Investigation of K1 bacterial capsular morphology and single molecules in capsular biosynthesis using super-resolution fluorescence microscopy (dSTORM)

A Thesis Submitted to The University of Manchester
for the Degree of Doctor of Philosophy
in the Faculty of Science and Engineering

2019

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Publications


Conferences

Physics meets biology 2019 (Institute of Physics, IOP)
Attended this meeting and made a poster presentation on the topic of “Investigation of K1 bacterial capsular morphology and single molecules in capsular biosynthesis using super-resolution fluorescence microscopy (dSTORM)”.

2nd Mechanobiology meeting in Vietnam (ICISE)
An oral presentation on the topic of “Investigation of K1 bacterial capsular morphology and single molecules in capsular biosynthesis using super-resolution fluorescence microscopy (dSTORM)”.

The Physics of Microorganism II (Institute of Physics, IOP)
Attended this meeting and made a poster presentation on the topic of “Investigation of K1 capsular polysaccharide, a permeable shield of E. coli against antimicrobial peptides”.

Quantitative approaches to antimicrobial resistance (Institute of Physics, IOP)
Attended this meeting and made a poster presentation on the topic of “Antibody labelling to study Escherichia coli K1-polysialic capsules using super-resolution fluorescence microscopy (dSTORM)”.

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Abstract

Bacterial capsules are a virulence factor, especially in uropathogenic Escherichia coli (UPEC) that cause urinary tract infections (UTIs). The study of K1 capsule morphology and biosynthesis provide valuable information for the development of alternative treatments to problematic antibiotics.

To resolve sub-diffraction limited details in the morphology of K1 bacterial capsule, direct stochastic optical reconstruction microscopy (dSTORM) with 50 nm lateral resolution was used to visualise the inhomogeneous thickness of the K1 bacterial capsules. The variation of capsule thicknesses (~49%) from polar and equatorial regions will affect bacterial adhesion and the infection of host cells. The investigation concluded the bimodal thickness was due to internal capsular biosynthesis in agreement with HPLC results. The heterogeneity of capsule thickness is independent of the bacterial curvature due to the dense grafting and strong polyelectrolyte properties of fully stretched K1 capsular brushes. The steric stabilization due to the osmotic pressure of bacterial capsular brushes was confirmed using atomic force microscopy (AFM). The K1 biosynthesis of capsular polysaccharide was investigated via polysialyltransferase (NeuS) dynamics using a novel Halotag self-labelling method. The rapid dynamics of NeuS in capsular biosynthesis compared with a CPS mutant revealed a sub-diffusive regime with a 0.2-0.5 ms characteristic time for interaction using fluorescence correlation spectroscopy (FCS). The variation of NeuS interaction influences the polydispersity of capsular chains. Although densely grafted K1 capsular brushes fully cover the bacterial surface, positively charged amphiphilic peptides, G(IKK)3-I-NH2, could penetrate the thick layer of the bacterial capsule. This is similar to permeability in the nutrient consumption pathway. The inhomogeneous landscape of the bacterial surface mixture, which comprised proteins, lipid islets and other elements, is one of the factors. Thus, the K1 bacterial capsule is a permeable shield with a bimodal thickness that protects the bacterial surface from antimicrobial peptide treatment by introducing aggregation on its surface, through partial interaction with the bacterial membranes.
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Acknowledgements

This thesis would have been impossible complete without the generosity and support from both of my supervisors, Thomas Waigh and Ian Roberts. I am grateful for their kind instruction, informative suggestions and straightforward comments to help me improve myself in the past 4 years. Learning all microbiological practices with care from Ian Roberts, Jane King, Marie Goldrick and other people from microbiology labs are valuable knowledge for a physicist who initially did not know how to culture bacteria and now can independently work with bacterial genetic modification. I am really enjoy working with the BBC 2 radio background besides Jane and Marie; thank you so much for continual prompt support.

I have to say thank you to my family for all their mental and spiritual support. Thanks for all my friends that I have met here including Thai friends for all gathering and enjoyable moments (especially Wisit and Nawapong), biological physics friends for interesting discussions and a nice Thursday bake-offs, my basketball friends for gold medals, intense exercising and stress relief and for all other friends to provide memorable experiences, e.g. having a chance to perform a gig live in UK, working in Thai restaurants, adventurous hiking and so on. These family and friends helped me to be happy during my PhD time. I have to say a special thanks to Pantelis Georgiadis, Ruiheng Li, Hanna Perkins, Johanna Blee, Henry Cox and Jack Hart for interesting discussion in our Tom’s group on a variety of topics including politics, culture, traveling, games, jokes and intensive science which help me to improve my perspectives in all these areas.

Finally, thank you to my sponsor, the Development and Promotion of Science and Technology Talents Project (DPST), Thailand from my financial support and opportunity to have a great experience during my PhD and a new journey that is awaiting.
Dedication

I would like to dedicate all my effort to my Phaphak family, including Mr. Suwit, Ms. Saksri and Mrs. Siwichaya who supported me through the toughest time of my life.

In memory of my uncles, aunts and grandmather who passed away during pursuing of a PhD, including uncle Sumruai, uncle Sumreng, aunt Mali, aunt Jarussri and grand mother Tongdee. This PhD has completely changed my life forever!

“Study hard what interests you the most in the most undisciplined, irreverent and original manner possible.”, RPF.
CHAPTER 1

Introduction

1.1 The importance of capsular polysaccharide (K-antigen) on an *Escherichia coli* surface

Bacteria are one of the simplest living systems and act as genetic factories that produce and control the expression of proteins. Research into these genetic factories involves the development of many complicated, up-to-date gene editing technologies, such as CRISPR-cas9 [1,2]. With only simple organelles and sophisticated gene manipulation for self-manipulation, bacteria can adapt themselves to survive in almost any harsh conditions, e.g. high-temperature resistant bacteria [3], MRSA bacteria [4] and capsule upregulated bacteria [5,6]. In addition, one of the several mechanisms that bacteria develop to protect themselves are physiological components [7] including flagellar chemotaxis proteins [8], fimbria adhesins [9], secreted proteins [10,11], lipopolysaccharides (LPS) or O-antigen [12,13], and capsules (CPS) or K-antigen [14], which allow them to survive under a wide range of environmental pressures.
The morphology and construction of bacterial capsules vary extensively between different strains despite the existence of analogous biosynthesis gene clusters [15]. Capsules are found on the surface of many pathogens in both gram-positive and gram-negative bacteria, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Actinobacillus pleuropneumoniae*, *Sinorhizobium meliloti*, *Pasteurella multocida*, *Haemophilus influenzae* type B and *Escherichia coli* [16]. These bacteria can cause lethal diseases, e.g. pneumonia, otitis, meningitis and urinary bladder cancer. One of the most common bacterial infections, which can cause morbidity to infant boys, elder men and women of all ages, is urinary tract infections (UTIs) [17–19].

Uropathogenic *E. coli* can adhere to epithelial cells in the bladder using *FimH* adhesin proteins (type 1 fimbriae) and then invade host cells [20]. The infected cells cover themselves with capsule [21] to limit desiccation in harsh conditions, to shield recognition by most bacteriophages, and to prevent phagocytosis and other host immune responses by restricting access to antigens. A higher dose of antibiotics is needed to eradicate encapsulated pathogens, so this is a major cause of antibiotic resistance and the recurrence of UTIs in patients when treatment with antibiotics is unable to completely cure the infection [22].

A fundamental understanding of the physiology of bacterial capsule (K-antigens) and their molecular biosynthesis are limited, although the capsular effects on antibiotics have been studied for decades. Alternative treatments, such as cationic antimicrobial peptides (CAPs), are ambiguous as to whether bacterial capsules protect the cells from threatening CAPs.
1.2. Theoretical aspects of capsular polysaccharide brushes

1.2.1 The stabilization of capsular polysaccharide: a scaling theory for capsular brushes

K1 capsular polysaccharides are anchored on the bacterial membrane and stabilized in electrolyte (ionisable) solutions. Their conformations can be modified dependent on their environment. From a microscopic point of view, the bacterial capsule is a smooth, homogeneous layer (a homogeneous thickness, \(H\)) and can cover the whole bacterial surface. The conformation and morphology of capsular polysaccharides is affected by the distribution of ions surrounding the bacterial capsules. With the assumption of a smooth bacterial capsule layer, the steric force can be approximated using a Derjaguin-Landau-Verwey-Overbeek (DLVO) method, so as to estimate the potential from a distant position with respect to the bacterial capsule layer. The DLVO theory can thus approximate the thickness \((H)\) of the bacterial capsule [23,24].

To explain interactions involved with encapsulated bacteria, it is possible to classify 4 different regimes from outside to inside the cells. From a distant position, the DLVO model can be used to calculate a long-range interaction defined as a regime 1 in Figure 1.1a.
Figure 1.1 a) Different length regimes of bacterial capsule stabilization. Interactions related to encapsulated bacteria are simplified according to 4 different regimes: 1) long-range DLVO interaction, 2) short-range interaction, 3) compressed interaction and 4) elastic interaction related to turgor pressure. b) The thickness of an extended polyelectrolyte brush according to a scaling theory. c) Curvature topology with different types of polyelectrolyte attached to spherical colloids: strong (quenched) and weak (annealed) polyelectrolyte brushes [25]. On the bacterial surface, $H$ is the thickness of capsular brushes and $\Delta H$ is the variation of thickness. For the electrostatic blob model, $L$ is the contour length of a polysaccharide chain, $l_p$ is the electrostatic persistence length, $r_D$ is the Debye screening length and $\xi_e$ is the size of an electrostatic blob. A polyelectrolyte chain is stabilised due to an external force $f$. From the curvature influencing the polyelectrolyte chain conformation, $\rho_q$ and $\rho_a$ are the thicknesses of the internal sublayer of quenched and annealed polyelectrolyte brushes, respectively.
Investigating at a finer length scale, the capsular layer is comprised of densely grafted polysaccharide chains which act as polyelectrolyte (PE) brushes [26,27]. In addition to the steric effects of polymer brushes due to the short-range extended volume interaction between monomers [28], polyelectrolyte brushes also demonstrate a long-range interaction due to ionizable charges on the polysaccharide chains. The conformation of polyelectrolyte chains involves nonlinear effects due to counterions, e.g. condensation effects, especially with weakly charged polyelectrolyte brushes [26,29,30]. The thickness of capsular, polyelectrolyte brushes is related to the osmotic pressure (regime 2) and follows the Hertz model (regime 3) when the polysaccharide brush is compressed [31]. In addition, to maintain bacterial rod shape, the turgor force in regime 4 from the cytosol is dominant. The bacterial capsule layer covers a relatively rigid bacterial surface. Rod-shaped bacteria have different curvatures at the equators (1/R) and the poles (2/R). These curvatures influence internal protein localisation [32] where the preferred region is around the poles and also influence the stretching polysaccharide brushes, which is significant in weak polyelectrolyte brushes. The heterogeneity of the capsular brush thickness (variation, \( \Delta H \)) is due to both variations in polysaccharide stabilization, e.g. salt concentration [26], and curvature of the surface [25,33] onto which the capsular polysaccharides are anchored.

There are several theories used to approximate polyelectrolyte conformation, e.g. mean-field Poisson-Boltzmann theory [34], self-consistent field theory [35] and scaling theory [36]. The scaling theory ignores scalable factors but tends to be the most successful, i.e. in best agreement with experiments [37].
Scaling theory is used to approximate the size of stabilized polymers or polyelectrolyte chains. In salt-free solutions, the size and conformation of the chains are approximated using the degree of polymerisation ($N$), a Kuhn length ($b$) and a Flory exponent ($\nu$). The end-to-end distance ($R_e$) is given by $R_e \sim bN^\nu$ according to the Flory approximation [38]. For an ideal polymer chain, the Flory exponents are different according to individual solution conditions, e.g. $\nu = 1/2$ for no-intermonomer interaction and $\nu = 3/5$ in a good solvent.

To understand the stabilization and conformation of a polyelectrolyte brush, its free energy can be approximated using the balance between the elastic force ($F_{\text{elastic}}$) and an electrostatic force ($F_{\text{Coulomb}}$, Coulomb repelling) [26] as

$$\frac{F}{k_B T d} \approx \frac{F_{\text{elastic}}}{k_B T} + \frac{F_{\text{Coulomb}}}{k_B T} \approx \frac{R_e^2}{bL} + \frac{l_B^2 (\alpha N)^2}{R_e},$$  

(1.1)

where $d$ is relative distance, $R_e$ is the averaged end-to-end distance of the polyelectrolyte chains (in salt free condition), $k_B$ is the Boltzmann constant, $l_B = e^2/(\varepsilon k_B T)$ is the Bjerrum length ($e$ is the charge, $\varepsilon$ is the dielectric constant, and $T$ is temperature), $\alpha$ is the fractional charge of monomers and the contour chain length, $L = aN$ ($a$ is a monomer size). Minimization of the free energy ($F$) gives the conformation of the polyelectrolyte ($R_e$). In salt-free buffers, the end-to-end distance (the averaged chain size) is $R_e \approx (b l_B (\alpha N)^2 L)^{1/3} \approx L(u \beta^2)^{1/3}$, where $u = \frac{l_B}{b}$ is the ratio of the Bjerrum length to the Kuhn length and $\beta = \frac{ab}{a}$ is the charge density per Kuhn length. The conformation of polyelectrolyte chains can be defined in term of electrostatic blobs (in analogy to an excluded
volume of polymer chains) and its size is \( \xi_e \approx bL/R_e \) \( (R_e \sim H) \) in a brush model, Figure 1.1b).

As an indication of the counterion screening effect after adding salt ions, the chain size \( (R_s) \) varies with the electrostatic persistence length \( (l_p) \) and the Debye screening length \( (r_D) \). The Debye screening length depends on the salt concentration \( (c_s) \) and the Bjerrum length \( (l_B) \) due to a monovalent salt, so \( r_D = (8\pi l_B c_s)^{-1/2} \), where \( c_s \) is the salt concentration. However, the persistence length of polyelectrolyte chains is still a hotly debated subject and they have been predicted to scale as \( l_p \sim r_D, l_p \sim r_D^2 \) and \( l_p \sim r_D^{2-\xi_e} \) \cite{26}. This in turn will affect the expected size of polysaccharides. An extensional force, \( f \approx k_B T r_D/l_p^2 \) can extend the Gaussian polyelectrolyte chain at thermal equilibrium and the chain is stabilised to variations in size if \( f > k_B T r_D/l_p^2 \) \cite{26}.

For bacterial capsules, the anchored polysaccharides are stretched under the influence of osmotic pressure which acts as an external force \( (f_{osm}) \) \cite{26}. The osmotic pressure has contributions from localized counterions, connected segments and chain elasticity \cite{37,39}. The contribution from connected segments is normally negligible compared to that from localized counterions. Following a scaling approximation, the screening of the Coulombic repulsion due to the salt concentration \( (c_s) \) between each monomer is negligible \cite{25,26,40}. As an approximation, mobile ions \( (c_\pm) \) are approximated according to the Boltzmann distribution \cite{26}, \( c_\pm = c_s \exp(\mp e \Delta \psi / k_B T) \) in Donnan equilibrium (where \( c_s \) is salt concentration). Charges on the polysaccharide chains interact with counter ions as required for electroneutrality, \( c_+ \approx \alpha c + c_- \), where \( c_\pm \) is the ion.
concentration of the positive or the negative charges respectively and $\Delta \psi$ is the electrostatic potential difference (between the outside and the inside of the brush). The counterion concentration is equated with the average concentration of the polysaccharide brush ($c = \frac{N\sigma}{H}$, where $\sigma$ is a grafting density, $N$ is the degree of polymerisation and $H$ is the thickness), and the difference in the osmotic pressure ($\Delta \Pi$) between the polyelectrolyte brush and the outside buffer can be calculated from [26] 

$$\frac{\Delta \Pi}{k_B T} = 2c_s \left[ \sqrt{\left( \frac{ac}{2c_s} \right)^2 + 1} - 1 \right] \approx \begin{cases} \frac{ac}{c_s}, & \text{when } c_s \ll ac, \\ \frac{(ac)^2}{c_s}, & \text{when } c_s \gg ac, \end{cases} \quad (1.2)$$

At a low salt concentration ($c_s \ll ac$), the gradient osmotic pressure ($\Delta \Pi$) depends on the fraction of charged monomers ($\alpha$) and the polysaccharide concentration ($c$) is related to the grafting density. However, at a high salt concentration ($c_s \gg ac$), the effect of charge screening is dominant. Hence, the osmotic pressure difference reduces when the salt concentration is higher.

Although the salt concentration affects the extension of the capsular polysaccharide, it also influences the bacteria’s metabolism and including the membrane potential of bacteria [41]. Live bacterial investigations require that the cells are cultured in an optimal salt concentration, e.g. 0.9 mM NaCl in M9 media. Since the K1 capsular polysaccharide chains comprise highly negatively-charged monomers, the specific case of strong polyelectrolytes in a buffer will be considered (salt-free and at low salt concentration).
The balance between the osmotic force, the conformational free energy of anchored polysaccharides and the screening effect of the buffer, \( f_{\text{osm}} = \frac{1}{a}(f_{\text{entropic}} + f_{\text{screening}}) \) determines the thickness of a capsular polysaccharide brush \((H)\) [26],

\[
H \approx \begin{cases} 
L \beta^{1/2} & \text{when } 0 < c_s < c_s^l, \\
L(\sigma b^2)^{1/3} (\beta^2 / b^3 c_s)^{1/3} & \text{when } c_s > c_s^l,
\end{cases}
\] (1.3)

where \( c_s^l = \alpha N \sigma / L \beta^{1/2} \) is the counterion concentration in the polysaccharide brush e.g. a concentration threshold, \( c_s^l \sim 0.1 \text{ M NaCl} \) when the Debye screening length, \( r_D \sim 1 \text{ nm} \).

With an increase of the salt concentration in the buffer, the height of capsular brush can be reduced in the low salt concentration regime. This is one of the conditions that could cause on inhomogeneous thickness for the bacterial capsule.

In addition to the effect of the salt concentration (in the low concentration regime) on the heterogeneity of the capsular brush, the surface curvature of densely grafted polyelectrolyte brushes can vary the capsular brush height [25,33,36]. Different types of strong (quenched) and weak (annealed) polyelectrolytes respond differently to the curvature of a surface [25], Figure 1.1c from top to bottom, respectively. The curvature effect influences the stiffness and degree of ionization of an annealed (weak) polyelectrolyte brush more than a quenched (strong) polyelectrolyte brush [25]. Although the effect of curvature is more important in sparsely grafted brushes, it is less significant in densely grafted-strong-polyelectrolyte brushes. According to Figure 1.1c, the thickness of the internal sublayer of quenched \((\rho_q)\) and annealed \((\rho_a)\) brush relates to the strength and the shear modulus of the polysaccharide brushes. A significant deviation of two types of the internal sublayers, \( \rho_a \gg \rho_q \), weakens the electrostatic interaction in weak electrolyte brushes. Hence the thickness of
weak polyelectrolyte brushes can be varied sensitively according to the curvature of the surface on which they are bound. On the contrary, for the strong polyelectrolyte brush, the thickness of the polyelectrolyte brush depends more on salt concentration than on the surface curvature.

1.2.2 Phenotypical investigation of capsular polysaccharide brushes

Theoretical explanations of the heterogeneous thickness of polyelectrolyte brushes motivate phenotypical investigations using optical techniques, electron microscopy and atomic force microscopy. Characterisation is also needed of the polydispersity and molecular weight of the capsular polysaccharides.

Some direct investigations using electron scattering to characterise the conformation of bacterial capsules have been performed. TEM images [42] (Figure 1.2a) and SEM images [31] have demonstrated that the capsule structure is heterogeneous. However, cells for electron microscopy require invasive fixation procedures. Previous high-resolution studies of bacterial capsules have thus been limited to dead bacteria.
Figure 1.2 Imaging technique on fixed and live bacteria: a) fixed-cell TEM image of K1 encapsulated bacteria [42] (the scale bar is 1µm), b)-d) Fixed-cell wide-field fluorescence images (the scale bar is 10 µm) [20] due to multi-colour labelling (e.g. AF647 and AF594) with different specific antibodies: b) antiK1 antibody for capsule labelling, c) antiEcoli antibody for bacterial membrane labelling and d) Dapi for chromosome labelling in combined image to visualise bacterial invasion of the host cell (blue), e) live-cell super-
resolution image of the cell wall of *D. radiodurans* using Point Accumulation for Imaging in Nanoscale Topography (PAINT) technique (the scale bar is 1 µm) [43] and f) live-cell AFM image in aqueous solution (the scale bar is 1 µm) [44].

It is difficult to measure the heterogeneity of bacterial capsules on live-bacterial cells due to the resolution limit of optical fluorescence techniques. Although fluorescence microscopy is a primary technique used to investigate capsular phenotypes ([Figure 1.2b](#)) via specific labelling antibodies [20], the optical diffraction limit (to be described more detail in chapter 2) makes the bacterial structure unclear. The thickness of the bacterial capsule ([Figure 1.2b](#)) is unresolvable in the membrane labelling image ([Figure 1.2c](#)). This wide-field fluorescence technique is suitable to investigate whole cell phenomena (e.g. invasion mechanism) of a single encapsulated cell ([Figure 1.2d](#)) rather than characterise the morphology of the bacterial capsule.

Recently, the development of super-resolution fluorescence microscopy [45] has been intensively studied on bacterial cells from protein dynamics [46,47] to bacterial structures [45], e.g. the bacterial cell wall ([Figure 1.2c](#)) [43]. Hence, super-resolution microscopy has the potential to resolve variations in bacterial capsule thickness. The existence of a heterogeneous capsule in super-resolution images which revealed the capsular layer will be presented for the first time in this thesis [48]. Fluorescence labelling of bacterial polysaccharides is directly representative of the presence of the bacterial capsule (overestimated by ~ 20 nm due to the size of antibodies) and the variation of the bacterial capsule thickness can be investigated.
The bacterial surface can also be mapped using atomic force microscopy (AFM) with specific probes and cantilever stiffnesses. Hence, a smooth surface across the bacteria has been observed (Figure 1.2d) [44]. As an assumption, AFM is unable to directly investigate the roughness of an inhomogeneous capsule. The force on capsular brushes is around 10-100 pN, so the variation in its value is too small to be measured in hydrated conditions [49,50].

Techniques can investigate the heterogeneity of capsular polysaccharides which depends on salt concentration and surface curvature. pH [51] and external forces [52] can also play a role. As an intrinsic characteristic of the polymer, the source of polydispersity is due to the biosynthesis pathway in the bacteria. Thus, molecular expression and gene regulation also influence the heterogeneity of polyelectrolyte chains in the capsule.

1.3 The size distribution of capsular polysaccharide chains controlled by molecular manipulation of K1 capsular biosynthesis

1.3.1 The regulation of group 2 capsular gene transcription due to thermoregulation at 37°C

Although the serotypes of capsules depend on the E. coli strain chosen, the gene clusters controlling capsule regulation are held in common and can be categorised. The capsules have been classified into 4 groups [14,15] based on their physical properties, including thermostability, gene cluster organisation and the polymerization system used.

K1 capsular polysaccharides are categorised as group 2 capsule (CPS) due to their thermoregulation where proteins involved in CPS production are expressed at 37°C (no capsule synthesis under 20°C) and the existence of an ATP-binding-cassette (ABC) transport
system [53] (*KpsM* and *KpsT*). On the contrary, group 1 and group 4 capsules depend on flippase proteins [15] for polysaccharide translocation (e.g. *Wzx* and *Wzy*). Group 3 capsule is similar to group 2 capsule, although it has no temperature regulation [15].

The organisation of gene clusters in group 2 capsule are coincident in 3 regions (Figure 1.3a) which are located near the serA locus in a wide variety of different bacterial strains, e.g. K1, K4, K5, K7, K12 and K92 [15]. To initiate transcription from the CPS clusters, two main promoters PR1 (for region 1) and PR3 (for region 3 and region 2 with reverse transcription of the gene order) control the regulation of protein expression and capsular biosynthesis coping with global regulators [15, 54, 55] including H-NS (histone-like nucleoid structuring protein), SlyA, IHF (integrated host factor) and RfaH (anti-terminator for a long transcription). For activation and repression of DNA transcription, the regulators work in synchrony at 37°C. As shown in Figure 1.3a, IHF proteins are used to activate transcription from PR1 (a tandem model called PR1-1). In addition, the model suggests tandem promoters (PR1-2 and PR1-3) also are repressed by IHF due to DNA conformation changes after IHF binding at +140 [55]. RfaH proteins proceed transcription from PR3 into region 2, and BipA increases the efficiency of transcription in conjunction with activating proteins which are similar to SlyA proteins. H-NS proteins activate the transcription at 37°C, but repress the transcription at 20°C [15]. The regulators affect the efficiency of transcription and capsular polysaccharide production. Consequently, this is one of the causes of polydispersity of capsular polysaccharides from a molecular perspective.
Figure 1.3 a) The regulation of transcription in the CPS cluster due to global regulators [55], b) Polymerization of a capsular (polysialic) polysaccharide chain involving several steps from Kdo binding to a lyso-phosphatidyl glycerol (lyso-PG) lipid, then conjugation with sialic acid monomers, before extension to become a high MW capsular polysaccharide chain (N ~200-400) [56]. c) Processive and distributive polymerisation (*in vitro*) for chain elongation with different distributions and polydispersities [57]. Neu5Ac (Sialic acid) is successively transferred from CMP-Neu5Ac onto a substrate (DMB-DP3).
1.3.2 E. coli group 2 capsular biosynthesis at 37°C

For the group 2 capsular biosynthesis pathway (Figure 1.3b), the CPS cluster contains important genes involved in polysialic (PSA) biosynthesis, modification and transportation. The CPS cluster includes kpsFEDUCS (region 1 for modification and transportation), neuDBACES (region 2 for biosynthesis, activation and polymerisation) and kpsMT (region 3 for ABC transportation) [6,42].

Several steps are required for lyso-PG glycosylation, including polysaccharide polymerisation and transportation. To initiate a new capsular polysaccharide chain anchored on the bacterial membrane, KpsS proteins utilise CMP-Kdo (3-deoxy-D-manno-oct-2-ulosonic acid) to phosphorylate a glycerol moiety in the lyso-PG and then extend it a few units with β-(2-4) and β-(2-7) linked Kdo units by KpsC (with C-N complexes) [58,59]. After the Kdo units conjugate with lyso-PG, an initial Neu5Ac unit nucleates on the Kdo residue using NeuE (bound to the inner membrane), and then the polysaccharide chain is extended by NeuS (polysialyltransferase), utilising mobile CMP-Neu5Ac units [42,56,60]. NeuS also works as a repressor of NeuE expression [6]. NeuS has a key role in extension for group 2 capsular biosynthesis. The mechanism of NeuS (GT38 family) controls the polydispersity of capsular polysaccharide chains [56]. Once a polysaccharide chain is fully synthesized, at around N ~ 200-400 (the degree of polymerisation), the lipid-linked capsular polysaccharide is translocated to the bacterial surface via the ABC transporter complex, KpsDE (combined with PCP3 and OPX proteins) and KpsMT, mainly driven by KpsMT which uses ATP [53,61]. The heterogeneity from a molecular level is due to gene regulation and biosynthesis, especially the action of glycosyltransferase, e.g. NeuS. The mechanism
and dynamics of NeuS on Neu5Ac polymerisation has experienced only a few studies [56,60,62,63].

1.3.3 The size regulation of capsular polysaccharides

Polysaccharide polymerisation is known to be regulated by polysaccharide copolymerase (PCP) proteins [64]. These proteins contribute to the modality of polysaccharide chain size in lipopolysaccharide (LPS) chains when combined with O-antigen polysaccharide (OPS) elements [13,65,66]. However, it is different in group 2 of CPS which have the ATP Binding Cassette (ABC) transport system. This ABC system requires ATP in order to translocate and synthesize polysaccharides. The polydispersity of polysaccharide production in ABC transport system is still unclear.

For Wzy-flippase dependence, the polysaccharide chain is synthesised before it ligates (using WaaL proteins) to lipid A in the periplasm [13]. The regulator proteins Wzz (PCP-1 protein) and Wzc (PCP-2a proteins) are used to control the chain length of the polysaccharides [67]. Due to the stochastic mechanism, the probability of termination increases when the size of the polysaccharide chain is longer. Although there are several theories a conclusive model about how polydispersity is controlled does not exist, although the complex PCP system has an important role in limiting the size of this system.

For the ABC transporter (KpsMT) dependence, the polysaccharide chain is synthesised on lyso-PG phospholipid [59]. To compare with OPS biosynthesis which requires a ligation step, CPS biosynthesis also requires a nascent polysaccharide chain polymerised by glycosyltransferase (GT) enzymes inside the cytoplasm before it is transported outside via the ABC transporter channel. There is no specific terminator in the
system despite the mechanism of the PST enzymes (e.g. *KpsC* and *NeuS*) [56,59]. Recently, the crystal structure of GTs (polysialyltransferase, PST) involved in K1 capsular biosynthesis has been characterised and the active sites have been determined. The sites control the switching mechanism of polysaccharide chain extension [56]. However, it is unknown whether a molecular clock controls the size of the polysaccharide chains [65,68].

The process of chain extension is due to both distributive and processive polymerisation [57] (**Figure 1.3c**). Distributive polymerisation depends on the ratio of donor and acceptor substrates. The size distribution of the polysaccharide can be described with a Poisson distribution due to the simple kinetic process involved (a single enzyme). The processive polymerisation requires a strong enzyme affinity to extend a long polysaccharide chain with multiple rounds of interaction with enzymes and substrates. Inside polysialyltransferase (PST), specific regions (e.g. K69 from NmBPST and MhPST) work as a molecular switch to control the size [56]. With its mutation, the processive polymerisation mechanism can be transformed into a distributive polymerisation. Several *in vitro* experiments observed the distributive mechanism in an abundant enzyme-substrate environment; however, *in vivo* studies observed the reduced concentration of local substrates (lyso-PG-sugars) due to lyso-PG anchored in the inner membrane and this causes the variation of polysaccharide size [56].

To investigate size regulation, the dynamics of glycotransferase proteins can be studied. With the processive mechanism in polysaccharide production of group 2 capsular biosynthesis, the time period that *NeuS* interacts with the substrate to elongate the capsular polysaccharide chain (with several rounds of CMP-Neu5Ac donors) can be investigated.
The size regulation of processive polymerisation (where there is no specific terminator to control the chain size) of the capsular polysaccharide as a source of polydispersity can thus be directly investigated. Because of the rapid dynamics of bacterial enzymes in the polymerisation process, fluorescence correlation spectroscopy (FCS) is a potential tool to directly investigate the mechanism involved.

The heterogeneity of polysaccharide chains is produced by stochastic mechanisms of gene regulation, proteins expression and biosynthesis (e.g. NeuS enzymes involved in chain extension of the capsular polysaccharide). This heterogeneity influences the pathogenicity of the encapsulated bacteria [16,70].

1.4 Challenging aspects of bacterial capsule studies and the thesis outline

1.4.1 Challenging aspects of bacterial capsule studies

Problems central to the biological-physics of capsules include: how is the heterogeneity of K1 capsule controlled (Chapter 3); how does the internal biosynthesis of K1 production work investigated via NeuSHT dynamics (Chapter 4); and how does the capsule protect the bacteria from short peptide invasion (Chapter 5)? The thesis is constructed around these intriguing questions.
Figure 1.4 Scope of the bacterial capsule studies investigated in the thesis: a) the heterogeneity of bacterial capsules, b) molecular dynamics in capsular biosynthesis and c) the ability of bacteria to protect themselves against invading substances.

1.4.2 Thesis outline

A brief introduction about bacterial capsular polysaccharides is summarized in this chapter 1, including polysaccharide chain properties (electrostatic stabilization) [26,27] and molecular controls of regulation and biosynthesis [15,54,55]. These pioneering studies led to a further investigation of bacterial capsule morphology from a phenotypic perspective and the dynamics of proteins involved in capsular biosynthesis.

The study of K1 bacterial capsule required genetic modification methods and several tools for characterization including fluorescence microscopy such as wide-field, confocal
and super-resolution microscopies; and atomic force microscopy for nanomechanical force studies. All methods used in the experiments are discussed in chapter 2.

There are 3 main key questions about the bacterial capsule which form the skeleton of this thesis (Figure 1.4). The application of bacterial capsule to prevent antimicrobial peptide is also considered. Firstly, in chapter 3, the main question is how heterogeneity of capsular polysaccharide occurs after they had been transported to the bacterial surface via an ABC transport system. Hence, the investigations used characterization techniques to directly visualize and investigate the bacterial capsule from nucleation (to approximate the rate of bacterial capsule coverage after temperature upshifting) to fully covered (to investigate the bimodal thickness distribution of bacterial capsules). Secondly, in addition to the external investigation, the dynamics of the polymerase (e.g. NeuS) has been investigated inside bacterial cells using confocal microscopy with fluorescent correlation spectroscopy (FCS). Chapter 4 demonstrates and characterizes the rapid moving enzyme NeuS tagged with a Halotag. Hence, the specific time domains related to the diffusion and the kinetics of the polymerase enzyme unveil the dynamics of single molecules in the capsular biosynthesis process. Finally, the heterogeneity of capsular polysaccharide thickness is thought to relate to the ability of the encapsulated bacteria to survive harsh environments. One harsh environment is during treatment with antibacterial substances, e.g. antimicrobial peptides. In chapter 5, the assumption that the bacterial capsule provides a completely protective layer preventing alien antimicrobial substances to interact with the bacterial membrane has been tested. The investigation was done using super-resolution microscopy to observe the antibacterial peptides (e.g. G3 peptides). With the super-
resolution technique, the location of peptide aggregation contradicts previous models that the bacterial capsule shields the bacterial surface from invading peptides [71,72].

To conclude all the studies, the final chapter (chapter 6) summarizes all investigations that have been presented in previous chapters and describe specific conditions and problems with bacterial capsule investigations of single-molecules and single-cell. The potential for further studies on bacterial capsules will also be considered.

