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A nanoscale characterisation of the interaction of OligoG with the cell surface and motility of *Pseudomonas aeruginosa*

Running title: Characterisation of OligoG-bacterial interactions

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Abstract

Pseudomonas aeruginosa (PA) biofilm-associated infections are a common cause of morbidity in chronic respiratory disease and represent a therapeutic challenge. Recently, the ability of a novel alginate oligomer (OligoG) to potentiate the effect of antibiotics against Gram-negative, multi-drug resistant bacteria and inhibit biofilm formation *in vitro* has been described. Interaction of OligoG with the cell surface of PA was characterized at the nanoscale using atomic force microscopy (AFM), zeta potential measurement (surface charge) and sizing measurements (dynamic light scattering). The ability of OligoG to modify motility was studied in motility assays. AFM demonstrated binding of OligoG to the bacterial cell surface, which was irreversible following exposure to hydrodynamic shear (5,500 *g*). Zeta Potential analysis (pH 5-9, 0.1-0.001 M NaCl) demonstrated binding was associated with marked changes in the bacterial surface-charge (-30.9±0.8 mV to -47.0±2.3 mV; pH 5, 0.01 M NaCl; P<0.001). Sizing analysis demonstrated alteration of surface charge was associated with cell aggregation with a 2-3 fold increase in mean particle size at OligoG concentrations >2% (914±284 nm to 2599±472 nm; pH 5, 0.01 M NaCl; P<0.001). These changes were associated with marked dose-dependent inhibition in bacterial swarming motility in PA and *Burkholderia* spp. The ability of OligoG to bind to a bacterial surface, modulate surface charge, induce microbial aggregation and inhibit motility, represent important direct mechanisms by which antibiotic potentiation and biofilm disruption is affected. These results highlight the value of combining multiple nanoscale technologies to further understanding of the mechanisms of action of novel antibacterial therapies.

Keywords: Cystic Fibrosis, Antimicrobial, Biofilm, Atomic Force Microscopy, Zeta Potential, Sizing, *Pseudomonas aeruginosa, Burkholderia* spp.

Introduction

Pseudomonas aeruginosa is a versatile opportunistic human pathogen often presenting in a range of chronic human diseases with serious clinical implications. It has a physical/genetic "pliability" that enables it to colonise and proliferate in a diverse range of habitats. *P. aeruginosa* is of particular importance in cystic fibrosis (CF) where it represents the dominant, persistent pathogen (1). The *Burkholderia cepacia* complex is also important in adult CF infections. The establishment of *P. aeruginosa* biofilms in the lungs results in the bacterial cells being encased in extracellular polymeric substance (EPS) and extracellular (e)DNA. These biofilms represent a formidable challenge to conventional antibiotic therapies (2) inducing chronic inflammation and, ultimately, reducing lung function (3). *P. aeruginosa* infection consequently represents a major cause of morbidity and mortality in CF patients. The increasing incidence of multi-drug resistant (MDR) Gram-negative biofilm-related infections, such as *P. aeruginosa,* is reflected in an urgent need to identify novel antimicrobial therapies.

Several potential candidate therapies for the treatment of biofilms, derived from the marine environment, have recently been described (4−6). OligoG is a low molecular weight antimicrobial agent, derived from the brown seaweed *Laminaria hyperborea* (7). We have recently demonstrated the ability of OligoG to potentiate the activity of conventional antibiotics against MDR bacteria including *Pseudomonas*, *Acinetobacter* and *Burkholderia* spp, where OligoG treatment reduced (by up to 512 fold) the minimum inhibitory concentrations (MICs) of a range of antibiotics, such as ceftazidime and macrolides, against MDR pathogens (5, 8). Although the mechanism of action is still unclear, *in vitro* studies have demonstrated that these effects are unrelated to membrane permeability, sequestration of divalent cations at the cell surface, or inhibition of efflux pumps (in MDR organisms; 5). Interestingly, OligoG has also been demonstrated to possess specific anti-biofilm properties;

reducing the formation of biofilm biomass as revealed by Confocal Laser Scanning (CLSM) and Scanning Electron Microscopy (SEM) images (5) and increasing the susceptibility of biofilms to physical disruption as measured through rheological and AFM force measurement techniques (8). Physical alteration of the Gram-negative cell-wall was also apparent following OligoG treatment, as observed by SEM images (5) but, to date, no characterisation of this surface interaction has been performed.

