SSB Binding to Single-Stranded DNA Probed Using Solid-State Nanopore Sensors

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ABSTRACT

Single-stranded DNA (ssDNA) binding protein plays an important role in the DNA replication process in a wide range of organisms. It binds to ssDNA to prevent premature re-annealing and to protect it from degradation. Current understanding of SSB/ssDNA interaction points to a complex mechanism, including SSB motion along the DNA strand. We report on the first characterization of this interaction at the single-molecule level using solid-state nanopore sensors, namely without any labelling or surface immobilisation. Our results show that the presence of SSB on the ssDNA can control the speed of nanopore translocation, presumably due to strong interactions between SSB and the nanopore surface. This enables nanopore-based detection of ssDNA fragments as short as 37 nt, which is normally very difficult with solid-state nanopore sensors, due to constraints in noise and bandwidth. Notably, this fragment is considerably shorter than the 65 nt binding motif, typically required for SSB binding at high salt concentrations. The non-specificity of SSB binding to ssDNA further suggests that this approach could be used for fragment sizing of short ssDNA.

INTRODUCTION

Single-stranded DNA binding (SSB) proteins are an important class of proteins that protect single-stranded DNA (ssDNA) from chemical or enzymatic degradation. SSB also prevents ssDNA from premature re-annealing, for example after helicase-induced splitting of double-stranded DNA during the replication process.¹⁻³ While many different types of SSB proteins exist in nature, *E.coli* SSB is perhaps the best characterised and is often used as a model system. It binds to ssDNA in tetrameric form in a rather sequence-unspecific manner with high affinity. It exhibits three different binding modes, labelled according to the number of nucleotides (nt) bound to each tetramer: SSB₃₅, SSB₅₆ and SSB₆₅. SSB₃₅ is dominant at low salt concentrations (monovalent salts $c < 0.1$ M) and is known to form closely packed coatings on the ssDNA strand; i.e. the inter-tetramer cooperativity of binding is high. On average, only two protein subunits interact with the strand. On the other hand, in the SSB₆₅ mode, ssDNA interacts with all four monomers. This mode is prevalent at high salt concentrations, such as used in this study, and is thought to form octamers (dimers of tetramers), rather than compact layers.² To some extent, this was confirmed in AFM studies (in air) by Hamon et al., even though structural alterations of the SSB/ssDNA during sample preparation (addition of high-valent ions, drying step) cannot be ruled out. The affinity of SSB₆₅ to ssDNA is high ($K = 1 \cdot 10^4 - 1 \cdot 10^5 \text{ M}^{-1}$),^{4,5} but at the same time the protein must remain mobile while on the strand, in order to allow other DNA processing enzymes to function. Significant progress has been made recently to elucidate the mechanism and dynamics of this motion, mainly based on single-molecule FRET studies using labelled SSB/ssDNA complexes.⁶ To this end, Lohman and Ha et al. proposed a diffusion mechanism and estimate the diffusion coefficient of the SSB₆₅ tetramer on an extended 65-T homopolynucleotide to be $270 \text{ nt}^2/\text{s}$ at 310 K with an apparent activation energy of 81 ± 7 kJ/mol. Subsequently, the same group investigated the actual mechanism of SSB motion in

significant detail and found a "reptation model" to be best in-line with experimental data. According to their model, thermal fluctuations spontaneously create a bulge of ssDNA around the SSB tetramer, which then propagates due to the (relative) rotational motion of the SSB. Notably, they also find that the SSB/ssDNA complex sustains forces of up to 6 - 13 pN, before it dissociates.

In this study, a label free strategy is used to study the SSB/ssDNA whilst at the same time enabling single molecule sensitivity. This is performed using solid-state nanopore sensors which encompass a thin, mechanically stable membrane made from materials such as silicon nitride (SiN_x) and features a nanometre-sized pore (typical diameter $\sim 5\text{-}25\text{ nm}$).^{7,8} Built into a fluidic cell, the membrane separates the cell into two compartments, with one electrode in each. Fluid and ion transport can only occur through the nanopore. Upon application of a bias voltage, an ion current I_0 is established that depends on the dimensions and surface properties of the nanopore, the conductivity of the electrolyte and the magnitude of the bias voltage (the "open pore" current). Since the nanopore normally represents the largest resistance in the current path, most of the voltage drops across membrane, setting up a large electric field at the nanopore ($10^6 - 10^7\text{ V/m}$). A more complex picture arises, if more than two electrodes are present in the system.⁹⁻¹² The large electric field not only drives ions, but also other charged species, such as DNA and proteins, through the nanopore ("translocation"). During the translocation process, the ion current is modulated in a characteristic way, which can typically be related to the length, diameter, conformation, excluded volume inside the pore and charge of the translocating molecule, as well as its overall structure and composition.¹³⁻²¹ A range of applications are discussed in references 22 and 23.

