The Bexsero *Neisseria meningitidis* serogroup B vaccine antigen NHBA is a high-affinity chondroitin sulfate binding protein

Tsitsi D. Mubaiwa, Lauren E. Hartley-Tassell, Evgeny A. Semchenko, Christopher J. Day, Michael P. Jennings & Kate L. Seib

*Neisseria meningitidis* is a Gram-negative bacterial pathogen that causes life threatening meningitis and sepsis. Neisseria Heparin Binding Antigen (NHBA) is an outer membrane protein that binds heparin and heparan sulfate and DNA. This protein is one of the four antigens in the meningococcal serogroup B vaccine Bexsero. In the current study, we sought to define the full glycan-binding repertoire of NHBA to better understand its role in meningococcal pathogenesis and vaccine efficacy. Glycan array analysis revealed binding to 28 structures by recombinant NHBA. Surface plasmon resonance was used to confirm the binding phenotype and to determine the affinity of the interactions. These studies revealed that the highest affinity binding of NHBA was with chondroitin sulfate ($K_D = 5.2$ nM). This affinity is 10-fold higher than observed for heparin. Analysis of binding with well-defined disaccharides of the different chondroitin sulfate types demonstrated that the most preferred ligand has a sulfate at the 2 position of the GlcA/IdoA and 6 position of the GalNAc, which is an equivalent structure to chondroitin sulfate D. Chondroitin sulfate is widely expressed in human tissues, while chondroitin sulfate D is predominantly expressed in human tissues, while chondroitin sulfate D is predominantly expressed in human tissues.

*Neisseria meningitidis* is a Gram-negative bacterial pathogen that causes life threatening meningitis and sepsis. The organism is susceptible to antibiotics; however, it is difficult to diagnose at early stages and can rapidly progress to a life-threatening disease. The combination of difficult diagnosis and rapid progression indicates that vaccination is the most effective and appropriate public health response to this organism. There are 13 serogroups of *N. meningitidis* based on the expression of different polysaccharide capsule structures. Serogroup A is the dominant serogroup in sub-Saharan Africa, where the highest burden of disease exists. Serogroups B and C predominate in the developed world, although serogroups X and W-135 are also emerging as a major cause of disease in several regions worldwide. The polysaccharide capsules that are the basis of these serogroups have been used as antigens in highly successful conjugate vaccines, including monovalent serogroup A and C vaccines, and four-valent vaccines comprising A, C, W-135 and Y capsule polysaccharides. The same capsule-based vaccine approach cannot be used for serogroup B strains, as the alpha-2-8 polysialic acid expressed by these strains is a structural mimic of human neural cell adhesion molecule (NCAM) and may induce immunopathology if used as a vaccine antigen. Therefore, serogroup B vaccine development had to move in a different direction, utilizing surface proteins of this highly variable pathogen.

In 2013, a serogroup B vaccine (4CMenB, Bexsero) was licensed for use. This vaccine contains four components; outer membrane vesicles from a serogroup B strain, formulated with three recombinant proteins NadA, fHBP and NHBA. These outer membrane proteins provide targets for complement-mediated serum bactericidal activity. They also provide an opportunity for functional blocking as each is proposed to play a key role in *N. meningitidis* pathogenesis, and understanding the functional roles of these surface antigens enables a better understanding of how the vaccine is functioning to protect against infection. NadA is an adhesin and fHBP binds to human factor H in serum reducing the efficiency of complement mediated killing. NHBA binds heparin and heparan sulfate proteoglycans via an arginine (Arg)-rich region. Two proteases, the phase variable meningococcal NalP and human lactoferrin have been shown to cleave NHBA upstream and downstream of the central
Arg-rich region, respectively\(^{11}\). The functional significance of these cleavage events is yet to be elucidated. NHBA binding to heparin mediates increased serum resistance\(^{11}\), potentially via interactions between heparin and factor H or C4b binding protein\(^{11,13}\). NHBA is also involved in meningococcal adherence to epithelial cells, via binding to heparan sulfate proteoglycans\(^{12}\). NHBA also binds to DNA, and although this interaction has not been fully characterized, NHBA-DNA binding promotes biofilm formation\(^{14}\). The \(nhbA\) gene is found in all meningococcal strains tested\(^{7,15}\).

