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# Effect of heparin and heparan sulphate on open promoter complex formation for a simple tandem gene model using *ex situ* atomic force microscopy

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ABSTRACT

The influence of heparin and heparan sulphate (HepS) on the appearance and analysis of open promoter complex ($RP_o$) formation by *E. coli* RNA polymerase (RNAP) holoenzyme ($\sigma^{70}$RNAP) on linear DNA using *ex situ* imaging by atomic force microscopy (AFM) has been investigated. Introducing heparin or HepS into the reaction mix significantly reduces non-specific interactions of the $\sigma^{70}$RNAP and RNAP after $RP_o$ formation allowing for better interpretation of complexes shown within AFM images, particularly on DNA templates containing more than one promoter. Previous expectation was that negatively charged polysaccharides, often used as competitive inhibitors of σRNAP binding and $RP_o$ formation, would also inhibit binding of the DNA template to the mica support surface and thereby lower the imaging yield of active RNAP-DNA complexes. We found that the reverse of this was true, and that the yield of $RP_o$ formation detected by AFM, for a simple tandem gene model containing two $\lambda_{PR}$ promoters, increased. Moreover and unexpectedly, HepS was more efficient than heparin, with both of them having a dispersive effect on the sample, minimising unwanted RNAP-RNAP interactions as well as non-specific interactions between the RNAP and DNA template. The success of this method relied on the observation that *E. coli* RNAP has the highest affinity for the mica surface of all the molecular components. For our system, the affinity of the three constituent biopolymers to muscovite mica was RNAP > Heparin or HepS > DNA. While we observed that heparin and HepS can inhibit DNA binding to the mica, the presence of *E. coli* RNAP overcomes this effect allowing a greater yield of $RP_o$s for AFM analysis. This method can be extended to other DNA binding proteins and enzymes, which have an affinity to mica higher than DNA, to improve sample preparation for AFM studies.


## 1. Introduction

A common application of biological atomic force microscopy (AFM) is molecular scale imaging of protein–nucleic acid interactions and has included studies of DNA transcription for the last twenty years or so [1–4]. Visualisation of molecular complexes through the force sensing AFM probe can be realised in hydrating air or aqueous liquids environments, depending upon the specific application, and whether or not the study is focussed mainly on structure or dynamics. Imaging of dried complexes in ambient air conditions can be termed *ex situ*, where the focus is investigating structural relationships between the protein and the DNA. Imaging of complexes *in situ* under aqueous buffers attempts to study dynamics in real-time or at least a time-lapse approach. Both of these two approaches requires the protein-DNA complexes to be adsorbed to a support surface, which is almost always mica, or mica which has been modified with a self-assembled monolayer or thin molecular film to promote binding of the DNA template [4,5]. Mica is an ideal support surface because it is atomically flat and its surface charge properties can be modulated using divalent cations to encourage DNA binding [6–9]. It should be noted that many studies indicate that the hydrophilic nature of the mica in typical ambient lab humidity keeps DNA hydrated [10–12].

*Ex situ* AFM imaging in air on mica has confirmed that *E. coli* RNA polymerase wraps the DNA template around itself during formation of the open promoter complex ($RP_o$) [13]. This effect has been quantified in detail by Rivetti et al. and can be used to establish the formation of $RP_o$s in AFM imaging by measuring contour length reductions of linear DNA templates [14]. *In situ* imaging

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has attempted to follow the process of transcription directly, but for the ground-breaking studies the scan speed of conventional AFM was too slow to capture more than a few frames [15,16]. This approach, however, imaged facilitated promoter location of *E. coli* RNAP on a linear template, demonstrating one-dimensional diffusion, hopping and inter-segmental transfer [17]. These were also the first imaging of the translocation of RNAP relative to DNA in the presence of NTPs, imaging transcription directly [15,16]. In one of the studies, the RNAP was bound stably to the mica surface and the DNA was free to move [15], but in different buffer conditions the RNAP was seen to move relative to the DNA, which was mobile but equilibrated onto the 2D surface of the mica [16]. Fine adjustment of buffer conditions is required for *in situ* experiments to allow the DNA sufficient movement for transcription to occur but restricting the DNA motion enough for the DNA backbone to be detected by the AFM tip [6–9].

More recently, the advent of higher scan speed AFMs has led to new attempts to follow transcription directly *in situ*. The major challenges of imaging DNA-dependent enzymes *in situ* by AFM arise from both the speed of the RNAP enzymes and the relative rotation of the DNA helix by a processive enzyme (such as RNAP). Higher frame rates partially address the first challenge and to address the second, these studies have tethered the DNA template strand by each end to a DNA origami nanotile as a way to overcome the conflicting requirements of DNA movement and localisation to the mica surface [18]. With this approach and frame rates of 1 image per second, promoter location and translocation of a single RNAP along the DNA template has been observed. Using real-time AFM imaging to analyse finer details of the transcription process may require further significant developments in the technology and methodology.

The *ex situ* approach can obviate, to a certain degree, these challenges: reactions are carried out *in vitro* and then the reaction can be quenched or run to completion and the outcomes imaged in a static manner [2]. DNA transcription is well suited to this approach, since $RP_o$s can be pre-formed and are very stable before deposition onto a support surface. Once the $RP_o$s are imaged, the NTPs can be added to the same *in vitro* reaction mix and incubated for a given period of time or quenched before the reaction mixture can be deposited and imaged again on mica [19].