While double-stranded DNA is routinely used as a model system in (solid-state) nanopore sensing, single-stranded DNA poses significant challenges, due to extensive secondary

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3 structure formation (folding, base pairing) and its comparatively low stability in solution.²⁴
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5 One possibility to overcome secondary structure formation is the use of high concentrations
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7 of denaturants such as urea,^{25,26} but urea is also known to decrease the DNA translocation
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9 frequency and the reduce the conductivity of the electrolyte solution.
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13 Since SSB binding both stabilises ssDNA in solution and prevents secondary structure
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15 formation, nanopore-based analysis of the complex may solve or at least alleviate this
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17 fundamental problem. The tetrameric complex is also rather large ($\sim 5 \text{ nm} \times 5 \text{ nm} \times 8 \text{ nm}$ (cf.
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19 fig. 1a),²⁷ compared to the diameter of the DNA strand, which increases the current blockade
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21 during translocation and thus the signal-to-noise ratio. The fact that the SSB₆₅/ssDNA
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23 complex is stable at high salt concentrations, makes it particularly amenable to nanopore-
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25 based detection.^{1,28,29}
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29 Here we explore, for the first time, how solid-state nanopore sensors could be used for
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31 the label-free detection of the SSB/ssDNA complex and the characterisation of SSB binding
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33 to very long (7249 nt) and short (37 nt, 100 nt) ssDNA to elucidate further information on the
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35 distribution of SSB along the DNA and conformation of the complex.
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Figure 1: Single-stranded binding protein -ssDNA complexes translocation through a nanopore. (a) Crystal structure of *E.coli* Single-stranded Binding Protein (PDB: 1QVC) showing surface charges and estimated dimension of globular protein (tetramer, ~5 nm x 5 nm x 8 nm). Blue colour represents positive charge and red colour represents negative charge. (b) Illustration of different ssDNA structures. Uncoated ssDNA forms globular (secondary) structure (top), whereas coating with *E.coli* SSB along the ssDNA strand leads to unfolding (bottom). Both are driven through solid state nanopores at a suitable applied potential. (c) 8% native polyacrylamide gel showing shifted bands of free ssDNA and *E.coli* SSB/ssDNA complexes of 17-, 35-, 50-, 75- and 100-nt ssDNA (data for M13mp18 ssDNA shown in the Supporting Information, fig. S1). All ssDNAs were incubated with *E.coli* SSB at 25 °C for 30 min before gel analysis. (d) Example current traces before and after addition of *E.coli* SSB-M13mp18 ssDNA complexes in the *trans* compartment and applied potential at -0.3 V and the inset figure showing one of 50-nm nanopores used in this experiment. The open pore current was 66 nA, translocation events appears as current decreases (blockage).

RESULTS AND DISCUSSION

Prior to performing translocation experiments, the binding properties of *E.coli* SSB with ssDNA in translocation buffer (1 M KCl, 10 mM Tris·HCl pH 8.0) were studied. High electrolyte concentrations are favourable from a sensing point of view, since the resulting larger currents may be recorded at higher bandwidth. We initially used linearized M13mp18 phage DNA, consisting of 7249 bases in length (see Supporting Information for details).³⁰ M13mp18 DNA was mixed with *E.coli* SSB in 1 M KCl, 10 mM Tris·HCl pH 8.0 at 25 °C for 30 min followed by performing gel electrophoresis using a 8% native polyacrylamide gel. Shifted bands of the SSB/ssDNA complex were observed (fig. S1), confirming that SSB indeed binds to M13mp18 ssDNA under the conditions used. These results are comparable with other reports on the effect of salt concentration on the binding of *E.coli* SSB with ssDNA,^{1,31,32} which also suggest that the SSB₆₅ binding mode is dominant. The latter is favoured at high ionic strength and low SSB_{tet}/ssDNA molar ratio. Here, $c(\text{SSB}_{\text{tet}}) = 6.61 \cdot 10^{-8}$ M, $c(\text{ssDNA}) = 2.65 \cdot 10^{-8}$ M (or $1.92 \cdot 10^{-4}$ M nucleotides), resulting in an SSB_{tet}/nt ratio of $\approx 3.4 \cdot 10^{-4}$.

To investigate this further, we also tested SSB binding to short ssDNA with 17, 35, 50, 75, and 100-nt in length, under the same electrolyte conditions as before ($c(\text{KCl}) = 1$ M). The concentration of SSB_{tet} was $6.61 \cdot 10^{-8}$ M, as above, and the oligonucleotide concentration $1 \cdot 10^{-6}$ M in all cases (SSB_{tet}/ssDNA = 0.066). Hence, the nucleotide concentration varied from $1.7 \cdot 10^{-5}$ (for 17 nt) to $1 \cdot 10^{-4}$ (for 100 nt) and the SSB_{tet}/nt ratio from $3.9 \cdot 10^{-3}$ to $6.6 \cdot 10^{-4}$. Due to the low SSB/ssDNA ratio and the high salt concentration, we expected to see binding of SSB in the conventional SSB₆₅ mode, namely a maximum of one tetramer to 75-nt and 100-nt ssDNA, but none to the shorter fragments.