1.5 References


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CHAPTER 2

Methodology

There were three main sections of methodology required for the experiments presented in this thesis: microbiological techniques (including genetic modification), fluorescence techniques [1,2] (including super-resolution microscopy and fluorescence correlation spectroscopy [3,4]) and atomic force microscopy (AFM) [5,6].

2.1 Microbiological methods for genetic modification

2.1.1 Bacterial strains and bacterial cultivation

In the study, several strains of bacteria have been used. The bacterial cells used in phenotypical investigations of bacterial capsule growth, genetic modification to create the Halotag labelling proteins and fluorescence investigations of modified proteins are listed in Table 2.1.

Table 2.1 List of E. coli strains and plasmid-transformed strains used during the study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Antibiotic resistance (Table 2.2)</th>
<th>Source/References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F-, Φ80 ΔlacZD M15, recA1, endA1, gyrA96,</td>
<td>N/A</td>
<td>(Hanahan et al., 1983) [7]</td>
</tr>
<tr>
<td></td>
<td>Characteristics</td>
<td>Source</td>
<td>References</td>
</tr>
<tr>
<td>---</td>
<td>-------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>UTI89</td>
<td>A cystitis-derived isolate of serotype O18:K1:H7</td>
<td>N/A</td>
<td>(Cusumano et al., 2010) [8]</td>
</tr>
<tr>
<td>MS101</td>
<td>PA360 (from E. coli K-12) serA+ K5+</td>
<td>N/A</td>
<td>(Stevens et al., 1994) [9]</td>
</tr>
<tr>
<td>MS101ΔE</td>
<td>MS101 ΔkpsE</td>
<td>Strep50</td>
<td>(Hudson et al, 2009) [10]</td>
</tr>
<tr>
<td>MS101ΔE kpsEdsREDpB33</td>
<td>MS101ΔE (kpsEdsREDpB33)</td>
<td>Strep50 and Cm34</td>
<td>de novo, this study</td>
</tr>
<tr>
<td>EV1</td>
<td>PA360 (kps+ serA+ malA+)</td>
<td>N/A</td>
<td>Ian Roberts [11]</td>
</tr>
<tr>
<td>PA360</td>
<td>EV1 with capsule deficiency</td>
<td>Strep50</td>
<td>Ian Roberts [12]</td>
</tr>
<tr>
<td>EV36</td>
<td>K-12/K1 hybrid (phage K1 positive)</td>
<td>N/A</td>
<td>Eric Vimr [13]</td>
</tr>
<tr>
<td>EV36pCOC2</td>
<td>EV136 (pCOC2)</td>
<td>Amp100</td>
<td>de novo, this study</td>
</tr>
<tr>
<td>EV136</td>
<td>EV36 neuS::tet (phage K1 negative)</td>
<td>Tet10</td>
<td>Eric Vimr [13]</td>
</tr>
<tr>
<td>EV136neuSpB33</td>
<td>EV136 (NeuS::Halotag pBAD33)</td>
<td>Tet10 and Cm34</td>
<td>de novo, this study</td>
</tr>
<tr>
<td>EV136neuSpBSK +</td>
<td>EV136 (NeuS::Halotag pBSK II+)</td>
<td>Tet10 and Amp100</td>
<td>de novo, this study</td>
</tr>
<tr>
<td>EV136neuSpHSK +kpsEdsREDpB33</td>
<td>EV136 (neuSpHSK+ &amp; kpsEdsREDpB33)</td>
<td>Tet10, Amp100, and Cm34</td>
<td>de novo, this study</td>
</tr>
</tbody>
</table>
The initial bacteria cultures were shaken at 200 rpm and grown in LB (Luria-Bertani) media [14] (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v sodium chloride (NaCl), supplemented with the appropriate antibiotic as required, Table 2.2). They were then incubated overnight at either 20°C or 37°C depending on the experiments.

**Table 2.2** List of antibiotics used during the study [14].

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Required Concentration (µg/ml)</th>
<th>Solvents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cm34</td>
<td>Chloramphenicol</td>
<td>34</td>
</tr>
<tr>
<td>Amp100</td>
<td>Ampicillin</td>
<td>100</td>
</tr>
<tr>
<td>Kan50</td>
<td>Kanamycin</td>
<td>50</td>
</tr>
<tr>
<td>Tet10</td>
<td>Tetracycline</td>
<td>10</td>
</tr>
<tr>
<td>Strep50</td>
<td>Streptomycin</td>
<td>50</td>
</tr>
<tr>
<td>Erm300</td>
<td>Erythromycin</td>
<td>300</td>
</tr>
</tbody>
</table>

For general genetic modification and morphological studies of bacterial capsules, after bacteria were initially grown overnight at 37°C (200 rpm shaking), the overnight culture was then 1:100 diluted into fresh media (Table 2.3) with LB or M9 media [15] supplement and the required antibiotics, amino acids and carbon-source for fluorescence imaging. The samples were then cultured (shaking at 37 °C) until they reached the exponential growth
stage (read out from OD<sub>600</sub> spectrometer). Cells were acquired by centrifuging and rehydration before their use in the experiments.

**Table 2.3** Buffer, culture media and agar recipes used in the study [14].

<table>
<thead>
<tr>
<th>LB media</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broth</td>
<td>For 200 ml, requires 2 g NaCl, 1 g yeast extract, and 2 g tryptone and antibiotic supplements.</td>
</tr>
<tr>
<td>1.5% Agar</td>
<td>For 200 ml, requires 2 g NaCl, 1 g yeast extract, 2 g tryptone, and 3 g agarose and antibiotic supplements.</td>
</tr>
<tr>
<td>ST agar</td>
<td>For 100 ml, requires 1 g NaCl, 0.5 g yeast extract, 1 g tryptone, 0.75 g agarose and antibiotic supplements.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M9 media</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x M9 salts buffer</td>
<td>In 1000 ml, requires 64 g Na&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt;.7H&lt;sub&gt;2&lt;/sub&gt;O (or Na&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt; 34 g), 15 g KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;, 2.5 g NaCl and 5 g NH&lt;sub&gt;4&lt;/sub&gt;Cl (autoclaved before stored at room temperature).</td>
</tr>
<tr>
<td>M9 Broth</td>
<td>For 50 ml, requires 5 µl 1 M CaCl&lt;sub&gt;2&lt;/sub&gt; (autoclaved), 10 ml 5X M9 salts buffer (autoclaved), 100 µl 1 M MgSO&lt;sub&gt;4&lt;/sub&gt;, 50 µl 10% w/v thiamine (filtered sterilised), 37.5 ml sterilised water, supplements of the carbon-source from (1 ml 20% w/v glucose/ 20% v/v glycerol, filter sterilised), amino acids (0.1% w/v casein) and antibiotics.</td>
</tr>
<tr>
<td>1% M9 agarose</td>
<td>Uses broth and mixes 1:1 with 2% w/v agarose. This was used for cell immobilisation and nutrient supplement in <em>NeuSHT</em> protein experiments.</td>
</tr>
<tr>
<td>1.5% M9 agarose</td>
<td>Uses broth and mixes 1:1 with 3% w/v agarose. This was used for cell immobilisation and nutrient supplement in <em>NeuSHT</em> protein experiments.</td>
</tr>
</tbody>
</table>

For a temperature upshift experiment (Chapter 3) for the evolution of the bacterial capsule, 20°C overnight growth in LB was specifically used. Then the culture was reinoculated in M9 media at 20°C. When the OD600 reached 0.3, 20 ml of cells were
harvested by centrifugation (3500 rpm for 15 minutes). The pellet was transferred to a fresh pre-warmed M9 media and then the temperature was up-shifted to 37 °C to induce capsule formation.

In addition to the aqueous media culture, strains were grown on LB agar (LB broth with 1.5% w/v agar), supplemented with the appropriate antibiotic and incubated at the static 37°C. For short term storage (no longer than 2 weeks), strains grown on LB agar were stored at 4°C. For long term storage, strains were stored as glycerol stocks at -80°C (900 μl of an overnight culture of the required strain was mixed with 600 μl of sterile 50% v/v glycerol).

Chemicals and reagents were from Sigma Aldrich and all premixed media (in addition to prepared media) were from Oxoid. Before use sterilisation was needed and reagents were autoclaved (120°C, 15 psi, for 20 minutes) or filtered through a 0.22 µm PES filter disc (Millipore).

2.1.2 Genetic modification procedures

Genetic modification is a common method in molecular biology [14–16]. Several techniques are involved in order to construct the designed proteins for the purpose of the investigation based on phenotypical variation, infection into host cells, control of expression level and fluorescence imaging dynamics of the labelled proteins.

In this study, most of the modified gene sequences were inserted into plasmids and the level of expression was controlled specifically depending on the types of the plasmid used. Then these plasmids were transformed to complement K1 capsule deficiency. All the modified plasmids and plasmid inserted strains used are listed in Tables 2.4 and 2.1, respectively.
### Table 2.4 List of plasmids used during the study.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description*</th>
<th>Reference/ Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBAD33 (pB33)</td>
<td>Arabinose-inducible cloning vector, 5.8 kb, Cm&lt;sup&gt;f&lt;/sup&gt;, Amp&lt;sup&gt;r&lt;/sup&gt;, p15a replication origin.</td>
<td>(Guzman et al., 1995) [17]</td>
</tr>
<tr>
<td>pBluescript II SK+ (pBSK+)</td>
<td>Multi-copy vector, MCS inside lacZ gene, 2.9 kb, Amp&lt;sup&gt;r&lt;/sup&gt;.</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pCoC2</td>
<td>Multi-copy vector, pBR322 origin, Amp&lt;sup&gt;r&lt;/sup&gt;, Kan&lt;sup&gt;r&lt;/sup&gt;.</td>
<td>(Corcoran et al., 2010) [18]</td>
</tr>
<tr>
<td>pJEBAN6</td>
<td>Contain dsRED gene.</td>
<td>Ian Roberts [19]</td>
</tr>
<tr>
<td>kpsEpB33</td>
<td>pBAD33 containing full-length kpsE cloned into XbaI and HindIII restriction sites.</td>
<td><em>de novo</em>, this study</td>
</tr>
<tr>
<td>kpsEdsREDpB33</td>
<td>kpsEpB33 containing full-length <em>dsRED</em> cloned into SacI and XbaI restrictive sites. Includes linker between kpsE and <em>dsRED</em>, and engineered RBS prior to the <em>dsRED</em> start codon.</td>
<td><em>de novo</em>, this study</td>
</tr>
<tr>
<td>pH6HTC</td>
<td>Full-length <em>Halotag</em> contained which allows C-terminal conjugation.</td>
<td>Promega</td>
</tr>
<tr>
<td>pH6HTN</td>
<td>Full-length <em>Halotag</em> contained which allows N-terminal conjugation.</td>
<td>Promega</td>
</tr>
<tr>
<td>pKT279</td>
<td>Full-length <em>neuS</em> contained in this plasmid.</td>
<td>Provided from Jane King</td>
</tr>
<tr>
<td>plasmid</td>
<td>description</td>
<td>origin</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>--------</td>
</tr>
<tr>
<td>neuSpH6HTC</td>
<td>pH6HTC containing full-length <em>neuS</em> cloned into pH6HTC into XbaI and SacI restriction sites.</td>
<td><em>de novo</em>, this study</td>
</tr>
<tr>
<td>neuSHTpB33</td>
<td>pBAD33 containing full length <em>neuS</em> conjugated with Halotag cloned into XbaI and HindII restriction sites. Includes engineered RBS prior to the neuSHT start codon.</td>
<td><em>de novo</em>, this study</td>
</tr>
<tr>
<td>neuSHTpBSK+</td>
<td>pBSK+ containing full length <em>neuS</em> conjugated with Halotag cloned into XbaI and HindII restriction sites. Includes engineered RBS prior to the neuSHT start codon.</td>
<td><em>de novo</em>, this study</td>
</tr>
</tbody>
</table>

*Cm r* is the chloramphenicol resistance, *Amp r* is the ampicillin resistance and *Kan r* is the Kanamycin resistance. The antibiotics used are shown in Table 2.2.

Using standard genetic modification protocols [14–16] (Figure 2.1), the kpsEdsREDpB33, neuSHTpB33, neuSHTpBSK+ plasmids were constructed and transformed into EV136 cells with a neuS deficiency (ΔneuS). They could then be used for the creation of proteins of interest (POIs), especially NeuSHT enzymes. There are 15 main steps from the initial cloning design to the final stage of protein expression.
**Figure 2.1** Genetic modification procedures for neuSHT cloning and expression from design to protein expression control. The back-loop arrows refer to diagnostic processes performed if problems occurred in each step from Step 1 to Step 15 [14–16].
2.1.2.1 The design of a DNA sequence for the protein of interest (POIs) using a DNA template and primers (Steps 1-3)

To create a protein construct (step 1), a DNA sequence is required. As a full DNA sequence resource, a gene bank from the NCBI website, which is a library of *E. coli* genes, was used. Although most DNA sequences were present, some additional sequences, e.g. SD site, digestive restriction sites, linkers, and start/stop codons, have to be included in sequence design to increase the efficiency of the protein transcription, translation and formation. To avoid self-digestion of the designed DNA template due to nuclease cleavage enzymes, an online application, a NEBcutter V2.0 or Webcutter V2.0 could be used to check internal restrictive sites. The complete designed DNA sequence should comprise of (1) the target DNA sequence (from 5’ to 3’), (2) restriction sites for digestion and plasmid insertion, (3) start (ATG)/stop (TAG, TAA or TGA) codons for initiation and termination of transcription, (4) plasmids (with specific restrictive sites) that control the level of protein expression and (5) additional components, e.g. linkers (GCC GCC GCC ) and ribosome binding sites (AGG AGG).

After the DNA sequence for the target proteins with all the additional active sites had been designed, the DNA template (step 2) from a cloning resource could be used with the specifically designed primers (step 3). The oligonucleotide primers were custom-made by Sigma-Aldrich. A list of primers used during this study is given in Table 2.5.
Table 2.5 List of primers used in this study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>DNA sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>kpsE-F (XbaI)</td>
<td>GCA TGA TCT AGA ATG TTA AAA GTG AAG TCT GCC</td>
</tr>
<tr>
<td>kpsE-R (HindIII)</td>
<td>TCG AGC AAG CTT TTA GTC TCG GTG ATC TTC AAC</td>
</tr>
<tr>
<td>DSred-F (SacI)</td>
<td>T CGA GCG AGC TCA AGG TGT GAG ATG GCC TCC</td>
</tr>
<tr>
<td>DSred-R (with XbaI and linkers)</td>
<td>TCG AGG TCT AGA GCC GCC GCC GCC GCC AGA GCC</td>
</tr>
<tr>
<td>DSred-R (with XbaI and linkers)</td>
<td>GCC GCC GCC CAG GAA CAG GTG GTG GCG GCC CTC GGC GCG</td>
</tr>
<tr>
<td>F-neuS (XbaI)</td>
<td>GCA CGA TCT AGA AGG AGG TGT GAG ATG ATA</td>
</tr>
<tr>
<td>R-neuS (SacI)</td>
<td>GCA TGA GAG CTC A CTC CCC CAA GAA AAT CCT TTT ATC GTG C</td>
</tr>
<tr>
<td>pH6HTC-MCS-F</td>
<td>TCG ATC CCG CGA AAT TAA TAC G</td>
</tr>
<tr>
<td>pH6HTC-MCS-R</td>
<td>AAC ATG CGG GAT GAT GTT GC GC</td>
</tr>
<tr>
<td>HaloT-pH6HTC-R (HindIII)</td>
<td>GTA CGA AAG CTT TTA ACC GGA AAT CTC CAG AGT AGA CAG CC</td>
</tr>
<tr>
<td>R-neuS_+200 (HindIII)</td>
<td>GAC TGC AAG CTT ATT TAT TGA TGA AGA CGC AGG TTA TTG GG</td>
</tr>
<tr>
<td>F-neuS+1129</td>
<td>GGA TTA ATA CGC TGC GTC TCC</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>F-neuSHTpB_SD</td>
<td>CTT GGG GGA GGG AGC TCT CGA</td>
</tr>
<tr>
<td>R-neuSHTpB_SD</td>
<td>AAA ATC CTT TTA TCG TGC AAA GAG GG</td>
</tr>
</tbody>
</table>

* Single underline indicates restrictive sites according to individual enzymes.

** Double underline indicates the linker sequence.

The DNA template was prepared using the plasmid extraction method with plasmid DNA purification [15] (or Qiagen kits’ instruction). Plasmid DNA concentration and purity were determined using a Nanodrop ND-1000 ultraviolet (UV) spectrophotometer (Labtech), reading at 260 nm. Alternatively, the DNA template could be used from bacterial nucleotide/plasmids inside bacterial cells from single colonies on solid media, e.g. grown on LB agar plate with selective antibiotics.

2.1.2.2 DNA template amplification by polymer chain reaction (PCR) (Steps 4-6)

Polymer chain reaction [15] is a technique that uses high temperature to denature a DNA template, then a lower temperature (due to primers) to anneal the DNA template with designed primers on 3’ complementary sites. Then the temperature is increased to allow thermostable Taq polymerase with excess dNTPs to extend the DNA. These steps are repeated for several cycles, e.g. 30 cycles, to amplify the specific DNA according to the chosen primers.

Using the polymer chain reaction (PCR) technique (step 4) with a DNA template, designed primers and reagents, the designed DNA fragments were amplified inside a thermal cycler machine (PxE 0.5 from Thermo Electron Corporation or Tc-412 from Techne) and a specific program of temperature and times was used in each cycle (Table 2.6). PCR
protocols and reagents are varied dependent on the condition of the primers and the DNA templates. The general PCR reagents used in the study are listed in **Table 2.7** for PCR cloning and screening from a single colony and the DNA templates used in aqueous solution.

**Table 2.6** List of the PCR programs used in the study.

<table>
<thead>
<tr>
<th>DNA target fragments</th>
<th>Original source</th>
<th>Forward/ reverse primers (Table 2.5)</th>
<th>Description*</th>
<th>No. cycles (T1-T3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>T0 °C (s)</td>
<td>T1 °C (s)</td>
</tr>
<tr>
<td>kpsE</td>
<td>UTI89</td>
<td>kpsE-F/kpsE-R</td>
<td>95 (300)</td>
<td>94 (60)</td>
</tr>
<tr>
<td>dsRED</td>
<td>pJBAN6</td>
<td>DSred-F/DSred-R</td>
<td>95 (300)</td>
<td>95 (60)</td>
</tr>
<tr>
<td>neuS</td>
<td>pKT279</td>
<td>F-nueS/R-neuS</td>
<td>95 (300)</td>
<td>95 (60)</td>
</tr>
<tr>
<td>neuSHT</td>
<td>neuSpH6 HTC</td>
<td>pH6HTC-MCS-F/pH6HTC-MCS-R</td>
<td>95 (300)</td>
<td>95 (60)</td>
</tr>
<tr>
<td>neuSHT_SDM (2.5kb)</td>
<td></td>
<td>F-neuSHTpB_SDM/F-neuSHTpB_SDM</td>
<td>95 (300)</td>
<td>95 (60)</td>
</tr>
</tbody>
</table>
where T0(s), T1(s), T2(s) and T3(s) are the initial denature, denature, annealing and extension temperatures, respectively, with the description showing the time used in each cycle for T1 to T3 in seconds. The annealing temperature depends on the melting temperature of the primers. The annealing times depend on the size of the DNA sequence, which follows the approximate rate: 500bps/30s.

Table 2.7 The reagents used in PCR for cloning and screening.

<table>
<thead>
<tr>
<th>PCR</th>
<th>Reagents used in PCR (for 1 unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA Template</td>
</tr>
<tr>
<td>Cloning PCR</td>
<td>1 colony/1 µl (100 ng)</td>
</tr>
<tr>
<td>Screening PCR</td>
<td>1 colony/1 µl (100 ng)</td>
</tr>
</tbody>
</table>

The PCR products were cleaned up using a Qiagen DNA purification kit (following Qiagen’s instruction) with a filter column (Step 5). To verify the PCR products, some of them (10 µl) were labelled with 6× DNA loading buffer (0.25% w/v xylene cyanol FFC, 0.25% w/v bromophenol blue, 15% v/v ficoll type 400) and loaded into 1% w/v agarose gels made up in TAE buffer (40mM Tris-acetate pH7.7, 1mM EDTA). The gels were added to 0.5 µg ml⁻¹ ethidium bromide, EtBr, (Sigma) before they set. The DNA samples loaded in the electrophoresis gels were run at 100V DC (Step 6) which drove amplified fragments of
DNA through the body of the agarose gels. The approximate size of the PCR products was calculated using comparison to a loaded ladder (7 µl) with a UV transilluminator (UVIpro Silver, UVItec).

2.1.2.3 Digestion and Ligation of DNA products (Steps 7-10)

After the size of the PCR products had been confirmed, the PCR product could be digested and conjugated into specific restrictive sites of the chosen plasmids. The chosen restrictive sites from the design process determine the restrictive enzymes used in the digestion step. In order to digest PCR products and plasmids in which the PCR clone is inserted, 1 µl of individually selected digestive enzymes (Table 2.8) were incubated with 1 µg of digested DNA in CutSmart buffer (NEB) or any other suitable buffers (according to the endonuclease cleavage enzymes) and water was added for a 30 µl total volume in a 37°C water bath for 90 minutes (Step 7). To check the digested DNA, electrophoresis of the loaded DNA was used (Step 8).

Table 2.8 A list of digestion and ligation products, plasmids, enzymes and regions used in the experiments.

<table>
<thead>
<tr>
<th>Inserting products</th>
<th>Inserted plasmids</th>
<th>Restrictive enzymes</th>
<th>Ligating region</th>
</tr>
</thead>
<tbody>
<tr>
<td>kpsE</td>
<td>pBAD33</td>
<td>XbaI/HindIII</td>
<td>MCS of pBAD33</td>
</tr>
<tr>
<td>dsRED</td>
<td>kpsEpBAD33</td>
<td>SacI/XbaI</td>
<td>5’ before kpsE in kpsEpBAD33</td>
</tr>
<tr>
<td>neuS</td>
<td>pH6HTC</td>
<td>XbaI/SacI</td>
<td>MCS of pH6HTC</td>
</tr>
<tr>
<td>neuSHT</td>
<td>pBAD33</td>
<td>XbaI/HindIII</td>
<td>MCS of pBAD33</td>
</tr>
<tr>
<td>neuSHT</td>
<td>pBluescript II SK+</td>
<td>XbaI/HindIII</td>
<td>MCS of pBSK+</td>
</tr>
</tbody>
</table>
After the cut DNA products (both digested plasmids and cleaved PCR products, Table 2.8) were confirmed from electrophoresis experiments and cleaned up using Qiagen kits (following their instruction), ligation reactions (Step 9) were performed in a total volume of 15 μl per reaction. 1 μl of T4 DNA ligase (Roche Diagnostics), 1.5 μl of 10x ligase buffer (Roche Diagnostics) and a 1:3 ratio of digested plasmids to digested PCR products were used. The concentration ratio of plasmids and PCR products could be deduced from the results of prior gel electrophoresis. The ligations were set at 10 °C overnight. 2 μl of ligated DNA products were checked via gel electrophoresis for a smeared band corresponding to variations in the DNA conformations (Step 10). The rest of the ligated DNA products were then used for Ca^{2+} transformation into chemically competent cells, e.g. Dh5α, to preserve the engineered DNA products.

2.1.2.4 Transformation of the DNA products (Steps 11-13)

The competent cells used in Ca^{2+} transformation and electro-transformation of DNA products (in plasmids) were differently prepared for the efficient uptake of DNA. The Ca^{2+} competent cells for chemical transformation were prepared from a 1:100 dilution overnight with the target hosts, e.g. DH5α, into 100 ml of LB. The reinoculated cells were cultured at 37°C, 200 rpm, until OD_{600} = 0.4 in the early exponential phase of growth. After cells were incubated on ice for 10 minutes and pelleted from 3000 g spinning for 15 minutes at 4°C, the pelleted cells were resuspended in 25 ml (ice-cold) transformation buffer 1 (30 mM potassium acetate, 10 mM rubidium chloride (RbCl), 20 mM calcium chloride (CaCl₂), 50 mM manganese chloride, 15% v/v glycerol, pH 5.8), then incubated on ice for 10 minutes. Again, cells were pelleted after centrifugation for 10 minutes (3000 g) at 4°C before the
pellet was resuspended in 1 ml (ice-cold) transformation buffer 2 (10 mM 3-(N-morpholino)propane sulfonic acid (MOPS), 150 mM CaCl₂, 10 mM RbCl₂, 15% v/v glycerol pH 6.5). Afterwards, it was stored on ice for 2 to 3 hours, the chemically competent cells were placed in 100 µl aliquots using 1 ml Eppendorf for immediate usage or snap-frozen using liquid nitrogen and stored at -80°C.

To perform a chemical transformation (Step 11), the prepared Ca²⁺ competent cells contained in an Eppendorf, e.g. Dh5α, were thawed on ice and mixed with the ligation mixture (5 µl or all product after electrophoresis) using at least 100 ng of plasmid DNA. Then it was left on ice for 10 minutes, heat-shocked at 42°C for 60 seconds and returned to the ice for 5 minutes. The transformed cells were recovered by adding 900 µl of pre-warmed LB broth and incubating them at the appropriate temperature (e.g. 37°C, 200 rpm) for no longer than 40 minutes. The cells were plated onto the appropriate selective solid medium with antibiotic supplements, left to dry, and were statically incubated overnight at 37°C. The colonies that emerged from solid agarose plates were then used in a screening step. The screening (Step 12) for successful cloning was initially investigated using a colony PCR test (Step 12.1) and a specific nuclease cleavage test (Step 12.2). The colony PCR test (Step 12.1) assumes that the colonies emerged on a selective agar plate are originally from individual mutated cells. The emergent colonies from overnight transformed plates were copied by patching them into fresh agarose plates and labelled for further usage. A tiny portion of the individual colonies were picked up and mixed with reagents (Table 2.7) to perform colony PCR and the PCR programs for each specific test in this study are shown in Table 2.6.
In addition, a specific nuclease cleavage test or a restrictive enzyme digestion test (Step 12.2) could be used for the identification of the potential, transformed clone. The emergent colonies from overnight transformed plates were copied by patching into fresh agarose plates and labelled for further usage after overnight culture in suitable media for later plasmid purification. After plasmids from individual labelled, selected colonies were acquired from Qiagen’s kits, the restriction enzymes (Table 2.8) were used for size quantification via an electrophoresis experiment.

After the potential colonies were found, the purified plasmids that represented individual colonies were sent for sequencing (Step 12.3) using an ABI PRISM 3100 Genetic Analyzer DNA sequencer (Applied Biosystems) by the University of Manchester’s Faculty of Life Sciences DNA Sequencing Core Facility. The sequenced samples were a mixture between sequencing DNA (e.g. potential plasmids) and 1:10 sequencing primers. To verify the mutant, the sequencing primers can be efficiently performed within 700bps. Hence, sequencing primers need to be designed to complete the full sequence of the original design template. The sequencing results were a 100% match to the designed template. Nucleotide BLAST application from NCBI can also be used to check the mutation that occurs.

However, when mistakes were observed in the sequence due to a mutation or slight design problem, site-direct mutagenesis (SDM, Step 12.4) could be used to alter small numbers of sequence mismatches for DNA sequence correction. Site-directed mutagenesis (SDM) was performed according to the Q5® Site-Directed Mutagenesis method (BioLabs). Forward and reverse primers coding for the point mutations were designed and used for PCR-based DNA manipulation, e.g. Table 2.5 for F-neuSHTpB_SDM and R-
neuSHTpB_SDM and the program used in Table 2.6. After the SDM step, the SDM products were checked for the potential adjustments via Steps 10–12 and confirmed via complete sequencing with Step 12.3.

After the correct sequence (a 100% match with the designed template) was confirmed, the successful clone inside the reservoir/hosts (e.g. DH5α) was stored in glycerol at -80°C (900 μl of overnight culture from a labelling copied plate was mixed with 600 μl of sterile 50% v/v glycerol). The cloned plasmids were purified via plasmid purification process (Qiagen’s) and then electro-transformed into prepared electro-competent cells (Step 13).

The electro-competent cells were prepared from an overnight culture of the desired strain, which was diluted 1:100 into 50 ml fresh LB and grown at 37°C, 200 rpm shaking, until the OD₆₀₀ reached 0.5. To be harvested, the cells were kept on ice for 15 to 30 minutes and then pelleted by cold centrifugation at 4000 g (3500 rpm) for 20 minutes at 4°C. The pellet was resuspended in 50 ml of ice-cold sterile distilled water and centrifuged at 4000 g for 20 minutes. It was then resuspended in 25 ml ice-cold sterile water and cold recentrifuged at 4000 g for 15 minutes before it was resuspended in 800 μl ice-cooled 10% glycerol in a 1ml Eppendorf. The mixture was again centrifugated at 3500 rpm for 5 minutes before the pellet was again resuspended with 100 μl ice-cooled 10% glycerol. The suspension contained electro-competent cells and was aliquoted in 50 μl quantities in 1 ml Eppendorf. The electrocompetent cells in aliquots could be snap-frozen using liquid nitrogen and stored at -80°C or immediately used for electro-transformation.
To perform electro-transformation (Step 13), the thawed aliquot of electrocompetent cells (50 μl) was mixed with 1-2 μl of the confirmed plasmid DNA in an electroporation cuvette (Bio-Rad). The electrocompetent cells are non-encapsulated. Hence, the successful transformation can be verified from capsular production after transformation with plasmids, testing using immune-fluorescence and phage K1 recognition methods. The electro-competent cells were electroporated using a Gene Pulser apparatus at 2.5 kV, 200 Ω and 25 μF. After the apparatus reached the characteristic time of capacitance at around 4.84 ms, the cuvette was immediately replenished with 1 ml of fresh LB before the suspension was transferred to 1 ml Eppendorf and incubated with 200 rpm shaking at 37°C for 1 hr.

After the shaking incubation step, 200 µl of cell suspension was plated on antibiotic specific agar plates for further selection. Since these plasmids had been confirmed via the sequencing step, the successful electro-transformed colony would inherit the original correct plasmids. To prove the complementary plasmid had been inserted and successful expression of the designed proteins, a K1-specific bacteriophage sensitivity assay (Step 14) and a protein expression test (Step 15) were used for verification.

2.1.2.5 Complementary test (Steps 14-15)

2.1.2.5.1 K1-specific bacteriophage sensitivity assay (Step 14)

A bacteriophage is a lytic coliphage with a double-stranded DNA genome, an icosahedral head, a base plate and tail spikes. The K1 phage degrades the K1 capsule using its tail spike protein, the lysate KflA, in order to expose phage receptors on the outer membrane of the *E. coli*. To test the K1-capsule complement strains, they were screened for the presence of K1 polysaccharide capsule using the K1-specific bacteriophage [20].
For the K1-bacteriophage sensitivity assay, overnight cultures were diluted 1:100 in fresh sterile 10 ml LB broth and grown at 37°C (200 rpm shaking) until they reached \( \text{OD}_{600} = 0.5 \), in the mid-log growth phase. Cells were collected by centrifugation at 3500 rpm for 10 minutes and the pellet was resuspended in 1 ml of ice-cold sterile 10 mM magnesium sulphate (MgSO\(_4\)) and stored on ice.

K1 bacteriophage dilution series from \( 10^{-4} \) to \( 10^{-8} \) were made up in sterile phage dilution buffer (100 mM NaCl (0.58 g), 8 mM MgSO4 (0.2 g), 50 mM Tris (5 ml 1 M Tris pH 7.5), 0.01 % v/v gelatine (0.5 ml 2% w/v gelatin) in 100 ml) and stored on ice. For each dilution, 100 μl of phage dilution was mixed with 100 μl of cell suspension and incubated at room temperature for 30 minutes. Before the incubation finished, 25 ml of agar plates with specific antibiotics and 3 ml ST agar (Table 2.3) with antibiotics (separate falcon tubes that contained 3 ml ST agar stored in a 40°C water bath to prevent solidification) were prepared. The incubated cell-K1 phage mixture was combined with 3 ml prepared soft-top (ST) agar, then plated on warmed, solid media plates and incubated overnight at 37°C.

2.1.2.5.2 Protein expression (Step 15): induction and quantification

To express proteins of interest from different plasmids requires different inducing reagents. For example, in pBAD33 plasmids, higher arabinose concentration caused higher proteins concentration to be produced by the P\text{BAD} promoters. In pBSK+ plasmids, proteins were expressed when they were induced by a certain concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG) according to P\text{lacZ} promoters. The level of protein expression was then quantified from a Comassive/Bradford protein assay, SDS-PAGE, Western blot (Step 15.1), and fluorescence techniques (Step 15.2).
2.1.2.5.2a Approximate protein concentrations using Comassive/Bradford assay (Step 15.1)

Protein concentration was determined using the Bradford protein assay. Serial dilutions of standard bovine serum albumin (BSA) solutions were prepared in H₂O (Table 2.9). Before mixing a Bradford reagent (Sigma, at room temperature) with test samples, diluted (e.g. 1:100) test samples and standardised series were loaded (5 µl) into 96 well plates, then the standard dilution series and samples were well mixed (without any bubbles) with 250 µl of Bradford reagent (Sigma). Mixtures were incubated for 10 minutes at room temperature (RT). Absorbance readouts at 595 nm could be converted to protein concentrations when compared with standardised BSA concentrations.

Table 2.9 List of chemical reagents used in the Bradford Assay.

<table>
<thead>
<tr>
<th>Tube</th>
<th>H₂O (µl)</th>
<th>[BSA] (mg/ml)</th>
<th>Tube</th>
<th>H₂O (µl)</th>
<th>[BSA] (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>/proteins (µl)</td>
<td></td>
<td></td>
<td>/proteins (µl)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0/300 (stock)</td>
<td>2</td>
<td>F</td>
<td>325/325 (tube E)</td>
<td>0.25</td>
</tr>
<tr>
<td>B</td>
<td>125/375 (stock)</td>
<td>1.5</td>
<td>G</td>
<td>325/325 (tube F)</td>
<td>0.125</td>
</tr>
<tr>
<td>C</td>
<td>325/325 (stock)</td>
<td>1</td>
<td>H</td>
<td>400/100 (tube G)</td>
<td>0.025</td>
</tr>
<tr>
<td>D</td>
<td>175/175 (tube B)</td>
<td>0.75</td>
<td>I</td>
<td>400/0</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>325/325 (tube C)</td>
<td>0.5</td>
<td></td>
<td></td>
<td>BSA stock*</td>
</tr>
</tbody>
</table>

* Stored at -20°C and stock solution is made of 2 mg/ml of BSA in sterile water.