The lectin (carbohydrate binding) activity of NHBA for heparin and heparin sulfate has been well characterized and is believed to be important for meningococcal pathogenesis. In the current study, we sought to investigate the full range of lectin activity of NHBA using glycan array analysis.

### Results

**Glycan array analysis reveals additional lectin activity of NHBA.** To determine the lectin activity of NHBA we performed glycan array analysis using an unencapsulated MC58 strain (Σ3) in comparison with an isogenic \(nhbA\) mutant strain (Σ3ΔnhbA). Fluorescently labelled bacteria were incubated on the Institute for Glycomics’ v3.0 glycan array\(^{15}\). The results shown in Fig. 1 (see also Supplementary Table S1) reveal that strain Σ3 binds to 61 structures that the Σ3ΔnhbA strain does not bind. Direct binding to glycans by NHBA was also investigated using purified recombinant NHBA, which revealed binding to 28 glycan structures on the array. Twenty-two of these structures were in common with those bound by the NHBA expressing strain. Seven

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### Table

| Glycan   | Strains | Protein |
|----------|---------|---------|
|          | Σ3      | Σ3ΔNHBA | rNHBA  |
| Terminal Gal | ![heatmap](image) | ![heatmap](image) | ![heatmap](image) |
| Terminal GlcNAc | ![heatmap](image) | ![heatmap](image) | ![heatmap](image) |
| Mannosyl  | ![heatmap](image) | ![heatmap](image) | ![heatmap](image) |
| Fucosyl   | ![heatmap](image) | ![heatmap](image) | ![heatmap](image) |
| Sialylated| ![heatmap](image) | ![heatmap](image) | ![heatmap](image) |
| Terminal Glucose | ![heatmap](image) | ![heatmap](image) | ![heatmap](image) |
| GAG digests | ![heatmap](image) | ![heatmap](image) | ![heatmap](image) |
| GAGs (High molecular weight) | ![heatmap](image) | ![heatmap](image) | ![heatmap](image) |
| Other     | ![heatmap](image) | ![heatmap](image) | ![heatmap](image) |

*Figure 1. Heat map showing glycan binding by NHBA. The glycan binding properties of unencapsulated *N. meningitidis* (Σ3) are compared to that of an isogenic NHBA mutant (Σ3ΔNHBA) and recombinant NHBA (rNHBA). Red represents binding (in three independent replicates) and white is no binding observed.*
structures bound both the recombinant NHBA and the wild type strain (but not the ΔnhbA strain) indicating NHBA-specific and -dependent binding (see Supplementary Table S1; Index 5D, 46, 12B, 13B, 13E, 13J, 14F). These data suggest that the binding observed by the recombinant protein is mainly consistent with that mediated by the protein expressed in its native state on the surface of *N. meningitidis*. However, some glycans that bound the whole cell bacteria in an NHBA-dependent manner did not bind recombinant NHBA, possibly due to the need for native folding of NHBA on the cell surface for binding to occur. Also, several of the recombinant NHBA-binding glycan structure were bound by both the wild type and the nhbA mutant strain, suggesting that more than one meningococcal lectin is involved in binding. In addition, some of the recombinant NHBA-binding glycan structures were not bound by the wild type, possibly due to interactions being blocked on the bacterial surface by the presence of other meningococcal components, such as lipooligosaccharide (LOS) or pili that can negatively affect interactions mediated by other structures. The observed NHBA-dependent lectin activity includes the glycosaminoglycans (GAGs) heparin, heparan sulfate, heparinase III digests, hyaluronan, chondroitin sulfate, as well as glucose and the ganglioside GT3 (Fig. 1, Supplementary Table S1).

Surface Plasmon Resonance analysis of NHBA lectin activity. To determine the affinity of NHBA interactions discovered in glycan array analysis, we used surface plasmon resonance (SPR). In these studies, recombinant NHBA was bound covalently to a biosensor chip and various glycans were analyzed in flow for interactions discovered in glycan array analysis, we used surface plasmon resonance (SPR). In these studies, recombinant NHBA was bound covalently to a biosensor chip and various glycans were analyzed in flow for interactions discovered in glycan array analysis, we used surface plasmon resonance (SPR). In these studies, recombinant NHBA was bound covalently to a biosensor chip and various glycans were analyzed in flow for interactions discovered in glycan array analysis, we used surface plasmon resonance (SPR).