Previous studies have used a combination of techniques to investigate the mechanisms of bacterial adhesion and aggregation to host tissues and biomaterial substrates (9−11). Zeta potential measurements, bacterial motilities and AFM force measurements have all been used to determine bacterial attachment to poly(ethylene oxide) brush coatings (10). However, few studies have used a comprehensive combination of techniques to determine the specific interactions between antimicrobial agents and their target bacteria.

Atomic Force Microscopy (AFM) has been previously used to study the nanoscale interaction of antimicrobial agents (such as colistin) and antimicrobial peptides, on the morphology and topography of the cell surface of MDR, Gram-negative bacteria, including *P. aeruginosa*, *Acinetobacter baumannii* and *Escherichia coli* (12-14). AFM represents a valuable characterisation tool to visualise alterations in the cell surface structure following exposure to antimicrobial agents.

In addition to changes in structure, bacterial surface charge is an important determinant of a number of key processes in bacterial adhesion and biofilm formation. Bacterial surface charge can be characterized by the zeta potential, which is defined as the electrical potential of the interfacial region between the bacterial surface and the aqueous environment (15). Electrophoretic Light Scattering (ELS) has become an established tool for zeta potential determination in bacteria (16, 17). Studies on the surface charge of *P. aeruginosa* strains have been performed under various conditions (18, 19). The net-negative surface charge of

P. aeruginosa strain PAO1 has been demonstrated in a range of physiological pH's (pH 4-9), which reflects the presence of carboxylate (COO^-) groups in the lipopolysaccharide (LPS) of the Gram-negative bacterial cell wall (9). ELS has also been employed to characterise the biophysical effects of the antimicrobial peptides BP100 and pepR on the surface charge of *E. coli* (20).

Dynamic light scattering (DLS) is a technique for the measurement of hydrodynamic size, and is another characterisation tool to test the effect of specific agents on bacteria cells. DLS has previously been used to demonstrate an increase in bacterial cell size after TiO_2 treatment due to nano TiO_2 biosorption (19). This technique allows measurement of bacterial size and bacterial aggregation.

The motility of Gram negative bacteria is crucial for colonization and is a major factor in the spread of infection *in vivo*. *P. aeruginosa* motility has been strongly implicated in its virulence (21). Unusually, *P. aeruginosa* possess three distinct means of motility which are habitat-dependent, namely; flagella-mediated swimming (aqueous motility); type IV pilus mediated twitching (solid surface motility); and flagella and type IV pilus mediated swarming (semi-solid surface motility) (22). We have previously shown that OligoG inhibits swarming in *P. aeruginosa*, *Proteus mirabilis* and *E. coli* (5).

Low molecular weight OligoG oligomers have been shown to disrupt biofilm formation (8) and modify the structure of mucin (25). They have an exemplary safety profile (5) and a nebulized formulation is currently in Phase II human trials in CF patients at a concentration of 6% OligoG (60mg/ml) (EudraCT number 2010-023090-19, www.clinicaltrials.gov identifier NCT01465529), however only a fraction of OligoG inhaled is estimated to reach the lung. While the anti-biofilm and antibacterial effects of OligoG offer promising new therapy for the treatment of chronic Gram-negative infection in CF, a better understanding of

the mechanism of action is needed to inform the design and delivery of future oligosaccharide-based therapies.

We sought to characterize the nanoscale interactions of these low MW OligoG oligomers with the cell surface of *P. aeruginosa* using a range of techniques including AFM to characterize cell surface and structural changes, ELS to monitor surface charge, and DLS to investigate cell size. The effect of OligoG treatment on cell motility of CF-relevant MDR bacteria was also investigated in models of bacterial cell "swarming motility". In addition, the stability of OligoG binding was studied by exposure of the treated cells to hydrodynamic shear.

MATERIALS AND METHODS

Alginate Oligosaccharide Synthesis

The OligoG particles used in these experiments were generated from alginate extracted from the stem of the brown seaweed *Laminaria hyperborea*. OligoG production involved purification, fractionation and characterization with NMR of low molecular weight (mean, 2,600 Mw) oligomers with a high guluronate content (90% to 95%) as previously described (5).

