acids and the DNA backbone [2] resulting into compact folded structures [3].

Protein mediated DNA bending has been studied with various techniques [4]. Atomic force microscopy (AFM) was used for observing conformations of DNA single molecules or DNA–protein complexes, providing important information about protein locations on DNA and DNA bend angles [5–7]. An optical biosensor was applied to monitor DNA/histone H1 interactions providing information about the thickness of the formed nucleoprotein complexes [8] while acoustic sensors were employed for the study of protein binding to DNA [9], and in order to derive qualitative structural information related to DNA bending driven by a transcription factor [10]. It was recently shown that label-free acoustic biosensors based on shear acoustic waves (QCM or Love wave devices) in combination with a novel mathematical treatment can be employed for determining the shape and size of surface bound DNA molecules [11–13]. This is possible by measuring the velocity (phase) and energy dissipation (amplitude) of the wave which largely depend on the mass and viscosity, respectively, of the sensing layer. In the case of surface bound molecules, interfacial viscosity changes that affect energy dissipation measurements depict changes in the conformation of the attached biomolecules. In addition, it was shown that any bound material can be characterized through the ratio of ΔAmplitude/ΔPhase (ΔA/ΔPh = energy dissipated per bound mass unit, at equilibrium) which provides an acoustic fingerprint for each molecule/layer, indicative of its structure.

The purpose of this study is to further exploit the potential of acoustic biosensors to yield information (a) on the structure of the formed complex/layer, (b) on the structural differences of the partaking DNA (i.e. chain size and shape) and (c) on the kinetics of the protein–DNA complex formation and all that from one experiment. DNA molecules varying in length and intrinsic curvature were employed in order to study their interaction with the positively charged Hv1 histone protein. Hv1 is a histone H2A variant from the ciliated protozoan *Tetrahymena thermophila*, lysine–arginine rich with a molecular weight of approximately 15 kDa [14]. Hv1 was selected as a model class 2 DNA bending protein that

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is known to interact with any DNA sequence, regardless of inherent sequence-dependent structure [15,16] via electrostatic forces [17].

2. Materials and methods

2.1. DNA design and preparation

Different sets of biotinylated and non-biotinylated primers were designed by FastPCR software and obtained from Metabion (Germany) in order to produce double-stranded DNA products of various lengths (75, 132, 167, 198 bp) by PCR amplification. The specific DNA sizes were previously studied and characterized with acoustic biosensors [12,13]. Plasmid pBR322 obtained from Minotech (Heraklio) was used as template in standard PCR reactions. PCR products were purified with a nucleospin kit following the manufacturer protocol.

Two 90-mer oligonucleotides were designed based on sequences that are known to create different degrees of intrinsic curvature. Each sequence was flanked by 20 bases in order to be amplified by a PCR reaction.

Histone Hv1 protein (H6881) and poly-L-lysine (P8920) 0.1% w/v water solution were purchased from Sigma–Aldrich (USA).

2.2. Preparation of AFM samples and AFM imaging

Aliquots of 5 μl containing 0.5 ng/μl or 5 ng/μl of the 198 bp DNA diluted in Tris/HCl pH 8.5 and 2.5 mM $MgCl_2$ were applied to freshly cleaved mica (RS company) for 2 min followed by rinsing with distilled water. Samples were dried with nitrogen and visualized immediately under the microscope. An aliquot of 5 μl containing 20 ng/μl of the Hv1 histone protein was applied directly to freshly cleaved mica or to the previously visualized DNA treated mica, rinsed, dried and immediately visualized. AFM images in air were collected using the NanoScope IIIa system. The instrument operated in tapping mode and in ambient temperature using Veeco tips (RTESP model, spring constant 20–80 N/m; resonance frequency 267–298 kHz; tip radius 10 nm) and a scan rate of 1–2 Hz. The scan size was 1–8 μm and images were obtained and analyzed using the Nanoscope and ImageJ software.