** If necessary, the cells could be lysed by adding 0.01% SDS.
2.1.2.5.2b SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Step 15.1)

To visualise total protein expression, Tris-glycine-SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to their sizes and conformations. **Table 2.10** shows the various compositions for making resolving gels and stacking gels used during the study. Alternatively, precast 12% acrylamide NuPAGE Bis-Tris gels (ThermoFisher) could be used for the SDS-PAGE with specific NuPAGE buffers: running buffer and membrane transfer buffer. After the SDS-PAGE gels were prepared, protein samples were denatured by resuspension in 4x SDS-loading buffer with 14 µl β-Mercapthethanol (β-ME) (**Table 2.10**) and boiling for 7-10 minutes. After the total concentration of proteins was calculated from Bradford assays, denatured proteins were loaded into prepared SDS-PAGE gels. Their loading volumes were adjusted to balance the total level of proteins. Protein markers (Bio-Rad) formed a ladder and were run alongside the samples to indicate molecular weights of test protein samples.
Table 2.10 List of chemicals used in SDS pages and Western blots (WB) [14].

<table>
<thead>
<tr>
<th></th>
<th>SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels</th>
<th>Other reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resolving gels (12% v/v acrylamide gels)</td>
<td>4X SDS loading buffer (5ml), later adding β-ME in 1ml</td>
</tr>
<tr>
<td></td>
<td>Stacking gels (5% v/v acrylamide gels)</td>
<td>WB transfer buffer (250ml)</td>
</tr>
<tr>
<td>Components</td>
<td>Final volume 5ml (ml)</td>
<td>Components Final volume 2ml (ml)</td>
</tr>
<tr>
<td>H₂O</td>
<td>1.6</td>
<td>H₂O 1.4</td>
</tr>
<tr>
<td>30% acrylamide mix*</td>
<td>2.0</td>
<td>0.1 ml 1% w/v Bromophenol blue in water</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>1.3</td>
<td>0.73 g Glycine</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
<td>4 ml glycerol</td>
</tr>
<tr>
<td>10% ammonium persulfate (APS)</td>
<td>0.05</td>
<td>50 ml Abs. methanol</td>
</tr>
<tr>
<td>TEMED (Bio-Rad)</td>
<td>0.002</td>
<td>Stock prepped and aliquoted before mixed with β-ME in 1 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 µl β-Mercapthethanol</td>
</tr>
</tbody>
</table>

* Carcinogenic, highly toxic, requires a fume hood.
When self-prepared gels are chosen (with stacking and resolving gels), proteins samples are electrophoresed in an SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% w/v SDS) at 80 V for 30 minutes and at 180 V for the remaining time (e.g. 1.50 hrs). However, when precast SDS-PAGE gels were used, protein samples were electrophoresed in MES running buffer (ThermoFisher) at 100-130 V until complete (e.g. 1-2 hrs). SDS-PAGE gels were visualized by rocking-staining with Coomassie-based Instant Blue stain (Invitrogen) for 2 hrs and rocking-destained using distilled water at room temperature.

2.1.2.5.2c Western Blots (Step 15.1)

In order to specifically quantify the concentration of expressed proteins, antibodies could be used to recognise a specific protein of interest. Western blotting [21] is a technique that transfers unstained proteins separated using an electric field from SDS-PAGE gels to specific membranes (e.g. PVDF or Nitrocellulose membranes) for further investigation, e.g. antibody recognition.

Before blotting proteins onto PVDF membrane, the PVDF membrane was pre-soaked in absolute methanol for 2 minutes. After that the membrane, thick-layered papers (Whatman 3MM paper) and SDS-PAGE gels that contained separated proteins were soaked in Western blot transfer buffer (Table 2.10) for 10 minutes. Without any bubbles, the transfer layers (from bottom to top: a layered paper, the PVDF membrane, the SDS-PAGE gel and a layered paper) were placed onto a Semi-Dry Transfer Cell (Bio-Rad). Proteins were transferred at 15 V for 20-30 minutes. The membrane was then processed for either chemiluminescence or near-infrared fluorescence detection for visualisation and
quantification after it was stained with specific antibodies and reporters, e.g. chemiluminescent or fluorescent antibodies.

Important properties of monoclonal antibodies are their high selectivity and low noise generation due to low nonspecific binding. However, polyclonal antibodies create more background noise due to more nonspecific binding and can aggregate. The pre-absorption of nonspecific antibodies with Acetone Powders was used to remove cross-reacting nonspecific antibodies.

Acetone powders were prepared from target bacteria that contained the expressed proteins, e.g. EV136. 500 ml samples of an overnight culture of the cells were collected by centrifugation in a Sorval SLA-3000 swing-arm rotor at 4°C, 8000 g for 20 minutes. A pellet was resuspended in 25ml LB and vigorously mixed with 100 ml of acetone at -20°C before incubation on ice (0°C) for 30 minutes with occasional mixing. Then the pellets contained in polypropylene (PPCO) tubes were spun down using a Sorval SS-34 rotor at 10,000 g for 30 minutes. After the supernatant was discarded, the pellet was again resuspended and well mixed in 100 ml acetone at -20°C before incubation for 10 minutes on ice (0°C). After repeated centrifugation, the cell pellet was spread onto clean Whatman 3MM paper and left to dry at room temperature. The bacterial acetone powders, e.g. EV136 acetone powders, could be used to improve the efficiency of polyclonal antibody recognition. A few mg of the acetone powders were mixed with antibodies (high concentration) in 3% w/v bovine serum albumin (BSA) in PBS and incubated at 4°C for 30 minutes. The supernatant after centrifugation could be used as acetone-powder-treated antibodies.
When expressed proteins were transferred to the PVDF membrane via a Western blot process, the membrane could be labelled with monoclonal or acetone-powder-treated polyclonal antibodies. For the labelling process, the PVDF membrane was soaked in PBS-Tween-3% BSA (PBS, 0.1% v/v Tween-20 supplemented with 3% w/v BSA) overnight with rocking at 4°C in a cold room to provide protein blocking (Figure 4.2c).

2.1.2.5.2d Chemiluminescent detection for western blotting using a negative film printing system (Step 15.1)

After an overnight rocking of proteins on the PVDF membrane in the blocking buffer, the membrane was washed three times (included two times with PBS-Tween20 (PBS, 0.1% v/v Tween-20) and one time with PBS for 10 minutes in each wash before incubation with 10 ml of 3% BSA in PBS-Tween20 supplemented with the specific primary antibody for proteins cognition, e.g. rAb anti-Halotag antibody, 1:500. The membrane was incubated for 1 hour with rocking at room temperature or overnight with rocking at 4°C. After incubation, the membrane was washed three times as before and was then sealed in a bag containing 12 ml of 3% BSA in PBS-Tween20 with rAb anti-HRP antibody (e.g. 1:12000) as a secondary reporter for chemiluminescence. The membrane was incubated for 1 hour with rocking at room temperature before it was rocking-washed three times (including two times with PBS-Tween20 and one time with PBS) for 10 minutes in each wash.

After three washing steps, the membrane was incubated with a chemiluminescent western blot detection kit, SuperSignal West Femto (ThermoFisher) for 1-5 minutes. The membrane was then exposed to a light-sensitive film (Biomax XAR, Kodak) and developed
using an X-ray film printing technique. An example film result is shown in chapter 4, Figure 4.2b.

2.1.2.5.2e Near-infrared fluorescence detection of the western blot (Step 15.1)

As an alternative to negative film printing techniques, the antibodies labelled PVDF could be visualised using a NIR fluorescence machine. Using a different type of reporter antibody, milk-powder blocking buffers were used.

Instead of using 3% w/v BSA in PBS-Tween20 buffers as a blocking media for the chemiluminescent reporters, 5% w/v skinned milk powder was used as a substitute for overnight blocking. All washing and incubation steps avoided direct exposure to light by covering them with tin foil or placing them in a black box. In addition, the secondary antibodies used for NIR fluorescence detection [22] were different due to the specific primary antibody, e.g. rAb IgG IRDye antibody or mAb IgG IRDye antibody (Licor). Using an LI-COR blocking buffer with a PBS-Tween20 (1:1) for blocking Li-COR secondary antibodies helped improve the signal. After labelling with antibodies and washing, the membrane was scanned using the Odyssey® Infrared Imaging System (LI-COR), according to the manufacturer’s instructions.

2.1.2.5.2f Fluorescent antibody investigation using fluorescence microscopy and spectroscopy techniques (Step 15.2)

A variety of fluorescent secondary antibodies and fluorescent proteins (which can be conjugated directly to a protein of interest) are commercially available [23–26]. Using fluorescence microscopy and spectroscopy for direct visualisation of expressed proteins is a potential quantification method.
Proteins conjugated with *Halotag* enzymes, e.g. *NeuSHT*, were designed for multiple labelling with fluorescent ligands. The proteins could be visualised using fluorescence microscopy and fluorescence correlation spectroscopy to confirm the success of protein expression.

### 2.1.3 Self-labelling HaloTag enzymes

HALotag [23,27] is a novel self-labelling technique which can be used for multiple applications, such as protein purification and fluorescent tracking. Due to the small size of the Halotag enzyme (33kDa), this enzyme can be genetically conjugated to the protein of interest without any disruption of the original structure. To identify the presence of the Halotag-tagged proteins, Halotag ligands (which are commercially available [23] with several choices of dyes and markers) are covalently bonded using an alkyl-enzyme reaction. This was originally discovered from *Rhodococcus rhodochrous* mutants that lacked a haloalkane dehalogenase enzyme for hydrolysis [28].

### 2.1.4 HPLC (High-performance liquid chromatography) for size exclusion chromatography

HPLC [29] exploits the use of rapid, high-pressure size exclusion chromatography (SEC) coupled with a light scattering detector and a refractive index detector. HPLC is a standard technique to analyse polysaccharide molecular weight (Mw).

Light scattering in the Rayleigh regime is proportional to the product of the molecular weight (Mw) and the solute concentration (c) based on Zimm’s model. Hence, Zimm’s formula [30] is
where \( c \) is solution concentration, \( R(\theta, c) = \frac{I_{\text{scattered}}(\theta, c)}{I_{\text{incident}}(c)} \) (the ratio of scattered intensity \( I_{\text{scattered}} \) and incident intensity \( I_{\text{incident}} \)), \( \theta \) is the scattering angle, \( M_w \) is the molar mass weight average, \( P(\theta) \) is the angular dependence of the scattered light, \( A_2 \) is the second virial coefficient (a measure of weak protein-protein interactions), \( c \) is the concentration of the solute and the optical constant is defined as \( K^* = \frac{4\pi^2 (dn/dc)^2 n_0^2}{N_a \lambda_0^4} \), where \( dn/dc \) is the specific refractive increment at chemical equilibrium, \( N_a \) is Avogadro’s Number, \( \lambda_0 \) is the wavelength of the incident light, \( n_0 \) is the solvent refractive index, the flexible coil form factor is \( P(\theta) = \frac{2}{u^2} (e^{-u} - 1 + u); \ u = (4\pi/\lambda_0)^2 \langle r_g^2 \rangle \sin^2 \frac{\theta}{2} \). At small angles, the coil form factor can be simplified to \( \frac{1}{P(\theta)} = 1 + \left( \frac{16\pi^2}{3\lambda_0^4} \right) \langle r_g^2 \rangle \sin^2 \frac{\theta}{2} + \cdots \), where \( r_g \) is a radius of gyration. Using Zimm’s equation with the polynomial fit of \( \sin^2 \frac{\theta}{2} \) (from the expansion of \( \frac{1}{P(\theta)} \)), the MW is derived as a fit parameter and \( \langle r_g^2 \rangle \) can be obtained from the intercept and slope at zero scattering angle.

2.2 Fluorescence imaging techniques

2.2.1 Fluorescence and phosphorescence

Fluorescence microscopy is a common technique used to visualise and quantify fluorescent molecules that specifically label objects of interest, for example proteins [31], polysaccharides [32], and lipids [33] of the host cells [3,34]. The specificity comes from the choice of markers used. The fluorescence process depends on several factors in the
molecules, e.g. excitation/emission wavelength, fluorescence lifetime and quantum yield are key for fluorescence microscopy [35,36]. Due to the excitation of electron populations, fluorescence (radiation) and phosphorescence (delayed radiation) can be induced [37,38]. Mechanisms of radiative and non-radiative emission are shown in Figure 2.2. Although a polyatomic model is used to define the electronic states and the molecular orbital states of actual fluorescent molecules due to the involvement of NH₂ and CO chemical group, the mechanism of electronic translocation can be simplified using Jablonski’s diagram. As an example, Figure 2.2a and 2.2c show the energy states and electron population transition in the diatomic model including when photoactivable light and imaging buffer are used in a super-resolution experiment to recover the dark state in fluorophores and to extend the lifetime of a dark state for the benefit of localisation precision.

Figure 2.2 Jablonski diagram for the fluorescence of a diatomic molecule. a) Various electronic transitions of electron populations from the ground state and their
recombination in a fluorescent molecule, where S (singlet) states are the electronic states of paired electrons \( s = +1/2, -1/2 \) and then \( S = 0 \) and T (triplet) states are the electronic states of parallel electrons \( s = +1/2, +1/2 \) or \(-1/2, -1/2 \) and then \( S = 1 \) and each vibrational energy state relates to \( E_{vib} = (\nu + 1/2) \hbar \omega \) where \( \nu = 0, 1, 2, \ldots \). b) Photo-switchable mechanism of cyanine fluorophore with thiol incubation which can recovery from thiol-bound polymethine bridge formation by UV light [39]. c) Additional energy states when thiols are included in an imaging buffer due to the reduction and the formation of thiol-fluorescence radicals (F-).

The molecular structure of fluorescent molecules (from nature or synthetic) defines a hierarchy of energy states [40] due to different vibrational states \( \nu \) (including bonding and antibonding of \( \pi \)- and \( \sigma \)-electrons) inside different electronic states \( n \). Hence, fluorescence and phosphorescence occur when excited electrons relax from excited states to different pathways before the electrons arrive in the ground state. Fluorescence is radiation of excited electrons relaxing from the excited singlet state (in 1-100 ns) while phosphorescence is from cascades from the triplet states (T) to the ground state (\( S_0 \)). The relaxation time of phosphorescence can be varied from 0.1 ms to 1 s.

To classify the efficiency of fluorophores, fluorescent quantum yield \( (Q_Y) \) and life time \( (\tau_{fl}) \) can be used. The rate of electron population transitions in radiative \( (k_r) \) and non-radiative \( (k_{nr}) \), the quantum yield \( (Q_Y) \) and the fluorescence life time \( (\tau_{fl}) \) are defined as

\[
Q_Y = k_r \tau_{fl},
\]  

(2.1)
\[ \tau_{fl} = \frac{1}{k_r + k_{nr}}, \]  

(2.2)

where the rate of radiative transitions \( (k_r) \) is inversely proportional to the electron transition times of fluorescence (1-100 ns) and the non-radiative rate is comprised of all non-fluorescence transitional processes according to Figure 2.2a. This non-radiative transition is a key for dSTORM super-resolution imaging since it extends the time in the dark state.

Not only do incident photons cause excitation, but they can also be used to reactivate dark fluorophores that can be excited causing fluorescence, due to the release of the bound thiol from the fluorophore after reactivation with 405 nm [39], e.g. Cy5, Figure 2.2b. This recreates the energy states which can be excited and re-fluoresce. For dSTORM super-resolution imaging, fluorophores also require a blinking ability to switch between bright (fluorescence) and dark states (phosphorescence). To make fluorophores blink without bleaching, additive chemicals [41,42] are used including oxygen scavenger systems: glucose oxidase and catalase (to remove oxygen causing photobleaching) and \( \beta \)-mercaptoethanol (to quench electron populations in the triplet state using thiol molecules). With the thiol in an imaging buffer to form the thiol-radical complex, this extends the dark-state lifetime due to the creation of additional energy states, Figure 2.2c. The excited electron population transits via intersystem crossing (ISC) to triplet states which are delayed due to reduction or oxidation processes [21,35] before recombining to the ground state. This radical formation can be reversed in the presence of reducing/oxidizing reagents and oxygen scavenger systems to reduce bleaching. Hence, within a specific acquisition time, super-resolution imaging has a better signal-to-noise ratio due to the lower background. The localisation precision is also improved.
2.2.2 Fluorophores: fluorescent proteins, fluorescent dyes and Halotag-ligands.

The main advantage of fluorescent proteins (FPs) is the ability to co-express the protein of interest using genetic modification, in which the FP is ligated to the proteins ready for observation, so it shows a high fidelity of labelling specificity [43,44]. Although fluorescent proteins can be genetically conjugated to proteins of interest, the size (2 – 5 nm, about 28 kDa for monomeric dsRED-RFP [45], for instance) and the low photon budget are a limitation. Synthetic fluorophores are therefore superior to FPs due to their smaller size (1 - 2 nm), higher photon yields and allow the creation of specific conjugated sites [40]. However, the synthetic fluorophore needs a chemical labelling procedure to bind the fluorophores to the molecule of interest [46]. To label the target, the immunofluorescent technique is widely used, but the size of Immunoglobin G (IgG) is quite large, about 8 nm; which may cause mis-localization of the observed nano-structures.

For super-resolution imaging, especially dSTORM [47], fluorophores that have an ability to switch, activate and convert between two states are common choices for super-resolution imaging techniques. dSTORM uses wide field laser excitation and sequentially acquires the fluorescent signal in each switching event to reconstruct the super-resolution image. For high-resolution images, the fluorescent probes have to be bright producing a large number of photons before switching off or bleaching. The STORM technique requires several frames of fluorescent emission from fluorophores which are sparsely labelled on the sample, so the switching events of the probes are crucial. Alternative dyes are available which are spontaneous, self-blinking, self-recovering or work without an oxygen scavenger system [48], e.g. HMSiR [49] and iFlour [50] dyes, without the support of any additive buffer.
interfering with the cell morphology. However, in practical experiments with live bacterial cells imaging, AF647 co-labelling with Cy3b provided the best super-resolution images in the current studies.

In addition to immunofluorescence, chemically self-labelling methods [51] are available using specific site recognition, e.g. SNAP and Halotag labelling [52]. In the following study, Halotag has been used to conjugate NeuS enzyme for dynamic observations. Small ligands can specifically recognise the Halotag enzyme and there are several types of ligands available for multiple fluorescent labelling observations.

2.3 Fluorescence microscopy and spectroscopy

2.3.1 The spatial resolution of optical microscopy

An image is reconstructed from a pattern of many Airy discs. The Airy patterns are diffraction patterns when fluorescent point sources in the sample are transmitted through optical elements after being excited with specific wavelengths of light.

According to the paraxial Far-field/Fraunhofer approximation [37,38], point sources can be described as fluorescent plane wave packages that diffract through an objective lens, pinholes (for confocal microscopy) and other optical elements in the detection path. The Airy disc pattern \( g(x, y; z) \) is viewed in a detection or an image plane \( h(x, y; z) \), in Figure 2.3. The pattern is the consequence of a convolution \( h(x, y) \odot \delta(x, y) = \iint h(x-x', y-y') \delta(x', y') \, dx' \, dy' \) between objects from the original plane (the fluorescent point sources, \( \delta(x, y; z) \)) and the point spread function (PSF, \( h(x, y; z) \)) at the image plane,
where the system point spread function, $h(x, y; z)$ is the inverse Fourier transform of an optical transfer function (OTF). In the case of Figure 2.3, $M = -z_2/z_1$, and $OTF(v_x, v_y) \approx p'(x, y) = p'(\lambda z_2 v_x, \lambda z_2 v_y)$ (according to a pinhole). This is a simplified version of the OTF approximation for an optical system at the focal plane. When including noise and blur effects ($\nu(x, y)$) of the image formation in the spatially invariant case [38], the Airy pattern or image can be defined as $g(x, y; z) = h(x, y; z) \odot \delta(x, y; z) + \nu(x, y)$.

This happens when fluorescent signals dissipate after travelling through optical components, e.g. a lens or a pinhole.

Figure 2.3 The Airy disc patterns ($g(x, y; z)$) from point sources ($\delta(x, y)$) on a sample plane travelling through optical elements ($p(x, y)$) and formed on an image plane, where $h(x, y; z)$ represents the point spread function on image plane, $z_1$ and $z_2$ are distances from the sample plane and the image plane to the optical elements, respectively [38]. Hence, a magnification of the image is defined as $M = -z_2/z_1$. 

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In addition to using a pinhole for removing background noise from fluorescence images, deconvolution can be used as a post-processing method. This deconvolution method uses the spatial frequency information to remove noise in Fourier space before it is converted back to an image [38],

\[
F(v_x, v_y) = \frac{G(v_x, v_y)}{H(v_x, v_y)} = F^{(0)}(v_x, v_y) + \frac{W(v_x, v_y)}{H(v_x, v_y)},
\]  

(2.5)

**Equation (2.5)** uses all the parameters in Fourier space, (spatial frequency components: \(v_x\) and \(v_y\)) including the object including noise \((F)\), the OTF \((H)\), the image \((G)\) and the object in the absence of noise \((F^{(0)})\) and the noise object \((W)\).

With the information of Airy pattern \((g(x, y; z))\) from conventional fluorescent imaging or the object pattern \((\tilde{F}(x, y; z) = FT\{F(v_x, v_y)\})\) from deconvoluted image post processing, the final image on the image plane can be reconstructed according to the intensity pattern. For the intensity pattern \(I(x, y)\) from a circular aperture (e.g. an aperture or a lens), it is related to the first kind of Bessel function [38] given by

\[
I(x, y) = |g(x, y; z)|^2 = \left(\frac{\pi D^2 I_0}{\lambda f^2}\right) \left|J_1(\frac{\pi Dr}{\lambda f})\right|^2, \tag{2.5}
\]

where \(D\) is the lens diameter, \(f\) is the focal length of the lens, \(\lambda\) is the wavelength, and \(r\) is the spatial distance and \(I_0\) is the maximum intensity. When the first minimum of an Airy pattern coincides with the maximum intensity of another Airy pattern \((r_{x,y})\), \(\pi Dr_{x,y}/\lambda f = 3.83\) and

\[
r_{x,y} = 1.22 \frac{\lambda}{2NA}, \tag{2.6}
\]
where $NA = nsin\alpha$, $n$ is refractive index and $sin\alpha \sim D/2f$. This defines a spatial resolution for the fluorescence image. Additionally, the spatial resolution in the axial direction, which is possible to calculate from the full width half maximum (FWHM) of the Airy pattern in the axial direction is

$$r_z \approx \frac{2n\lambda}{NA^2}$$  \hspace{1cm} (2.7)

This resolution barrier in both axial and lateral direction limits the investigation of many biological problems, especially in the study of bacteria, which include intricate hidden structures [53–55], e.g. MrB- actin homologues, Ftsz ring- tubulin skeleton structures and protein-protein interaction in both static and dynamic observations. To circumvent this optical diffraction limit, several optical techniques have been invented that can be used to observe bacterial structures, mechanisms and activities. For sub-diffraction limited resolution, confocal microscopy can be used to improve the spatial resolution (by a factor of $\frac{1}{\sqrt{2}}$ due to background noise removal using a circular pinhole), enhance the contrast and for 3D sectioning. However, many of the finer details of bacterial morphology were still not resolvable using confocal microscopy. Thus, super-resolution fluorescence, direct Stochastic Optical Reconstruction Microscopy (dSTORM) [47] was used to image live bacteria with a spatial lateral resolution $\sim$50 nm and an axial resolution of $\sim$200 nm.

### 2.3.2 Single-molecule localization microscopy

Single-molecule localization microscopy (e.g. dSTORM) uses switchable fluorophores [48] (switching between two different states) combined with a localization analysis to reconstruct super-resolution images. To acquire the super-resolution image,
several thousand frames have to be sequentially collected and the positions of the fluorophores are then localized to reconstruct the final image. A variety of software is now available to reconstruct images and it plays an important role in their final quality.

2.3.2.1 Localization method: Gaussian fitting

The dSTORM technique exploits sparsely localized fluorophores that emit light with diffraction-limited spots. The diffraction-limited image of single fluorophores is resolved from localisation and multiple localisations are reconstructed to get a super-resolution image. The position of fluorophores can be estimated from a Gaussian fit to the centroid intensity profiles from a few pixel areas [56,57],

\[ I(x, y) = I_0 \exp(-a k^2 [(x - x_0)^2 + (y - y_0)^2]) + b, \quad (2.6) \]

where \( k = \frac{2\pi}{\lambda} \), \( a \) is the width of the PSF, \( I_0 \) is the peak intensity, \( (x_0, y_0) \) are a fluorophore’s coordinates and \( b \) is the average background intensity per pixel. This Gaussian fit not only provides the position of the observed molecules, but also reveals how software analysis can gain spatial resolution that is independent of the excitation wavelength, mainly due to the intensity \( I_0 \).

2.3.2.2 Filtering method: localisation precision

After all possible fluorescent molecules are localised, precision and intensity filtering are used to get rid of the background noise. The resolution of the image mainly depends on the brightness of fluorophores, the density of labelled fluorophores, the size of the binding fluorophores and the reconstruction methods used. The fits without overlapping of
neighbouring fluorophores can be determined with high precision and quantified using the variance of position measurements ($\sigma$) [58],

$$\sigma^2 \approx \frac{s^2 + (a^2/12)}{N} + \frac{4\sqrt{n}s^3b^2}{aN^2}$$

(2.7)

where $s$ is the standard deviation of the point spread function (PSF), $a$ is the size of the imaged area on each detector pixel, $b$ is the standard deviation of the background and $N$ is the number of detected photons from the fluorophores. Clearly, the precision with which the position can be determined varies with the number of photons detected. The spatial resolution of this method is not directly limited by the type of wave used for excitation.

2.4 Fluorescence microscopy systems used

Several fluorescence microscopy techniques were used. A wide-field fluorescence microscope was mainly used to visualise fluorescent labelling cells for testing and optimisation before performing a super-resolution experiment. Also, confocal microscopy was used to investigate 3D localization of fluorescent labelling and to compare with super-resolution imaging. Last but not least, super-resolution microscopy (dSTORM) was used to perform static and dynamic imaging of labelled bacterial cells.

The wide-field fluorescence microscope was an Axio Imager.M2 Upright Zeiss microscope with a 63x Plan Apochromat (Oil, DIC) objective lens used at a low laser power with the appropriate filter set. For the confocal microscopy, images were collected using a Leica TCS SP8 AOBS Inverted gSTED microscope in a confocal mode with a 100x HC PL APO lens (Oil; STED WHITE). The excitation laser was a tuneable super-continuum source.
and was used at around 250mW. The Zeiss LSM880 with Airyscan was also used with 63x Plan-Apochromat 1.4NA (Oil, DIC) for confocal imaging.

In the case of dSTORM imaging (Figure 2.4), a bespoke STORM microscope was used with 6 main components: 1) **Laser sources**, 4 different wavelength sources (405 nm, 488 nm, 561 nm and 647 nm) could be selected from 4 lasers using the dichroic mirrors (DM) that were collimated through a multimode optical fibre. For the dSTORM experiment, 2 lasers were needed at the same time, one for excitation and the other with low power for fluorescence recovery, e.g. 647 nm and 405 nm, respectively, for the Alexa Flour 647 dyes used. 2) **Beam expansion and optimization** comprised of 2 plano-convex lenses for laser collimation (beam expansion), an oscillating motor for mixing the internal reflected lights inside the fibre (to scramble coherence effects) and two M2 mirrors for planar alignment before incidence on the back focal plane of the objective lens. 3) **Control units**, these interlocked boxes control oscillation motor, laser powers, temperature control, objective lens drifting correction and the translational piezo stage controller. 4) **sCMOS or EMCCD camera**, different cameras were used depending on the fluorescence sensitivity (EMCCD camera) and the rate of acquisition (sCMOS) required. 5) **Microscope with objective lens drifts correction**, within the bespoke incubator box. Bacterial cells could be imaged at 37°C while a 100x oil-immersive objective lens was stably focused using the drift correction lens holder and the stage translation was controlled with a piezo stage. 6) **PC for acquisition and analysis**, the image acquisition, laser control, objective lens drift correction and image analysis depended on software packages, e.g. Thunderstorm for image reconstruction [59]. The specific tools and software used in the following studies will be mention specifically in different sections.
Figure 2.4 Diagram of the bespoke super-resolution microscope including a temperature control unit. There are 6 main components: 1) laser sources, 2) beam expansion and optimization, 3) control units, 4) sCMOS or EMCCD camera, 5) microscope with objective lens drift correction and 6) PC for acquisition and analysis.

For example, to observe the dynamics of the capsular lyso-PG brushes, lyso-PG rafts were tracked with fluorescence microscopy using a sCMOS, Hamamatsu ORCA-Flash 4.0 V2, camera. Fluorophores were tracked after excitation by a 647 nm laser, with a power of less than 10 mW for long-duration tracking.

2.5 Fluorescence correlation spectroscopy (FCS)

Fluorescence correlation spectroscopy is a standard technique to quantify the dynamics of a few fluorescent molecules inside a small observable volume. Due to the potential to differentiate the mixed species that diffuse simultaneously, many studies on
proteins interaction \textit{in vivo} have been performed \cite{4,60–62}. This technique is thus suitable to investigate the dynamics of proteins inside bacterial cells.

2.5.1 \textit{Theory behinds FCS}

The dynamics of fluorescent particles could be investigated via the autocorrelation \((G(\tau))\) of the fluctuations of the fluorescence signals \cite{63}:

\[
G(\tau) = \frac{(F(t+\tau)F(t))}{(F(t))^2} = \frac{(F(t+\tau)+\delta F(t+\tau))(F(t)+\delta F(t))}{(F(t)+\delta F(t))^2} = 1 + \frac{(\delta F(t+\tau))}{(F(t))^2} (2.8)
\]

where \(F\) is a fluorescence intensity and \(\delta F\) is a fluctuation of the fluorescence intensity between times \(t\) and \(t+\tau\). The fluorescence fluctuations are related to the change of concentration \((\delta C(\vec{r}, t))\) as fluorescent molecules diffuse through a specific confocal volume \((\int dV)\) with a Gaussian spatial distribution \(S(\vec{r})\), so \(\delta F(t) = \int_V \delta C(\vec{r}, t) S(\vec{r})dV\) and \(S(\vec{r}) = \frac{2P}{\pi \omega_0^2} e^{-r^2/r_0^2} e^{-z^2/z_0^2}\), where \(r^2 = x^2 + y^2\); \(r_0\) and \(z_0 = wr_0\) are 1/e\(^2\) of the maximum intensity from lateral and axial distance of the confocal volume respectively; \(\omega_0\) is the beam width; \(\vec{P} = \epsilon_0 \chi^{(1)} \vec{E}(t)\) is the polarization of the light related to the permittivity \(\epsilon_0\), linear susceptibility \(\chi^{(1)}\), and electric field \(\vec{E}(t)\). The autocorrelation can be written as \cite{38}

\[
G(\tau) = 1 + \frac{\int \langle \delta C(\vec{r}, t+\tau) \delta C(\vec{r}, t) S(\vec{r}) S(\vec{r}) dV \rangle dV}{\langle C \rangle^2 \int_V S(\vec{r}) dV} (2.9)
\]

where \(\langle C(\vec{r}, t) C(\vec{r}', t + \tau) \rangle = \langle C \rangle (4\pi Dt)^{-3/2} \exp \left( \frac{(\vec{r} - \vec{r}')^2}{4Dt} \right) = \langle C \rangle (\pi r_0^2)^{-3/2} \exp \left( \frac{w^2 r_0^2}{\tau_0^2} \right) \).

After substitution into equation (2.9), the autocorrelation can be calculated

\[
G(\tau) = 1 + \frac{\pi^{-3/2}}{r_0^2 \epsilon_0 \langle C \rangle} \left[ 1 + \frac{(r_0)^2}{r_0^2} \right]^{-1} \left[ 1 + \frac{(r_0)^2}{w^2 r_0^2} \right]^{-1/2}, \quad (2.10)
\]

where \(w = z_0/r_0\) is a ratio between the axial and the lateral length of the confocal volume.
To cover whole diffusion scenarios, diffusing proteins can experience normal diffusion ($\alpha = 1$) due to a thermal fluctuation. Additionally, proteins can be sub-diffusive ($\alpha < 1$) when they interact with protein complexes or other molecules inside the bacterial cytoplasm. With the definition of mean square displacement in anomalous diffusion as $\langle r^2 \rangle = 4D\tau^\alpha$ and thus $\langle r_0^2 \rangle = 4D\tau_1^\alpha$, the autocorrelation trend can be fitted with a characteristic diffusing time ($\tau_1$): $G(\tau) = 1 + \frac{\pi^{-3/2}}{w r_0^2(C)} \left[ 1 + \left( \frac{\tau}{\tau_1} \right)^\alpha \right]^{-1} \left[ 1 + \frac{1}{w^2} \left( \frac{\tau}{\tau_1} \right)^\alpha \right]^{-1/2}$. Hence, the dynamic behaviour of anomalously diffusing fluorescent proteins can be investigated. Not only single diffusive species can be traced, the autocorrelation function of a mixed population (m different species) diffusing inside confocal volume can be written as

$$G(\tau) = 1 + \frac{1}{\sum_{l=1}^m N_l} \sum_{l=1}^m \left( Q_l \left[ 1 + \left( \frac{\tau}{\tau_l} \right)^\alpha \right]^{-1} \left[ 1 + \frac{1}{w^2} \left( \frac{\tau}{\tau_l} \right)^\alpha \right]^{-1/2} \right)$$  \hspace{1cm} (2.11)

where $\sum_{l=1}^m N_l$ is the total number of fluorescent molecules observed and $Q_l$ is the fraction of a multiple FCS population. The investigation of NeuSHT using FCS provided an autocorrelation curve with a lag time. Hence, equation (2.11) was used to fit for a mixed population and the anomalous behaviour of NeuSHT transport could be quantified.