Bacteria, Media and Culture Conditions

Strains used in this study included: *P. aeruginosa* (PAO1), *Burkholderia cenocepacia* (LMG 16656), *Burkholderia cepacia* (BCC 0001), *Burkholderia multivorans* (BCC 0011) and *Staphylococcus aureus* (NCTC 6571). *P. aeruginosa* (PAO1) was grown at 37˚C for 24 h in Mueller-Hinton broth (MH; Oxoid) in shake-flasks at 60 rpm. One ml of bacterial broth was then removed and washed twice at 5,500 *g* for 3 mins in deionised water to remove any growth media. Bacteria were then incubated in OligoG (0.2-10%) for 20 mins before the

sample was subjected to further washing and centrifugation at 2,500 g for 6 mins to remove excess OligoG. Then 20 µl of the bacterial suspension was added into 1 ml of the relevant electrolytic solution for zeta potential, sizing and AFM analyses. PAO1 was also grown at 37˚C for 24 h in the presence of OligoG (10%) in MH broth within shake-flasks at 60 rpm. Again, 1 ml of bacterial suspension was removed and subjected to hydrodynamic shear by washing in deionised water and centrifugation at 5,500 *g* (x3) for 3 mins. The zeta potential, sizing and AFM analyses were performed before and after each centrifugation step in pH 5 and 0.01 M NaCl to determine the effect of hydrodynamic shear (washing) on the interactions of OligoG with the PAO1 cells.

Bacterial Motility

Two methods (a plate and stab assay) were utilised to assess the effect of OligoG on bacterial motility (see online supplement for details).

AFM Imaging

AFM was performed on PAO1 cells grown with/without OligoG (10%) and on cells treated with OligoG (0.2-0.5%) after growth in MH (24 h) as described above. Bacterial suspensions (7 µl drops) were dried onto 0.01% poly-L-lysine coated mica plates for imaging. A Dimension 3100 AFM (Bruker) was used to achieve AFM images, using tapping-mode operation in air and a scan speed of 0.8 Hz. AFM images were processedusing Nanoscope data-processing software.

Zeta Potential and Sizing Measurements

Zeta-potential and sizing analyses were performed on PAO1 cells grown with/without OligoG (10%) and on cells treated with OligoG (0.2-10%) after growth in MH (24 h) as

described above. Bacteria were examined at a range of salt concentrations (0.1-0.001 M NaCl) and pHs (5, 7 and 9). A Zetasizer Nano ZS (Malvern Instruments) and disposable capillary cells (DTS1061 Malvern instruments), were used to measure ELS and DLS. The zeta potential of PAO1 was calculated by applying the Smoluchowski's model (15).

Statistical Analysis

Minitab (v.14) statistical software was used for all statistical analyses presented. Group-wise comparisons were analysed by the non-parametric Kruskal-Wallis test, followed by the Mann-Whitney test to determine significant differences for pairwise comparisons if appropriate. A P-value of <0.05 was considered statistically significant. To account for multiple testing, the Bonferroni-Holm correction was applied to adjust P-values.

RESULTS

Effect of OligoG on Cell Motility

OligoG interactions with the important CF pathogens *P. aeruginosa, B. cenocepacia, B. cepacia, B. multivorans* and also *S. aureus*, were examined to determine effects on bacterial motility *in vitro*. OligoG inhibited swarming motility of PAO1 in a dose-dependent manner at concentrations between 0.2 and 6% (Figure 1A and 1B). This effect was only evident in the presence of OligoG. Bacteria exposed to OligoG in broth conditions demonstrated a diminished motility impairment when subsequently plated onto agar containing no OligoG. Treatment with OligoG (6%) also inhibited swarming motility of all the *Burkholderia* strains studied (Figure 1C). The negative control (*S. aureus*) indicated no bacterial motility with or without OligoG.