Somewhat surprisingly, SSB also interacted with the shorter fragments, except the 17-mer, as shown by the shifted bands in figure 1c. Based on our data, we cannot distinguish whether SSB binds to those short ssDNA in the conventional SSB₃₅ mode, where only two of the four SSB monomers interact with the DNA (*vide supra*), or in a different, perhaps "truncated" SSB₆₅ mode. A similar effect is, however, observed for the 100 nt ssDNA, where we observed two shifted bands. The lower band ran approximately at the same speed as the SSB bound to the shorter ssDNA fragments and disappeared for higher SSB/ssDNA ratios (not shown). Thus, the lower band most likely corresponds to the ssDNA-(SSB_{tet}) complex. Accordingly, the upper band is likely to be due to an ssDNA species with two SSB_{tet} bound, possibly in an SSB₆₅+SSB₃₅ configuration (six monomer units interacting with the DNA). A significant amount of ssDNA remains unbound, according to the gel data, in line with the low SSB_{tet}/ssDNA ratio. This suggests that the average binding density ν (in mol SSB_{tet} bound/mol nt) is low, for both the short fragments and the long ssDNA. A comparatively small fraction of the DNA will carry one, two or a small number of SSB_{tet} complexes; the majority of the DNA is still unbound.

This is confirmed by AFM imaging data (fig. 2) and by modelling SSB binding for our experimental conditions, based on the tetramer/octamer model developed by Lohman et al.³³

Figure 2: Tapping-mode AFM images in air of (A) SSB_{tet}, (B) 7249 nt (circular) ssDNA, and (C) after incubation of the ssDNA with SSB_{tet}. Image size: 1 μm $\times$ 1 μm . Note that the cyclic plasmid was used for the imaging studies, while the linearized form was employed in the nanopore translocation experiments. See text for further discussion.

SSB_{tet} appears as blob-like structures with an approximate diameter of 15 nm. This is larger than the actual physical dimension of the protein, due to tip/sample convolution. We thus analyzed the average height of the different species and obtained 6.1 ± 2.0 nm for the SSB, 7.2 ± 1.4 nm for the protein bound to ssDNA, and 0.7 ± 0.3 nm for the straight sections of ssDNA. The average protein diameter of approximately 6 nm is thus in excellent agreement with the dimensions of SSB_{tet} obtained from the crystal structure ($5 \times 5 \times 8$ nm).

The images of ssDNA consistently show coiled structures with a characteristic sizes of approximately 100 nm; this is similar to previous results in the literature.³⁴ The coiling is a consequence of the low persistence length of ssDNA and its tendency to "self-base pair" to form locally double-stranded DNA segments. After incubation of the ssDNA with SSB_{tet}, most species on the surface appear to be either unbound SSB_{tet} or protein-free ssDNA. SSB/ssDNA complexes appear occasionally, as shown in fig. 2 (C), in accordance with the equilibrium binding simulations below. Interestingly, the ssDNA species appear less compact, sometimes even circular, potentially as a consequence of SSB binding. This would suggest that SSB is not only capable of preventing base pairing, but that it can also disrupt base pairing once it is bound.

According to the tetramer/octamer model, the cooperativity of binding in the SSB₆₅ mode is restricted to the formation of octamers (dimers of tetramers); close-packed protein coatings are thought not to form (albeit they do in the SSB₃₅ binding mode). The binding cooperativity may be characterised by a cooperativity parameter ω and equilibrium constant K , which in high salt (0.2 M NaCl, 10 mM Tris·HCl, 0.1 mM Na₂EDTA; pH 8.1, T = 298 K) was found to be 420 ± 80 for poly(dA) ($K = 8.8 \cdot 10^3 \text{ M}^{-1}$) and poly(U) ($K = 1.15 \cdot 10^5 \text{ M}^{-1}$).³⁵ The sequence dependence of K is relatively weak; we take $K \approx 1 \cdot 10^4 \text{ M}^{-1}$ as an approximate value for the mixed sequences and salt conditions used here. The concentration of free (unbound) SSB_{tet} is given by

$$[\text{SSB}_{\text{tet}}^{\text{free}}] = \frac{v \cdot q^{2n-1}}{K \cdot [q^2 - 2vq + (1-\omega) \cdot v^2]^n} \quad (1)$$

where

$$q = [1 - (n-1) \cdot v] + \{[1 - (n-1) \cdot v]^2 - v \cdot (1-\omega) \cdot [2 - (2n-1) \cdot v]\}^{\frac{1}{2}}$$

n is the length of the binding site (65 nt/SSB_{tet}). v ranges from 0 to $1/n$, i.e. at a maximum of 100% protein coverage every nucleotide carries $1/65^{\text{th}}$ of SSB_{tet} on average. The concentration of bound SSB_{tet} is simply

$$[\text{SSB}_{\text{tet}}^{\text{bound}}] = [\text{nt}]_{\text{tot}} \cdot v \quad (2)$$