2.5.2 FCS optimisation and calibration

For the investigation of bacterial proteins, e.g. NeuSHT, a confocal microscope (LSM 880, Zeiss) was used to acquire the fluorescent fluctuation signals, which were then converted to autocorrelation values with different time steps (equation (2.8)). With this system, a calibration procedure was performed to measure the size of the confocal volume with different sized apertures (e.g. 1AU with a 33 μm pinhole size) using 100 nm Cy3B
coated with PS beads. With the calibrated confocal volumes, the FCS results were verified to represent the dynamics of fluorescent molecules rather than random background noise.

2.6 Atomic force microscopy (AFM)

Atomic force microscopy (AFM) can image and characterise forces at the nanoscale including biological cells [64]. There are several modes that are useful for bacterial investigation: tapping, contact and force spectroscopy [65]. Force spectroscopy can investigate the tiny force responses of the oscillating tip-bacterial interaction when it indents bacterial cells [66].

To optimise the signal to noise, an AFM tip with a cantilever stiffness is selected according to the range of force response required. The force resolution ($F_{RMS}$) of a cantilever is given by [67]

$$F_{RMS} = k x_{RMS} = \sqrt{k_b T \kappa}$$

(2.12)

where $\kappa$ is the cantilever stiffness, $k_b$ is the Boltzmann constant and $T$ is a temperature. To investigate bacterial capsule response to an indenting force, a spherical probe (2.5 µm in diameter) with a soft cantilever ($k = 0.3$ N/m) in room temperature was chosen. The force resolution ($F_{RMS}$) was approximately 180 pN.

Response forces ($F$) directly relate to the deflection of the laser after the cantilever was deflected. The response (indentation force) can be measured according to Hooke’s law,

$$F = \kappa a V$$

(2.13)
where $\kappa$ is the calibrated spring constant (N/m), $\alpha$ is deflection sensitivity (nm/V) and $V$ is a voltage from the deflected laser on the photodetector (V). The deflection response is characterised in a calibration with an axial displacement ($\Delta z = \alpha V$) using the piezo stage.

To investigate bacterial capsules (including hydrated polysaccharides), AFM measurements were performed in water, Figure 2.5. In the calibration process, without bacterial cells, the spherical probe was moved to touch a poly-l-lysine coated mica glass-slide from a distant point. The natural frequency ($f_0$) of the probe with the cantilever, the spring constant ($\kappa$) and the deflection response ($\alpha$) were determined. After the probe was calibrated in water, the cantilever was moved from a faraway position to immediately touch the bacterial sample ($z_0$ displacement) with individual scan steps of around 10 nm. The axial displacement of the cantilever corresponded to different values of the voltage on the photodetector. AFM mapping was also used to visualise the bacterial cell location (allowing the bacterial size to be measured, $h = 1 \mu$m) and to mask spots for indentation.
Figure 2.5 AFM indentation mode on the bacterial surface. The bacteria cell was deposited on mica/glass slide in contact with an aqueous solution, where V1 and V2 are the sums of the deflection voltages corresponding to the positions of the cantilever Z1 and Z2, respectively. Z0 is the distance that the cantilever moved before it started to bend.

In force response measurements, Z1 and Z2 are axial displacement positions. They are directly proportional to the signals V1 and V2, respectively. With specific deflection thresholds (~10-20 nm), the noise from force spectroscopy can be minimized. During the investigations, force curves (forces as a function of distance) were produced and characterised under various conditions.
2.7 References


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CHAPTER 3

Super-resolution fluorescence microscopy study of the production of K1 capsules by *Escherichia coli*: evidence for the differential distribution of the capsule at the poles and the equator of the cell.

3.1 Introduction

*E. coli* infections can cause disease in humans. A major factor in their virulence is often the coatings of the bacteria which are called capsules. Recent developments in fluorescence microscopy is used to make much higher resolution images of capsules on living bacteria than were previously possible. *E. coli* that cause urinary tract infections were studied. The technique of super-resolution fluorescence microscopy (STORM) with graphene oxide coated coverslips to image the capsules was developed [1]. Without the graphene oxide coating, it would be very challenging to image the bacteria using antibody labelling due to the large background of non-specifically bound fluorophores. This background fluorescence is conveniently extinguished by the graphene oxide (GO) using
resonant energy transfer, without requiring invasive cleaning or blocking techniques. The bacteria were cylindrical with hemispherical caps (super-ellipsoidal) and the lengths of the molecules in the capsules were also measured using chromatography. Statistical analysis of atomic force microscopy measurements with spherical colloidal probes (including control experiments) was then used to explore the forces the capsules exert on the bacteria and their surroundings. A model based on the polymeric nature of the capsules was then used to explain the origin of the force regimes measured.

Many clinically important bacteria have polysaccharide capsules attached to their surfaces which increase their virulence [2,3]. The primary physical mechanism behind this virulence is thought to be the creation of a repulsive steric potential that allows the bacterial cells to resist phagocytosis by the immune cells of the host organisms. The exact molecular mechanisms involved in the physical chemistry of the repulsive steric brushes are not well understood and there are also a number of gaps in our understanding of their biochemistry, such as how the capsular polysaccharides are synthesised and transported through the inner and outer cell membranes [4–7].

K1 capsular polysaccharide is one of more than 80 K-antigen serotypes [8,9] that are found on the surface of pathogenic Escherichia coli and K1 capsules occur on bacteria that cause urinary tract infections (UTIs) [4,10,11]. The K1 capsular polysaccharide chains are made of nine-carbon N-acetylneuraminic monomers, with α-2,8 keto-glycosidic linkages (with a degree of polymerization less than 200) linked with few β-linked poly-3-deoxy-D-manno-oct-2-ulosonic acid (KDO) linker on lyso-phosphatidylglycerol (lyso-PG) [12] (Figure 3.1).
Figure 3.1 Schematic diagram of a single chain of polysialic acid comprised of sialic monomers (orange circles) with α-2,8 keto-glycosidic linkages linked with a few β-linked poly-3-deoxy-D-manno-oct-2-ulosonic acid (KDO) (green circle); a monomer structure is shown in the green box, anchored on to lyso-phosphatidylglycerol (lyso-PG), in the red circle; a lyso-PG structure is shown in the red box. The lyso-PG is surrounded with lipid A in the bacterial outer membrane (the O-antigens are omitted for clarity).

Capsular polysialic polysaccharides on bacterial surfaces have been investigated previously in terms of both their biosynthesis and for applications, in terms of targets for new antimicrobials and as vaccine candidates [11]. However, detailed descriptions of capsular polysaccharide morphology and studies of their dynamics on live-bacterial surfaces are relatively limited.
Due to the high virulence of bacteria with capsular polysaccharides, the manipulation of polysaccharide production could have important medical benefits. Most studies have been focused on the biochemical synthesis of capsular polysaccharides and the molecular machines used to control the biosynthesis pathways [13]. Recent models have concluded that in the case of the *E. coli* K1 polysaccharide, the capsular polysaccharide chains are made inside bacterial cells before transport across the periplasmic space onto the outer membrane [14]. In addition to biosynthesis mechanisms, bacterial capsular morphology has been observed on fixed bacterial surfaces using scanning electron microscopy, transmission electron microscopy [15,16] and atomic force microscopy [17,18]. These studies confirmed a physical model of the bacterial capsule as a capsular polyelectrolyte brush anchored on to the underlying phospholipid membrane. Brush is a term adopted from soft-condensed matter physics, meaning an array of surface-tethered polymer chains. Polymer brushes are commonly used in chemistry to stabilize colloidal phases of matter against aggregation, for example in paints, to reduce surface adsorption, such as in anti-biofouling coatings, and to reduce frictional forces between surfaces in lubricants [19,20].

A recent paradigm in membrane structure is that of lipid rafts i.e. the lipids that form cellular membranes are not uniformly mixed and have a well-defined heterogeneous structure [21]. The idea of lipid raft formation has been recently applied to bacterial cells [22]. In the current study, the formation of K1 capsular lyso-PG rafts that merge together to form the capsular structure during its maturation was explored. It was found that their nucleation occurs randomly across the bacterial surface and the rafts move slowly from their initial point of nucleation.
Synthetic polymeric brushes have been extensively studied in the literature both theoretically and experimentally [23,24]. Models can describe polymer brush morphology and the forces they experience. Extensions have also been made to when the polymers are charged (polyelectrolytes) and in this case, the counterions can also play a major role [25–27]. Such models for polyelectrolyte brushes have been adapted to describe capsular polysaccharides and allowed intracellular forces measured in atomic force microscopy (AFM) to be explained. There have been only a small number of quantitative studies of bacterial capsules with AFM which used *Klebsiella pneumoniae* [18,28]. Here *E. coli* capsules are studied for the first time, using a colloidal probe cantilever (that improves the signal to noise ratio over standard cantilevers) and demonstrate quantitative agreement with super-resolution fluorescence measurements (previously the brush thickness was used as an unsubstantiated fit parameter with *K. pneumoniae*).

Super-resolution fluorescence microscopy techniques are rapidly becoming a standard tool in biological physics laboratories, such as STORM [29,30], PALM [31], SIM [32] and STED [33]. These methods can provide sub-diffraction limited images with little or no disruption of biological processes. Samples can be hydrated, at room temperature and require minimally invasive sample preparation procedures [17]. Recent developments can provide relatively fast data acquisition (fast cameras, bright fluorophores and optimal software analysis) enabling live cells to be imaged and these developments have been used in the current research on *E. coli*. Super-resolution studies on bacteria are still relatively limited, but research has been performed on chromosome organization [34], DNA repair [35] and peptidoglycan architecture [36].
Due to the small thickness of K1 polysaccharide capsules (≤ 200 nm), the resolution of conventional optical microscopy is at its very limit \( R = \lambda / 2NA \approx 200 \) nm, where \( \lambda \) is the wavelength and \( NA \) is the numerical aperture. Thus to visualize the morphology of bacterial capsules using conventional staining techniques is challenging and the fine structure of bacterial capsules cannot be resolved due to the diffraction limit. However, conventional fluorescence microscopy does allow the dynamics of diffraction limited capsular polysaccharide rafts to be tracked as they move around bacterial surfaces and it was therefore performed in parallel with other studies. Furthermore, confocal microscopy could be used to provide a \( \sqrt{2} \) improvement on the resolution (\( \sim 200/\sqrt{2} \) nm), contrast enhancement and improved 3D sectioning, although finer details in the images were still not resolvable. Therefore super-resolution fluorescence, direct Stochastic Optical Reconstruction Microscopy (dSTORM) was used to image live bacteria [29,37]. The stochastic emission of excited fluorophores with specific imaging buffers allowed us to create static super-resolved images (50 nm resolution). Previous studies of live bacterial capsules using super-resolution fluorescence imaging in the literature have not been experienced and the work builds on the previous study using graphene oxide coatings with dSTORM that allows high contrast quantitative imaging [1]. Transparent GO films spin-coated on to coverslips extinguished non-specifically bound fluorophores, that otherwise would provide a large fluorescence background and low contrast (low SNR) on images of the capsules.

In this chapter, an integrative picture of capsule formation based on a combination of fluorescence microscopy techniques and AFM was demonstrated. Lyso-PG capsular rafts
initiated at a relatively small number of nuclei (~10) in each bacterium and the rafts grew to cover the whole bacteria with limited diffusional motion. The thicknesses of the lyso-PG rafts at the bacterial poles were substantially larger than those at the equator from dSTORM, which was in agreement with chromatography experiments on identical specimens. Quantitative agreement was found between the AFM and dSTORM experiments implying that microscopy experiments can now be used to predict the mesoscopic forces experienced by the bacteria. The repulsive inter-particle potential provided by the capsules will thus be longer ranged on the poles than on the equator of the bacteria by a significant factor (49%) providing an anisotropic modulation of bacterial interactions e.g. they are less likely to adhere to any other surface (including another bacterium) at their poles than on their equators.

3.2 Methodology

3.2.1 K1 encapsulated E. coli strains and their cultivation

*Escherichia coli* strains: EV36 (encapsulated strain) and EV136 (capsule deficiency, generously provided by Eric Vimr) were cultured in two different media: LB (Luria-Bertani) [38] and M9 (Minimal) [39] with supplements of 0.4% (v/v) glycerol and 0.1% (w/v) casein, to observe bacterial polysaccharide capsule formation. For fully encapsulated bacteria, EV36 were cultured in LB at 37 °C overnight before inoculation in fresh LB media and further incubated at 37 °C until they were imaged. To observe bacterial capsule expansion over time, 1:100 of the bacteria were cultured in LB at 20°C overnight and inoculated in fresh M9 media at 20°C. When the OD$_{600}$ reached 0.3, 20 ml of cells were harvested by
centrifugation (3500 rpm for 15 minutes) and the pellet was transferred to a fresh pre-warmed M9 media, and then temperature up-shifted to 37 °C to induce capsule formation.

3.2.2 Immunofluorescent techniques for labelling K1-capsular polysaccharide

Live cells were collected at well-defined time points, washed three times with PBS and deposited on poly-L-lysine-and-graphene oxide (PLLGO) coated 8-well chambers. This PLLGO coating improves the signal to noise ratio (i.e. the contrast) for fluorescence imaging by a factor of 100 [1].

The K1, α2-8 N-Acetylneuraminic (Polysialic) acid in the polysaccharide capsules can be recognised by mAb735 (monoclonal IgG2A) antibodies [40–42]. For live cell imaging using wide-field fluorescence microscopy, cells were incubated with 1% w/v BSA in PBS and then incubated with a 1:400 dilution from a 4 mg/ml stock in a blocking buffer, then washed before incubation with a 1:500 dilution with a secondary antibody (anti-mouse Fab’2 AF647, Invitrogen #A-21237). In addition, confocal and super-resolution (dSTORM) microscopy were used without the washing step with the immunostaining methods by incubating both primary (1:8000) and secondary (1:10000) antibodies at a lower concentration with the bacteria due to the benefits of using PLLGO coated slides i.e. the PLLGO coating removed the background due to non-specifically bound fluorophores and provided a 100 factor of improvement in contrast [1]. The poly-l-lysine coating has a negligible effect on the proliferation of the bacteria over a period of 3 hours (the time for a complete capsule to form, Appendices A5).
3.2.3 Optical fluorescence microscopes and imaging buffers

For wide-field fluorescence microscopy, ready-to-visualize cells were supplemented with Mowiol buffer to stabilize them. Samples were sealed to prevent evaporation. In confocal fluorescence microscopy, cells were deposited on coverslips of 8 well-chamber slides with M9 media (supplemented with 0.4% (v/v) glycerol and 0.1% (w/v) casein) mixed with both primary (1:8000) and secondary (1:10000) antibodies for the observation of bacterial capsule development. These 8 well-chamber covers were sealed to prevent evaporation when observed at 37°C.

In addition to M9 media and antibodies (no washing step was used), super-resolution fluorescence microscopy (dSTORM) also required an imaging buffer, which was GLOX [43]: 10mM MEA (cystamine), 1/100 dilution from 20 mg/ml catalase and 1/10 dilution of 150 mg/ml glucose oxidase. This helped prevent photobleaching of the fluorophores. Since the acidity of the GLOX buffer reduces significantly after a few hours, fresh GLOX buffer and M9 media were refilled every 1 hr in longer experiments to prevent photobleaching, toxicity and acidification (Appendix A4).

3.2.4 Image acquisition with the fluorescence microscopes

For capsular polysaccharide brush formation, a wide-field fluorescence microscope was used; an Axio Imager.M2 Upright Zeiss microscope with a 63x Plan Apochromat (Oil, DIC) objective lens at a low laser power with the appropriate filter set. In confocal microscopy, images were collected using a Leica TCS SP8 AOBs inverted gSTED microscope in confocal mode with a 100x HC PL APO (Oil; STED WHITE) objective lens; selected laser source at 250 mW was used. In the case of dSTORM imaging, a bespoke
STORM microscope [44] was used with 405 nm (0.6 mW) and 647 nm (77 mW) excitation lasers and a 100x oil immersive with 1.49NA, TIRF objective lens (Olympus UAPON 100xOTIRF). The depth of focus was calibrated using 100 nm polystyrene beads coated with Cy3B, deposited on glass-slides coated with poly-L-lysine. The z-stack images were taken at 10 nm/step for 200 steps using a piezo stage with an acquisition rate of 100 frame per seconds (fps) and analysed with the virtual light sheet (vls) plug-in [45].

In order to observe the dynamics of the capsular lyso-PG rafts, lyso-PG rafts were tracked with fluorescence microscopy using a sCMOS, Hamamatsu ORCA-Flash 4.0 V2, camera. Fluorophores were tracked after excitation by a 647 nm laser, with a power of less than 10 mW for long-duration tracking.

dSTORM was used to observe a gradual expansion of the capsular polysaccharide rafts during their growth. Up to ten thousand frames were used to construct each dSTORM image, which were followed by times with no illumination to reduce photobleaching. Thus, the expansion of K1 capsular brushes on bacterial membranes could be investigated by taking images at defined times.

3.2.5 High pressure light-scattering chromatography (HPLC)

Purified K5 (group 2) capsular polysaccharide was analysed using multi-angle light scattering (Wyatt). The purified polysaccharide was diluted in 500 μl of PBS 1 hr prior to loading and then centrifuged at 14k RPM for 10 minutes. The extraction method of Clarke et al. was used [46]. A superdex 200 (separation range 5,000 – 600,000 Da) was equilibrated in PBS and the capsular polysaccharide was injected using a NGC (BioRad) HPLC onto the column. The detectors used were a Wyatt Helios 8-angle light scattering detector, an Optilab
rEX refractometer and a QELS dynamic light scattering detector. The molar mass of the lyso-PG was acquired using this system.

3.2.6 Bacterial capsule analysis and image processing methods

Several pieces of software were used to construct images and to interpret the results. The Thunderstorm plug-in in Fiji was used to localize the blinking fluorophores and reconstruct super-resolution images [47]. Origin (OriginLab, MA) was predominantly used for plotting and fitting functions. Capsular brush thicknesses, mobilities and expansions were analysed via MATLAB (The MathWorks, Inc., MA). Newly written MATLAB code was used to find the capsules’ boundaries and the distribution of capsule thicknesses. The polysaccharide rafts were tracked using the TrackMate plugin [48] and MATLAB code was used to construct the mean square displacement as a function of lag time. The depth of focus of the microscope was analysed with the virtual light sheet plug-in [45] and it was found to be 347 ± 22 nm (Appendix A2).

3.2.7 Nano-mechanical measurements using AFM with a spherical probe

Bacteria were prepared overnight in M9 media and then deposited on a poly-L-lysine-mica coated glass slide, one hour before an experiment. Nano-mechanical forces were observed using a Bruker Catalyst AFM system with spherical borosilicate colloids, 2.5 µm in diameter (sQUBE, 2017NM310/1), with a cantilever spring constant (κ) of 0.3 N/m. The probes together with their cantilevers were thermally calibrated before they were used to characterise the hydrated capsular brush. Bacterial cells were submerged and observed under UHQ water within 4 hours. Nano-mechanical forces between the capsular brush and the probe were optimised and multiple measurements were made on each position of the
bacterial surface. Raw data were analysed using the NanoScope Analysis toolbox, Matlab codes and Origin. The contact positions of the AFM probe with the capsules were found using exponential fits (Appendix A3).

3.3 Results

3.3.1 Morphology of the E. coli K1 capsule

The K1 capsule is constructed via the assembly of K1 polysaccharides (polyneuraminic acid) arranged on the cell surface, that are not chemically cross-linked but may be bridged by multivalent cations. To visualize the presence of a capsule on a bacterial surface, capsules can be dyed and observed using bright field microscopy [49], stained with ruthenium red and visualised by electron microscopy or immunoelectron microscopy [16,50,51] or detected via fluorescence microscopy using specific antibodies [42]. Initially E. coli K1 capsules were observed with different spatial resolutions: a diffraction-limited image at around 400 nm from conventional fluorescence microscopy (Figure 3.2a), a sub-diffraction limited image at around 200 nm from confocal fluorescence microscopy (Figure 3.2b), and a super-resolution imaging from dSTORM (Figure 3.2c), see also Appendix A1a. The super-resolution image has 50 nm spatial resolution (analysed using the Fourier Ring Correlation (FRC) method [52] and shown as an inset in Figure 3.3d) and the capsule thickness from the polar ends of the E.coli is seen to be larger than the equatorial ends. The thickness of bacterial capsules that were only sectioned through the middle of the cells was considered. When the sectioning plane was not at the middle of the cells, the curvature surface of the bacterial capsule could be observed (Appendix A1b) and the measurement of capsule thickness was omitted in these cases. There is also a possibility of an axial projection
discrepancy; however, the axial sectioning was calibrated with 100 nm polystyrene beads dyed with Cy3B. Within the 300 nm depth of focus (calibrated using virtual light sheet method [45]), the variation of bead size due to an axial projection within this sectioning region is negligible, as shown in Appendix A2. Thus the effect on bacterial projections will also be negligible.

Figure 3.2 Images of bacterial capsules labelled with anti-K1 antibodies conjugated with AF647 created using: (a) diffraction-limited fluorescence microscopy, (b) confocal fluorescence microscopy and (c) dSTORM super-resolution fluorescence microscopy. (d) Shows the spatial resolution of the bacterial capsule images from the different microscopes calculated using the Fourier ring correlation technique (FRC) [52]; the inset shows the FRC as a function of spatial frequency from the super-resolution images (the red lines show the
threshold value of 1/7). The scale bars on a), b) and c) are 1 µm. The spatial resolution of image c) from the FRC was 50 nm.

Furthermore, the thickness distribution of the polysaccharide brushes around the capsules was carefully investigated to see whether bacterial capsules hold a homogenous thickness or not. Two image processing methods were used to quantify the thickness ($H$) of the bacterial capsules from the dSTORM images: (1) a method based on the capsule area called the ratio method i.e. $H = 2A/(C_1 + C_2)$ ($A$ is the area of the capsule, and $C_1$ and $C_2$ are the inner and outer ellipsoidal contour lengths) and (2) elliptical fits with line sectioning normal to the ellipse called the profiling method. Although the initial assumption is the bacterial capsule thickness covered the bacterial surface with a uniform thickness in the first method, the results from the second method showed two qualitatively different domains of bacterial capsular brush height: at the poles (long) and at the equator (short).

Using the assumption of uniform thickness (the ratio method), the capsule thickness ($H$) was calculated from a ratio between the area ($A$) of the projected capsule image in two dimensions and the average contours from inside and outside the capsule boundaries ($(C_1+C_2)/2$) (Figure 3.3a). The average thickness of the K1 capsule was 200-300 nm and the distribution is shown in Figure 3.3c. In addition, using normal vectors scanning along elliptical fits in which purple and cyan mark the equatorial and the polar regions respectively (the profiling method, Figure 3.3b). A histogram of bacterial capsule thicknesses could be constructed at different positions around the capsule (Figure 3.3d). The outermost extent of the bacterial capsule at the equatorial regions of the cell is around 200 nm from the cell surface (Figure 3.3e), whereas at the polar regions the capsule extends further out with a
broader range between 250-400 nm, (Figure 3.3f). The results of Figures 3.3e and 3.3f were calculated from more than 200 K1 encapsulated *E. coli* cells.

**Figure 3.3** Histograms of bacterial capsule thicknesses were created using two different methods: a) the ratio between the area (yellow) and the averaged contour lengths \((C1+C2)/2\) (red) called the *ratio method*, and b) the normal vector profiles from elliptical
fits called the *profiling method*: the purple and cyan dashed lines correspond to equatorial and polar regions respectively. **c)** A histogram of the thicknesses of the capsular brush from the ratio method shown in **a)**. A bimodal distribution can be seen. **d)** A histogram of the thicknesses of the whole capsular area created using the profiling method (using a scatter plot to show all histogram from different individual cells). **b)** The histogram bars are randomly coloured to improve visibility. **e)** A histogram of the thicknesses from the equatorial regions (purple region, purple arrow indicated, in the inset) and **f)** a histogram of the thicknesses of the polar regions (cyan regions in the inset), both created using the profiling method **b)**. The polar regions contain a bimodal population of thicknesses that contains longer polysaccharide chains in the brushes than the monomodal population observed at the equator.

The dSTORM technique provided high-resolution images of the capsular brush to probe their morphology. The uneven surfaces were predominantly due to variations of the brush itself i.e. the molecular weight of the capsular polysaccharide anchored on lyso-PG. The variations in the images were reproducible and defects in immunofluorescent labelling are thought to be a secondary effect.

The variation of the thickness of the capsular brush (**Figure 3.3f**) was similar to its polydispersity measured by HPLC (**Figure 3.4**). Two populations of polysaccharide length were measured peaking at (ii) $24.4 \pm 1.3$ kDa and (i) $47.2 \pm 4.3$ kDa (magenta curve), where 1 g/mol corresponds to 1.008 Da. These results reveal the potential of using super-resolution
technique to quickly identify the polydispersity of polysaccharides on living cells since both HPLC and dSTORM methods demonstrate a similar bimodal distribution.

![Chromatography results from purified K5 capsular polysaccharide showing the two main fractions of lipopolysaccharide molecular weight (i and ii, indicates molar masses of 47 kDa and 27 kDa, respectively, according to the different injected volumes). These scattering signals were detected using different detectors: a Wyatt Helios 8-angle light scattering detector (blue), an Optilab rEX refractometer (green), and QELS dynamic light scattering detector (cyan). The molar mass ($M_w$) was interpreted using Zimm plot analysis [53,54] (magenta).](image)

**Figure 3.4** Chromatography results from purified K5 capsular polysaccharide showing the two main fractions of lipopolysaccharide molecular weight (i and ii, indicates molar masses of 47 kDa and 27 kDa, respectively, according to the different injected volumes). These scattering signals were detected using different detectors: a Wyatt Helios 8-angle light scattering detector (blue), an Optilab rEX refractometer (green), and QELS dynamic light scattering detector (cyan). The molar mass ($M_w$) was interpreted using Zimm plot analysis [53,54] (magenta).

### 3.3.2 Nano-mechanical properties of capsular brushes measured with AFM

A borosilicate spherical probe (diameter 2.5 μm) was attached to the AFM cantilever which interacted with the bacterial capsular brushes during force measurements. The force
curves (Figure 3.5a) for the encapsulated strain EV36 (red) and the capsule-deficient strain EV136 (blue) show that the encapsulated bacteria had much longer ranged forces curves of up to 500 nm compared with 225 nm for the capsule-deficient strain. Additionally, the encapsulated data is replotted in Figure 3.5b to show different nanomechanical force regimes: i) the double layer charge interaction, ii) the osmotic brush regime, iii) the Hertzian regime and iv) the Hookean regime [18,28].

The double layer interaction occurs at the longest distances (~ 400 nm) due to screened (i.e. in a salty environment) electrostatic repulsion between the AFM probe and the charge lyso-PG brush. The osmotic brush regime occurs when the probe contacts the lyso-PG and experiences a steric repulsion. It is concluded that the capsular brush beneath these bacteria is completely collapsed, since it provides consistent thicknesses between AFM and STORM, and was observed in previous studies [55]. In the Hertzian regime, the probe considerably perturbs the structure of the brush and it is modelled as a deformable elastic continuum. Finally, in the Hookean regime, the probe indents the cell membrane and it is resisted by the cytoplasmic turgor pressure. The Hookean regime in Figure 3.5 is shown with a semi-log scale. Due to a small indentation depth of a spherical probe, AFM force spectroscopy responses to both compressed bacterial capsules and a restoring force from bacterial shape deformation. Hence, the turgor force measurements were interfered with the inelastic compressed capsular brush. This introduced noise in the investigation around Hookean regime.
Figure 3.5 Force as a function of separation distance from AFM measurements, showing the nano-mechanical properties of capsular brushes. (a) Comparison between encapsulated and capsular deficient strains on a log-linear scale (the inset shows a linear-
linear scale). (b) Nano-mechanical regimes of the interaction between the spherical probe and the encapsulated bacteria. The insets show schematic diagrams of the position of the probe and the encapsulated bacteria from non-contact (i) to contact modes (ii - iv). Different colours in b) show the fits to the different regimes: (i) the double layer charge interaction (red) (ii) the osmotic brush model (green), (iii) the Hertzian regime (magenta) and (iv) the Hookean regime (cyan).

The bacterial capsule thicknesses and their nano-mechanical interaction were observed from fully encapsulated bacteria that were cultured at 37°C. The K1 encapsulated *E. coli* can switch on and off their capsules in response to temperature changes. Upshifting the temperature from 20°C to 37°C stimulates transcription of the kps and neu genes leading to the synthesis and transport of the K1 capsular polysaccharide to the cell surface [8,56]. In order to observe the emergence of the capsules on the bacterial surfaces and their dynamics, both upshifting and constant temperature controlled experiments were used.

### 3.3.3 Dynamics of bacterial capsular raft formation

The linkage of K1 capsular polysaccharides to lyso-PG in the outer membrane means that this array of negatively charged molecules can be regarded as having brush-like properties [25,57]. These K1 capsular polysaccharides, covalently anchored to the bacterial cell surface via lyso-PG molecules, were specifically labelled with antiK1 mAb735 after upshifting the temperature from 20°C to 37°C for 1 hour (Figure 3.6a) in M9 media. Lyso-PG capsular brush rafts were defined due to aggregated regions of lyso-PG that form discrete regions on the bacteria [21,22]. Implicitly with the definition of a membrane raft is the possibility of microphase separation defining the sizes of the rafts i.e. they are aggregated
because the interaction between lyso-PG chains is more favourable than that with other membrane components [22]. Once the capsular brush had been labelled, the dynamics of the early stage capsular brush rafts were observed in different regions of the bacterial surface and then tracked with sub-pixel resolution using standard fluorescence microscopy. The tracked rafts are shown in yellow on Figure 3.6b. In Figures 3.6c and 3.6d, the mean square displacement (MSD) of the rafts with lag time ($\tau$) was analysed from their tracks at 1 hr and 1 hr 30 minute time points. The motion of the labelled capsular brush was in a sub-diffusive regime over the entire duration of the movies based on a power law fit, $\langle r^2 \rangle = 4 D_\alpha \tau^\alpha$, where $r$ is the displacement, $D_\alpha$ is the fractional diffusion coefficient and $\alpha$ is the power law exponent. The power law exponents of the capsular brush are shown in Figure 3.6e from different time points after temperature upshifting. The average is $\alpha = 0.33 \pm 0.23$ at 1 hour and $\alpha = 0.36 \pm 0.20$ at 1.5 hours. After 180 minutes (the time for a complete capsule to form), extrapolation of the averaged MSD as a function of lag time implies the capsular raft would have moved around 114 nm. This is a much smaller distance than that required to cover the whole bacterial surface of approximately 2 $\mu$m. Thus, the position of the lyso-PG rafts appears to be relatively invariant over the time scale of capsule growth and the capsular covering is created by raft nucleation at multiple random sites, rather than diffusive transfer from isolated sites.
Figure 3.6 Particle tracking of fluorescently labelled capsular polysaccharide rafts in standard fluorescence microscopy experiments. **a)** K1 capsular rafts (labelled with anti-K1 conjugated with anti-mouse AF647, red) emerge from *E. coli* cell labelled with GFP (green). **b)** A pixelated image used for sub-pixel tracking. Tracked lyso-PG rafts are shown in yellow. The position of the *E. coli* outer membrane is shown by the green dotted line. **c)** and **d)** show the mean square displacement (MSD) as a function of lag time for times of 1hr and 1hr 30m respectively; different colours show the results from individual K1 rafts. **e)** The
sub-diffusive power-law coefficients, $\alpha$ (from the scaling of the mean square displacement, $<r^2> \sim \tau^\alpha$, where $\tau$ is the lag time), with different times after temperature upshifting. The scale bars are 1 µm on (a) and (b).

Capsule formation could be imaged from the point at which there was no capsule to the fully encapsulated state. With a typical fluorescence microscope, the quantification of capsule size is overestimated due to the diffraction limit. The gradual growth of the capsular brushes was therefore calculated from super-resolution microscopy (dSTORM). The radius of gyration ($R_g$) of the antibody labelled regions on the dSTORM images (Figure 3.7), provides a robust metric to describe the size of the irregularly shaped lyso-PG rafts. In Figure 3.7d, the radius of gyration of the capsular brush rafts is plotted with time. A logistic function (red) provided a good fit (equation (3.7) shown later). With M9 media as a nutrient source, after the temperature was upshifted to 37°C, *E. coli* EV36 became fully encapsulated after 3 hours. In order to minimize the toxicity of dSTORM imaging buffer, we discarded and refilled mixed media imaging buffer after every hour.
Figure 3.7 dSTORM images of the capsular brush rafts after temperature upshifting from 20 to 37°C. The nucleation and expansion of the capsular brush rafts are shown in the sequential images a) and b). c) Shows the rafts from three sequential times superposed. Red is the initial time point (20°C), yellow 10 min after temperature upshifting (37°C) and magenta 20 min after temperature upshifting (37°C). The scale bars are all 1 µm. d) The radius of gyration ($R_g$) of the lyso-PG rafts as a function of time after upshifting the temperature. The expansion process is well described with a logistic function (red line). The error bars are from repeat experiments and are due to variations of capsule emergence on the bacterial cells.
Despite the relatively low number of photons emitted and the photo-bleaching of the fluorophores that labelled the early capsular brush rafts, the rafts could be observed with sub-diffraction resolution after the localization and image reconstruction processes. In Figures 3.7a and 3.7b, the capsular brush rafts emerged in different locations within a 10 minutes time difference and Figure 3.7c is a combined image after 30 minutes, developing from red (from \( t_0 \) time point), yellow (at \( t_0 + 10 \) mins), and magenta (at \( t_0 + 20 \) mins). In addition to this emergence of the lyso-PG rafts, the further expansion of capsular brush rafts could be observed until the capsule completely covered the bacterium (Figure 3.7d).