Effect of OligoG on Modulation of Surface Charge (Zeta Potential)

OligoG treatment induced a marked alteration in the surface charge of the bacteria, as determined by zeta potential measurements using ELS. OligoG treatment increased the overall negative bacterial surface charge (-30.9±0.8 mV to -47.0±2.3 mV; pH 5, 0.01 M NaCl) at all pH values (pH 5–9; $P<0.001$) and at salt concentrations of 0.001 M and 0.01 M NaCl (Figures 2A and 2B respectively). Comparison of the zeta potential distribution of PAO1 cells with OligoG-treated PAO1 cells (Figure 2D), revealed a secondary, more negative, zeta-potential peak in the measurements (-57.8±2.7 mV; pH 5, 0.01 M NaCl).

Effect of OligoG on Modulation of Cell Size

Sizing analysis using DLS showed a 2-3 fold increase in measured size for 10% OligoG-treated cells (914±284 nm to 2599±472 nm; pH 5, 0.01 M NaCl) at all pH values (pH 5-9; $P<0.001$) and in the two salt concentrations of 0.001 M and 0.01 M NaCl (Figure 3A and 3B respectively). Both zeta potential and sizing analyses demonstrated the strong nature of OligoG-cell binding (Figures 2 and 3).

Effect on PAO1 grown in the presence of OligoG

The strength of the interactions between PAO1 and OligoG was also demonstrated by PAO1 cells grown in the presence of OligoG (Figure 4). These cells were subjected to hydrodynamic shear by washing in deionised water and centrifugation. The zeta potential values revealed that PAO1 cells grown in OligoG exhibited an overall more negative bacterial surface charge which was significant ($P<0.005$) for each of the three hydrodynamic shear tests (washes) when compared to PAO1 alone (30.0±0.9 mV to -33.1±1.1 mV; pH 5, 0.01 M NaCl, 3rd Test) (Figures 4A and 4B). The zeta potential distributions revealed that the secondary, more negative zeta potential peak remained after application of hydrodynamic shear, although the secondary peak size reduced in magnitude after the first test (Figures 4A).

Increase in the size of PAO1 bacteria when grown in the presence of OligoG (869±74 nm to 1423±145 nm; pH 5, 0.01 M NaCl, 3rd Test), was evident following the hydrodynamic analysis (Tests 1-3; $P<0.005$) (Figures 4B). AFM analysis confirmed the results obtained from the zeta potential and sizing analyses. Although the majority of the cell surface associated OligoG was removed by the initial exposure to hydrodynamic shear, OligoG remained bound to the cell surface, was evident after multiple washing steps and was associated with cellular aggregation (Figure 4C).

Effect of reduced OligoG concentrations on Surface Charge and Cell Size

Zeta potential and sizing analyses was used to examine the effect of reduced, more clinically relevant, concentrations of OligoG (0.2% and 2%) on PAO1 cells. Again, OligoG treatment resulted in a increase in the overall negative bacterial surface charge (-31.5±0.6 mV to -34.4±0.6 mV; pH 5, 0.01 M NaCl, 0.2% OligoG; Figure 5) in both 0.2% and 2% OligoG ; ($P<0.001$). The effects of 0.2% and 2% OligoG treatment on cell size were less apparent (Figures E2); a finding which reflects the lack of aggregation observed in treated PAO1 bacteria at these concentrations.

Effect of OligoG on Bacterial Cell Morphology

The direct surface interaction of OligoG with PAO1 was studied using AFM to visualize the effect of OligoG on the morphology of the PAO1 cells. AFM clearly demonstrated the binding of OligoG to the cell surface of PAO1 (Figures 6A and 6B). In comparison to the untreated control (Figure 6A), Figure 6B shows OligoG not only binding to the cell wall, but demonstrates its interaction with the bacterial flagella.

DISCUSSION

The study utilised AFM, zeta potential measurements, cell sizing and motility assays to investigate the mechanisms by which OligoG modifies the Gram-negative bacterial cell-surface, structure, charge and function. OligoG was demonstrated to bind to the surface of PAO1 thereby inducing specific changes, including decreased bacterial motility and swarming, a more negative cell-surface charge and increased cellular aggregation. The results revealed that OligoG had a similar effect on PAO1 populations, whether it was present during or after the growth phase. In addition, the application of hydrodynamic shear showed that this binding was strong, and not readily physically reversible.