$[\text{nt}]_{\text{tot}}$ is the total concentration of nucleotides in the sample. The total concentration of SSB_{tet} is the sum of eqs. (1) and (2), and also known experimentally. Thus, we use the latter as an input parameter and solve numerically for v . More detailed modelling results are shown in the SI. For the average number of SSB_{tet} bound/ssDNA under the respective experimental conditions, we obtained values of 2 for the 7249 nt ssDNA ($[\text{ssDNA}] = 2.65 \cdot 10^{-8} \text{ M}$), and 0.03 and 0.06 for the 37 nt and the 100 nt ssDNA ($[\text{ssDNA}] = 1 \cdot 10^{-6} \text{ M}$), respectively. This

confirms that the density of SSB_{tet} on the DNA is low in all cases - most of the DNA in the population are in fact "protein free" under the conditions used.

We then performed nanopore translocation events with the protein, ssDNA and the respective complex. Translocation events from the *trans* to the *cis* compartment were detected for both long ssDNA (7249 nt) and SSB/ssDNA complex at negative applied potentials, figure 1d (bottom) - both species move towards the positively biased compartment. This is in accordance with the expected molecular charge of ssDNA and the complex at low SSB density, and was furthermore confirmed by zeta potential measurements (cf. Supporting Information). The electrophoretic force is thus dominant during the translocation process (as opposed to electroosmosis).³⁶

For the translocation of (uncoated) M13mp18 ssDNA through a nanopore with a diameter of 50 nm, the observed ion current modulation was 0.82 ± 0.2 % (current drop) with a mean duration τ of 0.54 ± 0.30 ms at -0.3 V bias (data not shown). We define the ion current modulation as the maximum change in current during a translocation event, relative to the open pore current (cf. section 3 of the SI for further illustration).

This is comparable to previous results reported by Kowalczyk and colleagues,³⁴ who studied the translocation behavior of M13mp18 ssDNA through 8-10 nm SiN nanopores. In their study, the translocation time at similar applied bias was approximately 1 ms, where the smaller pore diameter and the thinner membrane (20 nm, larger local electric field) have opposing effects on τ . These authors also found that the large ionic current blockades were caused by unravelling of the folded ssDNA structure in solution and the same argument is likely to hold here. Using the freely jointed chain (FJC) model (thus neglecting intramolecular hybridization), we estimate the diameter of uncoated, linear M13mp18 ssDNA in solution from the radius of gyration R_g based on eq. (3).^{34,37,38}

$$D_{\text{DNA}} = 2R_g = 2 \cdot b \cdot \sqrt{\frac{N}{6}} \quad (3)$$

where b is the Kuhn length (1.6 nm for ssDNA)³⁴ and N the number of segments/bases ($N = 7249$). This yields $D_{\text{DNA}} = 111$ nm, which is also in accordance with our AFM imaging data, fig. 2, and the literature.³⁴ Hence, even for the relatively large pore size used here, some compression/unfolding of the DNA is required during the translocation process.

SSB alone could not be detected under positive and negative applied bias voltage over 1 h under otherwise the same experimental conditions. This is probably due to the translocation events being too fast and/or too small in magnitude to be detected.^{36,39} In fact, the ratio between the volumes of SSB_{tet}, $V_{\text{SSB}} = 5 \times 5 \times 8 \text{ nm}^3 = 200 \text{ nm}^3$, and a cylindrical pore with $d = 50$ nm and $L = 100$ nm, $V_{\text{pore}} \approx 200,000 \text{ nm}^3$, is on the order of $1 \cdot 10^{-3}$, giving an indication of the current modulation expected for the translocation of a single protein. This would clearly be too small to be detected.

On the other hand, the translocation events could be detected for the complex and, under certain conditions, exhibited some interesting sub-structure that warrants further analysis. Namely, for small biases (0.1 V), the translocation events could be subdivided into two classes, fig. 3a.