| Glycan | Index | Name | Structure | $K_D$ (μM) (mean ± standard deviation) |
|--------|-------|------|-----------|----------------------------------------|
| 46     | β-Glc6P | 6-H_2PO_4Glcβ-sp4 | 0.056 ± 0.025 |
| 2E     | P1 antigen | Galα1-4Galβ1-4GlcNAc | 14.93 ± 3.48 |
| 8A     | Sulfotransferase | SO_3β-Galβ1-3(Fucα1-4)GlcNAc | 33.4 ± 2.54 |
| 12B    | Neocarzinostatase | C_9H_18O_6SnA | 0.44613 ± 0.1511 |
| 12C    | Neocarzinostatase | C_9H_18O_6SnA | 0.44613 ± 0.1511 |
| 13E    | Hyaluronan disaccharide | GlcA/IdoA -GlcNAc | 0.13 ± 0.03 |
| 17M    | GT3 ganglioside sugar | Neu5Acα1-2-Neu5Acβ1-2-Neu5Acα1-3Galβ1-4Glc | 0.21 ± 0.056 |
| 13K    | Chondroitin sulfate | (GlcA/IdoAβ1-3±(±4/6S)GlcNAcβ1-4)n (n < 250) | 0.0052 ± 0.0024 |
| 14J    | Heparan sulfate | (GlcA/IdoAβ1-3-4(±NS))n (n = ~200) | 1.362 ± 0.200 |
| 13J    | Heparin | (GlcA/IdoAβ1-3-4(±NS))n (n = ~200) | 0.052 ± 0.026 |

Table 1. SPR analysis of NHBA-glycan interactions. NCDB: No concentration dependent binding observed within the range of the instrument’s detection.

NHBA-chondroitin sulfate interaction is a high affinity interaction that requires high, mixed sulfation. Chondroitin sulfate may have sulfation at different positions on the repeating disaccharide, and the highest affinity interaction observed was between NHBA and chondroitin sulfate with a dissociation constant($K_D$) of 5.2 nM. NHBA was named based on its interaction with heparin, and binding to heparan sulfate has subsequently been described. The affinity of NHBA with heparin (13J) was $K_D = 52$ nM, and its affinity with heparan sulfate (14J) was $K_D = 1.4$ μM. Heparin is more sulfated than heparan sulfate, and these data suggest that the sulfation of heparin may be required for high affinity binding by NHBA. We conducted studies with disaccharides representing all possible sulfations of heparin, indicating that NHBA requires a larger binding epitope for the higher affinity interaction observed with the heparin polymer structure (Fig. 2). The unsulfated GAG, hyaluronan, is bound by NHBA with affinity of $K_D = 130$ nM, and binding was also observed to the ganglioside GT3 ($K_D = 210$ nM; Table 1).
at all three positions (2S on GlcA/IdoA and GalNAc with 4S and 6S) or with disaccharides sulfated at a single position (i.e. either 2S on GlcA/IdoA only or GalNAc with 4S only or GalNAc 6S only; Fig. 3). This indicates that the binding of NHBA to chondroitin sulfate is highly specific and that the position of the sulfation affects the affinity of the interaction.

Competition between chondroitin sulfate, heparan sulfate and heparin for NHBA binding. To investigate the nature of binding sites on NHBA, a competition SPR assay was performed with the three main GAG substrates of NHBA using the A-B-A function of the Biacore S200 software. The assay is designed to show if a cumulative response is observed when a second analyte (B) is flown across the bound protein while the first analyte (A) is present. Analysis of the resultant sensorgrams showed that the binding of chondroitin disaccharide D to NHBA was not able to inhibit the subsequent binding of heparin or heparan sulfate polymers to the NHBA protein, as an additive effect was observed when the disaccharide was added (Fig. 4(i)). When heparin is added first, it inhibits binding of both the chondroitin disaccharide D and heparan sulfate (Fig. 4(ii)). However, heparan sulfate cannot inhibit the binding of either of the chondroitin disaccharide D or the heparin, with additive binding occurring in each case (Fig. 4(iii)). The competition assay suggests that there are two GAG binding sites. The first is a disaccharide specific site, responsible for the high affinity chondroitin sulfate D and heparin binding. The second site is a polymer binding site responsible for the low affinity binding of heparan sulfate, but this region also associates with the high affinity GAG polymers.