3.4 Discussion

To date, a few recent studies using the indentation of an AFM cantilever have examined the nano-mechanics of *K. pneumoniae* capsules to quantify their thickness and explored the influence of fimbria on capsular brushes that induce biofilm formation [28,55]. These results confirm the morphological structure of these capsules as polyelectrolyte brushes anchored on the lipid membranes. The current super-resolution imaging experiments on live *E. coli* in a hydrated environment overcome many morphological artefacts from sample preparation and cell damage found in other high-resolution imaging techniques, such as in SEM and TEM experiments [15]. By using super-resolution fluorescence images, particle tracking and AFM with a large colloidal probe, the morphology and dynamics of bacterial capsular brushes could be investigated in more detail than previously possible.

The bacterial capsule morphology found in super-resolution images (Figure 3.2c), reveals details of how the capsular polysaccharide brush is organised with a resolution beyond the diffraction limit. The variation of brush density along the bacterial surface relates to the localization of the membrane translocation proteins and enzymes (biosynthetic export
complexes) for capsular polysaccharide production that are arranged at discrete points on the E. coli membranes. Although mathematical models [25–27] for synthetic brushes have examined the variation of polyelectrolyte brush heights attached to nanoparticles as a function of surface curvature, the capsular polysaccharide brushes were expected to be independent of curvature due to its high grafting density, large radius of curvature and relatively short capsular polysaccharide chains (in comparison to the radius of curvature). Thus, the height differences in Figures 3.3e and 3.3f from the equatorial and the polar regions of the bacteria are deduced to be predominantly due to different lengths of K1 capsular polysaccharides and not their curvature or grafting density effects (i.e. the surface density of lyso-PG chains) or salt concentration variation (in low salt environment).

Specifically, the osmotic brush model indicates the lyso-PG chains are in fully stretched conformations (Appendix A1) and the chain size does not depend on membrane curvature or grafting density. Thus, changes in brush size measured on images are directly due to changes in lyso-PG lengths.

The morphology of polyelectrolyte brushes anchored on to phospholipid membranes can be described by the Pincus polyelectrolyte model for an osmotic brush [25], which uses a balance of the entropy due to osmotic pressure ($f_{osm}$) with the loss of entropy during its expansion. This model was extended by Zhulina and Borisov [27], who considered the capsular brushes grafted onto a nanoparticle of small curvature (e.g. a bacterial surface) with a high charge density (e.g. provided by the sialic acid groups), so the layer of counterions was much smaller compared to the thickness of capsular brush ($H$). The compressive pressure in the capsular brush tends to stretch the polymer chains and is balanced by the
osmotic pressure of the counter ions inside the brush, which gives

$$\frac{f_{\text{OSM}}}{k_B T} \cong \frac{\alpha N}{H},$$  \hspace{1cm} (3.1)

where $N$ is a degree of polymerization of the chains, $T$ is the temperature, $H$ is the thickness of the polyelectrolyte brush and $\alpha$ is the charge fraction of the polyelectrolyte chains. Conveniently the thickness of the capsular polyelectrolyte brush can be calculated independently of the tethering density of the capsular brush in this osmotic brush regime using

$$H \cong Na(\alpha)^{1/2},$$  \hspace{1cm} (3.2)

where $a$ is the monomer length.

A single chain of K1 α-2, 8 polysialic acids that is extended from the point where its lyso-PG is anchored is shown in Figure 3.1 together with a polymeric blob model representation (the basis of the osmotic brush model). Thicknesses from Figures 3.2a, 3.2c, 3.2d were measured of $242 \pm 18$ nm for the average areal approximation, $217 \pm 29$ nm for the equatorial region, and $238 \pm 41$ nm and $323 \pm 62$ nm for the bimodal polar regions. These regions are highlighted in Figure 3.8. Thus, the number of monomers per chain could be approximated as 200-300 units. This length of polymer would predict a molecular weight closer to 70 kDa (chromatography gave two fractions at 47 kDa and 27 kDa, Figure 3.4). This apparent discrepancy is likely a consequence of the arrangement of the capsular polysaccharide on the bacterial surface. Specifically, ionic bridges between the capsular polysaccharide and the lipopolysaccharide molecules also decorate the bacterial cell surface [58]. These interactions will cause swelling of the capsule [59] and hence the discrepancy in the perceived capsule width as determined via imaging and the actual molecule weight of
the polysaccharide.

Figure 3.8 A schematic diagram of the polysaccharide anchored onto phospholipids of the external bacterial membrane forming the K1 capsular lyso-PG raft. A single type of K1 capsule anchored on lyso-PG is shown to form a raft. The capsular brush rafts created at the polar and the equatorial regions (the dashed circle/rectangle show the different regions of the capsular brush rafts), then spread out to cover the whole bacterial surface.

Previous studies have demonstrated that purified *E. coli* K5 capsular polysaccharide also has two molecular weight peaks [60]. This earlier observation may now in part be explained by the observations in this chapter in terms of the polar and equatorial distribution of *E. coli* capsular polysaccharides. This size difference of polysaccharide from the poles to the equator of the cell may reflect differences in transport of the polysaccharide at these two sites in the cell and different proteins complexes involved in this polysaccharide biosynthesis. Several reports on sub-cellular localization had explained how proteins tend to
gather around polar regions [61], including proteins involved in capsular biosynthesis [62–64]. Additionally, a model in which polysaccharide export is initiated while biosynthesis is still taking place means that slower transport could result in increased polymer length before the molecule is finally extruded i.e. different reaction kinetics at the poles and the equators cause the changes in the lyso-PG lengths. Further quantitative comparison to the osmotic brush model is included in the Appendices A1 and A3 and additional information on the biosynthesis will be explained more in chapter 4.

In addition to non-contact observations using super-resolution fluorescence imaging of live bacterial cells, AFM with large spherical probes provides insights into the nanomechanical properties of the capsular brushes. The superior signal to noise ratio compared with the Strugnall AFM studies [28,65] was attributed to the use of a colloidal probe, which averages over a larger region of the capsule, effectively increasing the measured signal. Not only could the data differentiate between capsule-deficient and encapsulated strains of E. coli (in Figure 3.5a), four force regimes involved in the interaction of the encapsulated bacteria could be determined (Table 3.1).

Table 3.1 Force–distance regimes used to describe AFM data in Figure 3.5b. $A_0$ describes the short range van der Waals force (negligible when compared with the electrostatic force in this scenario), $A_1$ is the force magnitude for the long-range electrostatic interaction, $d$ is a separation distance, $d_0$ is used to correct the relative separation distance; $a_1$ and $b_1$ are reduced parameters of the full osmotic brush model for grafted polyelectrolyte brushes [28] (where $\ln(\delta_c^L) = a_1/b_1$), $\delta$ is an indented distance after probe contact, $c$ is a
correction term of the indented distance from a relative separation distance, $R$ is the effective radius ($R_{probe}R_{bac}/(R_{probe}+R_{bac})$), where $R_{probe}, R_{bac}$ are the radius of the probe and the indented bacteria respectively, and $\nu$ is the Poisson ratio of the bacterial cells, e.g. assumed equal to 0.5 as a first approximation for an isotropic elastic solid [55]. The main parameters were the bacterial capsule thickness, the Debye screening length ($\lambda_D$), the effective Young’s modulus ($E_{eff}$) and the elastic constant of the K1 bacterial cells ($k_{bac}$).

<table>
<thead>
<tr>
<th>Regimes</th>
<th>Approximate Fitting function</th>
<th>Parameters</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Double layer (DLVO)</td>
<td>$F_{DL} = A_0 + A_1 \exp\left(\frac{d-d_0}{\lambda_D}\right)$</td>
<td>$\lambda_D$</td>
<td>Debye screening length (nm)</td>
</tr>
<tr>
<td>II. Osmotic brush</td>
<td>$F_P = a_1 - b_1 \ln(\delta + c)$</td>
<td>$\delta_L^0 = \exp(a_1/b_1)$</td>
<td>The onset of linear compliance, capsule thickness: $H \approx 2\delta_L^0$ (nm)</td>
</tr>
<tr>
<td>III. Hertzian</td>
<td>$F_H = \frac{4\sqrt{R}}{3(1-\nu^2)} E \delta^{3/2}$</td>
<td>$E$</td>
<td>Young’s modulus (Pa)</td>
</tr>
<tr>
<td>IV. Hookean</td>
<td>$F_{Hook} = k_{bac}\delta$</td>
<td>$k_{bac}$</td>
<td>Elastic constant of bacteria cells (N/m)</td>
</tr>
</tbody>
</table>

From the non-contact regime to the contact regime (regime I, the indentation of both the capsular brush and the bacterial membrane, the Double layer regime in Table 3.1), the Debye screening length ($\lambda_D$) was found to be equal to $39.4 \pm 2.1$ nm, which is the length scale over which the electrostatics is felt. In regime II from the compression of the brush,
the bacterial capsule thickness was approximated using $\delta_L^0 = \exp(a/b)$, which gave a total thickness ($H$) of $241 \pm 34$ nm (Table 3.1). It is assumed that the lower capsular brush in contact with the poly-L-lysine coating is completely collapsed as observed in previous studies [55]. The bacterial capsule thickness is in reasonably good agreement with the thickness of the single component equatorial brush measured with dSTORM i.e. $217 \pm 29$ nm. In regime III, Young’s modulus was found to be $11.36 \pm 0.22$ kPa, indicating a soft effective modulus for the compression of the membrane. In regime IV the elastic constant was $k_{bac} = 4.14 \pm 0.06$ mN/m, which is related to the cell turgor pressure. Although the spherical probe was large compared to the size of the bacteria, identical force curves were observed after several measurements, so their reproducibility was good. See the Appendix A3 for AFM images of encapsulated bacteria.

In addition to static morphological observations, the dynamics of bacterial capsular brush formation were investigated after temperature upshifting from 20 to 37°C. In Figure 3.6, the mean square displacements show that the K1 capsular brush had low mobility and the rafts of the capsular brush were unable to diffuse appreciably between regions of the bacterial surface over time scales of bacterial capsular raft growth (~ 114 nm displacement over 3 hours). In conclusion, the lyso-PG capsular brushes nucleate from the polysaccharide transport channels and then spread over the bacterial surfaces as additional material is pushed through the channels, but their position remains closely correlated with that of the original channels [62]. These results on the low mobility of the capsule rafts are in good agreement with separate fluorescence correlation spectroscopy experiments of our group.
performed with a confocal microscope (including GFP labelled proteins in the synthetic pathway that act as raft nucleation sites) and will be presented in more detail in the future.

Considering the rate of capsular brush expansion (Figure 3.6), the increase of the brush sizes using $R_g$ (the radius of gyration of the capsular brush raft) were calculated. The number of lyso-PG translocation channels ($N_{ABC}$) was relatively low at ~10 channels (Figure 4.3c, chapter 4) when lyso-PG was produced after temperature upshifting. There was slow growth of bacterial capsule initially and the full growth curve could be described by a logistic model, i.e.

$$R_g = \frac{R_{g\infty}}{1+e^{-f(t-t_0)}},$$

where the time constant ($t_0$) was $157 \pm 18$ minutes, $R_{g\infty}$ is the radius of a fully formed capsule at long times and $f$ is the steepness parameter. The smallest $R_g$ that was included in this trend was $76.7 \pm 11.2$ nm which was set by the resolution of the dSTORM technique. The bacterial capsular brush rafts randomly emerged at positions on the bacterial surfaces, around 30 minutes after temperature upshifting. However, due to the low emission and photobleaching from the high power of the dSTORM acquisition process, the very early stage rafts were omitted from the analysis. The imaging buffer perturbed the growth of bacteria cells (Appendix A4) causing a substantial decrease of proliferation at long time scales (>2 hours). In order to reduce this problem, cells were replenished with M9 media and fresh imaging buffer every hour to prevent the acidification process reducing bacterial division. Within these limitations bacterial capsule expansion was investigated, mainly focusing on the later stage development of large rafts. The bacteria can switch from no capsule to fully covered capsules within 3 hours. In addition to the rate of bacterial capsule production, the
polysaccharide translocation channels are important for creating a relatively uniform capsule that covers the whole bacterium. A random distribution of the nucleation of capsular biosynthesis was observed on the bacterial surfaces [62,66], which also occurred at the septum when the bacteria divided [64].

A number of fundamental questions still exist on the export of the K1 capsular polysaccharides through the two membranes of Gram-negative bacteria. More work is needed to understand the biosynthesis (Chapter 4) and polymer translocation of large capsular polysaccharide molecules from their site of synthesis in the cytoplasm through the external membranes onto the cell surface, which is likely to involve a multiprotein complex [8,9,12,14]. Treatments could be found to compromise the steric potential created by the capsular brushes and thus help the immune system to combat an infection more quickly e.g. the addition of multivalent cations. It would also be useful to employ genetic synthetic biology techniques to modulate the size of the capsular brush to create a quantitative model of capsular physiology.

3.5 Conclusions

K1 Bacterial capsules labelled with antiK1 antibodies allowed the characterisation of capsular polysaccharide polydispersity using super-resolution fluorescence microscopy (dSTORM) with graphene oxide coated coverslips. The distribution of capsule thicknesses varied with localization on different regions of the bacterial surfaces. Capsular polysaccharide brushes were longer at the poles than the equator of the bacterial cells by a factor of 49%. The capsular brush could be indented by a large spherical probe attached to an AFM to determine the nano-mechanical properties of the bacterial capsule and results
were in agreement with dSTORM images based on the osmotic brush model for the steric forces created by a polyelectrolyte brush.

For dynamic investigations, capsular brushes emerged from a few tens of capsular biosynthesis channels to cover whole bacterial cells during their growth. The capsular polysaccharides could be rapidly labelled after transportation to the bacterial surface. Super-resolution fluorescence microscopy provides non-invasive observation of live bacteria that could visualise dynamic events during lyso-PG raft production when the rafts emerged from their channels on the *E. coli* surfaces.

### 3.6 References


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CHAPTER 4

The roles of proteins and enzymes that are involved in the capsular biosynthesis pathway: *KpsD, KpsE* and *NeuS*

4.1 Introduction

4.1.1 Proteins and enzymes that are involved in bacterial capsule biosynthesis and recent polysaccharide biosynthesis studies

Urinary tract infection is a recurrent chronic disease that has become more important in recent times since we are unable to treat it easily with antibiotics [1–3]. One of the main virulence factors is due to uropathogenic encapsulated *Escherichia coli* (*E. coli*) [2,4]. This pathogenic *E. coli* has a K- antigen or capsule, which covers the whole bacterial surface. The capsule prevents the recognition by antibodies in the immune system that prevents infection, so bacteria can avoid, survive, and proliferate to cause severe damage to bladder cells [5,6].

Uropathogenic encapsulated *E. coli* are categorized into 4 groups due to the function of proteins involved in their capsular biosynthesis pathway [7–11]. Group 2 capsules use ABC transport proteins. In the biosynthesis pathway of K1 capsule [8–10] (group 2),
capsular biosynthesis requires at least 14 proteins to produce the polysaccharide on the outside of the bacterial cell (Figure 4.1a).

The function of capsular proteins expressed from gene clusters in region 1 and region 3 is to support the translocation of K1 polysaccharides through the ABC transporter, i.e. the KpsMT complex (Figure 4.1b). Additionally, enzymes encoded in region 2 behave as polymerases [10]. An understanding of the role of NeuS will provide an ability to trace the whole activity of the biosynthesis pathway. NeuS is a polysialyltransferase enzyme which plays a key role in polysaccharide polymerization and chain extension as mentioned in section 1.3.1 (Figure 1.3b). How these proteins interact with each other is still puzzling. Recent studies of the biosynthesis of the K1 capsule indicate that the polysaccharide is fully synthesized before it is exported through the ABC transporting channels to the outer membrane [12,13]. In the following work, the focus is on how KpsD, KpsE and NeuS interact [14] with each other (Figure 4.1b) 3 key proteins in the biosynthesis pathway. Genetic modification was used to label these proteins with fluorescent markers, Figure 4.1c.
Figure 4.1 a) The proteins (KpsD, KpsE and NeuS) expressed from capsular gene clusters. b) A schematic diagram of the capsular biosynthesis machinery of group 2 K1 capsules is shown from the cytoplasm to the bacterial surface. c) Techniques used for fluorescent labelling. KpsD were labelled with anti-KpsD antibodies and then immunolabelled with antirabbit AF488; KpsE were fused with dsRED fluorescent proteins with a linker; NeuS were conjugated with a Halotag enzyme from the C terminal and then labelled with the fluorescent ligands. For the functions and roles of other proteins in capsular polysaccharide synthesis, there are several reviews [7,10,11,15,16].
For decades, the biosynthesis of capsular polysaccharide has been studied [9,10,17]. However, no definitive models have yet been developed based on real-time analytic tools to observe the mechanism of NeuS action during the synthesis of capsular polysaccharide. From an understanding of the dynamics of NeuS enzymes, the rate of capsular polysaccharide production inside the live uropathogenic bacteria could be predicted.

NeuS is an α-Neu5Ac α-2,8-sialyltransferase which polymerises α-2,8-linked Neu5Ac to form polysialic acid (PSA) [18]. The residues of the K1 neuS gene on the N terminus (for the first 52 base pairs) do not affect the function of NeuS; however, the first 85 base pairs on the C terminus do alter the enzyme function [16,19]. This information was used to create a genetic modification (NeuS::Halotag, NeuSHT, was created) without interrupting the function of the NeuS enzyme.

There have been several indirect observations of the production of polysaccharides; such as biomass information of the extracted polysaccharide based on a batch investigation [12,20]. Instead, in the current work, the fluorescent intensity was observed to understand the time scales over which NeuS enzymes interact with other protein complexes and capsular polysaccharides.
4.1.2 **Fluorescence microscopy with bacterial proteins that are involved in capsule production**

A non-invasive method to observe protein interactions and enzyme kinetics is fluorescence microscopy [21–24] using both imaging [22] and spectroscopy [25]. Fluorescence microscopy allows us to visualize the presence and to localise the position of labelled fluorescent molecules moving inside bacterial cells. The resultant tracks allow the dynamics to be studied. Nevertheless, the technique has a number of limitations [26,27]: the number of collectable photons (fluorescent yield), signal to noise ratio (related to the background noise and the photon yield from the fluorophores) and the rate of image acquisition (limited to 1000 fps by current imaging technologies, e.g. the limitation of sCMOS camera acquisition rate).

In the experiments, *NeuS::Halotag (NeuSHT)* were produced in small amounts (less than 50 molecules per bacterial cell) and the efficiency of the system to collect photons was quite low. The measurement approached the resolution limit of fluorescence imaging microscopy and the dynamics of these rapidly moving enzymes could not be tracked. Instead, fluorescence correlation spectroscopy [28–34] (FCS) was used due to its improved sensitivity, dynamic range (a few ms) and small measurement volume. This spectroscopy technique was thus expected to be more suitable for studies of *NeuSHT* dynamics and their activity in polysaccharide biosynthesis.

In general, bacterial proteins can be tracked with their fluorescent signals if the fluorescent proteins are sufficiently bright. However, single-molecule characterization of unstable proteins and enzymes is challenging. The common tools to label proteins of interest
using fluorescence are by labelling with antibodies or genetic modification with fluorescent proteins. One promising technique is tagging proteins using Halotags [23,35–37] which can then be linked with fluorescent ligands that have a variety of excitation/emission wavelengths. We investigated NeuS enzymes (43 kDa) using Halotags (33 kDa), as a conjugating glue was not expected to affect the mechanism of the NeuS glycolysis reaction.

When bacterial cells are fixed, proteins often have less mobility and can improve the resolution of static images. This allowed longer exposure times to be used and more photons could be collected to identify the localization of fluorescent proteins. In contrast with live bacterial cells, the mobility of bacterial proteins and enzymes are more rapid and can only be tracked if these molecules are very stable and very bright.

4.1.3 Fluorescence correlation spectroscopy (FCS)

4.1.3.1 Gaussian beam profile, \(I(\vec{r})\), and fluorescent intensity variation, \(\delta F(\tau)\)

Fluorescence correlation spectroscopy (FCS) is a technique to quantify the change of fluorescent molecule concentration inside a specific focal volume. A confocal volume was used in the experiments since an Airy scan confocal fluorescence microscope was available for the FCS investigation. A pinhole was used to limit the size of the confocal volume and to remove background noise. Inside the confocal volume, the Gaussian beam was focused with an intensity profile \(I(\vec{r})\) to excite the fluorescent molecules [34],

\[
I(\vec{r}) = I_0 \exp\left(-\frac{2(x^2+y^2)}{w_{xy}^2} - \frac{2z^2}{w_z^2}\right),
\]

where \(I_0\) is an intensity amplitude, \(w_{xy}\) is the beam waist, \(w_z\) is an axial beam elongation and \(\vec{r} = x\hat{x} + y\hat{y} + z\hat{z}\) is a directional vector in xyz coordinates.
To physically interpret the dynamics of fluorescent molecules [34], the fluctuations of the measured photons (fluorescent intensity fluctuation, \( \delta F(\tau) \) that varies in \( \Delta t \), time) can be expressed in terms of concentration variations \( \delta C_i(\vec{r}, \tau) \).

\[
\delta F(\tau) = F(\tau) - \bar{F} = \Delta t \int I(\vec{r}) \sum_{i=1}^k Q_i \delta C_i(\vec{r}, \tau) \, d^3\vec{r},
\]

(4.2)

where \( \delta C(\tau) \) is the concentration difference of fluorescent molecules at time \( \tau \), \( \bar{F} \) is the averaged fluorescent intensity from all the time points, \( \Delta t \) is the total observation (sampling) time, \( \vec{r} \) is the radius of the confocal volume, \( Q_i \) is the absorption cross section of component \( i \) and \( k \) is the total number of molecular species. Although only NeuS::Halotag (NeuSHT) conjugated with fluorescent ligands was investigated, some unbonded and aggregated fluorescent ligands contributed to the number fluctuations. The normalized autocorrelation of fluorescent intensity fluctuations is

\[
G(\tau) = \frac{\langle \delta F(t) \, \delta F(t+\tau) \rangle}{\langle F(t)^2 \rangle}. 
\]

(4.3)

When equation (4.2) is substituted into equation (4.3), the autocorrelation (according to Krichevsky, 2002) becomes [34]

\[
G(\tau) = \frac{(\Delta t)^2}{\bar{F}^2} \iint I(\vec{r})I(\vec{r}') \sum_{j,l=1}^k Q_j Q_l \, \delta C_j(\vec{r}, 0) \delta C_l(\vec{r}', \tau) \, d^3\vec{r} \, d^3\vec{r}'. 
\]

(4.4)

The solution for \( \delta C_j(\vec{r}, \tau) \) will be called \( \delta C_l(\vec{r}, \tau) \), using the subscript for the diffusive molecule \( l \) to avoid confusion, when considers \( t = 0 \) for a simplification and \( r' \) is another position relative to \( r \). \( \delta C_j(\vec{r}, \tau) \) can be solved using Fick’s first law for diffusion,

\[
\frac{\partial}{\partial \tau} \delta C_j(\vec{r}, \tau) = D_j \nabla^2 \delta C_j(\vec{r}, \tau), 
\]

(4.5)
where $\delta C_j(\vec{r}, \tau) = C_j(\vec{r}, \tau) - \bar{C}_j$, $\bar{C}_j$ is an average concentration of the molecular species $j$ and $\tau$ is the time for the molecule to diffuse across the confocal volume. $D_j$, the diffusion coefficient, is taken constant in normal diffusion. However, this parameter can be time-dependent in anomalous diffusion.

Fourier transformation of equation (4.5) gives an expression for the concentration dependence on the spatial frequency $\vec{q}$ and time $\tau$,

$$ \frac{\partial}{\partial \tau} \tilde{C}_j(\vec{q}, \tau) = \sum_{k=1}^{m} M_{lk} \bar{C}_j(\vec{q}, \tau), \quad (4.6) $$

since $\tilde{C}_l(\vec{q}, \tau) = (2\pi)^{-3/2} \int \exp(i\vec{q}.\vec{r}) C_j(\vec{r}, \tau) d^3\vec{r}$ is a Fourier transform of $C_j(\vec{r}, \tau)$ and the Fourier transform of Fick’s law changes the operation to $M_{lk} = -D_l q^2 \delta_{lk}$, where $m$ is the number of dimension ($m = 3$). The $l$ and $j$ subscripts were introduced to differentiate the components in the real and Fourier transform domains respectively.

After using algebraic analysis ($M_l X^{(s)} = \lambda^{(s)} X^{(s)}$), the $M$ matrix has eigenvalues $\lambda^{(s)} = -D_l q^2$ and eigenvectors $X^{(s)}$. When combined with the solution of the initial condition ($\tilde{C}_l(\vec{q}, 0)$) $\tilde{C}_l(\vec{q}, 0) = \sum_{s=1}^{m} X^{(s)}_l h_s$, the solution for the concentration in the time-dependent spatial frequency domain $\tilde{C}_l(\vec{q}, \tau) = \tilde{C}_l(\vec{q}, 0) \exp(\lambda^{(s)} \tau)$ becomes

$$ \tilde{C}_l(\vec{q}, \tau) = \sum_{s=1}^{m} X^{(s)}_l \sum_{k=1}^{m} (X^{-1})^{(s)}_k \tilde{C}_k(\vec{q}, 0) \exp(\lambda^{(s)} \tau). \quad (4.7) $$

To calculate the autocorrelation function, the Fourier correlation is introduced,

$$ \langle C_j(\vec{r}, 0) C_l(\vec{r}', \tau) \rangle = \langle C_j(\vec{r}, 0) \{ (2\pi)^{-3/2} \int \tilde{C}_j(\vec{q}, \tau) \exp(-i\vec{q}.\vec{r}') d^3\vec{q} \} \rangle. $$

The correlation length is very small, so the Kronecker delta function can be used $\langle C_j(\vec{r}, 0) C_k(\vec{r}'', 0) \rangle = \delta_{jk}$.
\[ \bar{C}_j \delta_{jk} \delta(\vec{r} - \vec{r''}), \] where \( \vec{r''} \) is a dummy variable used for small correlation lengths. This can reduce equation (4.7) to

\[ < C_j(\vec{r}, 0)C_l(\vec{r'}, \tau) > = \left( (2\pi)^{-3/2} \left\{ \exp(-i \vec{q} \cdot \vec{r'})d^3 \vec{q} \right\} \bar{C}_j \sum_{s=1}^{m} X_l^{(s)} \exp(\lambda^{(s)} \tau)(X^{-1}_j)^{(s)} \right) \]

(4.8)

Alternatively, it can be simplified as

\[ < C_j(\vec{r}, 0)C_l(\vec{r'}, \tau) > = < C_j > (4\pi D \tau)^{3/2} \exp \left( -\frac{(\vec{r} - \vec{r'})^2}{4D \tau} \right). \quad (4.9) \]

Although equation (4.9) is an approximation, for a general calculation of diffusive molecules, the autocorrelation equation, equation (4.4) can be combined with equation (4.8) to give

\[ G(\tau) = \left( \frac{\Delta t}{\tau} \right)^2 \int |\tilde{I}(\vec{q})|^2 \sum_{j,l=1}^{k} Q_j Q_l \bar{C}_j \sum_{s=1}^{m} X_l^{(s)} \exp(\lambda^{(s)} \tau)(X^{-1}_j)^{(s)} \, d^3 \vec{q}. \]

(4.10)

Using the Fourier transformation of the excitation intensity in equation (4.1) \( \tilde{I}(\vec{q}) = \frac{I_0 w_{xy} w_z}{8} \exp\left( -\frac{w_{xy}^2 (q_x^2 + q_y^2)}{8} - \frac{w_z^2 q_z^2}{8} \right) \), and Fourier transform of the average intensity of the collected signal \( F = \Delta t \int I(\vec{r}) \sum_{l=1}^{k} Q_l C_l(\vec{r}, \tau) d^3 \vec{r} = (2\pi)^{3/2} \tilde{I}(\vec{q}) \Delta t \sum_{l=1}^{m} Q_l \bar{C}_l \) the general form of autocorrelation can be written as

\[ G(\tau) = \left( \frac{2\pi}{\Delta t} \right)^{3/2} \frac{w_{xy} w_z^2}{64} \int \left\{ \exp \left( -\frac{w_{xy}^2 (q_x^2 + q_y^2)}{4} - \frac{w_z^2 q_z^2}{4} \right) \right\} d^3 \vec{q} \]

\[ \times \sum_{j,l=1}^{k} Q_j Q_l \bar{C}_j \sum_{s=1}^{m} X_l^{(s)} \exp(\lambda^{(s)} \tau)(X^{-1}_j)^{(s)}. \quad (4.11) \]
With this general equation, the autocorrelation function can be determined from the eigenvalue (s), \( \lambda^{(s)} \), in the Fourier spatial frequency domains \((q_x, q_y, q_z)\) for the dynamics of fluorescent molecules.

4.1.3.2 Models of FCS curves as a predictive tool to investigate NeuS in the capsular polysaccharide biosynthesis pathway

By adapting the general equation for the autocorrelation function, from equation (4.11) the time constants \( \tau_D \) and \( \tau'_D \) can be extracted for lateral \((w_{xy})\) and axial \((w_z)\) diffusion inside the confocal volume \( V \approx \frac{4}{3} \pi w_{xy}^2 w_z \). In equation (4.5), \( j \) defines the number of species in the mixture. If there is only one species \((j = 1)\) of fluorescent molecules in this confocal volume, the definition of time constants can be defined as the periods that fluorescent molecules diffuse through the lateral and axial beam waist. To calculate the autocorrelation, the eigenvalue \( (\lambda^{(s)}) \) and eigenvector \( (X_i^{(s)}) \) are calculated. The term inside the integral of equation (4.10), \( \sum_{j,l=1}^{k} Q_j Q_l \bar{c}_j \sum_{s=1}^{m} X_l^{(s)} \exp(\lambda^{(s)} \tau)(X^{-1})^{(s)}_j \) becomes \( Q^2 \bar{c} X^{(s)} \exp(\lambda^{(s)} \tau)(X^{-1})^{(s)} \), since \( k \) and \( m \) are equal to 1. With only one eigenvector and one eigenvalue (the trivial solution is using \( X = 1 \) and \( \lambda^{(s)} = -D(q_x^2 + q_y^2 + q_z^2) \)) it becomes \( Q^2 \bar{c} \exp(-Dq^2\tau) \) and is substituted back to equation (4.11), where \( q^2 = q_x^2 + q_y^2 + q_z^2 \).

Hence, the mean square displacement of these molecules could be found as \( <r^2>_{\text{lateral}} \approx w_{xy}^2 \approx 4D'\tau_D \alpha \) and \( <r^2>_{\text{axial}} \approx w_z^2 \approx 4D'\tau'_D \alpha \). The autocorrelation function for sub diffusive transport becomes

\[
G(\tau) = \frac{1}{cV} \left( 1 + \left( \frac{\tau}{\tau_D} \right)^\alpha \right)^{-1} \left( 1 + \left( \frac{\tau}{\tau'_D} \right)^\alpha \right)^{-1/2}, \quad (4.12)
\]
where $\bar{CV} = \bar{N}$ the average number of molecules inside the confocal volume, $\alpha$ is a constant exponent and $D'$ is the diffusion coefficient. When molecules move with normal diffusion ($\alpha = 1$), $D' = D$ represents the standard diffusion coefficient. If the exponent constant is not equal to 1, this means the molecules are in the anomalous diffusive regime, so $D'$ is called the anomalous diffusion coefficient and has fractional units. From in vivo investigations, anomalous diffusion may happen when proteins of interest interact with cells’ components (e.g. lipid raft and cytoskeleton) to cause the diffusion coefficient $D_\alpha(t)$ to vary with time [29]. There are several models for anomalous diffusion, including obstruction, continuous time random walk (CTRW) and fractional Brownian motion (fBM) to name just a few [38].

With multiple species of fluorescent molecules ($j = 1, 2, 3, \ldots$) in the confocal volume, more diffusion equations are required (equation (4.5)). Consequently, the number of eigenvalues and eigenvectors varies due to the involvement of each fluorescent molecular species. In the case of a mixed population, equation (4.11) can be used to derive

$$G(\tau) = \frac{1}{(\Sigma_{k=1}^m Q_k N_k)^2} \left( \Sigma_{j=1}^m Q_j^2 \bar{N}_j (1 + \left( \frac{\tau}{\tau_{Dj}} \right)^\alpha )^{-1} (1 + \left( \frac{\tau}{\tau_{Dj}} \right) )^{-1/2} \right), \quad (4.13)$$

where $m$ is the number of species. Equation (4.13) is equivalent to $G(\tau) = \frac{1}{(\Sigma_{i=1}^m \bar{F}_i)^2} \Sigma_{i=1}^m \bar{F}_i^2 G_i(\tau)$, where $\bar{F}_i = Q_i \bar{N}_i$. Hence, this autocorrelation function can be applied to characterise the dynamics of molecules in mixed systems. However, the ability to resolve the different components is dependent on how strong the fluorescent signal is in comparison to background noise, called the signal-to-noise ratio.
To have an optimal resolution with the FCS measurements, there are several requirements to enhance the signal-to-noise ratio (S/N). For example, the number of fluorescent molecules in the confocal volume $\bar{N} = \bar{C}V$ and the rate of photon acquisition from each fluorescent molecules, $v$, in a $\Delta t$ acquisition time are important. This signal-to-noise ratio (S/N) can be stated as

$$\frac{S}{N} = \frac{G(\tau)}{\sqrt{\text{var}G(\tau)}} = \langle \frac{\delta F(0) \delta F(\tau)}{F^2} \rangle / \left\{ \frac{1}{TF^4} \text{var}(\delta F(0) \delta F(\tau)) \right\}^{1/2}, \quad (4.14)$$

where $T$ is the total acquisition time of the FCS measurement. According to equation (4.14), $G(\tau) \propto 1/\bar{N}$ and the variance of $G(\tau)$ ($\text{var} G(\tau)$) is dependent on $\bar{N}$ and $v$, where the FCS experiments observed in $v \sim 1 \mu m^3$. After using the Taylor’s expansion of 2$^{nd}$ moment for a definition of variance, $\text{var} f(x) \approx (f'(E[x]))^2 \text{var}(x)$, the variance of the autocorrelation function is

$$\sqrt{\text{var} G(\tau)} = \begin{cases} \frac{1}{\sqrt{N}} & \text{small } \bar{N} \\ \frac{1}{\sqrt{TN}} & \text{large } \bar{N}. \end{cases} \quad (4.15)$$

The variance of the FCS measurement can be used to determine the S/N in the system and approximate the emission efficiency of the fluorescent ligands used to determine the dynamics of the protein of interest, e.g. NeuS::Halotag (NeuSHT).
4.1.3.3 Monte Carlo simulation of a mixed population of fluorescent molecules in experimental FCS measurements (in collaboration with Jack W. Hart)

There are several methods to simulate anomalous diffusion and reproduce FCS correlation functions, e.g. obstructed walks, continuous time random walks (CTRW) and fractional Brownian motion (fBm) [38]. The fBm model was used for anomalous diffusion since this could mimic the NeuS::Halotag interacting with proteins complexes inside bacterial cells.