Figure 3: Translocation data for long M13mp18 ssDNA (7.2 kb); 50-nm SiN nanopore in 1 M KCl, 10 mM Tris·HCl pH 8.0. The open pore current was 22 nA at -0.1 V bias. (a) Illustration of two event classes (I) and (II) (b) Scatter plot of the translocation events, namely current drop vs. event duration. Event type (I) ("spikes") in black, open squares; event type (II) in two sub-sets: spikes in red, full circles, and low-amplitude events in red, open circles. (c) Detailed analysis of events class (II), where the spike occurs at different times during the event (relative spike position 'o' = beginning of event; 'l' = end).

Event type (I) is a single, short spike (current decrease) in the current-time trace. The duration and modulation amplitude are similar to the events observed for uncoated ssDNA, which suggests that these events are indeed due to uncoated ssDNA, in accordance with equilibrium binding simulations, fig. 3b (open, black squares).

Event type (II) exhibited a spike feature combined with a relatively long, but small current blockade. The total event duration at this voltage was 1.0 ± 0.1 ms and thus substantially longer than for event type (I). However, the duration and magnitude of the spike were similar to the uncoated ssDNA (red, full circles), implying that this part of the current modulation is again due to the protein-free segments (red, open circles). Note that the red open and the full circles in fig. 3b both belong to the same event type (II); we plot spike and low-amplitude event separately for further analysis, *vide infra*.

The relatively low-amplitude, but long part of the event does not occur for uncoated ssDNA and must thus be related to the presence of the protein. Since SSB_{tet} is significantly smaller than the pore (see above), physical blockade is unlikely to cause significant delay in entering the pore channel. Note also that the pore is not small enough to force the ssDNA into a linear conformation during the translocation process. On the other hand, if protein

adsorption to the inner pore walls indeed plays a significant role during the translocation process, as reported previously for individual protein species,¹⁷ then the translocation time would be greatly affected by adsorption and desorption of ssDNA-bound SSB inside the pore. The low amplitude of the signal during this period would thus be due to the relatively small volume of SSB (but now with some ssDNA attached), relative to V_{pore} .

We did not observe events with two or more spikes, perhaps as a result of the limited time resolution in our experiments and the smaller relative magnitude of the current modulation from fractional parts of the uncoated ssDNA. It is also possible that in a "dumbbell"-shaped structure (coiled ssDNA/SSB_{tet}/coiled ssDNA), the protein is more efficiently shielded from the pore wall and is thus less likely to adsorb. Finally, we have also observed the low-amplitude events without spike occasionally, but they remained low in numbers and statistically insignificant.

Further analysis of the composite nature of event type (II) revealed that the spike feature did not occur randomly, but with the highest probability at the beginning of the translocation event, fig. 3c. This is in-line with the fact that the uncoated part of the complex exhibits a higher negative charge density (see table S1), compared to the coated part, which orients towards the positively biased compartment (and the pore) during translocation. Events with the spike feature closer to the centre of the event) were detected, albeit at lower probability. These may correspond to ssDNA bound to two SSB_{tet}, i.e. at the beginning and at the end of the DNA strand.

As shown in fig. S6 A) (Supporting Information), there is no correlation between the amplitudes of the spike and the low-amplitude part of the translocation events ($R^2 = 0.00$), the ratio is about 3-4. A weak correlation seems to exist between the duration of the two event features ($R^2 = 0.34$), fig. S6 B), but further work is clearly needed to investigate this aspect

in more detail. Following the interpretation above, the weak correlation (if any) would suggest that protein adsorption inside the pore does not strongly affect the translocation of the uncoated part of the ssDNA (including partial unravelling).^{34,40}

For bias voltages larger than 100 mV, such complex events could not be detected anymore and only "spike-like" events were observed. The events were much faster, namely $\tau = 0.173 \pm 0.031$ ms for $V_{\text{bias}} = 0.2$ V and $\tau = 0.169 \pm 0.026$ ms for $V_{\text{bias}} = 0.3$ V. Unfortunately, we were unable to record any data for larger V_{bias} , due to signal-to-noise limitations. If the long, low-amplitude features in event type (II) (at $V_{\text{bias}} = 0.1$ V) indeed results from the adsorption of bound SSB to the inner pore walls, then this process either no longer occurs or is no longer rate-limiting. In that case, the translocation process would be governed by (partial) unravelling of the ssDNA, prior to entering the pore channel.³⁴

In order to explore this further, we performed translocation experiments with SSB_{tet} bound to short fragments of ssDNA, which consequentially lack larger-scale, globular ssDNA structures. Translocation experiments with the uncoated 37- and the 100 nt ssDNA were performed in the same way as above (cf. SI for sequence information; 1 M KCl, 10 mM Tris·HCl pH 8.0; [ssDNA]: 150 ± 50 nM). Translocation events could not be detected for bias voltages between ± 0.5 V. This is not surprising, given the already fast translocation time of 7249 nt ssDNA and the much smaller size of the short ssDNA samples. Hence, it is likely that the corresponding translocation events are simply too short and/or too small to be detected in our experiments.