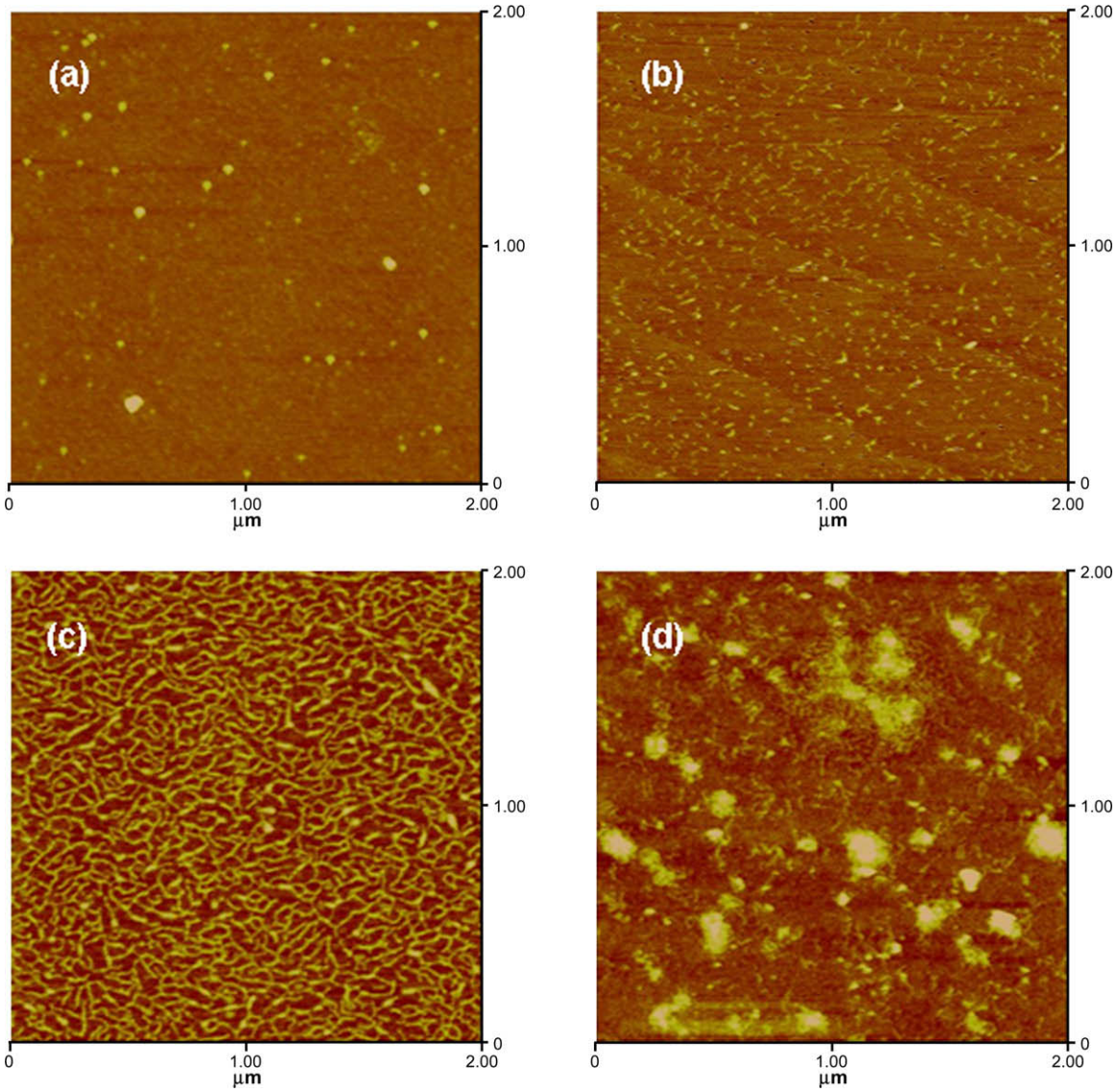

Fig. 1. AFM images of DNA and Hv1 on mica. Surface roughness (RMS) of mica is <0.1 nm. (a) Hv1 protein, RMS 0.188 nm, average cluster height 1–2 nm, width 40 nm, (b) low concentration of 198 bp DNA, RMS 0.131 nm, average height 0.5 nm and width 8 nm, (c) high concentration of 198 bp DNA, RMS 0.477 nm, average height 0.6 nm and width 16 nm, (d) 198 bp DNA/Hv1 complex, RMS 0.610 nm, average height of clusters 2–4 nm and width up to 220 nm.