Analysis of NHBA-DNA binding. NHBA has been reported to bind to DNA so this was further investigated using SPR and ITC. SPR analysis demonstrated interaction between DNA and NHBA with a $K_D$ of 0.071 ± 0.017 μM, but saturation was not reached indicating an interaction that did not have 1:1 stoichiometry (Supplementary data S2). ITC analysis was therefore used, determining the affinity between DNA and NHBA was 3.33 μM (±1.1) with a stoichiometry of 30.73 (±10.89) NHBA bound per 503 bp DNA molecule. This is a relatively low affinity interaction due to the high stoichiometry, suggesting that NHBA has no preference for a specific DNA sequence and is interacting based on the negative charge and the polymeric nature of the DNA.
Discussion
Glycans are important for host pathogen interactions, and several pathogens that colonize the upper respiratory tract and central nervous system have been shown to target host glycans for their adherence/invasion into host cells\(^24\,25\). Recent work has shown that the meningococcal surface protein NHBA binds to the GAGs heparin and heparan sulfate, and these interactions mediate meningococcal serum resistance\(^11\) and adherence to host epithelial cells\(^12\), respectively. In the current study, we sought to fully characterize the lectin activity of NHBA using a comprehensive glycan array, as well as SPR and ITC analysis.

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**Figure 3.** SPR analysis of NHBA-chondroitin interaction. NCDB: No concentration dependent binding observed within the range of the instrument's detection.

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| Glycan                                                      | Structure | K_0 (μM) (mean ± standard deviation) |
|-------------------------------------------------------------|-----------|---------------------------------------|
| Chondroitin sulfate from shark cartilage (mix of chondroitin sulfate A, C and D) | ![Structure](image1) | 0.0052 ± 0.0024 |
| Chondroitin sulfate A                                      | ![Structure](image2) | NCDB |
| Chondroitin sulfate B (dermatan sulfate)                   | ![Structure](image3) | NCDB |
| Chondroitin sulfate C                                       | ![Structure](image4) | 71.8 ± 16.914 |
| Chondroitin disaccharide ΔDi-disD                          | ![Structure](image5) | 0.0095 ± 0.0029 |
| Chondroitin disaccharide ΔDi-disB                          | ![Structure](image6) | 0.52 ± 0.045 |
| Chondroitin disaccharide ΔDi-triS                          | ![Structure](image7) | NCDB |
| Chondroitin disaccharide ΔDi-UA2S                          | ![Structure](image8) | NCDB |
| Chondroitin disaccharide ΔDi-disE                          | ![Structure](image9) | NCDB |
| Chondroitin disaccharide ΔDi-4S                            | ![Structure](image10) | NCDB |
| Chondroitin disaccharide ΔDi-6S                            | ![Structure](image11) | NCDB |

![Legend](image12)

Glycans are depicted in different colors: Glucuronic acid (GlcA), Iduronic acid (IdoA), N-Acetylgalactosamine (GalNAc)

NCDB: No concentration dependent binding within instrument’s range of detection.
The initial screen using glycan array analysis and the *N. meningitidis* wild type and ΔnhbA mutant strains revealed loss of binding to 61 structures by the mutant relative to the wild type, which indicates glycan binding by NHBA. Interestingly, 41 additional interactions on the glycan array were observed with the mutant, that were not seen with the wild type strain. These results suggest a possible inhibitory or modulatory role for NHBA, one similar to that reported for meningococcal antigens such as lipooligosaccharide (LOS)\(^1\), capsule\(^1\) and pili\(^1\); which have been shown to negatively affect interactions mediated by other structures.

NHBA lectin activity was confirmed using recombinant NHBA protein. The recombinant NHBA protein bound to 28 structures on the glycan array, and binding to 22 of these structures was also observed by the wild type *N. meningitidis* using whole fluorescently labelled bacteria. These data indicate that NHBA has a much broader lectin activity than binding to heparin; the ligand binding activity after which it was named. The majority of the newly identified glycans bound by NHBA are sialylated and/or sulfated, which is consistent with the previously described negatively charged ligands of NHBA, which include heparin\(^1\), heparan sulfate\(^1\) and DNA\(^1\). Furthermore, the majority of the glycans to which NHBA bound were GAGs and included chondroitin sulfate polymers and digests as well as hyaluronan.