On the other hand, translocation of SSB/100 nt ssDNA complex did cause ion current blockades of $0.49 \pm 0.08\%$ in magnitude at -0.3 V bias, figure 4a (top panel). The ion current modulation is similar in magnitude to the low-amplitude modulation for the long

ssDNA, suggesting that they are indeed due to the SSB-coated segment. . The short event duration made it impossible to detect any sub-features in the event signal even at lower V_{bias} .

Figure 4: Translocation of SSB/short ssDNA through SiN nanopores. (a) The top left figure is showing typical blockade events of SSB/ssDNA (100 nt) translocation through a 50-nm SiN nanopore (open pore current: 66 nA) and through a 20-nm SiN nanopore (open pore current: -20 nA), both at -0.3 V bias. Inset: SEM images. (b) Voltage dependencies ($\tau \propto e^{-V/V_0}$) of the blockade durations for SSB/ssDNA (100 nt), which is red line and SSB/ssDNA (37 nt), which is black line, in 1 M KCl, 10 mM Tris·HCl pH 8.0. (c) Scatter plot of ionic current blockades caused by SSB/ssDNA (100 nt) translocation through 20-nm SiN nanopores in 1 M KCl, 10 mM Tris·HCl pH 8.0, when ssDNA was added in the *trans* compartment and applied voltage at -0.3 V. (d) Scatter plot of ionic current blockades caused by SSB/ssDNA (37 nt) translocation through 20-nm SiN nanopores under the same conditions as in (c).

Decreasing the pore diameter to 20 nm, as expected, increased the signal-to-noise ratio (increased excluded volume) and the translocation time (increased pore wall

interactions), fig. 4a (bottom trace).⁴⁰ Accordingly, translocation of SSB/(100 nt) ssDNA at -0.3 V bias produced ionic current blockades of larger relative magnitude ($2.1 \pm 0.4\%$) and longer mean duration (1.40 ± 0.14 ms), fig. 4b/c. We observed only one event cluster for the 100 nt sample, indicating that (SSB_{tet})/ssDNA_{100nt} is the majority species, in accordance with our modelling studies. Again, no sub-structure was detected in the translocation events, confirming that the spike features detected for the complex with long ssDNA were indeed due to uncoated parts of the DNA.

When translocating the SSB/ssDNA_{37nt} complex at the same V_{bias} , we observed ionic current blockades of $1.8 \pm 0.7\%$, which is the same as for the 100 nt fragment within experimental error. This confirms that both ssDNA species had the same number of SSB_{tet} bound. The mean duration of the blockade events was ~ 1.5 times shorter than for the 100 nt complex, which is somewhat surprising, given that the electrophoretic mobility is very similar, fig. 1c.

For both species, the translocation time decreases exponentially as a function of applied voltage, fig. 4b, implying that translocation is an activated process.³⁴ In contrast to the complex with the long 7249 nt ssDNA, however, this can no longer be related to the unravelling of the ssDNA. Rather, it is most likely associated with the adsorption of the protein, as discussed above.

The voltage dependence of the translocation time is different for SSB-coated 37 nt and 100 nt ssDNA. Using $\tau = \tau_0 \cdot \exp(-V/V_0)$ in a range from 0.2 V to 0.5 V, we obtained $\tau_0 = 6.8 \pm 1.7$ ms and $V_0 = 0.14 \pm 0.02$ V for SSB-bound 37 nt ssDNA, and $\tau_0 = 5.49 \pm 0.02$ ms and $V_0 = 0.21 \pm 0.10$ V for SSB-coated 100 nt ssDNA. Within experimental error, the translocation times for SSB-coated 37 nt and 100 nt ssDNA in the absence of an external electric field, τ_0 , are the same, suggesting that the diffusional rate is the same for both

species. Interestingly, the 37 nt fragment appears to display a stronger voltage dependence, which explains the faster translocation times at finite V_{bias} . This is most likely rooted in different binding characteristics to the pore inner walls and the response of the (adsorbed) complex to the local electric field.

Finally, it is interesting to compare the electrophoretic force exerted on the SSB/ssDNA complex with the force needed to unravel the complex itself. Based on the work by Keyser et al. and assuming similar line charge densities for the SSB/ssDNA complex, this force is estimated to be on the order of 20 pN.⁴¹ This is somewhat larger than the unravelling force of the SSB/ssDNA complex measured by Lohmann, Ha et al. (6-13 pN).^{4,5} Thus, with a sufficiently small nanopore (diameter < ~8 nm), this process could be induced by translocation through the pore and studied in further detail. In fact, given that SSB_{tet} remains mobile once bound to the ssDNA, it might even be possible to control the speed of translocation of ssDNA, which is a long-standing challenge in the field of solid-state nanopore sensing. Such experiments have been successfully performed with biological pores, for example by covalently attaching a DNA processing enzyme to the pore⁴² or by pre-assembly in solution.⁴³