In biofilm infections, bacterial motility and swarming are important pathogenicity-associated traits. High cell densities and bacterial swarming are believed to be effective strategies by which bacteria evade antimicrobial attack (26). Whilst Khan et al (5) demonstrated that OligoG treatment almost completely inhibited swarming of the normally motile *E. coli, P. aeruginosa*, and *P. mirabilis*, we have shown in the present study, that this effective inhibition of bacterial motility is evident in the important *Burkholderia* spp. CF pathogens. The inhibition of bacterial motility and swarming observed in these experiments may result from both direct (i.e. physical) and indirect mechanisms. Importantly, the changes observed in the stab-assay were not the result of increased physical resistance to cellular migration in the substrate, as OligoG decreased the viscosity of the medium. In support of this physical inhibition of bacterial movement, AFM studies showed OligoG binding to the flagella (Figure 3B) inducing cellular aggregation of the bacteria. This may reflect one of the direct mechanisms of action for OligoG in the inhibition of bacterial cell motility.

Indirect mechanisms of action are also important and could include specific changes in gene expression induced by OligoG in PAO1. Although not featured in the present study, preliminary expression profiling suggests that *pilE* is down-regulated in OligoG-treated *P. aeruginosa* (unpublished data), which would indeed support that hypothesis. The observed

inhibition of swarming motility with OligoG in the present study may therefore, reflect down-regulation of the expression of quorum-sensing genes, which are essential for biofilm development (27, 28, 29). Interestingly, Gram-negative bacteria (including *P. aeruginosa*) exhibit elevated resistance to various antibiotics under swarming conditions (21, 30) and the reduced motility of MDR *Pseudomonas* and *Burkholderia* spp. may be linked to the antibiotic potentiation that we have previously described (5, 8).

Biofilm growth is governed by a number of physical, chemical and biological processes. The initial stages of bacterial adhesion involve the transport of cells to a surface. This occurs either by physical interactions, such as hydrodynamic forces, Brownian motion or, by bacterial appendages e.g. flagella-mediated locomotion. Initial (reversible) adhesion is governed by "long range forces" including: electrostatic (double-layer) interaction (repulsive due to negative charges of the cells and surface), steric interactions, van der Waals forces (attractive) and hydrophobic/hydrophilic interactions (31, 32). Determination of the surface charge properties of PAO1 in the presence of OligoG in an aqueous environment may facilitate our understanding of potential cell-surface interactions in these early stages of biofilm growth.

In the lung, epithelial surfaces are coated with mucin, which is negatively-charged due to the presence of N-acetylneuraminic acid and sulphated sugars (33, 34). The importance of electrostatic charge in surface adhesion has been highlighted by the failure of a *S. aureus* mutant, (with increased negative charge due to the lack of D-alanine esters in its teichoic acids) to colonise material surfaces (35, 36). Interestingly, the CF epithelium has also been shown to exhibit a reduced negative charge (37) which may favour microbial adherence in the CF lung. The greater negative charge on *P. aeruginosa* induced by OligoG, may effectively increase electrostatic repulsion between OligoG-treated PAO1 and mucin in the CF lung (38), thereby reducing bacterial adherence, and subsequent biofilm formation.

Alteration of the bacterial surface-charge, was evident following OligoG treatment, and may arise from its binding directly to LPS on the bacterial cell outer membrane, thereby making the LPS more negatively charged. Electrostatic interactions play an important role in the mechanical stability of biofilms (39−41). Alterations in electrostatic repulsion between negatively-charged moieties within a biofilm (in the presence of a negatively-charged bioelectric field) may effectively modulate the resultant mechanical structure and thickness (42). This observed change in charge following OligoG treatment may therefore, in addition to mediating alterations in bacterial adhesion, result in the altered structural assembly of pseudomonal biofilms. The altered structural assembly of OligoG treated biofilms has been previously quantified through AFM force measurements which revealed a significantly lower Young's moduli for OligoG treated biofilms and rheological techniques which revealed a observed increase in phase angle which reflected a decreased ability of the OligoG-treated biofilm to resist structural rearrangement under stress (8). Also the altered structural assembly of OligoG treated pseudomonal biofilms may explain how OligoG treatment was able to reduce the MICs of a range of antibiotics against Gram-negative pathogens (5) and also how OligoG was able to potentiate antimicrobial treatment in dental biofilm infection (Roberts., 2013). DLS demonstrated clear differences in the size of OligoG-treated PAO1. DLS however, assumes Brownian motion of the cells and *Pseudomonas* spp. are motile. To ensure that the observed increase in cell size did not simply reflect decreased motility, direct cell measurements were performed using AFM. This confirmed that the increase in size was due to cellular aggregation following OligoG treatment.