2.3. Acoustics instrumentation and experimental set-up

Surface acoustic wave devices (SAW) operating at 155 MHz were prepared by photolithography. These devices were used to support a Love wave in a configuration employing a 0.4 μm thick PMMA waveguide layer. A 20 nm gold layer was deposited on the region between the IDTs by sputter-coating with a Bal-Tec SCD 050 sputter-coater. The gold layer was etched immediately prior to the acoustic experiments to ensure a clean surface. An Agilent E5061A network analyzer was used to measure the amplitude and phase of the output signal with respect to a reference signal at 25 °C. Tris buffer (50 mM Tris pH 7.5, 2.5 mM $MgCl_2$ and 10 mM KCl) was pumped over the surface of the gold-coated devices at a flow rate of 0.02 ml/min using a peristaltic pump. The signal was allowed to equilibrate prior to the first addition and all samples were added in the same buffer. Neutravidin (Pierce) was added at a concentration of a 100 μg/ml for 10 min, long enough to saturate the surface with a large excess of protein. After a buffer rinse, DNA and the histone were added sequentially as specified in the results section.

3. Results

3.1. AFM images of DNA–Hv1 complexes

Adsorption of Hv1 (20 ng/μl) on mica (Fig. 1a) resulted in the observation of protein clusters rather than single molecules [18]. Magnesium mediated binding of a low concentration 198 bp dsDNA sample to mica (Fig. 1b) resulted in discrete straight or curved rods with measured lengths that agree with published data [7]. A 10-fold increase of DNA concentration (Fig. 1c) caused the formation of a DNA network with molecules that appear to interact with each other and aggregate in an end-to-end manner, again in agreement with reports on short DNA molecules [7]. The formation of DNA networks is facilitated by molecules with ss-ends overhangs [19]. Such molecules are known to be produced by the action of Taq polymerase, used in our experiments, which leaves dA overhangs at the 3′-end of synthesized PCR products.

The formation of DNA–Hv1 complexes (Fig. 1d) was observed after the addition of 5 μl of the Hv1 (20 ng/μl) on a mica surface that was previously treated with high concentration of the 198 bp DNA preparation. In contrast to the DNA network observed earlier, histone binding resulted in the formation of DNA–protein aggregates and entangled mats of nucleoprotein [5]. DNA molecules seemed to gather around histone cores with a diameter of 100–200 nm, aggregating into large, densely packed configurations. Free DNA molecules could also be observed.

3.2. Acoustic measurements

Experiments were performed in a flow-through system which allowed continuous additions of DNA or protein samples and buffer in an alternating way. Real-time binding curves were obtained during sample addition monitoring amplitude (ΔA) and phase change (ΔPh) (Fig. 2). Addition of DNA mass (biotinylated 198 bp DNA, 5 μg/ml) on the surface resulted in phase and amplitude decrease, as expected.

Hv1 protein was added to the DNA-modified surface either as a one concentration (250 μg/m) aliquot (on 75, 132 and 167 bp) or as a series of concentrations for complete titration (on 90 "straight", 90 "bent" and 198 bp). Fig. 2 shows typical results for the addition of Hv1 on to DNA covered sensor surface; while phase decreases (as mass is loaded) the amplitude, interestingly, increases. This unexpected amplitude response cannot be considered as a charge effect, since it is observed only in the particular case of Hv1–DNA electrostatic interaction and not when the order of the addition of the interacting partners is reversed. Further investigation attributed this particular amplitude response to DNA conformational changes caused upon interaction with the Hv1.

3.2.1. The effect of DNA size, shape and surface coverage

The interaction of the Hv1 protein with the DNA was investigated by monitoring together the ΔPh (Hv1) and ΔA (Hv1) signals during the sequential addition of the protein (10–400 μg/ml) on top of immobilized biotinylated DNA (b-DNA) populations varying in size, shape and percentage of surface coverage. Direct comparisons were performed between: (a) a 198 bp b-DNA population covering fully the surface and a 198 bp b-DNA population covering only 50% of the surface, (b) a "straight" 198 bp b-DNA population with a "straight" 90 bp b-DNA and (c) a "straight" 90 bp b-DNA with a "bent" 90 bp b-DNA.