In order to generate anomalous diffusion from a high-order fractional Gaussian process, the Weierstrass-Mandelbrot (WM) function was used [38],

\[ W_x(t) = \sum_{n=-\infty}^{+\infty} \frac{1}{\gamma^{nH}} (\cos \phi_x(n) - \cos[\gamma^n t^* + \phi_x(n)]), \]  \hspace{1cm} (4.16)

where \( t \) is the time, \( t_{\text{max}} \) is the total time and \( t^* = 2\pi/t_{\text{max}} \). \( \gamma = \sqrt{\pi} \) and \( \phi_x(n) \) are uniform phases between 0 and 2\( \pi \). This function can generate both normal and anomalous behaviour depending on the Hurst exponent \( H \), where the mean square displacement is \( <r^2> \propto t^\alpha = t^{2H} \). When \( \alpha = 1 \), there is a normal diffusive regime, whereas when \( \alpha < 1 \) there is a subdiffusive regime. The \( x \) position at time \( t \) was generated from equation (4.16) and the noise was generated as the sum of the differences of this function. In addition to \( x \) generation, \( y \) was also generated using the same method with a different phase. By using this function to generate fractional Brownian motion, the FCS curve from the subdiffusive motion of simulated particles could be calculated.
4.2 Methodology

4.2.1 Genetic modification to express KpsE::dsRED and NeuS::Halotag proteins

4.2.1.1 KpsE::dsRED in pBAD33 plasmids

The fragments of kpsE DNA (1.1 kbs) were amplified using the polymerase chain reaction (PCR) from the UTI89 nucleotide using kpsE-F (forward primers) and kpsE-R (reverse primers) with a proof reading (Pwo) DNA polymerase (in chapter 2, section 2.1.2). They were then digested with SacI and XbaI restriction enzymes for 1hr at 37°C, with both kpsE PCR products and pBAD33 plasmids. After cleaning up the digested enzyme, a ligation reaction was used at 10°C overnight to make a KpsE::dsRED in pBAD33 plasmid construction. Figure 4.2a (left) shows how this DNA fragment was constructed in pBAD33 plasmids.
4.2.1.2 NeuS::Halotag in pBAD33 or pBSK II + plasmids

The fragments of neuS DNA were multiplied using PCR from UTI89 nucleotides or pKT279 plasmids. In the PCR, NeuS-F and NeuS-R were the forward and reverse primers
(in chapter 2, Table 2.5) that were mixed with Pwo DNA polymerase and pKT279 plasmids in 30 cycles of 95°C for denaturing, 60°C for annealing and 72 °C extension over 1.5 minutes for each cycle. The amplified PCR products of neuS were purified and digested with XbaI and SacI enzymes for 1hr at 37°C, with both the neuS PCR products and the pH6HTC plasmids (this plasmid contained the DNA sequence of a Halotag enzyme). After cleaning the digested enzyme, a ligation reaction at 10°C was performed overnight to insert into pBAD33 and pBluescript (pBSKII+) plasmids. The plasmids from neuS::Halotag in pH6HTC were purified using plasmid purification kits (Qiagen). The products of neuS::Halotag in pH6HTC, pBAD33 and pBSKII+ plasmids were digested with XbaI and HindIII enzymes before cleaning. This was followed with a ligation process (with DNA ligase enzyme at 10°C overnight) to make neuS::Halotag in pBAD33 and pBSKII+. Figure 4.2a (right) shows how this DNA fragment was constructed in pBSKII+ plasmids.

4.2.1.3 Plasmid selection and K1 capsule compliment confirmation

The inserted kpsE::dsRED in pBAD33 and neuS::Halotag in pBAD33/ pBSK II+ were chemically transformed into DH5α E.coli for multiplication and storage. To store the transformed plasmids, colony PCR and electrophoresis gels were used before fragment dropping tests and DNA sequencing. The potential colonies were picked up and then their transformed plasmids were purified. The confirmation of plasmid transformation was made using dropping size measurements (fragments were digested with specific enzymes before running through a 1% agarose gel) and 100% agreement was found in sequencing results with that expected. After their sequence had been confirmed, the plasmids were electro-transformed into an EV136 (K1 capsule negative) strain of E. coli. The colonies that survived
on specific antibiotics were selected as potential, positive plasmids. EV136 (neuS::Halotag in pBAD33) survived and continued to multiply in bacterial culture media with tetracycline (10 µg/ml, tet10) and chloramphenicol (34 µg/ml, cm34). Furthermore, EV136 (neuS::Halotag in pBSK II+) would grow in bacterial culture media with tetracycline (10 µg/ml, tet10) and ampicillin (100 µg/ml, AMP100). Double electro- transformations of EV136 (neuS::Halotag in pBSK II+; kpsE::dsRED in pBAD33) would proliferate in culturing media with tet10, cm34 and AMP100.

To confirm K1 capsule production, a K1 phage assay was tested on selected colonies and they were 100% positive. These K1 phage viruses can specifically recognise K1 capsular polysaccharide and only lyse the bacterial cells that are encapsulated with K1 polysaccharide. Since EV136 is a K1 capsule negative strain, there were no void regions due to phage recognition. However, EV36 (wild type and K1 capsular polysaccharide positive) showed void regions that were spread over the whole culture plate. In the plasmid inserted strains, they were recognised by the K1 phage: EV136 (neuS::Halotag in pBAD33) with arabinose (ara) induction, EV136 (neuS::Halotag in pBSK II+) with isopropyl β-D-1 thiogalactopyranoside (IPTG) induction and EV136 (neuS::Halotag in pBSK II+; kpsE::dsRED in pBAD33) with arabinose and IPTG induction (an example of K1 phage results is shown in Appendix B1).

4.2.1.4 The expression of KpsE:: dsRED and NeuS::Halotag from pBAD and pBSK+

induced by arabinose and isopropyl β-D-1 thiogalactopyranoside (IPTG)

kpsE::dsRED inserted in pBAD33 and neuS::Halotag inserted in pBAD33/pBSK II+ were produced and then analysed using Western blot techniques to quantify the number of
proteins expressed. According to Figure 4.2b, the KpsE::dsRED proteins (~60kDa) appeared after induction with 0.05 %, 0.1 %, 1% and 2% w/v of arabinose. The NeuS::Halotag enzymes (~50kDa) appeared after incubation with 1mM IPTG for 2 hrs and 17 hrs. Despite having been expressed for a longer time (17hrs), NeuSHT enzymes had low copy numbers. The KpsE::dsRED proteins and NeuS::Halotag enzymes were produced in small amounts and no significant difference occurred when induced with higher concentrations of arabinose or IPTG, or for a longer period of time.

4.2.2 Halotag with TMR/AF488 ligand labelling on pre-expression proteins

Before incubation with TMR/AF488 ligands, bacterial strains were grown overnight in LB at 20°C, with antibiotic supplements, before inoculation in M9 media. Bacterial strains: EV36 (wt, K1 capsule positive) and EV136 (neuS mutant, K1 capsule negative with tet10 antibiotics), EV136 (neuS::Halotag in pB33/pBSK II + with tet10 and cm34/AMP100 antibiotics) and DH5a (neuS::Halotag in pB33 with cm34 antibiotic) were used with their different antibiotic reagents.

TMRs (2,6-dideoxy-4-thiomethyl-beta-d-ribohexopyranoside) or Alexa Flour488 ligands with chloroalkane conjugation with Halotag enzymes were used to label NeuSHT inside encapsulated K1 bacteria. Due to the small size of these ligands, they can permeate through bacterial membrane and can label the bacterial cytoplasm. These bacteria expressed the compliment plasmids (NeuS::Halotag pBSK+II plasmids) by the following procedure. After cells were cultured in LB media at 20°C overnight (OD600 1.2), they were re-inoculated at 1:100 in M9 media with glycerol as a carbon source and a casein supplement. It is possible to induce proteins of interest by arabinose and IPTG for pBAD33 and pBluescript SK+ II
This step could produce enough \textit{KpsE::dsRED} proteins and \textit{NeuS::Halotag} enzyme before applying the Halotag ligands. When bacterial growth was in the exponential phase (OD$_{600}$ 0.2), bacteria were incubated with TMR/AF488 ligands (with 5\( \mu \)M concentration) in an Eppendorf with shaking at 37 \( ^{\circ} \)C. After incubation for an hour, labelled bacteria were washed with PBS and moved to a fresh Eppendorf to reduce the background noise from unbound ligands.

Labelled and washed \textit{E. coli} were deposited on a poly-L-lysine pre-coated glass slide and left for 2 minutes before immobilization with 4\% agarose dissolved in M9 media, ready for observation using confocal microscopy and fluorescence correlation spectroscopy.

\textbf{4.2.3 Fluorescence microscopy and fluorescence correlation spectroscopy (FCS)}

For TMR/AF488 ligand optimization, wide-field fluorescence microscopy, with an Axio Imager.M2 Upright (Zeiss) microscope with 63x Plan Apochromat (Oil, DIC) and 150X Olympus UApO 1.4NA Oil immersion lens, was used at low power with selected filters from 405 to 990 nm.

For confocal microscopy, images were collected using a Leica TCS SP8 AOBs Inverted gSTED microscope in a confocal mode with a 100x HC PL APO lens (Oil; STED WHITE). The excitation laser was a tuneable super-continuum source and was used at around 250mW. The Zeiss LSM880 with Airyscan also was used with 63x Plan-Apochromat 1.4NA (Oil, DIC) for confocal imaging. With this system, a calibration procedure was done to measure the size of the confocal volume with different sized apertures (e.g. 1AU with a 33 \( \mu \)m pinhole size) using 100 nm Cy3B coated with PS beads. The images of fluorescent beads were corrected from different axial plane to approximate the point spread function.
(PSF) after fluorescent beads were activated i.e. a calibration in confocal microscopy. With the calibrated confocal volumes, the FCS results were verified to represent the dynamics of fluorescent molecules rather than random background noise. Images from wild-field and confocal fluorescence microscopy were analysed using a Fiji plugin and MATLAB codes. The spectroscopic data from FCS also was analysed using MATLAB and Origin.

4.3 Results

4.3.1 The presence of KpsD, KpsE and NeuS::Halotag (NeuSHT) proteins in capsular biosynthesis

4.3.1.1 The localization of KpsD and KpsE recognised with their specific rAb antibodies

There are 14 proteins expressed from the CPS clusters involved in capsular biosynthesis pathways [8,10,11]. In this chapter, the focus is on KpsD, KpsE, and NeuS. They play a key role in the biosynthesis and transfer of the K1 capsule from the inside of the cytoplasm to the outside of the bacterial surface. KpsD and KpsE are membrane proteins that transport capsular polysaccharide chains from the cytoplasm to the cell surface [8].

KpsD (cytoplasmic, polysaccharide co-polymerase protein) and KpsE (outer membrane polysaccharide protein) were observed using fluorescence microscopy labelled with anti-KpsE and anti-KpsD antibodies in live and fixed cells. To identify the localization of these proteins, the bacterial chromosomal DNAs were labelled with DAPI dyes internally while anti-K1 antibody (mAb735) labelled the K1 bacterial capsule on the outside of the cells. Since anti-KpsD and anti-KpsE antibodies have polyclonal specificities (rabbit Ab), both could not be labelled simultaneously. The KpsE proteins were predominantly labelled
in the cytoplasm (**Figure 4.3a**), while the *KpsD* proteins were labelled in the outside of the bacterial membrane and colocalised with the K1 bacterial capsule (**Figure 4.3b**).

The localization of *KpsD* proteins is shown in **Figure 4.3a** (green spots) and the blue regions represented the DAPI counter staining on the chromosomes inside the bacterial cytoplasm. In addition, in **Figure 4.3b**, the localization of *KpsE* proteins are shown as green spots, where the red labelled regions represent the K1 bacterial capsule. Since protein complexes for capsule polysaccharide synthesis were produced after a temperature change from 20 to 37 °C, the average number of viable complexes changed with time. However, there is no significant difference in the numbers of proteins, in **Figure 4.3c** implying that their stoichiometry is reasonably well matched, e.g. 1:1(from this investigation). Also, protein complexes were observed all over the bacterial surface. This would imply that, although they are needed for capsule production, the degradation rate of these proteins was slow.
Figure 4.3 Fluorescence images of proteins involved in K1 capsular polysaccharide biosynthesis: KpsD and KpsE imaged, from scanning confocal microscopy. a) KpsD proteins labelled with rAb antiKpsD-AF533 (green) were labelled on the bacterial membranes and counter-stained with DAPI (blue); white dash lines are approximate bacterial positions. The right-hand side of Figure 4.1a) shows the 3D constructed image used for quantifying the amount of protein aggregation. b) KpsE proteins labelled with rAb antiKpsE-AF533 (green) and K1 capsular polysaccharides were labelled with mAb antiK1-AF647 (red). The adjacent image shows the shape of healthy bacteria during labelling. c) The time independence of
protein expression showing the number of proteins inside K1 encapsulated bacteria as a function of time after temperature upshifting.

4.3.1.2 The coexistence of NeuS::Halotag (NeuSHT) and others proteins: KpsE and KpsD in K1 capsular biosynthesis

NeuS is a glycosyltransferase enzyme, which elongates sialic chains of the capsular polysaccharide (K1 PSA). NeuS enzymes are unstable and only a few of them per bacteria are produced naturally during the biosynthesis process. When neuS DNA was genetically modified to conjugate with Halotag enzyme DNAs, NeuSHT enzymes were produced. These could be tagged or labelled with a range of fluorophores, AF488 (green, 488nm excitation) or TMR (orange, 533 nm excitation). In order to investigate the fluctuations of fluorescent signals, a high concentration (~ nM concentrations) of NeuSHT was needed. This high expression often disturbed the metabolism of cells causing cell elongation. KpsD-anti-KpsD rAb AF488 colocalization with NeuSHT TMR-ligand and K1 bacterial capsule were investigated and are shown in Figures 4.4a and 4.4b.

Due to the low expression of NeuSHT (Figure 4.2c), it was difficult to track the movement of fluorescent proteins inside bacterial cells using imaging. The probability of successful labelling inside the bacteria cells also varied. Often there was no NeuSHT expression, and TMR/AF488 ligands permeated inside bacteria with no expressed NeuSHT. This will be described in more detail in a further section.

As an alternative method to fluorescence tracking, KpsE::dsRED and NeuSHT were expressed simultaneously. The mobility of KpsD, KpsE::dsRED, NeuSHT and K1 PSA rafts
were observed using the fluorescence correlation spectroscopy (FCS) mode of an Airy confocal microscope. The calibration and control experiment for the FCS curve will be shown in the following section. In Figures 4.4a to 4.4c, the fluorescent labelling on proteins and enzymes, including KpsD (Figure 4.4a), TMR-labelled NeuSHT and K1 PSA rafts (Figure 4b), KpsE::dsRED (Figure 4.4c), were analysed to identify their mobilities via statistical analysis of the autocorrelation of the fluorescence fluctuations. In Figure 4.3d, these FCS curves revealed the mobility of different fluorescence signals, showing the low mobility of KpsD, KpsE and K1 PSA rafts and the high mobility of NeuSHT. According to equation 4.13, when molecules are localised at short lag time (μs), the high correlation is revealed as a large amplitude of the FCS curve. However, when long lag times occur (s to min), low correlation appear as the foot of FCS curve. Intermediate lag time (ms) represent the dynamics of NeuSHT mobility according to their activities.
**Figure 4.4** Comparison of the immobilised and mobilised proteins using confocal microscopy and FCS. **a)** The presence of *KpsE* proteins (green) in live bacterial cells was demonstrated with confocal imaging. **b)** A merged image of K1 bacterial capsular rafts (K1 PSA, red) and NeuS::Halotag conjugated with TMR ligands (NeuSHT::TMR, yellow) from confocal imaging. **c)** Additional confocal imaging experiments that show the functioning
NeuSHT::TMR coexisted with KpsE::dsRED proteins while the bacteria produced K1 capsular polysaccharide. d) The FCS autocorrelation function versus lagtime of KpsE proteins (orange), KpsD proteins (red), NeuS::Halotag enzymes (green) and K1 capsular polysaccharide raft (black).

4.3.2 FCS analysis of the dynamics of NeuS::Halotag (NeuSHT)

4.3.2.1 Calibration of FCS curves from different confocal volumes

TMR and AF488 ligands were used to label NeuSHT enzymes to investigate the mechanism of glycosylation of bacterial capsule polysaccharide. The initial expectation was to track fluorescent signals from NeuS enzymes to investigate their dynamics using imaging. However, after several attempts using a variety of fluorescence imaging techniques, it was difficult to clearly identify and simultaneously track the proteins.

Due to the low fluorescence intensity and high background noise inside the bacterial cells, fluorescence correlation spectroscopy (FCS) was used. A schematic diagram of the confocal detection volume is shown in Figure 4.5a and demonstrates how KpsD and KpsE are localized and bound to the protein complex (Figure 4.1b) that then interacts with NeuSHT in the capsular biosynthesis pathway.

Using the FCS technique, the fluctuations of the fluorescence signals from bacterial cells were measured and autocorrelated. These curves reflected the dynamics of fluorescent molecules moving across the confocal volume. The fluctuation of fluorescence intensities from molecular diffusion across the confocal volumes was also analysed, Figure 4.5b. With the assumption that the FCS results should not depend on the size of the confocal volume,
control experiments of the 0.5 and 1 Airy unit (AU) were performed (Appendix B2), where the Airy unit is defined as the width of point spread function (the lens resolution multiplied with the lens magnification) which is proportional to the pinhole size. The inset of Figure 4.5b shows as an invariant parameter: a time constant ($t_d$), which is the time that the fluorescent particles moved across the specific volume ($v$ confocal volume). Even if the confocal volume varied, the motility of the fluorescent proteins should be similar. Since the large confocal volume $\sim 1$-$2 \, \mu m^3$ is about the size of bacteria cells, the experiments sampled fluorescent proteins that were attached to the bacterial membranes, interacted with the protein complexes and freely diffused inside the cytoplasm.

**Figure 4.5** A schematic diagram of fluorescence correlation spectroscopy (FCS) measurements on *NeuSHT* labelled with TMR ligands and the confocal volume calibration using different sized pin holes. **a)** Schematic of how the confocal volume occupied the space during FCS experiments with *KpsD*, *KpsE* and *NeuS::Halotag* inside bacterial cells. **b)** The
FCS autocorrelation function versus lag time from two different confocal volumes with 2 aperture sizes: 0.5 Airy unit (AU) and 1 AU. The inset shows the time constants calculated.

Attempts to visualise moving fluorescent *NeuSHT* enzymes before FCS measurements were difficult due to their instability and low molecular weight. There were a small number of cells containing fluorescent ligands; these ligands were inside bacterial cells bound to *NeuSHT*, but some were unbound. The unbound ligands for *NeuSHT* were observed in the bacterial cytoplasm even though the cells were washed several times. However, the populations of fluorescent cells associated with the ligands varied. In the case with no expression of *NeuSHT* (EV136, EV136pB33 and DH5α), the number of fluorescent cells was significantly less than the cells where *NeuSHT* had been expressed (EV136pB33_ara1, EV136pBSK+, EV136pBSK+_IPTG1). The *NeuSHT* also interacted with capsular proteins complexes (which form ABC transport channels) involved in the K1 polysialic glycosylation (PSA) process.

4.1.3.2 FCS curves for different conditions of *NeuSHT* expression

In addition to the labelling of TMR/AF488 ligands, the FCS observations required the fluorescence signals to be sufficiently bright. The statistical analysis of the FCS curves was performed on several repeats. Several conditions of *NeuS::Halotag (NeuSHT)* expression were investigated, shown in Table 1. The observations are categorised dependent on different conditions of *NeuSHT* expression, including: i) no *NeuSHT* expression (EV136 and EV136pB33), ii) few/leakage expression (EV136pB33_ara01, and EV136pBSK), iii) over expression (EV136pBSKIPTG) and iv) low *NeuSHT* expression with no capsular protein complexes surrounding.
Table 4.1 The conditions for *NeuSHT* expression inside encapsulated and non-encapsulated bacteria that were used in FCS measurements.

<table>
<thead>
<tr>
<th>Expressed NeuS::Halotag (NeuSHT) Conditions</th>
<th>Description</th>
<th>The level of <em>NeuSHT</em> expression</th>
<th>Capsular proteins complex</th>
<th>NeuSHT expression condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>■ EV136</td>
<td>Non-encapsulated bacteria</td>
<td>No <em>NeuSHT</em></td>
<td>Yes</td>
<td>I</td>
</tr>
<tr>
<td>■ EV136pB33</td>
<td>Non-encapsulated bacteria that were complimented with pneuSHTpB33 plasmids</td>
<td>No <em>NeuSHT</em></td>
<td>Yes</td>
<td>I</td>
</tr>
<tr>
<td>■ EV136pB33 ara01</td>
<td>Encapsulated bacteria where NeuSHT were expressed from pneuSHTpB33 plasmids with 0.1% arabinose induction</td>
<td>Few copy numbers</td>
<td>Yes</td>
<td>II</td>
</tr>
<tr>
<td>■ EV136pBSK</td>
<td>Encapsulated bacteria where EV136 cells were complimented with pneuSpBSK+ plasmids</td>
<td>Few copy numbers (due to leakage expression)</td>
<td>Yes</td>
<td>II</td>
</tr>
<tr>
<td><strong>EV136pBSK</strong>&lt;br&gt;<strong>IPTG1</strong></td>
<td>Encapsulated bacteria where NeuSHT were expressed from pneuSpBSK+ by 1mg/ml IPTG induction</td>
<td>High copy numbers (due to the over-expression)</td>
<td>Yes</td>
<td>iii</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>DH5α</strong>&lt;br&gt;<strong>neuSHTpB33</strong>&lt;br&gt;<em>at 20°C</em></td>
<td>Non-encapsulated DH5α bacteria that were complimented with pneuSHTpB33 plasmids</td>
<td>Few copy numbers</td>
<td>No</td>
<td>iv</td>
</tr>
<tr>
<td><strong>EV136</strong>&lt;br&gt;<strong>neuSHTpB33</strong>&lt;br&gt;<em>at 20°C</em></td>
<td>Non-encapsulated EV136 bacteria that were complimented with pneuSHTpB33 plasmids</td>
<td>Few copy numbers</td>
<td>No</td>
<td>iv</td>
</tr>
</tbody>
</table>

The autocorrelations of TMR/AF488 signals at different lag times are shown in Figure 4.6. These curves illustrate the different conditions of NeuSHT expression and were analysed to explore the dynamics of NeuSHT and their interaction with protein complexes during K1 capsular biosynthesis. The dynamics of these curves was independent of the amplitudes of the FCS curve $G_0$, which is inversely proportional to the number of observable proteins ($N$), $G_0 \approx 1/N$. The number of observable proteins explains the variance of the autocorrelation function according to equation (4.14). In addition, the possibility of TMR/AF488 ligands labelling inside was tested via the FCS curves. The higher the $G_0$, the lower the $N$, which implied that the lower amplitudes had less TMR/AF488 conjugated proteins.
NeuSHT due to the expression level of these enzymes. The availability of the fluorescent tagged NeuSHT was difficult to discern.

Figure 4.6 FCS correlation function versus lag times from different NeuS::Halotag (NeuSHT) expression levels and conditions. The inset shows the condition that NeuSHT had been expressed without any other proteins that are involved in capsular biosynthesis. The samples included: EV136 (non-encapsulated bacteria, orange), EV136pB33 (non-encapsulated bacteria that were complemented with pneuSHTpB33 plasmids, yellow), EV136pB33_ara01 (encapsulated bacteria that NeuSHT were expressed from
pneuSHTpB33 plasmids with 0.1% arabinose induction), EV136pBSK+ (encapsulated bacteria with NeuSHT from a leakage expression of pneuSpBSK+ plasmids), EV136pBSK+_IPTG1 (encapsulated bacteria where NeuSHT were expressed from pneuSpBSK+ by 1mg/ml IPTG induction), DH5αNeuSHTpBara (non-encapsulated bacteria where NeuSHT were expressed from pneuSpB33 plasmids by 0.1% w/v arabinose, red). The inset was a control experiment when cells were cultured and observed at 20°C with NeuSHT expression from pneuSpB33 plasmids by 0.1% arabinose inside EV136 (black) and DH5α (red) cells. The fit parameters and their distributions are presented in Figure 4.7. The two grey dashed lines represent different times of observation, $td_1$ and $td_2$, respectively.

The data was separated into 2 time domains: less than milliseconds (< ms) and greater than milliseconds (> ms). In the first domain, the time constant ($td_1$) was fit from µs to ms, while the second domain ($td_2$) was from ms to s. The dynamic regimes for $td_1$ and $td_2$ were normal diffusion and sub diffusion, respectively. However, sometimes a mixed regime was observed (with both normal diffusion and sub diffusion) in the $td_1$ domain. The fit parameters were analysed using equation (4.12). Based on the assumption of the interaction of NeuSHT with proteins complexes involved in capsular biosynthesis, the FCS curve revealed a sub-diffusive regime in the longer time domain.

In addition to NeuS::Halotag (NeuSHT) interaction with the capsular protein complex (Figure 4.6), the dynamics of expressed NeuSHT in the absence of capsular protein complexes, from EV136 (neuSHTpB33, 20°C) and DH5α (neuSHTpB3, 20 °C), were observed and they shared similar FCS curves (inset of Figure 4.6). The bound TMR/AF488
ligands and unbound ligands to NeuSHT enzymes could be differentiated by a statistical analysis of their autocorrelation curves. The distribution of diffusion coefficients (and time constants) could be differentiated due to size variation (around 77 kDa) after ligands bound to NeuSHT. Fit constraints were used to separate the two possible domains of fluorescent molecule dynamics. When TMR ligands travelled inside bacterial cells and in the case of no NeuSHT expression (i), normal diffusion could be observed in the sub-millisecond domain, Figures 4.7a and Appendix B3a. Nonetheless, with NeuSHT production (ii and iii), they interacted with capsular protein complexes and the TMR ligands conjugated to NeuSHT sub-diffused within sub-millisecond, Figure 4.7b and Appendix B3b.

4.1.3.3 Analysis of FCS fit parameters: diffusive exponents (\(\alpha\)), characteristic times (\(\tau_D\)) and approximate populations (\(N\)).

After statistical analysis using equation (4.12), the FCS curves provided the parameters: \(\alpha\), \(\tau_{D1}, \tau_{D2}\), and \(N\) (Appendices B3, B4 and Figure 4.8). Although the fit results from \(td_1\) regime were obviously comparable in time scale, the results in \(td_2\) were broadly spread from ms to s time scale. This made the comparison more difficult (Appendices B3b, B3d, and B4b). In order to differentiate fit results, a characteristic length \((L_{MSD})\) from mean square displacement definition \((MSD)\) was introduced.

The characteristic lengths \((L_{MSD})\) were calculated. Using the definition of \(MSD\), a diffusion coefficient was approximated \(D_\alpha = MSD/4 \tau_D^\alpha\) inside the confocal volume, where \(MSD \sim 1 \mu m^2\), \(\tau_D\) and \(\alpha\) are from fit parameters. With approximate diffusion coefficients and other parameters: \(\alpha\) and \(\tau_D\), the characteristic lengths were calculated using \(L_{MSD}^2 = 4D_\alpha \tau^\alpha\) (where \(\tau\) is a lag time) in all NeuSHT expression. This characteristic length
\( L_{MSD}^2 \) is different from the general MSD. It is used as a normalised parameter to compare the states of diffusion in all conditions, including normal diffusion and sub-diffusion.

In Figure 4.7a, the characteristic lengths of individual lag time were calculated from the parameters acquired from the sub ms range \((td_1)\) and the average of \( L_{MSD}^2 \) were plotted versus lag times. In addition, Figure 4.7b shows the characteristic lengths calculated using parameters fitted from the \( td_2 \) range (longer than ms). The relations in Figure 4.7 were fit with power laws to determine the averaged power exponent \((\alpha)\) and their magnitudes in different NeuSHT expression. The magnitudes of the characteristic lengths could explain how fast the NeuSHT enzymes diffuse in different observable times. This result implied a mixed population existed and the subdiffusive behaviour of NeuSHT occurred over a longer time scale, a few 10 ms.
Figure 4.7 Plots of the characteristic length ($L_{MSP}^2$) versus the lag time ($\tau$). a) A characteristic length plot where parameters (e.g. $D_\alpha$ and $\alpha$) were analysed from fits to the FCS autocorrelation in $td_1$ (< ms) range. b) The plot where parameters (e.g. $D_\alpha$ and $\alpha$) were analysed from fit FCS autocorrelation in the $td_2$ (> ms) range. Power laws were used for fits and are shown as solid and dashed lines. The averaged power exponent determines the state of subdiffusion.

In addition to the explanation of the dynamics of NeuSHT, the populations ($N$) were calculated from two domains of the FCS curve without normalization to approximate the concentration of observable fluorescent NeuSHT, Figure 4.8. The aim was to determine the fluorescent signals that originated from inside the bacterial cells: specifically, the cytoplasm and the inner membrane regions. In Figure 4.8a and 4.8b, information on the populations from two different time domains is shown. $N_1$ and $N_2$ from $td_1$ (< ms) and $td_2$ (>ms) show lower numbers of $N_2$ compared to $N_1$. This means that most of the particles freely diffused
in the cytoplasm, including TMR/AF488 ligands bound to *NeuSHT* and unbound ligands, and only a small minority were attached to the membrane.

The scatter plot of variances of the 2 populations, $N_1$ and $N_2$, have a potential to identify the origin of the signals, where $N_1$ inhabit a region in the cytoplasm and $N_2$ a region closes to the inner membrane that interacts with capsular protein complexes. The data shows they were all from the inside the bacterial cells and that higher variances occurred in conditions where *NeuSHT* were overexpressed, i.e. in EV136pBSKIPTG. Fit lines also show the correlation of the $1/N_1$ and $1/N_2$.

**Figure 4.8** The distribution of populations from 2 time domains showing a) $N_1$ and b) $N_2$ with different *NeuSHT* expression: EV136 (non-encapsulated bacteria, orange), EV136pB33 (non-encapsulated bacteria transformed with pneuSHTpB33 plasmids, yellow), EV136pB33ara (encapsulated bacteria in which *NeuSHT* were expressed from pneuSHTpB33 plasmids with 0.1% arabinose induction), EV136pBSK (encapsulated bacteria in which *NeuSHT* did a leakage expression from pneuSpBSK+ plasmids, implying the K1
phage assay results in Appendix B1c, shown as a small void region due to phage K1 recognition) and EV136pBSK1PTG (encapsulated bacteria in which NeuSHT were expressed from pneuSpBSK+ by 1mg/ml IPTG induction). The means of the population are shown in thick lines. c) A scatter plot of the variance of $N_1$ plotted against the variance of $N_2$. Linear fits in the figure show the correlation of the scatter plot. The fit lines in Figure 4.8c show the correlation trend of $1/N_1$ and $1/N_2$ parameters in different NeuSHT expression whether they are positive or negative correlation.

4.1.3.4 FCS curves represent the dynamics of NeuSHT enzymes: simulation and variance comparison

The variance of the FCS observations was key for the confirmation of the state of NeuSHT sub-diffusion when it interacted with proteins complexes and can be used as an optimization parameter. The variance from the experiment (Mean square residual) was compared with the variance from the fit parameter ($N^{-1}$). The variance information (Appendix B5) is linked to equation (4.14), for small confocal volumes. Thus, the variance of the autocorrelation function is proportional to $1/N_{total}^2$. This can use for optimisation of FCS measurement conditions.

Additionally, a simulation was used to recreate the scenario that NeuSHT inside bacteria cells had a mixed population exhibiting both normal diffusion and sub-diffusion without any exchange involved (as a simplification). The simulated fractional Brownian motion with no exchange represented the state of TMR/AF488 ligands as they diffused in
bacterial cells, for both the random walk and sub-diffusion. This code was written in collaboration with Jack Hart.

In **Figure 4.9**, the simulation and experimental results are compared. The input parameters (e.g., the Hurst exponents) were applied to equation 4.16 to create a mixed population from different diffusive regimes. The fit parameters agreed with both normal and subdiffusive motion with diffusion time constants of around milliseconds ($10^{-4}$ to $10^{-1}$ s), where $\alpha_1 = 0.94$ and $\alpha_2 = 0.37$; $t_{d1} = 0.0001$ s and $t_{d2} = 0.53$ s. The residual of the fit referred to the variance of the autocorrelation results and is shown in the inset of **Figure 4.9**.

**Figure 4.9** Simulation results of the correlation function as a function of lag time measured in FCS for a mixture of normal and anomalous diffusion compared with the experimental data when *NeuSHT* is expressed. fBM tracks were generated using the WM function (**equation (4.15)**) and the resultant FCS curve, including its variance (inset), is shown in
blue. The experimental FCS curve and its variance (inset) with *NeuSHT* expression are shown in red. To simplify, the model was fitted with the translational dynamics (both normal and anomalous) without the consideration of the rate of exchange between cytoplasmic membrane adsorbed states.