CONCLUSIONS

We have demonstrated that complexes between SSB protein and ssDNA can be studied using solid-state nanopore sensors. In all cases, the presence of the SSB protein in the complex appeared to have a significant effect on the translocation speed and details of the translocation characteristics. For complexes with long 7249 nt ssDNA, the translocation speed seems to be affected both by (partial) ssDNA unravelling upon entering the pore, and the protein/surface interaction with the inner pore walls. For shorter ssDNA fragments (37

and 100 nt), translocation is still an activated process, but no longer affected by structural changes in the ssDNA. Interactions with the pore walls seem to play a dominant role here. Since uncoated short ssDNA translocated too fast and thus remained undetected, the use of protein coatings could be a more general strategy for enhancing the capabilities of nanopore sensors. Finally, we note that this approach may be extended towards short fragments of RNA, e.g. miRNA.

METHODS

Preparation of linear M13mp18 (7249 nt) ssDNA: Circular M13mp18 single-stranded DNA (N4040S) was obtained from New England Biolabs (Hitchin, UK) and hybridized with a 22-base oligonucleotide (5' TACCGAGCTCGAATTCGTAATCA 3', Sigma-Aldrich, Dorset, UK) to generate an *EcoRI* recognition site. The hybridization reaction contained 7 μL of 250 $\mu\text{g mL}^{-1}$ circular M13mp18 ssDNA and 3 μL of 100 μM of the 22-base oligonucleotide. After incubating the hybridization reaction at 65 $^{\circ}\text{C}$ for 5 min, followed by 25 $^{\circ}\text{C}$ for 5 min, the reaction was kept at 10 $^{\circ}\text{C}$ for 10 min to stabilize dsDNA part. The hybridized DNA was then digested with *EcoRI* restriction enzyme. The digestion reaction contained 11 μL of hybridized DNA, 2 μL of 10XBufferH, 2 μL of 10 mg mL^{-1} Bovine Serum Albumin (BSA), 1 μL of 50 U mL^{-1} of *EcoRI* enzyme (Promega, Southampton, UK) and 4 μL of sterile water to make 20 μL total reaction volume. After incubating at 37 $^{\circ}\text{C}$ for 2 h, the digested DNA was analysed on 0.8 % agarose gel and the band for linear M13mp18 ssDNA was cut and purified using QIAquick Gel Extraction Kit (28704, Qiagen, West Sussex, UK). The purified linear ssDNA was eluted with sterile water; the purity was checked on a 0.8 % agarose gel. DNA concentrations were measured by UV Vis

spectroscopy (NanoDrop ND2000, Labtech International Ltd., East Sussex, UK); samples were kept at -20 °C until use.

Binding assay for long, 7249 nt ssDNA: The binding reaction contained 1.25 µg of M13mp18 ssDNA and 100 ng of SSB (Promega, Southampton, UK) in 20 µL of 1 M KCl, 10 mM Tris·HCl, pH 8.0. The reaction was incubated at 25 °C for 30 min and analysed on 8% native polyacrylamide gel in 1XTBE buffer (Tris-Borate-EDTA, Bio-Rad, Hertfordshire, UK) and stained with Fast Blast DNA stain (Bio-Rad, Hertfordshire, UK) for 10 min and destained with sterile water overnight. DNA bands were photographed and analysed using Gel Doc XR System (Bio-Rad, Hertfordshire, UK).

Binding assay for short DNAs: The binding reactions contained 1 µM of the 6 different oligonucleotides (Sigma-Aldrich (Dorset, UK)), namely 17-nt, 35-nt, 37-nt, 50-nt, 75-nt and 100-nt ssDNA (cf. Table S2 for sequence information) and 100 ng of SSB in 20 µL of 1 M KCl, 10 mM Tris·HCl, pH 8.0. After incubation at 25 °C for 30 min, all reactions were analysed on 8% native polyacrylamide gel in 1XTBE buffer and stained with 1 µg mL⁻¹ ethidium bromide for 10 min and destained with sterile water for 2 min. DNA bands were analysed using Gel Doc XR System (Bio-Rad, Hertfordshire, UK).

AFM images were obtained in air at 23°C with an Agilent 5500 AFM/SPM microscope operating in tapping mode using commercial “Super Sharp Silicon” AFM probes (Windsor Scientific) with following parameters: 1024 × 1024 pixels (0.2 -0.5 lines/s). Images were processed with third-order “flatten filter” (PicoView 1.10, Agilent Technologies) and ImageJ 1.48v.