The measured ΔPh (Hv1) signal as a function of the Hv1 concentration was found to be almost identical upon interaction with the various DNA populations regardless of their size, surface coverage or shape, indicating in all cases almost the same amount of bound Hv1 protein. For this reason, the isothermal curves from each different DNA experiment where combined into an average one.

The measured ΔA (Hv1) signal as a function of the Hv1 concentration is summarized in Fig. 4. In contrast to the previous observation concerning the phase change, the amplitude increase is

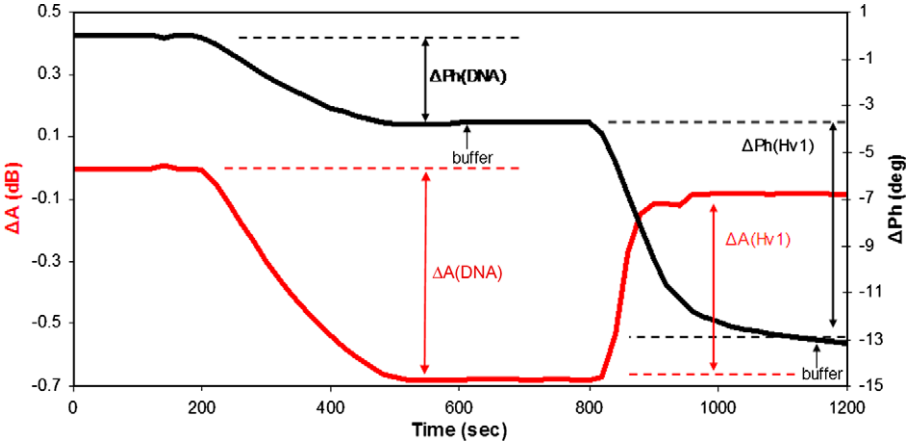

Fig. 2. Real time binding curves of the 198 bp DNA and Hv1 protein (250 μg/ml). The black line corresponds to the phase change (ΔPh) and the red line to the amplitude change (ΔA) of the DNA and Hv1.

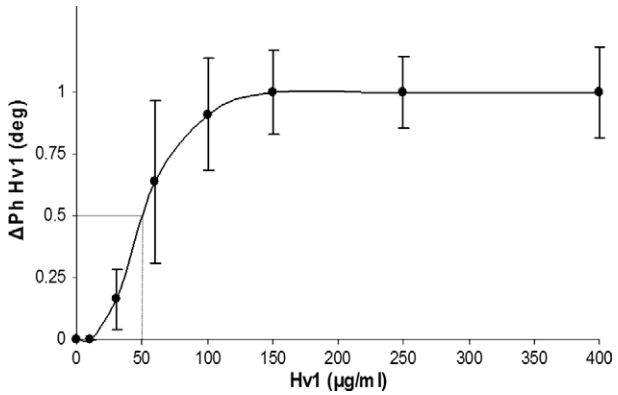

Fig. 3. Average binding isotherm of Hv1 to various DNA populations tethered on the sensor surface. Note that in all cases data are corrected for the non-specific binding of the protein to the sensor's surface.

affected by the DNA size, intrinsic curvature and percentage of surface coverage, as four different isotherms can be clearly observed. No effect on amplitude is displayed by non-specific binding ("no DNA" curve).

3.2.2. Complex formation measurements

Quantitative information on the structure of the DNA/Hv1 complex can be derived by calculating the acoustic ratio ΔA/ΔPh at equilibrium. Since the complex formation is monitored in two steps, the formation of the nucleoprotein complex is described by Eq. (1):

$$\text{Complex } (\text{dB}/\text{deg}) = \frac{\Delta A(\text{DNA}) + \Delta A(\text{Hv1})}{\Delta \text{Ph}(\text{DNA}) + \Delta \text{Ph}(\text{Hv1})} \quad (1)$$

Interestingly, applying Eq. (1) for the complex of Hv1 with the various DNAs results in the same acoustic ratio with an average value of 0.018 ± 0.009 dB/deg, regardless of the size, amount and conformation of the DNA used.