The interaction of *NeuSHT* with other protein complexes involved in capsular biosynthesis production was probed. From FCS curves with a range of *NeuSHT* expression, MSDs were compared. With the use of the variances of experimental and simulated results, the FCS technique has the potential to identify the dynamics of *NeuSHT* which could not be tracked directly due to the low concentration of TMR/AF488 fluorescent ligands tagged to *NeuSHT*. In the following section, the robustness of these experiments will be discussed.

4.4 Discussion

4.4.1 The presence of TMR/AF488 ligands bound to *NeuSHT* coexisting with other protein complexes: *KpsE* and *KpsD* involved in K1 capsular biosynthesis

To verify the function and pathogenic competency of transformed bacteria, the immunofluorescence technique was used on *KpsE*, *KpsD* and K1 PSA rafts. The fluorescence images for the coexistence of these proteins are presented in sections 3.1.1 and 3.1.2. Since the proteins, *KpsD* and *KpsE*, bound to the protein complex create a conduit to transport capsular polysaccharide from the cytoplasm to the bacterial surface, the localization of these complexes was clearly observed with *KpsE* and *KpsD* spread over the whole surface to produce encapsulated K1 capsules and they were static rather than moving around the bacterial surface. On the contrary, *NeuS* is a glycosyltransferase
(polysialyltransferase) which moves much more rapidly. The catalyst role of NeuS needs these enzymes to interact with the capsular biosynthesis complex.

In addition to labelling with antibodies, genetic modification of KpsD and Halotag methods with NeuS were introduced to directly label KpsD (as KpsD::dsRED) and NeuS::Halotag (NeuSHT). However, the NeuSHT modification slightly inhibited the performance of NeuS to synthesize K1 capsular polysaccharides. This result was demonstrated via a phage K1 experiment (Appendix B1). Although the yield and efficiency were lower, this was superior to the use of genetically conjugated fluorescent proteins which can interrupt the folding of NeuSHT. Nevertheless, the aim of this chapter is to demonstrate that NeuSHT enzymes coexist with other protein complexes during the synthesis of capsular polysaccharides. The NeuSHT enzymes diffused with high mobility compared to the protein complexes and K1 PSA capsular rafts, as shown in Figure 4.4d.

4.4.2 The dynamics of TMR/AF488 ligands bound to NeuSHT reveal a mechanism of capsular biosynthesis

To optimize the confocal volume and FCS autocorrelation results, the TMR fluorescent bacteria (containing TMR conjugated with expressed NeuSHT and freely diffusing TMR) were tested with different confocal volumes using pinhole diaphragm between 0.5 and 1 Airy unit (AU). The confocal volume, after the calibration, was found to be around 1µm² for 1 AU (Appendix B2). The size of the 1AU confocal volume was most suitable to investigate bright fluorescent proteins inside bacterial cells. With the low power excitation of a few mW and reasonably large confocal volume, photobleaching was prevented by reducing the acquisition times to 1s (Appendices B2a and B2b). In addition,
with different sizes of confocal volumes, time constants were observed of a similar order of magnitude, inset of Figure 4.5. This revealed their independence on the confocal volumes ($v$) which is important for modelling anomalous transport. The concentration (relating to the amplitude of FCS curves) was also varied, Appendix B2c. We mainly used 1AU for all of FCS investigation, because of the improved brightness of fluorescent signals from low excitation laser powers.

4.4.3 The dynamics of NeuSHT enzymes as a function of different expression conditions

The FCS measurements were performed on fluorescent bacterial cells, which contained unbound ligands and NeuSHT bound ligands mixed in the bacterial cytoplasm. With the different conditions of NeuSHT expression from Table 4.1, the dynamics and mechanism of fluorescent ligand binding to NeuSHT could be differentiated from unbound NeuSHT, while K1 capsular PSA rafts were being synthesized and emerged on the bacterial surface.

In Figure 4.6, the FCS curves show the dynamics of different levels of NeuSHT expression. Although the amplitude of the curves could approximate the concentration of fluorescent proteins, the primary focus was on the dynamics to differentiate bound ligands from unbound ligands. The unbound fluorescent ligands were observed in EV136 and EV136pB33 conditions (which were no NeuSHT expression).

According to the assumption that NeuSHT bound ligands are slower than unbound ligands, the longer characteristic times of bound ligands (including EV136pBara, EV136pBSK, and EV136pBSKIPTG) in the td1 case were observed in Appendix B3a and most of the unbound ligands were normally diffusive, $\alpha = 1$ (Appendices B4 and
Appendices B5) with sub-ms time constants (from 0.25ms to 0.70ms). In contrast, the bound fluorescent ligands had mixed distributions of lag times with mixed regimes between normal diffusive and subdiffusive. The distributions of the characteristic times ($\tau_D$) in td2 were ambiguous, Appendices B3b and B3d. The mixed population of normal and anomalous diffusion in all NeuSHT expression showed a distribution of the power exponents: $\alpha_1$ and $\alpha_2$ (Appendix B4). The ambiguity of these measurements can be resolved using the characteristic length ($L_{MSD}^2$) in different lag times ($\tau$) (Figures 4.7a and 4.7b).

The value of characteristic length ($L_{MSD}^2$) reveals how fast and slow diffusion of each NeuSHT expression. The slowest diffusion of NeuSHT happened when observed in 20°C in both case of Dh5aNeuS20°C and EV136pB20°C. In this case, there is no capsular protein complex for the interaction of expressed NeuSHT. The fastest diffusion is when few NeuSHT had been expressed due to a leakage expression in EV136pBSK case. However, there was no evidence that NeuSHT interacts with capsular protein complexes in this case because the average exponent showed normal diffusion in both $td_1$ and $td_2$, Figure 4.7a and 4.7b respectively.

4.4.4 Sub-diffusive behaviour of NeuSHT interacting with proteins complexes that are involved in the K1 capsular biosynthesis pathways

The amplitudes of the FCS curves are inversed proportional to the population of fluorescent molecules ($G(\tau) \propto 1/\bar{N}$). The distributions of these populations in Figure 4.8a and 4.8b confirmed the consistency of the FCS observations with a few fluorescent molecules inside a specific confocal volume. The population also linked to the variation of
FCS measurement (equation (4.14)). Thus, the scatter plot of reciprocal populations on Figure 4.8c shows that higher variances are observed in over-expressed NeuSHT conditions (iii), which was comparable to the few and leakage expression (ii). These conditions (ii and iii) show a mixed population between normal diffusion and sub diffusion. The normal diffusion included both fluorescent ligands bound to NeuSHT and free unbound ligands; the sub diffusion is due to NeuSHT interacting with the capsular protein complexes, Appendix B4.

Evidence of sub-diffusive behaviour can also be seen in Appendix B4 and Figure 4.7b which observed in both short ($td_1$) and long time periods ($td_2$). In contrast to the case of $td_1$ in Figure 4.7a the average power exponents showed normal diffusive behaviour, the molecular population in $td_2$ was in a subdiffusive regime (Figure 4.7b), especially in the case of EV136pBara that had low NeuSHT expression similar to EV136pBSKIPTG. However, the sub-diffusive regime was observed between 1 to 10 ms lag time with averaged power exponents: $\alpha \sim 0.3$ and 0.7, for EV136pBara and EV136pBSKIPTG, respectively.

The schematic in Figure 4.10 is used to clarify the different mechanisms observed in the FCS experiments. Although mixed mechanisms occurred in all cases, with no NeuSHT (case i, from EV136 and EV136pB33) expression the FCS result show more normal diffusion (Figure 4.10a). In the case of low or over expression (case ii and iv, from Table 4.1), normal diffusion of unbound ligands mixed with NeuSHT bound ligands occurred (Figure 4.10a and 4.10b). At 20°C, NeuSHT bound ligands seemed to diffuse slower than in 37°C according to Figure 4.7. This relates to an increased viscosity inside the cytoplasmic regions. Although the NeuS bound ligands would be sub-diffusive when they interact with
protein complexes (Figure 4.10c, for the case iii from Table 4.1), all possible diffusive mechanisms occurred in FCS signals (Figure 4.10a - 4.10c). Thus, there are limitations on the FCS technique to resolve the mechanism of NeuSHT when interacting with capsular protein complexes.

**Figure 4.10** Different diffusive states of fluorescent ligands from FCS. a) Freely diffusive, unbound ligand (case i, Table 4.1). b) Freely diffusive, NeuSHT bound ligand (case ii, iii, and iv). c) Sub-diffusive, NeuSHT bound ligand (case ii and iii). The fluorescent ligand diffuses along the orange path whether bound to NeuSHT (green) or not. Sub-diffusive behaviour is possible when NeuS bound ligand interacts with the capsular proteins complex (cyan),
comprised of various proteins, e.g. \textit{KpsE} and \textit{KpsD}. Dashed orange lines represent the tracks of molecule diffusion.

In addition to experimental confirmation that \textit{NeuSHT} behaved in a subdiffusive manner, fractional Brownian motion (fBm) was artificially generated using the Weierstrass-Mandelbrot (WM) function, \textbf{equation (4.16)}. This function simulated a mixed population scenario of coexistence of normal diffusion and subdiffusion fluorescent particles, \textbf{Figure 4.9}. Consequently, the simulation and experimental results were in good agreement and the variance also confirmed the state of FCS observation. It was confirmed that subdiffusion was observed in the $td_2$ domain with an exponent $\alpha$ of 0.5.

We investigated whether the \textit{NeuSHT} interacting with proteins complexes behaved subdiffusively over the 30ms time frames. However, it was difficult to confirm the hypothesis due to the indirect measurement of FCS signals that comprised of low signal to noise ratios (SNR). Additionally, the instability of \textit{NeuSHT} and the challenging labelling with TMR/A488 ligands made this experiment difficult. Improvement of genetic modification methods (e.g. direct conjugate fluorescent proteins to the protein of interest, \textit{NeuS::mCherry}) would be needed to increase the possibility of long-lasting investigations of bright fluorescent \textit{NeuS} enzymes.

\textbf{4.5 Conclusions}

The dynamics of \textit{NeuS} enzymes were investigated in combination with two other capsular protein complexes: \textit{KpsD} and \textit{KpsE}. Although \textit{NeuS} had been genetically modified as \textit{NeuS::Halotag (NeuSHT)}, these glycotransferase enzymes were functional and diffused
rapidly in comparison to the other synthesis proteins studied and the K1 capsular polysaccharide rafts. This has been observed using the FCS technique. The FCS curves were analysed (equation (4.12)) to generate the parameters: the diffusive exponent ($\alpha$), the characteristic time ($\tau_D$) and the population ($N$). To investigate the dynamics of NeuSHT enzymes, 4 types of NeuSHT expression were used to isolate the background noise and to identify the subdiffusive behaviour of NeuSHT in the FCS curve fits. A source of background noise in the FCS was the signal due to unbound TMR/AF488 ligands, that freely diffuse without interaction with NeuSHT and other proteins. Thus, when compared with no NeuSHT expression (i), the free ligands moved with a single population in a normal diffusive regime with a time constant of around 0.2-0.5 ms. The conditions with NeuSHT expression (ii and iii) showed more varied behaviour and both normal and sub diffusion was observed. Although the comparison of MSD and $\alpha$ could differentiate the range of average time constants, it was not absolutely quantitative. What could be confirmed was that a mixed population was found when NeuSHT was expressed.

To optimize the FCS experiments, the variances of the autocorrelation function from each FCS curve were investigated. The FCS curves were analysed using the autocorrelation function of the fluctuations of the fluorescent signal, so the number of fluorophores and confocal volume size are the main sources of noise. Thus, with the expressed NeuSHT conditions (ii and iii), the higher the NeuSHT population, the higher the variance of the FCS curves. The simulation based on fBM for subdiffusion recreated the FCS curve with the same variance to explain the subdiffusive behaviour of NeuSHT (with a time constant about 530 ms) inside the encapsulated bacteria.
Although the experiment could not conclude the exact nature of NeuSHT interactions with proteins complexes in K1 capsular biosynthesis, these results have revealed the dynamics of NeuSHT which was rapidly moving inside bacteria cells. This would be useful for further investigation of other proteins involved in other biosynthesis systems and chemical reaction inside bacterial cells, in real-time, without damaging the bacteria. Most previous intracellular experiments on fluorescence correlation spectroscopy (FCS) used eukaryotic cells [39–42], and the observation of FCS inside bacterial cells is challenging. Hence, the enhanced technique with smaller confocal volume looks promising. The STED-FCS [43] system has thus been shown to allow the investigation of the dynamics of fluorescent proteins and enzymes inside live bacteria cells.

4.6 References


CHAPTER 5

Investigation of K1 capsular polysaccharide, a permeable shield for *E. coli* against antimicrobial peptides

5.1 Introduction

5.1.1 Properties of K1 bacterial capsule

Bacterial capsule is one of several protective mechanisms possessed by bacterial cells [1–3]. The bacteria cover themselves with specific polysaccharides on their surface to avoid recognition by the immune cells of the host organism and to avoid phagocytosis [4]. The physical mechanism behind this capsular virulence is a repulsive steric potential due to polyelectrolytes in the capsules [5].

According to the previous chapter on capsule characterisation, K1 capsular polysaccharide chains are made of N-acetylneuraminic monomers linked with α-2,8 ketoglycosidic bonds (with a degree of polymerization less than 200) and conjugated with a β-linked poly-3-deoxy-D-manno-oct-2-ulosonic acid (KDO) linker on lyso-phosphatidylglycerol (lyso-PG) [6,7]. In addition, the polysaccharides randomly generate on
the bacterial surface. From the points of nucleation, bacterial capsular rafts were found to expand and cover the bacterial surface (Chapter 3 [5]).

K1 capsular polysaccharide was studied to see how the steric force of the capsular brush interacts with foreign peptides. The polysialic capsular polysaccharides behave as polyelectrolyte brushes in aqueous solutions. From the capsular structure, the capsule is known to be 90% water which hydrates and swells it [3]. The steric force due to the osmotic pressure can be used to explain the extension from the bacterial surface and the adsorption of positively charged peptides.

5.1.2 Properties of G3 antimicrobial peptides

The evolution of antibiotic resistance in pathogenic bacteria is a global health concern [8,9]. Our immune systems combined with low doses of antibiotics are now unable to cope with these evolved bacteria. As an alternative treatment, cationic antimicrobial peptides (AMPs or CAPs) have the potential to quickly eradicate bacterial cells with a much lower possibility of bacterial resistance [10,11]. A result of antibiotic resistance has been in urinary tract infection (UTI) patients who have a high recurrence rate. A key virulence factor in this infection is the K1 bacterial capsule.

Several natural and synthesised peptides are good candidates for bacterial treatment. However, no CAPs have yet gone through a clinical phase for patients [11]. With several possible sequences and structures of peptides available, a short helical, cationic peptide was chosen in this study due to the cheap cost of peptide synthesis. To have a conformation with a facile amphiphilic structure similar to natural antimicrobial peptides (e.g. magainin), α-helical G(IIKK)_3I-NH₂, G3 antimicrobial peptides were synthesized (they have cationic
charges and amphiphilic, hydrophilic/hydrophobic residues) [12,13]. Since the G3 peptides are small, they are adsorbed, deform the bacterial membrane structure and permeabilise the membrane to the cytoplasm (Figure 5.1a). However, due to the combination of charge and hydrophobic properties of G3 peptides, the interaction with the K1 capsule is unclear. It is possible that the strong negative charge of the K1 capsular polysaccharide (comprised of polysialic acids with 200 degrees of polymerisation) could electrostatically attract the positive charge on the cationic G3 peptides (Figure 5.1b). However, some of the G3 peptides are adsorbed to membrane lipids and penetrate the lower layers.
Figure 5.1  a) A diagram of G3/G4 peptides (grey helical peptides labelled with green Cy3b dyes) invading a K1 encapsulated *E. coli* (orange). b) The deposition of CAPs on K1 capsular polysaccharide and on the bacterial outer membrane (blue lipids). c) The 3D molecular structure of G(IIKK)₃l-NH₂, G3 CAPs peptides rendered using the Avogadro software package [14]. d) The Schiffer-Edmundson wheel projection of the G3 peptides [12]. The peptide images were provided by Haoning Gong.
In addition, the short G3 peptides have the potential to avoid an attractive force from hydrated K1 capsules due to the heterogeneity of the bacterial surface. Although bacterial capsule is formed continuously on the surface, previous observation of K1 capsular rafts [5] (Chapter 3) shows that it is an inhomogeneous landscape (lipid rafts were observed [15,16]) on the bacterial surface. Hence the gaps are large enough to allow small G3 peptides (a few nm in size) to reach the surface without interacting with the negatively charged polysaccharide of the capsule i.e. the capsule acts as a permeable shield.

The 3D molecular structure of G(IKK)3-I-NH₂, G3 CAPs is illustrated in Figure 5.1c showing the hydrophobic backbone and the hydrophilic sites. In addition, the designed sequence is shown in the Schiffer-Edmundson wheel diagram (Figure 5.1d). The amino sequence of G3 comprises of Glycine (G), Isoleucine (I) and Lysine (K). The ratio between charge (hydrophilic) and hydrophobic is 1:1 corresponding to the order and sequence of G, K and I residues; this contributes to amphiphilic properties.

5.1.3 How the K1 capsule is believed to protect bacterial cells as a shield

Natural antimicrobial peptides (AMPs) are well known to disrupt bacterial membranes. However, when bacterial cells are shielded with external polysaccharides: lipopolysaccharide (LPS), capsular polysaccharides (CPS) and other extracellular matrices, e.g. in biofilms, the activity of the AMPs is lower [17,18]. In the current study, the effects of bacterial CPS to AMPs will be described.

Although antimicrobial peptides can immediately kill invading bacterial cells and are invisible to host cells, they can trigger bacterial capsule expression to improve AMP
resistance. For example, in *K. pneumonia*, CPS upregulation and an increase in the amount of CPS was observed when the bacteria were exposed to the AMPs [19].

To lower peptide activity during the disruption of the bacterial membrane, some bacteria can eject capsular polysaccharides to neutralise the peptide activity. Evidence was found in the studies of extracted and released CPS from different bacteria: *K. pneumoniae*, *S. pneumonia* and *P. aeruginosa*. An increase of the minimal inhibitory concentration (MIC) of polymyxin B and human neutrophil α-defensin (HNP-1) antimicrobial peptides (AMPs) was observed when interacting with CPS for non-encapsulated bacteria, both *in vitro* and *in vivo*. Also, the study found that AMPs released CPS out of the bacterial surface in different concentrations due to their differing activities [20]. In addition to the thin layer of polysaccharides on the bacterial surface, the extracellular matrix also affects the antimicrobial peptide activity [21,22].

For live bacterial cells interacting with AMPs, several techniques have been used, e.g. super-resolution imaging, AFM and TEM, to observe the peptides on single bacterial cells [23,24]. Not only do the peptides disrupt the membranes of bacteria, some peptides can penetrate the outer layer of the bacterial surface and aggregate inside the cytoplasm and form complexes with bacterial nucleotides. Using a super-resolution tracking technique, LL37 human antimicrobial peptides have been observed in the cytoplasm and dense linkages were observed with nucleotide strands of the chromosomal DNA and 70s-polysomes [25]. Using AFM to probe the variation of turgor pressure and cryo-TEM to visualise the internal structure, the rupture of membranes was investigated after encapsulated *K. pneumonia* cells were treated with natural antimicrobial peptides [24,26]. There was evidence that peptides
could penetrate through the CPS layer, although the AFM technique can cause damage to cells, so this evidence is debatable.

To visualise the localisation of G3 antimicrobial peptides in live bacterial cells, fluorescence super-resolution imaging was used with image analysis to classify the formation of G3 peptides on the bacterial capsule surface and how K1 capsular polysaccharide brushes protect the cells from peptide invasion.

5.1.4 The mechanism of G3 adsorption on the bacterial surface

The interaction between the antimicrobial peptides and the K1 bacterial capsules can be divided into 3 steps: peptide self-aggregation, peptide adsorption on K1 bacterial capsular polysaccharides and pore formation after peptides become embedded in the bacterial membranes.

The oligomerisation and aggregation of peptides are mainly determined by intermolecular interactions, such as hydrogen bonds, electrostatic forces, hydrophobic interactions and π−π stacking [27]. According to MD simulations, the AMP intermolecular interaction via interpeptide bonding aggregation will lower the efficacy of adsorption on bacterial membranes [27]. However, this effect can also increase the propensity of the aggregated AMPs to adsorb on K1 capsular polysaccharides. The free energy landscape with ensemble averaging for peptide aggregation can be simplified according to the 2nd law of thermodynamics:

$$
\Delta F = \Delta U_{aggregate-monomer} - k_B T \ln \left( \frac{p_{aggregate}}{p_{monomers}} \right),
$$  

(5.1)
where $k_B$ is the Boltzmann constant, $T$ is the temperature, $p_{\text{monomers}}$ is the probability of the lowest energy state of monomer peptide, $p_{\text{aggregation}}$ is the probability of the observed aggregate and $\Delta U_{\text{aggregate-monomer}}$ is an internal energy difference, which is negligible since the entropy change (the second term) is dominant in the process of peptide aggregation.

In equilibrium at room temperature, the process of peptide formation (aggregate or non-aggregate) will minimise its free energy. The individual steps of peptide folding during aggregation [28] are neglected in this simple equation. At low peptide concentrations, the peptides do not aggregate very much, i.e. below their critical micelle concentration (CMC value).

The mechanism that amphiphilic peptides with positive charges use to invade bacterial cells is predominantly electrostatic in origin due to the highly negative charge on the bacterial surface [29], both on the extended polysaccharides and membranes. According to chapter 4 which discussed bacterial capsule thickness, the stretching of the polyelectrolyte brushes can be described by the osmotic pressure model [5]. When bacterial capsular brushes are in low ionic strength media, they can thus attract more positive charged peptides due to the stronger electrostatic interaction. However, at high salt concentration, these capsular polysaccharide brushes tend to repel the peptides due to a steric mechanism (the entropy of the polysaccharide chains causes this interaction). This is similar to the polyelectrolyte mediated protein adsorption phenomenon (PMPA) [30,31].

In low salt concentration, peptides are easier to localise on polysaccharide chains due to low counterion condensation and a small Gouy-Champman length $\lambda$. If peptides are
attached to the capsular polysaccharides, the free energies involved are from the peptides $(F_{\text{peptides}})$ and capsular brush chains $(F_{\text{capsular brush}})$ [31],

$$F_{\text{peptides}} = l_B \sigma^2 \lambda, \quad (5.2)$$

$$F_{\text{capsular brush}} = l_B \sigma^2 D, \quad (5.3)$$

where the Bjerrum length is defined as $l_B = e^2/4\pi\varepsilon k_B T$, $\varepsilon$ is the dielectric constant of media, $\sigma$ is the charge density and $D$ is the thickness of the adsorbed layer. The total change of the free energy for peptide adsorption involves contributions from both the counterions and the charge distribution of the peptides.

Despite strong electrostatic attraction from capsular polysaccharides, small amphiphilic peptides can penetrate through the hydrated, thick layer of the polysaccharides (200-300 nm) to enter the bacterial membrane (based on experimental investigations in this chapter). The hydrophilic side of the peptides makes them soluble. On the other hand, the hydrophobic regions of the peptides allow them to interact with bacterial membranes. Embedded peptides can perturb bacterial membrane stability and a pore can be created. The pore formation energy $U(r)$ can be expressed as [32–34]

$$U(r) = 2\pi r \gamma - \pi r^2 \sigma_0, \quad (5.4)$$

where $r$ is the radius of the pore formed by the peptides, $\gamma$ is the intrinsic boundary line tension and $\sigma_0$ is the external lateral tension. The energy barrier per unit area for pore creation is $\pi \gamma^2/\sigma_0$. After the membrane pore is created, the bacterial cell can burst due to the pressure imbalance.
The above mechanisms are key for G3 peptide-K1 capsular polysaccharide interactions. In addition, the conformation and aggregation of peptides can be differentiated depending on bacterial surface conditions: non-capsular polysaccharide coverage or capsular polysaccharide coverage.

In the case of non-encapsulated bacteria, antimicrobial peptides adsorb directly to the bacterial membrane. Since the peptide size is comparable to the lipid bilayer membrane thickness, peptides can penetrate into the inner layers such as the peptidoglycan, inner membrane and cytoplasm. The tiny peptides can translocate from the outer membrane to the cytoplasm within a few µs [35]. With plenty of peptides to adsorb on the bacterial cells, there are several mechanisms for peptides aggregation on bacterial membranes in either the inner or the outer membranes. The mechanisms of G3 aggregation on the bacterial membrane can be categorised as following 1) a pore model, 2) a carpet model or 3) a combined model [36].

For encapsulated bacteria, antimicrobial peptides not only adsorb on the bacterial membrane, but they also adsorb on the bacterial capsular polysaccharides. Hence, the mechanisms of G3 adsorption and aggregation are similar to non-encapsulated bacteria, but a capsular binding model also needs to be considered. Using the evidence of G3 peptide and bacterial capsule localisation, a more detailed understanding of the mechanisms of G3 adsorption is presented in the current chapter. This chapter also emphasizes the weakness of the bacterial capsule as a permeable shield which allows G3 CAPs to penetrate through the capsular layer and disrupt the bacterial membrane.
5.2 Methodology

5.2.1 Bacteria culture and sample preparation

*Escherichia coli* strains: EV36 (encapsulated strain) and EV136 (capsule-deficient strain, generously provided by Eric Vimr) were cultured in two different media: LB (Luria-Bertani) [37] and M9 (Minimal) [38] with supplements of 0.4% (v/v) glycerol and 0.1% (w/v) casein, to observe bacterial polysaccharide capsule formation. For fully encapsulated bacteria, EV36 were cultured in LB at 37 °C overnight before inoculation in fresh LB media and further incubated at 37 °C until they were imaged. When fully encapsulated EV36 and non-encapsulated EV136 were observed with G3 conjugated Cy3B, 1:100 of the bacteria were cultured in LB at 37°C overnight and inoculated in fresh M9 media at 37°C. When the OD$_{600}$ reached 0.4, 20 ml of cells were harvested by centrifugation (3500 rpm for 15 minutes) and the pellet was transferred to a fresh M9 media before they were deposited on a glass slide coated with a poly-L-lysine/GO (PLLGO) substrate.

5.2.2 K1 bacterial capsule immunofluorescence and G3 peptide conjugation with Cy3B

*NHS ester fluorescent dyes*

Live cells were collected at well-defined time points, washed three times with PBS and deposited on poly-L-lysine-and-graphene oxide (PLLGO) coated 8-well chambers. The K1 α 2-8 N-Acetylneuraminic (polysialic) acid in the polysaccharide capsules can be recognised by mAb735 (monoclonal IgG2A) antibodies [39,40]. For live cell imaging using wide-field fluorescence microscopy, cells were incubated with 1% (w/v) BSA in PBS and then incubated with a 1:400 dilution from a 4 mg/ml stock solution in a blocking buffer, then washed before incubation with a 1:500 dilution with a secondary antibody (anti-mouse
F(ab’)2 AF647, Invitrogen #A-21237). In addition, super-resolution fluorescence (dSTORM) microscopy was used without the washing step with the immunostaining methods by incubating both primary (1:8000) and secondary (1:10000) antibodies at a lower concentration with the bacteria due to the benefits of using PLLGO coated slides i.e. the PLLGO coating removed the background due to non-specifically bound fluorophores and provided a 100 fold improvement in contrast [41].

For α-helical G3 peptides, the NHS ester Cy3B (GE) dyes were used to label the antimicrobial peptides. Due to the active N-hydroxysuccinimide ester site of the NHS ester Cy3B dyes, an amide bond could be formed with the open amine group at room temperature and pH 7- 9 (Figure 5.2a). In order to avoid excessive unconjugated Cy3B dyes, a low concentration of Cy3B dyes was used with 0.1% v/v of G3 concentration, then it was incubated in RT for 2 hours and stored in a fridge. The samples could be purified using HPLC; however, in this experiment, this step was not applied.

Due to the MW of Cy3B dyes (772 g/mol) compared to the G3 peptides (1635 g/mol), labelled G3 peptides could be conjugated with a few Cy3B, containing less than the maximum 7 fluorophores per peptide due to the maximum number of binding sites. This labelling did not affect the amphiphilic properties of the peptides if the labelling occurs at an N-terminal (G) residue. However, the labelling occasionally happens on K (lysine) residues which affect the charge properties of G3 peptides (Figure 5.2b).
Figure 5.2 a) Reaction scheme of the Cy3B NHS ester dye conjugated with an amine group causing amide bond formation. b) The possible residues on the G3 peptides that could be labelled with Cy3B. G3 peptides comprise of G (Glycine), I (Isoleucine) and K (Lysine) amino acids. For G3 peptides: G(IIKK)I-NH2, the amine open bonding (R-NH2) reactions can possible to happen at the N terminal of G (Glycine) or at the intermediate KK sequence of the Lysine.
**5.2.3 Buffer and media for fluorescence imaging**

For fluorescence imaging, the deposited cells were hydrated with M9 media supplemented with essential amino acids. To investigate the interaction of G3 peptides with bacterial surfaces, Cy3B conjugated G3 peptides were added immediately before the experiments. In addition to M9 media and antibodies (no washing step was used), super-resolution fluorescence microscopy (dSTORM) also required an imaging buffer, which was GLOX [42]: 10mM MEA (cystamine), 1/100 dilution from 20 mg/ml catalase and 1/10 dilution of 150 mg/ml glucose oxidase. This helped prevent photobleaching of the fluorophores. Since the acidity of the GLOX buffer reduces significantly after a few hours, fresh GLOX buffer and M9 media were refilled every 1 hr in longer experiments to prevent photobleaching, toxicity and acidification.

**5.2.4 Diffraction limited and super-resolution fluorescence microscopy**

For initial bacterial capsule and G3 peptide observations, wide-field fluorescence microscopy, with an Axio Imager.M2 Upright (Zeiss) microscope with 63× Plan Apochromat (Oil, DIC) and 150× Olympus UApO 1.4NA Oil immersion lenses was used at low power with selected filters from 405 to 990 nm.

In the case of dSTORM super-resolution imaging, a bespoke STORM microscope [43] was used with 405 nm (0.6 mW), 533 nm (77 mW) and 647 nm (77 mW) excitation lasers and a 100× oil immersion 1.49NA, TIRF objective lens (Olympus UAPON 100×OTIRF). The depth of focus was calibrated using 100 nm polystyrene beads coated with Cy3B, deposited on glass-slides coated poly-L-lysine, in Chapter 3 [5]. The z-stack
images were taken at 10 nm/step for 200 steps using a piezo stage with an acquisition rate of 100 fps and analysed with the virtual light sheet (vls) plug-in.

dSTORM was used to observe the localisation of antimicrobial G3 peptides on *E. coli* surfaces (EV36 and EV136). Up to ten thousand frames were used to construct each dSTORM image, which was followed by times with no illumination to reduce photobleaching.

### 5.2.5 Image processing and analysis

Several pieces of software were used to construct images and to interpret the results. The Thunderstorm plug-in in Fiji was used to localize the blinking fluorophores and reconstruct super-resolution images [44]. Origin (OriginLab, MA) was predominantly used for plotting and fitting functions. The data were analysed via Matlab (The MathWorks, Inc., MA). Newly written Matlab code was used to find G3 sizes and their probability distributions.

### 5.3 Results

#### 5.3.1 Conventional fluorescence images of G3 peptides on bacterial surfaces

A primary investigation of how G3 cationic antimicrobial peptides (CAPs) adsorbed on the bacterial membrane was performed on the wide field fluorescence microscope with high magnification (150×). In **Figure 5.3**, multicolour fluorescence images of bacteria (both encapsulated and non-encapsulated *E. coli*) are shown. The G3 CAPs were incubated long enough until the whole bacterial cells were covered with G3 peptides that permeated the bacterial cytoplasm. The multicolour images were combined from different channels (**Figure 5.3a**). G3 conjugated Cy3B is shown in green, while antiK1 conjugated with AF647
antibodies labelled on the K1 bacterial capsules is shown in red. Different concentrations of G3 were used to incubate with encapsulated bacteria: Figure 5.3b (for 100 µM) and 5.3c (for 10 µM). The white triangles indicate the concentrated localisation of Cy3B conjugated G3 peptides. This demonstrates peptide aggregation on bacterial membranes, although bacteria are already covered with the capsules. For non-encapsulated bacteria (EV136, Figure 5.3d), the image shows the deposition of G3 CAPs on bacterial membranes. In Figure 5.3b-d, bacterial cells were counterstained with DAPI (blue) to investigate the compactness of bacterial nucleotides when a higher concentration of CAPs permeated bacteria cells, for both EV36 and EV136 strains.
Figure 5.3 Wide-field fluorescence microscopy on K1 capsule (red), G3 CAPs (green) and DAPI (blue). a) A merged image of encapsulated E. coli (EV36). b) and c) The merged image of encapsulated E. coli with different G3 concentrations: 100 µM and 10 µM, respectively. d) The CAPs deposited on the non-encapsulated bacterial surface (EV136). DAPI stained the bacterial nucleotide in the cytoplasm (blue). White triangles indicate the aggregation of G3 peptides after adsorption on bacterial cells.
5.3.2 Super-resolution fluorescence imaging reveals the localisation of G3 peptide aggregation on the bacterial surface and inside the cytoplasm.

Fluorescence super-resolution images reveal the localization of aggregated CAPs after adsorption. Example reconstructed images are shown in Figure 5.4a and 5.4b for G3 (G(IKK)_3-I-NH₂) and G4 (G(IKK)_4-I-NH₂), respectively. The antiK1-AF647 antibodies (red) were labelled on K1 capsule and Cy3B (green) labelled on G3 peptides via amide bonding. To compare with Figure 5.3 where G3 peptides fully cover the bacterial cells, the aggregation of the peptides can be resolved using super-resolution imaging. Labelled G3 peptides were observed in several regions including the capsular brush layer, the bacterial membranes and the cytoplasm. They are indicated by white triangles.