Heparan sulfate and chondroitin sulfate are found on a range of host cell surfaces and in the extracellular matrix\(^2\). Heparan sulfate proteoglycans are typically found on epithelial cells, endothelial cells and fibroblasts\(^2\), while chondroitin sulfate proteoglycans are typically found on lymphocytes\(^2\), within the central nervous system\(^2\), and are highly expressed on endothelial cells\(^2\). It is important to note that the expression of different chondroitin sulfate types is tissue and host specific and is affected by factors such as age and disease\(^2\). Chondroitin sulfate D is found in the adult human brain, while chondroitin sulfate A and C are typically found in cartilage and other tissues\(^2\).

In this study, for the first time, we present a quantitative measure of the binding affinity to the known ligands of NHBA, heparin and heparan sulfate, and also to the newly discovered glycan structures. The highest affinity interaction was between NHBA and chondroitin sulfate (\(K_D = 5.2\) nM). The chondroitin sulfate used is a natural product that contains a mixture of sulfation patterns (chondroitin sulfate A, C, D). Therefore, alternative sources of chondroitin sulfate polymers were also used to address the question of whether the alternate sulfation patterns played a role in NHBA affinity. Results indicate that NHBA prefers chondroitin sulfate D (2S on GlcA/IdoA and GalNAc with 6S), as there was a 10,000-fold decrease in affinity to chondroitin sulfate C and no binding to chondroitin sulfate A or B. A source of pure chondroitin sulfate D was not available, however studies using the repeating subunit disaccharide structures that make up the chondroitin sulfate polymer confirmed that the chondroitin sulfate type D disaccharide was the preferred substrate with a \(K_D\) of 9.5 nM. This is a remarkably high affinity considering that it is a small disaccharide subunit that does not have the polyvalency of high molecular weight chondroitin sulfate polymer.

The affinity of NHBA for chondroitin sulfate is 10-fold higher than for the previously described ligand heparin (\(K_D = 52\) nM), and 200-fold higher than for heparan sulfate (\(K_D = 1.362\) µM). It is interesting to consider that the binding to heparin with an affinity of 52 nM can only be achieved with the polymer. This is unlike chondroitin sulfate, which has nanomolar affinity for both polymer and disaccharide. Heparin disaccharides all had affinities approximately 500-fold weaker (\(K_D > 2.5\) µM) than we observed for the equivalent chondroitin sulfate disaccharide. Unlike chondroitin sulfate, this indicates the high affinity interaction observed in SPR with heparin requires polyvalent binding. Previous studies have indicated that NHBA-mediated adherence to
epithelial cells (endometrial adenocarcinoma cell line called Hecl-B) is heparan sulfate-dependent and chondroitin sulfate-independent\(^2\). This observation is not consistent with the high affinity NHBA-chondroitin sulfate interaction that we observed herein. However, the expression of different chondroitin sulfate types is variable between different tissues\(^3\) and all cancer cell lines that have been investigated do not express the preferred NHBA ligand, chondroitin sulfate D\(^{10,31}\). As a result, one would not expect to see NHBA binding to the atypical chondroitin sulfate types expressed by transformed cell lines typically used in cell association studies.

NHBA has a positively charged, arginine-rich region that is required for binding to heparin\(^11\) and heparan sulfate\(^2\). When the phase variable meningococcal protease NalP is expressed, NHBA is cleaved and the Arg-rich C terminal fragment is released\(^11\). The C terminal fragment has been shown to alter endothelial permeability and promote vascular leakage\(^32\), but its target receptor on endothelial cells is unclear. Competition by SPR revealed that the region of NHBA where chondroitin sulfate D disaccharide binds is also bound by heparin. However, this region does not overlap with the heparan sulfate binding region. Interestingly, the binding of heparin and heparan sulfate seem to overlap, suggesting that there may be two binding sites; a specific glycan recognizing pocket that binds to a preferred disaccharide portion of the GAGs, and a positively charged region that increases the affinity of binding to the rest of the polymer.

The only other high affinity NHBA interaction in the nanomolar range that was observed was with a non-GAG structure, the ganglioside GT3 (Kd = 210 nM) that is transiently expressed in the brain during embryonic development\(^33\) and is found on adult oligodendrocyte progenitor cells in the human adult brain\(^34\). GT3 consists of three negatively charged side branching sialic acid residues that may mimic the presentation of the sulfated GAGs and explain the binding of NHBA to this otherwise unrelated structure.

The interaction between NHBA and DNA is also likely to be charge dependent, since binding does not appear to be linked to a specific DNA sequence, with multiple NHBA proteins binding to a single double stranded piece of DNA. Future structural studies are required to determine the role of specific NHBA regions, including the Arg-rich region, in binding to the newly identified glycan structures and to DNA.