The samples were prepared as follows: Solutions of each ssDNA sample or complex (for binding assay see above) were freshly prepared at a concentration of 1.5 ng/µL in 1.5 mM EDTA (pH 8.0), 10 mM HEPES (pH 7.6), 4 mM MgCl₂ in a total volume of 20 µL. 4

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3 μL of this mixture was deposited on freshly cleaved 9.9 mm diameter mica (Agar Scientific)
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5 and left to adsorb to the surface for 5-10 min. To remove excess buffer salt, the substrate was
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7 rinsed with 1-2 mL of nuclease free H_2O and dried with flow of dry N_2 . All imaging
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9 conditions were reproduced independently at least three times.
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12 Zeta potential measurements: Three samples, which were (1) 1.25 μg of circular
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14 M13mp18 ssDNA, (2) 100 ng of SSB and (3) 1.25 μg of circular M13mp18 ssDNA and 100
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16 ng of SSB, were incubated in 2 mL of 1 M KCl, 10 mM Tris-HCl, pH 8.0 at 25 °C for 30 min
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18 and measured zeta potential using DelsaTM Nano (Beckman Coulter, High Wycombe, UK).
19
20 We measured the zeta potential of SSB, M13mp18 ssDNA and *E.coli* SSB/M13mp18 ssDNA
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22 complex in translocation buffer to be 35 ± 8 mV, -74 ± 4 mV and -47 ± 9 mV, respectively
23
24 (Table S1). This indicates that SSB has a net positive charge under sensing conditions and
25
26 may in fact translocate through the pore in the opposite direction, compared to ssDNA and
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28 the SSB/ssDNA complex (if transport is dominated by electrophoresis).³⁶
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34 Nanopore preparation: SiN_x membranes (100 nm thick) were fabricated using
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36 standard photolithography and KOH wet etching.⁴⁴ Nanopores with diameter of 45-50 nm
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38 were milled and imaged using a focus ion beam (FIB)/scanning electron microscope (SEM)
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40 instrument (Carl Zeiss XB 1540 Cross-Beam; ion acceleration voltage at 30 kV; milling
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42 current at 1 pA).
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46 Nanopores with diameter of 20 nm were fabricated using an ion beam lithography
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48 (IBL) system (Raith ionLiNE) at 40 kV acceleration voltage and 1 pA beam current.
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50 Equipped with a laser interferometer stage for positioning with nm-precision, focus
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52 correction by laser height sensing and automation capabilities, such as automated mark
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54 recognition for overlay alignment, this instrument allows higher quality pores to be produced
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56 on the wafer scale, in a reproducible fashion and with automated patterning capabilities.
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Nanopore analysis: Membrane devices with 20-50 nm pores were assembled in a Teflon cell and sealed with PDMS rings. 900 μL of 1 M KCl, 10 mM Tris·HCl, pH 8.0 was filled in both sides of the cell. 0.25 mm Ag/AgCl electrodes were connected to the cell and a patch clamp amplifier (Axopatch 200B, Molecular Devices, California, US). IV curves were measured between -0.5 V and +0.5 V to determine the pore conductance and hence the diameter, according to eq. (4). For a cylindrical pore with negative surface charge, where the length of the pore or membrane thickness (L) is much greater than the diameter (d), the pore conductance (G) is given by:¹¹

$$G = \frac{\pi d^2}{4 L} (\mu_K + \mu_{\text{Cl}}) n_{\text{KCl}} e + \pi \frac{d}{L} \sigma \mu_K \quad (4)$$

where μ_K and μ_{Cl} are the electrophoretic mobilities of K^+ and Cl^- ions, n_{KCl} is the charge density of the electrolyte, σ is the surface charge density and e is elementary charge. Then 1.25 μg linear M13mp18 ssDNA or 1 μM short oligonucleotides or SSB/ssDNA complexes (as described in the Methods section) were added to the *trans* compartment and negative voltages (-0.5 V to -0.1 V) were applied to drive these molecules through nanopores. The ionic current was filtered at 10 kHz and sampled at 50 kHz. Ionic current blockades and translocation times were further analysed with Clampfit 10.2 (Axon Instrument), Matlab and Origin 8.5.

ASSOCIATED CONTENT

Supporting Information. Gel electrophoresis data for SSB binding M13mp18 (7249 nt) ssDNA; tetramer/octamer model for SSB binding to ssDNA; calculation of ion current blockade from nanopore data; further statistical analysis of translocation data for 7249 nt ssDNA/SSB complexes; zeta-potential data for SSB, M13mp18 ssDNA and SSB/ssDNA

complex; sequence information for short oligonucleotides. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

DJ performed the experiments, drilled nanopores using dual-beam FIB/SEM, and produced an early draft of this manuscript. AB performed further experiments (AFM, gel electrophoresis). AN, LP and SB fabricated nanopores using ion beam lithography. JBE designed the sensor devices and supervised the chip fabrication. TA supervised the overall project and wrote the paper.

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