**Figure 5.4** Super-resolution fluorescence image of K1 interacting with CAPs: **a)** G(IKK)_3-I-NH₂, G3 (green) and **b)** G(IKK)_4-I-NH₂, G4 (green), where K1 capsules were recognised by anti K1 antibodies. White triangles indicate the aggregated residues. The scale bars are 1 µm.
5.3.3 Verification of G3-membrane aggregation on E. coli surfaces: peptide number and aggregate size

To characterise the adsorption of labelled G3 cationic antimicrobial peptides (CAPs), the number of molecules \( (N) \) and the size of aggregated peptides \( (R_g) \) were calculated. The number of localisations \( (N) \) was used to represent the number of G3 molecules observed on the bacterial surface and inside the bacterial cytoplasm. According to the Figure 5.2b, Cy3B dyes could be conjugated with the G3 peptides. With the super-resolution imaging method, the number of localisations can be used to approximate numbers of G3 peptides. In Figure 5.5a, different concentrations of G3 CAPs were added to encapsulated (EV36) and non-encapsulated (EV136) E. coli.

The higher concentration of 100 µM G3 peptides adsorbed in larger quantities on bacterial cells when compared to the lower concentration 10µM and 1 µM. However, in the case of no CAPs, adsorption of unconjugated Cy3B dyes on bacterial cells was also observed. The adsorption of fluorophores and antibodies were further compared (Figure 5.5b). Encapsulated bacteria were labelled with mAb antiK1- F(ab)’AF647 labelling on K1 bacterial capsule, while non-encapsulated E. coli were labelled with rAb antiE.coli – F(ab)’AF647 on the bacterial surface. Two colour super-resolution images were constructed for the relative localisation of the fluorescent dyes. Control experiments were also performed on the adsorption of Cy3B dyes and mAb antiK1- F(ab)’AF647 antibodies. The experiments cross-checked the number of localization and compared the number of peptide molecules to fluorophores adsorbed on the bacterial membranes. These show the 100 µM G3 CAPs’ absorption was predominantly due to G3 peptides, not to unfunctionalized Cy3B dyes.
**Figure 5.5** The results of image analysis of CAP aggregation on the encapsulated and non-encapsulated surfaces of *E. coli*. **a)** The variation of the density of G3 deposition due to different concentrations of G3 on encapsulated bacteria compared with Cy3B dyes. **b)** Encapsulated and non-encapsulated surfaces were independent of the density of G3 deposition.

In addition to the number of molecules adsorbed on bacterial cells, the average size of aggregated G3 was quantified using the radius of gyration ($R_g$), in **Figure 5.6**. Although the interaction of CAPs on the bacterial membrane is thought to be very fast around ns to µs [35] (from MD simulation studies), the aggregation of CAPs can be observed after 10 minutes using super-resolution imaging. Different concentrations of G3 peptides (100 µM and 10 µM) were observed in both K1 encapsulated (EV36) and non-encapsulated (EV136) *E. coli* (**Figure 5.6a**). With the lower concentration, more aggregated peptides were observed in bacterial cytoplasm (in similar to Cy3B internalisation). The adsorption of G3 after 40 minutes (G3 evenly distributed and saturated in a solvent) were similar to short
incubation times (5 minutes), \textbf{Figure 5.6b}. The populations of each set of conditions is shown in their insets. The histograms were fitted with exponential decay distributions according to the cut off from super-resolution limit at 50 nm and the size of the peptide radius of gyration ($R_g$).
Figure 5.6  a) A histogram of G3 aggregate sizes with different adsorption times.  b) A histogram of G3 aggregate sizes with different peptide concentrations (different colours shown in the key). The insets present the distribution of $R_g$ in different conditions in box plots.
5.3.4 K1 bacterial capsule acts as a permeable shield on the E. coli surface

Due to the electrostatic interaction of G3 CAPs and K1 capsular polysaccharide, it is possible to have G3 attached to K1 capsular polysaccharide chains. Using super-resolution imaging, the colocalisation of G3 and K1 bacterial capsule could be observed. With the Pearson colocalisation coefficients (PCC), histogram plots show the distribution of PCC in different G3 concentrations that attached to K1 bacterial capsules (Figure 5.7) and the inset shows the total population of PCC. Zero and negative correlation are found at the lower peptide concentrations of 10 µM and 1 µM.

![Figure 5.7](image.png)

**Figure 5.7** A histogram of the colocalisation coefficients between G3 and K1 capsules with different G3 concentrations. Inset is shown the distribution of PCC from each G3 concentration.
5.4 Discussion

5.4.1 The localisation of G3 peptides on K1 encapsulated and non-encapsulated bacteria

The localisation of peptides on bacterial surfaces depends on the properties of antimicrobial peptides and the morphology of bacterial surfaces. K1 capsular polysaccharide is a major barrier that prevents the peptides from getting into bacterial membranes. The strong negative charge (due to the sialic acid units) of the K1 bacterial capsular polysaccharides interact with the amphiphilic properties of G3 peptides which G (Glycine ) and K (Lysine) which are positively charged. This main electrostatic force could drive G3 peptides to adsorb on K1 capsular polysaccharides before reaching the bacterial membrane.

From conventional fluorescence microscopy, G3 CAPs were localized in the cytoplasm and on bacterial surfaces of encapsulated bacteria (white triangles). However, in non-encapsulated bacteria, G3 CAPs were localised on the bacterial membrane and were evenly distributed. The fluorescence images also show the compactness of the nucleotides (shown in blue DAPI staining) when G3 CAMPs permeated and become saturated inside the bacterial cells. Although more aggregation inside encapsulated bacterial cytoplasm is shown in Figure 5.3a, 5.3b and 5.3c, G3 CAPs also aggregated and covered the bacterial surface, Figure 5.3d. The surface aggregation on K1 encapsulated and non-encapsulated bacteria were investigated in more detail using super-resolution microscopy.

5.4.2 Super-resolution imaging reveals the localisation of G3 peptides on the bacterial surface and inside the cytoplasm.

To eradicate K1 encapsulated E.coli, minimum inhibitory concentration (MIC) assays of EV36 (in LB media) were performed and the MIC value was less than 6.9 ±
1.2 µM. However, in order to visualise the peptides using super-resolution fluorescence microscopy, a higher concentration of G3 peptides was used in live cell experiments. The ratio between the bacterial concentration (~100 cells per field of view) and the G3 concentration (1-100 µM) was not extreme in comparison to the MIC value.

The super-resolution images (e.g. **Figure 5.4**) can resolve the detailed morphology of the K1 bacterial capsule. How G3 CAPs disrupt or interact with bacterial capsule can be investigated beyond that possible with diffraction limited resolution. Using the high-power excitation laser, the graphene oxide coated glass (PLLGO) slides, low concentration of antibodies and imaging buffer, the background noises were minimized. When the images of the bacterial capsule from previous work on K1 capsule imaging were compared, the K1 capsule-G3 peptide images showed more uneven K1 capsule boundaries. Although cross-linking of antibodies could blur the reconstructed image of the bacterial capsules, the uneven boundaries were due to G3 peptides disrupting the bacterial surface to induce more roughness on the membrane. The localisations of G3 CAPs covered the whole bacterial surface.

With super-resolution techniques and noise reduction by PLLGO slides, the reconstructed images can be used to determine the interaction between the bacterial surface and G3/G4 peptides. The focus of this study was to use G3 peptides to investigate the aggregation of the CAPs on the bacterial membrane and K1 capsules. The Cy3B dyes could perturb the original charge configuration of G3 peptides. However, the Cy3B conjugated on G3 peptides still maintain their amphiphilic properties, since the localisations of G3 peptides were observed on both bacterial membranes and capsules.
5.4.3 The aggregation of G3 CAPs on E. coli surface: membranes and capsules

The consideration of peptide concentration used in the study needs to be stressed. Although the MICs of EV36 and EV136 are around 10 µM, using 100 µM peptide is not an excessively high concentration because of the ratio between the high density of bacterial cells deposited on poly-L-lysine, GO coated slide to the number of G3 peptides. Under this consideration (Figure 5.5a), the absorption of G3 CAPs on K1 encapsulated bacteria varied from 100 µM to 1 µM. However, in order to perform super-resolution imaging with a high-powered laser (50 mW), the lower concentration (less than 10 µM) would have a problem with quick photobleaching that affects the reconstructed images. The super-resolution technique requires high numbers of localisations for a reconstructed image, so the high concentration (100 µM) is more suitable to observe the localisation of the aggregated CAPs on the bacterial surfaces in different conditions.

Hence, the absorption of the 1 µM G3 CAPs had a higher standard deviation compared to the higher peptide concentration, since they are a mixture between non-specific Cy3B dyes and bacterial surface specific G3-Cy3B conjugated peptides. From this comparison, it can be concluded that only Cy3B adsorbed on the bacterial surface. The standard deviations of G3-Cy3B and only Cy3B cases were also similar.

The number of localisations (N) in the case of high concentration (100 µM G3 CAPs) were mainly due to specific binding on the bacterial surface. This is seen in Figure 5.5b where mAb antiK1-F(ab’)^2 AF647 and rAb antiE.coli AF647 antibodies were specifically bound on bacterial capsules and membranes, respectively. The number of localisations (N)
of 100 μM G3 and those of the antibodies were similar in magnitude, while non-specific Cy3b deposition had a lower number of localisations.

The efficiency of small, α-helical peptides can be lower when they aggregate. With incubation times (5 and 40 minutes) in which G3 CAMPs were equally distributed, the aggregation sizes ($R_g$) of G3 in all condition ($Rg$ values) were at around 30.0 ± 6.5 nm, based on an exponential fit (Figure 5.6a). The different incubation times seemed to have no effect on peptide aggregation. With high concentrations of the peptide, $R_g$ from 100 μM G3 had a larger size when they were adsorbed on the non-encapsulated surface at around 71 nm, Figure 5.6b. However, the averaged $R_g$ from the rest of population (inset of Figure 5.6b) were similar when the G3 CAPs were adsorbed on both of bacterial strains: EV36 (with K1 capsule) and EV136 (no K1 capsule). Larger $R_g$ size (more than 500 nm) of G3 aggregation is due to the larger coverage on the bacterial surface. The model of G3 adsorption involves the properties of G3 CAPs, including electrostatic and amphiphilic interactions to form aggregated peptides on bacterial membranes and on bacterial capsules, Figure 5.8. The aggregation is thought to be associated with pores that were created on the bacterial membrane (for both encapsulated and non-encapsulated bacteria). Occasionally, peptide aggregation could happen on K1 bacterial capsules from the observation.

5.4.5 Evidence that K1 capsule acts as a permeable shield

To investigate how much peptides can permeate through thick capsular brush layers, the number of peptides ($N$) and aggregation size ($R_g$) were useful indicators. In addition, the colocalization between K1 bacterial capsule and adsorbed G3 peptides (Pearson correlation coefficients, $PCC$) was also considered,
\[
PCC = \frac{E[(I_{\text{capsule}} - \bar{I}_{\text{capsule}})(I_{G3} - \bar{I}_{G3})]}{\sigma_{\text{capsule}} \sigma_{G3}},
\]

where \( E[...] \) is the expectation value with \( I_{\text{capsule}} \) and \( I_{G3} \) the individual pixel intensity of capsule and G3 images, respectively. \( \bar{I}_{\text{capsule}} \) and \( \bar{I}_{G3} \) are the mean intensity of capsule and G3 images, respectively. \( \sigma_{\text{capsule}} \) and \( \sigma_{G3} \) are the standard deviations of the intensities from the K1 capsule and the G3 aggregation images, respectively.

The PCC values could explain the aggregation of G3 adsorption on the K1 encapsulated bacterial surface. When a high concentration of G3 peptides was used, most of the G3 aggregation could be described by either the pore or the combined models, since a positive correlation was observed in the PCC histogram (Figure 5.7). However, a negative correlation was observed at the lower concentration. This implies that low concentrations of G3 can penetrate the bacterial membrane. However, within the limitations of super-resolution imaging, whether G3 was at the outer or the inner membrane cannot be distinguished (around 20 nm). Hence, the negative PCC could be due to the G3 localisation on the outer membrane, peptidoglycan, inner membrane and cytoplasm.

The models illustrated in Figure 5.8b and 5.8d are summarised from all super-resolution images. To simplify, Figure 5.8a represents K1 encapsulated \textit{E. coli} and Figure 5.8c represents non-encapsulated \textit{E. coli}. In Figure 5.8a, when the PCC values of aggregated G3 peptide blobs (green) against K1 capsule from mAb antiK1-AF647 antibodies (red) were zero, it is difficult to confirm the condition of an aggregated G3 localisation. However, the region that G3 aggregates close to the K1 capsule layer. This aggregation is assumed to be on the bacterial membranes, including outer membrane, peptidoglycan and inner membrane.
The data is in agreement with both (i) the pore and (ii) the carpet models. The positive PCC values (PCC > 0) were used to indicate (iii) the capsule binding and (iv) the combined models. Although some available dyes can be used for specific labelling on bacterial membranes, e.g. FM4-64 dyes [45,46], it cannot be used to perform super-resolution imaging effectively. Alternatively, to confirm the assumption of peptide-membrane localisation, rAb anti-	extit{Ecoli} antibodies conjugated with AF647 or AF488 dyes were used. The super-resolution fluorescence reconstructed images of peptide aggregation on bacterial membrane were observed.
Figure 5.8 Model of G3 CAPs on the bacterial surface. a) Super-resolution image of K1 encapsulated EV36 where antiK1 labelled the K1 capsule (red) and G3 CAP (green) were colocalised. B) The localisation of G3 CAPs according to 4 different models: (i) pore, (ii) carpet, (iii) capsule binding and (iv) combined models. C) Super-resolution image of no
capsule EV136 where rAb ant- E. coli-AF647 antibodies were labelled on the E. coli surface (red) and G3 CAP (green). d) The localisation of G3 CAPs on bacteria with no capsule according to 3 different models: (i) pore, (ii) carpet and (iii) membrane aggregated models. The carpet and pore models cannot be resolved using super-resolution microscopy. However, neutron scattering experiments and molecular dynamics (MD) simulations can be used to differentiate between these models.

When used with 100 µM concentration of G3 CAPs on encapsulated bacteria, around 10-40% of the G3 peptides were found on the K1 bacterial capsule due to the PCC results. This is due to the pre-adsorption of a few peptides that then nucleated with other incoming peptides to form the aggregates.

In the non-encapsulated case, when membranes were labelled with rAb anti-E. coli-AF647 antibodies (red), if positive PCC were observed, either the pore and carpet models occurred e.g. Figure 5.8c. However, in the zero PCC case, there is internal aggregation in the cytoplasm. This cytoplasmic aggregation was observed in both capsule and non-capsule bacteria. However, most of the aggregation was observed from membranes which extended toward the outside of the bacterial cells. This observation indicates that unaggregated, short peptides can nucleate on bacterial membranes according to (iii) a membrane aggregated model. There is no pre-aggregation with the low concentration (1-100 µM) used.

In this study, the aggregated peptides on the bacterial surfaces (both membranes and K1 capsules) were from low peptide concentrations from 1 – 100 µM. The critical micelle concentration (CMC) of the self-assembly of similar peptides was observed on G4 CAPs at
1000 times higher concentrations [47]. So, in this case, it is difficult to have aggregated G3 peptides form before adsorption (Equation (5.1)). Other conditions that induce G3 peptide aggregation are due to the electrostatic and pore formation potentials. In the case of capsule binding model, the electrostatic force is predominant (Equation (5.3)). However, when the pore is created on the bacterial membrane and filled with aggregated peptides the pore formation energy barrier (Equation (5.4)) is key to explain how encapsulated bacteria can hold their rod shape, without bursting. Based on repeat investigations, non-encapsulated bacteria were observed at lower densities in contrast to encapsulated bacteria.

From several repeat experiments from both encapsulated and non-encapsulated bacteria, the population of encapsulated bacteria that adsorbed G3 peptides was larger than the non-encapsulated bacteria. One assumption is the K1 bacterial capsules support and reinforce the bacterial shape, but allow G3 peptides and other small molecules (e.g. nutrients) to reach the bacterial membrane to permeate into the cytosol. The highest concentration of G3 aggregation was observed at the poles of the bacteria. This correlates with the previous observation that the capsule at the poles is longer than at the equators (Chapter 3) [5].

5.5 Conclusions

Due to the strong negative charge and thick bacterial capsules, the capsular polysaccharide brushes prevent invasive charged molecules from reaching their membranes. The effect of K1 capsular polysaccharides on peptide aggregation has been observed in the current study. For non-invasive, live bacterial visualisation, fluorescence super-resolution imaging technique was used to investigate the aggregation of G3 peptides on the bacterial
surface. The amphiphilic G3 CAPs can reach the bacterial membrane, although some of them were adsorbed on the K1 capsular polysaccharides.

The number of G3 molecules adsorbed ($N$) and the aggregate size ($R_g$) were quantified. This showed G3 peptide aggregation was observed after the adsorption on bacterial membranes and K1 capsules. From colocalization experiments, about 10-40% of the G3 peptides were found aggregated on the bacterial capsule. Different models of G3 aggregation were postulated according to 1) pore, 2) carpet and 3) capsule binding models. These models were together observed on bacterial surfaces.

The localisation and aggregation of G3 peptides provide evidence that the K1 bacterial capsules were unable to completely protect bacterial cells from short, $\alpha$-helical antibacterial peptides. This information is useful for drug design [35,48] in order to kill encapsulated bacterial cells. Since the G3 peptides could penetrate after K1 capsule adsorption and cover the whole surface, aggregating on the membrane, the K1 bacterial capsules behave as a permeable shield protecting bacteria from immediate bursting but are unable to fully protect them from the CAPs’ invasion.

5.6 References
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CHAPTER 6

Conclusions and prospective works

Various aspects of K1 bacterial capsules are described in the thesis, including studies from single molecules to single cells, from the nucleation of bacterial capsular rafts to fully coverage capsules and from individual encapsulated cells to encapsulated cells interacting with antimicrobial peptides. This thesis demonstrates the use of super-resolution fluorescence microscopy in corroboration with other techniques, including AFM and HPLC, to investigate the unresolved morphology attainable through conventional fluorescence microscopy and the rapid dynamics of proteins involved in capsular biosynthesis of uropathogenic E. coli.

6.1 From single molecules to single cells

A key understanding of bacterial capsules is from capsular biosynthesis. In total, 14 proteins (from the CPS gene cluster) involved in bacterial capsule biosynthesis have been studied. However, the importance of polymerases, specifically a sialyltransferase NeuS, is underreported in the literature and it is ambiguous how rapidly it interacts with capsular substrates for chain extension during polymerization. The single molecule studies in this thesis help to remedy this omission.
By tagging NeuS with fluorescent ligands using the Halotag enzyme, the diffusive and sub-diffusive intracellular movements of NeuS enzymes have been observed using fluorescence correlation spectroscopy (FCS). With two different time domains, i.e. \( t_d_1 \) and \( t_d_2 \), the results (Chapter 4) show the period over which NeuS interacts with and promotes polysaccharide extension inside bacterial cells is 0.2 - 0.5 ms. When NeuS was compared to other static proteins in the translocation complex e.g. KpsDE (which also formed a complex with ABC transporters, KpsMT), the rapid interaction was seen to be significant. From this investigation in vivo, the mechanistic role of NeuS during capsular biosynthesis can be described using a processive polymerase model, which influences the polydispersity of capsular polysaccharide chains prior to completion and transportation to the outer membrane.

Although the dynamics of NeuS can explain the temporal interaction of NeuS with polysaccharide chains and/or proteins complexes, no further conclusions can be made concerning the period over which complete capsular chain synthesis occurs. This is due to the lack of specific labelling on Neu5Ac monomers and the inability to directly visualize the NeuS enzyme during the extension process. However, the capsular polysaccharide chains during translocation are anchored to the outer membrane, leading to the formation of the lyso-PG capsular raft.

In addition to single molecule studies, the nucleation time of K1 capsular rafts (composed of K1 capsular polysaccharides recognized by mAb735, antiK1 antibodies) was visualized with super-resolution imaging after temperature upshifting with the support of graphene oxide coated slides to improve the signal to noise ratio [1]. The stochastic
transportation of capsular polysaccharide chains and the photobleaching of the fluorophores inherent in the super-resolution fluorescence technique limited the potential to investigate the emergence of the bacterial capsule. Therefore it was necessary to use dense fluorophore labelling in conjunction with higher concentrations of capsular rafts on the bacterial membrane to improve the visualization of small nucleation events (which are sparsely localized on the outer membrane) after 1 hour. Dense bacteria deposition in a field of observation was required to further improve the statistical weight of the subsequent analysis. Super-resolution imaging buffer introduces environmental stress that can influence bacterial growth and capsular development, but this was reduced by replenishing the growth media and imaging buffer (to prevent acidification).

6.2 From the nucleation of lyso-PG capsular rafts to full capsule coverage

Emergence of bacterial capsular rafts on the outer membrane is initiated by stochastic events during internal biosynthesis, which is limited by nutrient availability and environmental stress. The time course observations carried out in this study suggest that bacterial capsules initially start as small nucleated regions (i.e. rafts) that expand to completely encapsulate the outer membrane. Furthermore, the initial raft locations randomly covered the membrane, contrary to the notion that capsule nucleation is mainly concentrated at the poles of the bacteria [2]. Slow diffusion (as its expansion from nucleated regions) of capsular rafts on the membrane was also observed.

Following capsular raft development and whole cell encapsulation, the properties of the polyelectrolyte brushes that compose the bacterial capsule were tested using super-resolution imaging (dSTORM) and atomic force microscopy (AFM). The heterogeneity of
polyelectrolyte brush thickness was resolved in the equatorial and polar regions of the bacteria cell. Brushes in the polar regions consisted of a mixed population of different thicknesses but were overall 49% longer than at the equatorial regions (this was corroborated with HPLC results) [3].

To confirm the brush model, the stabilization of bacterial polysaccharides was investigated using AFM with a 2.5 µm spherical probe on a soft cantilever (with the stiffness ~ 0.3 nN/nm). The results confirmed the osmotic pressure contributed to steric stabilization and confirmed the thickness of the bacterial capsule extrapolated from the super-resolution images. However, the heterogeneity of bacterial capsule thickness was unable to be measured by AFM due to the inadequate signal-to-noise level.

**6.3 From individual cell studies to a mixed environment with antimicrobial G3 peptide treatment**

A primary function of the polysaccharide capsule is to protect the bacteria from potentially harmful extracellular substances. It is believed that bacterial capsules can protect bacteria from certain antibodies, antibiotic substances and prevent serum recognition. However, the investigations presented in this thesis show antimicrobial peptides with amphiphilic properties (e.g. 10-100 µM of G3 peptides) can penetrate the bacterial capsule layer and aggregate on bacterial membranes revealing the insufficient protection bacterial capsules provide against invasive cationic antimicrobial peptides (CAPs).

The distribution of peptide aggregation on the bacterial membrane was quantified in terms of the number of molecules (N) and the aggregation size (Rg). Although some aggregated peptide localised on the bacterial capsule can be attributed to a purely
electrostatic interaction, most of the peptide aggregates are due to the combination of charge attraction and bacterial membrane hydrophobicity. On the bacterial surface, a combination of the pore, carpet and capsule binding models was observed. A potential reason for the permeable nature of the K1 capsule could be the inhomogeneous landscape of polysaccharide brushes (i.e. cavity formation due to chain stretching).

6.4 From the problems of present studies to prospective studies

6.4.1 Problems with bacterial capsule studies

Several problems encountered when studying bacterial capsules were solved by experimental optimisation, post-processing correction and meticulous sample preparation. As an example, a significant hurdle that was overcome in the course of this investigation was the optimisation of fluorescent intensity and fluorophore conditioning to facilitate single molecule visualisation and tracking in a single cell. The choice and specificity of fluorophores are reflected in the quality of reproducible results, consistent long-term stability and high quantum yields, which are essential for both dSTORM and fluorescence correlation spectroscopy experiments.

In order to support the reactivation of the photoswitchable fluorophores, imaging buffer is a necessity when performing live-cell imaging over long periods (up to several hours). However, it tremendously affects bacterial metabolism (Figure A4), which subsequently delays bacterial growth. This effect can be reduced by replenishing with fresh media or using a less prohibitive buffer. A trade-off of using an alternative buffer is the reduced efficiency with certain fluorophores, for example Alexa Flour 647 dyes performed better in the GLOX buffer rather than the OxEA buffer [4,5].
In the single molecule study, using a self-labelling technique such as Halotag involves a high concentration of labelling ligands (e.g. TMR-Halotag ligands) to label the Halotag-conjugated proteins (e.g. NeuSHT) which required a careful washing procedure. Additionally, successful tracking and spectroscopic investigation are difficult when there were low copy numbers of the expressed proteins due to insufficient fluorescent intensity. Different expression methods were investigated, such as the pBAD33 and pBluescript SK+ II system. However, the number of observable proteins was quite low, as seen in Western blot results. Thus, several repeats with controls and attentive protocols were required for the NeuS dynamic studies. As the NeuS method directly conjugates fluorescent proteins (FPs) onto the proteins of interest, it ultimately reduces the difficulty of the labelling process [6]. However, a drawback of this technique is the difficulty when changing the fluorescent proteins to another specific excitation/emission state when the multi-fluorescence investigation is redesigned. In contrast, for the Halotag system, the commercial ligands are available with various functions and excitation/emission choices [6–8].

In the study of peptide-capsule interaction, the amphiphilic properties of the G3 peptide could be adversely affected by the labelling of NHS fluorophores. However, the peptide aggregation on the bacteria membrane still occurred due to remaining charge properties, despite the potential charge neutralisation caused by the Cy3B dyes.

6.4.2 Prospective works on bacterial capsule studies

This thesis demonstrates several interesting aspects of bacterial capsule studies. There are many potential future works that could clarify the understanding of bacterial capsule formation and protection (Figure 6.1).
Figure 6. 1 Flow chart summary of the problems and prospective works for bacterial capsule studies including the study of bacterial capsule morphology, the study of single molecules involved in capsular biosynthesis and the effect of the bacterial capsule with regard to the efficacy of antimicrobial peptides.

The *de novo* discoveries in this thesis lay the groundwork for more nuanced questions involving more realistic and complicated live systems. This includes extended studies into bacterial capsule morphology, capsular polysaccharide biosynthesis and the interaction of bacterial capsule with other invasive antibiotic treatments.

The results presented in this thesis demonstrate bimodal capsule thicknesses occur at the bacterial poles and the underlying cause could be part of the biosynthesis pathway. This assumption can further be tested in different environments, such as different nutrient loads [9] (from external supplement Neu5Ac or cell-synthesized *de novo* Neu5Ac) and *in vivo* host.
cells after bacterial infection [10]. The assumption could also be tested in different encapsulated strains [9]. Many studies have shown the significant interplay between capsule-deficient strains and the ability to form a biofilm. Bacteria without capsules form more robust biofilms [11] due to the efficiency of adhesins used in initial bacterial colonization [12,13]. Counterintuitively, bacteria found in vivo during infection studies were found to have bacterial capsules mixed with biofilm material [14,15]. Characterization of the bacterial capsule morphology when inside the complex extracellular matrix of the biofilm (both in vitro and in vivo) is challenging, but clinically relevant, since bacterial capsules increase the survivability in environmental stressed conditions [16].

The FCS investigation of NeuS dynamics concluded that the interaction periods occurred in sub-diffusive regime at around 0.2-0.5 ms for different conditions, including the presence and absence of the capsular biosynthesis complex. However, from the understanding of other proteins involved in bacterial capsule biosynthesis, NeuS proteins have the potential to interact with NeuE, KpsM and KpsT during the biosynthesis pathway. For example, the effect of capsular polysaccharide chain extension without the pioneer sugar Kdos [17,18] and the first unit of Neu5Ac can resolve an ambiguous result of NeuS dynamics. The studies on different knocked-out gene conditions (e.g. ΔkpsS, ΔkpsC, ΔneuE, ΔkpsM and ΔkpsT) bacteria could precisely describe the chain elongation period in the bacterial capsule biosynthesis process. The comparison can exclude and identify individual factors in capsular biosynthesis. Hence, the investigation could only focus on capsular polysaccharide extension processes and other specific conditions. The use of Halotag can be substituted for traditional genetic conjugation of NeuS to other specific high quantum yield
fluorescent proteins [19] (e.g. mCherry) to identify the observable proteins and even conjugated with specialized photoswitchable fluorescent proteins [20–23] (e.g. PAmCherry) to enable blinking and thus super-resolution microscopy [22,24].

From the bacterial capsule and peptide interaction studies, it was shown that amphiphilic G3 peptides aggregated on the surface of encapsulated bacteria. Therefore, in addition to preventing direct adsorption of peptides onto the bacterial surface, another property of the bacterial capsule could be to filter large particles [25–27] that would otherwise disrupt the stability of the membrane potential. These factors could be used to characterize the interaction between bacterial capsules and peptides [28]. The predominant effects of charges and sizes of invasive peptides could be used to investigate the efficiency of the protective capsule and to prove the intrinsic permeability of the capsule is also affected by other peptides. This could be a central model for antimicrobial peptide treatment on encapsulated bacteria, especially uropathogenic Escherichia coli.

6.5 References


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APPENDIX A

The physiological investigation of bacterial capsules and control experiments

Figure A1. Examples of wide-field super-resolution images of live *E. coli* bacteria, the capsular structures were labelled with anti-K1 conjugated with anti-mouse-AF647 placed in imaging buffer and M9 media. The images were created using dSTORM [1] (50 nm resolution) with PLLGO coated coverslips at different focal planes. a) Shows example data used for the analysis of capsular brush thicknesses and their polydispersity. b) Shows bacterial cross-sectioning at a level above their mid-point and we omitted this type of image for capsule thickness measurement to avoid projection artefacts. The scale bars are 5 \( \mu m \).
Figure A2 Axial projection calibration using 100 nm polystyrene beads coated in Cy3b for z-stack scans with the super-resolution microscope. a), b) and c) Show plots of the point spread function (PSF) with positions at $z = -0.8$, 0, and 0.8 $\mu$m. d) The FWHM and standard deviations (sigma) of Gaussian fits to the data shown in the inset. The inset shows the profiles from all 200 steps of the z scan image stack.

The depth of focus (DOF) of our super-resolution microscope was analysed using a virtual light sheet software package [2] and it was found to be $347 \pm 22$ nm. We used this DOF to quantify its effects on the bacterial capsule thickness measurements and it was found to be negligible e.g. on the results shown in Figure 3.3.
Figure A3  

**a)** Force as a function of separation distance from AFM for live encapsulated K1 *E. coli* under hydrated conditions, with filtered miliQ water. The force was fitted with an exponential decay to determine the contact points of the probe with the bacterium (coloured curves). **b)** and **c)** are 3D and 2D images respectively of encapsulated bacteria using AFM scanning with a 2.5 µm spherical probe.
Figure A4 Growth curve of E. coli bacteria (EV36-wild type and EV136-capsule mutant strains) showing OD600 as a function of incubation time with different carbon sources in the growth media. a) and b) are the same data plotted over different times. The media abbreviations are GG is 0.4% Glucose and 0.4% Glycerol, GGIMB is 0.4% Glucose and 0.4% Glycerol with imaging buffer, and blank is a control media without bacteria. The imaging buffer (IMB) has a significant impact on bacterial proliferation after 2 hours.

Figure A4 shows that the imaging buffer with the oxygen scavenger did perturb the growth of bacterial cells and reduced the population of the bacteria cells over time. However, to circumvent this problem in super-resolution microscopy, we first deposited a high density of bacteria cells on the PLGO coated substrate and then changed the media including the imaging buffer and the M9 media with the carbon source supplement every 1 hour [3]. This allowed us to observe the expansion of capsular lyso-PG raft over 3 hours (the time scale of capsule growth) with no significant artefacts.
Figure A5 The effect of positive charge substrate (poly-L-lysine) on bacterial growth. Bacteria were grown on the substrate without poly-L-lysine coated (red) and with poly-L-lysine coated (blue) from 0 to 6hrs. Then the deposited cells were lifted off using 0.1% triton X100 and grown on a culture plate to perform a viable count.

The proliferation of bacteria was also examined in contact with poly-L-lysine coatings (Figure A5). No significant changes were observed over the time scales of our experiments (3 hours) compared with the glass substrate control.
APPENDIX B

Genetic modification results and the control experiments for fluorescence correlation spectroscopy (FCS).

Figure B1 The K1 phage assay. a) The phage assay on EV36 strain revealed small plaque spots due to K1 phage recognition K1 capsule. b) The control phage assay on EV136 showed no plaque because of no capsule production. c) The phage assay revealed phage spots of EV136 neuSHTpBSK. This showed leakage expression of NeuSHT that produced K1 capsule without IPTG induction. d) The K1 phage spots observed from EV136 neuSHTpBSK induced with 1mM IPTG. White arrows show the location of phage spots and appeared on soft-top agar LB with antibiotic supplement. The scale bar is about 1 cm.
Figure B2 The calibration of FCS measurements on TMR labelled bacteria cells and Cy3B labelled 100 nm polystyrene beads. a) A fluorescence image of encapsulated *E. coli* contained *NeuSHT* conjugated with TMR ligands before performing FCS experiment. b) The fluorescence image after performing FCS over 1AU confocal volume. c) The population ($N$) observed by different confocal volumes. d) The calibration of confocal volumes using 100 nm fluorescent beads reconstructed the stack image to approximate different confocal volumes. The arrows indicate the locations of the confocal volumes investigated.
Figure B3 The distribution of characteristic lag times: $td_1$ and $td_2$. The box plots with data shows the distribution of lag time fitted from the FCS curves: a) $td_1$ and b) $td_2$. The histograms of the parameter value distribution were plotted with c) the kernel overlay $td_1$ population and d) the kernel overlay $td_2$ population.
Figure B4 Alpha distributions of FCS correlation function in different conditions of *NeuSHT* expression. They were observed on 2 different domains: from sub ms (\(\alpha_1\)) and beyond ms (\(\alpha_2\)).
Appendix references