The ability of \textit{N. meningitidis} to bind several glycan structures suggests that NHBA may target different host glycans in different microenvironments. However, the high affinity of NHBA-chondroitin sulfate D interactions suggest NHBA has evolved to preferentially bind this structure, indicating that it may have a key and unique role in meningococcal pathogenesis. If vaccine induced antibodies are able to block NHBA-chondroitin sulfate D interactions, then this may increase the efficacy of the vaccine.

**Materials and Methods**

**Bacterial strains and growth conditions.** \textit{N. meningitidis} \(\mathcal{L}3\) (a siaD knockout mutant derived from the serogroup B clinical isolate MC58\(^8\)) and the isogenic \(\mathcal{L}3\Delta nhbA\) mutant (generated as described previously\(^33\)) were grown on BHI supplemented with 10% Levinthal’s base and incubated overnight at 37 °C at 5% CO\(_2\). Levinthal’s base is prepared by adding defibrinated horse blood to three volumes of BHI broth, heating at 95 °C for 40 mins, followed by centrifugation (4500 × g, 4 °C, 15 min) to remove to remove insoluble material from the lysed blood.

**Expression of recombinant NHBA.** The nhbA gene was amplified from \textit{N. meningitidis} MC58 (primers 5′-attactcggTGCGGCGATGTCAAGTC-3′ and 5′-tgaatcatccCGGCCGATGCATCAATC-3′; underline indicates restriction enzyme sites) and cloned into pET19b (Invitrogen). Protein expression and purification was performed as described previously\(^35\).

**Glycan array analysis.** Glycan array analysis was performed as per Mubaiwa et al. using array v3.0 from the Institute for Glycomics and a ProScan Array Microarray scanner (Perkin Elmer) 555ex/568em\(^19\). Briefly, 125 μL of Bodipy558-succinimidyl ester labelled bacteria (OD 600 = 0.1) were added to the array slide and hybridized for 30 min. Analysis of recombinant NHBA was performed as per Day et al.\(^36\), using 1–2 μg of His-tagged protein. Binding was defined as positive if the average fluorescence intensity of the glycan spots was greater than one-fold above the adjusted background (average of the slide background plus three standard deviations) in three independent replicates (Student's t-test \(p < 0.001\)).

**SPR analysis of recombinant protein or DNA.** SPR was performed using a Biacore T200 instrument and Series S CM5 sensor chips (GE Healthcare). Recombinant NHBA (100 μg/ml) was immobilized by amine coupling (10 μL/min flow rate onto flow cells 2–4), resulting in ~8000 response units (RU) of immobilized protein. The reference surface (flow cell 1) underwent the same treatment, without protein injection. Single cycle kinetics were analyzed using the BIAcore T200 evaluation software 2.0.2; sensorgrams were double reference subtracted.

**SPR NHBA competition assays.** SPR competition assays were performed by using a Biacore S200 instrument and the A-B-A inject function. Recombinant NHBA was immobilized as above. The A-B-A was used with combinations of each of the glycan substrates (at concentration 10 × \(K_d\)) and PBS controls, with 120 second injections of analyte A to ensure saturation or near-saturation was reached prior to competition with analyte B. The results were analyzed using Biacore S200 evaluation software.

**ITC analysis of NHBA with DNA.** A 503 bp DNA fragment was amplified from \textit{N. meningitidis} genomic DNA (using primers 5′-GGTCTGTGTTCACGCAAGACC-3′ and 5′-GAGGCTGATTCTTCCAGC-3′), cleaned up using a QiAGEN PCR purification kit and quantified using a nanodrop. ITC was performed using a nano-ITC (TA instruments) with 10 μM NHBA titrated into 1 μM of DNA (20 injections of 2.5 μL NHBA at
300 second intervals). PBS-NHBA interactions were run as a negative control. This was also background sub-
tracted. Affinity data was determined for a minimum of two repeats and the average KD values and stoichiometry 
(n) are reported.

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Author Contributions
M.P.J. and K.L.S. conceived the study, all authors contributed to the design of experiments and analysis of data, T.D.M. performed the experiments, T.D.M., M.P.J. and K.L.S. wrote the paper, all authors reviewed and approved the final version of the manuscript.

Additional Information
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