The bacterial RNA polymerase from *E. coli* is the most widely studied by AFM, due to its relative simplicity and large size. This negates the need for additional factors, which might confuse interpretation of AFM images and gives confidence that the proportion of active complexes is high when NTPs are added. It is also noted that *E. coli RNAP* provides a model system for eukaryotic RNAPs due to the similarities in structure [20–23]. Our previous work on viral T7 RNAP showed that the *ex situ* AFM approach did not work particularly well because T7 RNAP spent the majority of the time off the DNA templates [24]. T7 is a fast and highly processive RNAP that re-initiates efficiently and is therefore able to perform multiple rounds of transcription *in vitro*. In our work, the probability of observing T7 RNAP on a DNA template by *ex situ* AFM was negligible. In the case of *E. coli* RNAP, it was generally expected that one enzyme will only perform one round of transcription, since the sigma factor sub-unit is not covalently attached to the holoenzyme and is expected to leave the complex shortly after or during initiation stages of elongation (also referred to as promoter escape). To date, however, the issue of sigma factor release has not been resolved with any degree of certainty [25–27]. This raises the uncertainty that *E. coli* RNAP may be able to perform multiple rounds of transcription complicating *ex situ* AFM analysis. Additionally, excess or free RNAP that has not formed an $RP_o$ can non-specifically bind to the DNA template, other RNAP molecules and/or an RNA transcript leading to ambiguity in AFM images.

AFM studies of *E. coli* RNAP $RP_o$ formation by Rivetti et al. and Crampton et al. have shown that there is shortening of the DNA contour length as well as a bend in the DNA giving a measurable angle of ~120°, which define this wrapping [13,14,19]. Even though these $RP_o$s have a noticeably different appearance to non-specific interactions in *ex situ* AFM imaging, issues arise with the presence of other RNAP molecules attached to the DNA still undergoing their search for a promoter. Non-specifically bound RNAP can be confused for $RP_o$s or active elongation complexes (ECs) [19]. There is therefore a need to inhibit this non-specific RNAP binding to optimise AFM analysis.

One of the simplest methods to reduce non-specific RNAP-DNA interactions is to increase salt concentration or ionic strength of the buffers used which leads to a decrease in the net electrostatic potential of the DNA. It is noted, however, that the rate of promoter binding decreases with increased salt concentration [28]. This change would mean that for *in vitro* transcription reactions to be analysed by AFM there would be a low number of RPOs for analysis. The effect of monovalent salts at high concentration can also alter the binding of the DNA and DNA-protein complexes to the mica surface and so may not be feasible for use in AFM [29–32]. This approach would also not solve the issue of multiple rounds of transcription occurring.

Biochemical methods overcome the issue of non-specifically bound RNAPs by the addition of the molecule heparin, which competes with DNA to bind RNAP in the DNA binding channel [33]. Heparin is a polyanionic polysaccharide of the glycosaminoglycan family (GAGs) which includes the closely related macromolecule heparan sulphate (HepS). Both heparin and HepS are linear polysaccharides made up of chemically similar monosaccharides but with varying degrees of sulphonation. Heparin is more highly sulphated than HepS, but HepS contains more N-acetylated monosaccharides. The exact chemical structure of any given heparin or HepS molecule can vary due to their non-templated production [34,35]. Both heparin and HepS are produced in the same manner and are made up of two repeating disaccharide units, the most common of which for each is shown in Fig. 1.

Heparin is produced by mast cells and has a molecular weight range of 60–100 kDa but when purified for biochemical uses has a size distribution of 12–15 kDa as purified from porcine intestine. HepS is produced by all cell types in the form of a proteoglycan, attached to a protein core. Free chains of HepS are rarely found *in vivo* but can be purified from bovine kidney cells free of the attached protein. The molecular weight of HepS has a similar range to that of heparin, but the average molecular weight of purified chains is slightly higher at approximately 20 kDa and it is less well characterised than heparin. The major biochemical difference between HepS and heparin is the number of GlcN-sulphate groups that occur. Both molecules have homologous structures and often are considered to display the same properties, and used as models of each other when necessary. Heparin and HepS chains adopt one of two right handed helical structures with a helical repeat of approximately 1.67 nm over a tetra saccharide sequence [36].

The use of heparin for *in vitro* transcription assays is a consequence of its comparable helical structure to DNA as well its polyanionic nature. Both DNA and heparin/HepS have a negatively charged backbone and adopt a helix with a residue rise of 0.4 nm for Heparin/HepS in comparison to 0.34 nm for DNA. Both are able to mimic DNA and bind to RNAP via its DNA binding domain located in the active site [33]. The similar structure of HepS means that it is also able to bind to DNA binding proteins in the same manner but is not commonly used due to its less well characterised chemical composition. The binding of heparin/HepS occludes the DNA from the active site thereby preventing the formation of $RP_o$s. If an RNAP has already formed an $RP_o$, then heparin/HepS are unable to bind as they cannot gain access to the binding site