3.3. Calculation of k_a, k_d and K_{eq} constants

Kinetic and equilibrium constants can be derived from mass-related phase real-time measurements [20]. Here, k_a was calculated to be $2.9 \times 10^3\ M^{-1}s^{-1}$ while k_d was $2.88 \times 10^{-3}\ s^{-1}$ resulting in $K_{eq} = 11.3 \times 10^5\ M^1$. From the isotherm curve (Fig. 3) K_{eq} is estimated to be $3 \times 10^5\ M^{-1}$, in agreement with the kinetic analysis value.

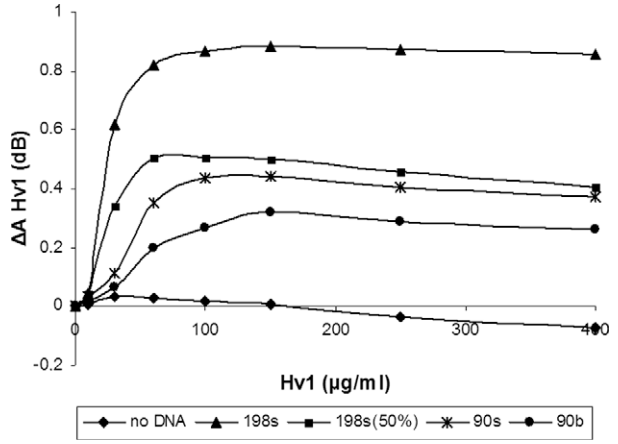

Fig. 4. Amplitude changes of sequential addition of Hv1 on various DNA populations tethered on the sensor surface. "s" stands for the straight and "b" for the bent molecules. The reproducibility, for each DNA molecule, is ~90%.

4. Discussion

In this work, acoustic experiments revealed that Hv1 interacts with DNA and aggregates into nucleoprotein complexes. Mass-related phase measurements showed that the amount of Hv1 bound to various DNA molecules was the same; conformation-based amplitude measurements clearly discriminated between the binding of Hv1 to various DNA molecules. Interestingly, the formation of nucleoprotein complex resulted in a constant acoustic ratio (0.018 ± 0.009 dB/deg, see Fig. 5) for all the DNA molecules used, comparable to that of a neutravidin layer (0.015 ± 0.008 dB/deg). Thus, the various DNA/Hv1 complexes form layers which are rather compact and rigid just like those of tightly adsorbed proteins on the sensor surface. This acoustic ratio is distinctly different to the values obtained for surface-attached DNAs, for example 0.260 ± 0.006, 0.110 ± 0.006 and 0.086 ± 0.004 (dB/deg), derived for the 198 bp, the 90 bp "straight" and the 90 bp "bent" DNA molecules, respectively [12]. This five to fifteen-fold difference is expected on the basis of the greatly different structural features between the different DNA molecules protruding from the surface and the collapsed DNA/H1v aggregates (Fig. 5). Note that detailed mathematical analysis presented elsewhere has correlated the acoustic ratio to the intrinsic viscosity of the surface bound molecules [12].

The observation that this value is identical of all DNAs used in this work, regardless of their surface coverage, size and shape of DNA is indeed not surprising since binding and subsequent aggregation is a non-specific electrostatic event. Compact rigid layers are formed on the sensor's surface, since each outward protruding DNA molecule will be forced to bend and collapse into a low energy dissipating structure (Fig. 5). AFM imaging (Fig. 1) proved the formation of such amorphous accumulations of molecules which are known to be created in the presence of multivalent ions and with short DNAs [21]. This behavior of the Hv1 is described theoretically by the asymmetric neutralization model which proposes that any protein with a high concentration of positive charges at its surface should interact with DNA, induce a bend and force it to condense. A co-operative interaction mechanism where, at the particular ionic strength, Hv1 binds to only some of the DNA molecules leaving the rest free (Fig. 6) is also plausible [22].

Furthermore, acoustic experiments followed the titration of surface bound DNA molecules with Hv1 by measuring the ΔA (Hv1) signal. The ability of amplitude to discriminate between the collapse of DNAs of various sizes and shapes resulting from the binding of the same protein mass (Fig. 5) is a clear indication

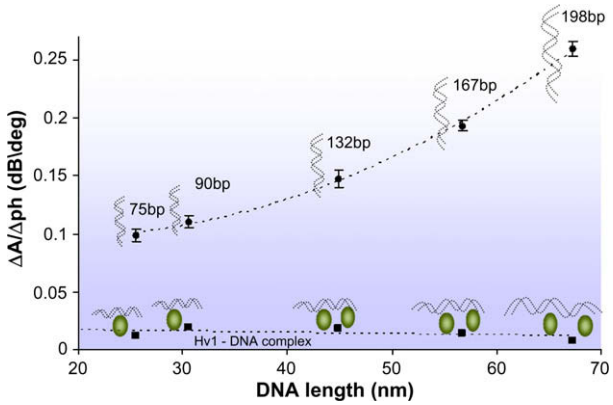

Fig. 5. Acoustic ratio results for various DNA molecules attached on the sensor surface before and after Hv1 addition. For clarity, the 90 bp "bent" molecule is not shown.

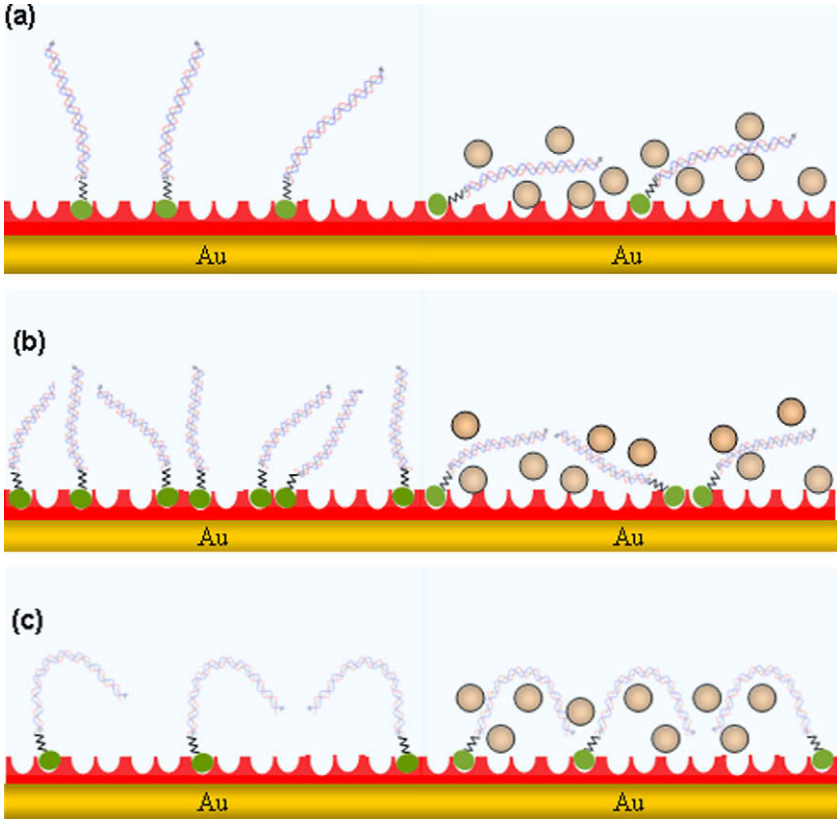

Fig. 6. Schematic representation of the Hv1-DNA complex formation as depicted in a co-operative mechanism. (a) 198 bp "straight" DNA, (b) 90 bp "straight" DNA, (c) 90 bp "bent" DNA.

of the high sensitivity of this method to the conformation of surface-bound molecules. This ability can be extremely useful in studies with sequence specific DNA targeting/bending proteins and can compete with standard gel assays in speed, simplicity and reliability. Kinetic analysis performed based on the phase change signal is also a benefit from the acoustic experiments. However, the calculated K_{eq} value cannot be directly compared with binding constants of other histone proteins [23,24] since it is strongly related to the ionic strength of the solution used in the experiment and can vary from 10^{-2} to 10^{13} M^{-1} [25].

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