An important finding was that the observed changes in surface charge and cell aggregation in *P. aeruginosa* were evident whether bacteria were grown in the presence of OligoG, or established cultures were exposed to OligoG. This was unsurprising, as we had previously shown effects on *Pseudomonas* spp. in planktonic minimum inhibitory

concentration (MIC) assays and SEM studies of OligoG treated, established pseudomonal biofilms (5). Hence, OligoG may have potential application in both preventing biofilm development (on host or material surfaces) following debridement and bacterial decolonization (43) and also as a treatment for established biofilms.

The physical, surface-charge and structural effects on *P. aeruginosa* described here may partly explain the observed action of OligoG on bacterial assembly, biofilm formation and antibiotic potentiation that have previously been described (5, 8). The benefits of using a combination of nanoscale technologies to characterize interactions between bacteria and antibacterial compounds, is also evident from this study. These findings, together with the potentiation of antibiotics against a range of MDR bacteria, and the previously described human safety in Phase I chronic inhalation studies, all highlight the potential utility of OligoG as an adjunct in the treatment of chronic biofilm-associated lung infections in conditions such as CF and chronic obstructive pulmonary disease (COPD).

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Figure Legends

Figure 1. Effect of OligoG on bacterial motility (*A*) *P. aeruginosa* PAO1 cultures grown in MH broth with 0, 0.2, 0.5, 2 or 6% OligoG and plated on BM2 agar containing no OligoG. (*B*) PAO1 cultures grown in MH broth without OligoG and plated on BM2 agar containing 0, 0.2, 0.5, 2 or 6% OligoG. (*C*) Motility test agar stab cultures supplemented with 0 or 6% OligoG, inoculated with *S. aureus* (NCTC 6571; negative control), *B. cenocepacia* (LMG 16656)*, B. cepacia* (BCC 0001)*, B. multivorans* (BCC 0011).

Figure 2. Mean zeta potential values for 10% OligoG, untreated PAO1 or PAO1 treated with 10% OligoG (post-wash) at various pH values in (*A*) 0.001 M NaCl and (*B*) 0.01 M NaCl. Typical zeta potential distributions of (*C*) 10% OligoG and (*D*) Untreated PAO1 cells only (black solid line); PAO1 treated with 10% OligoG (post-wash) (grey solid line).

Figure 3. Cell size analysis of PAO1, PAO1 with 10% OligoG (pre-wash) and PAO1 with 10% OligoG (post-wash) in (*A*) 0.001 M NaCl and (B) 0.01 M NaCl. (*C*) Typical size distribution by volume of OligoG (black solid line), PAO1 (grey solid line) and PAO1 treated with 10% OligoG (grey dashed line).

Figure 4. Effect of a hydrodynamic shear test at 5,500 *g* for 3 mins on OligoG treated cells tested in pH 5, 0.01 M NaCl. (*A*) Typical zeta potential distribution by volume of PAO1 grown in 10% OligoG after 1st test (black solid line), after 2nd test (grey solid line); after 3rd test (grey dashed line). (*B*) Their corresponding mean zeta potential values (mV) and mean sizing values (nm). (*C*) Corresponding AFM images of PAO1 grown in 10% OligoG after each of three hydrodynamic shear tests.

Figure 5. Typical zeta potential distributions at pH 5 and 0.01 M NaCl of (*A*) Untreated PAO1 cells only (black solid line); PAO1 treated with 0.2% OligoG (pre-wash) (grey solid line); PAO1 treated with 0.2% OligoG (post-wash) (grey dashed line) and (*B*) Untreated PAO1 cells only (black solid line); PAO1 treated with 2% OligoG (post-wash) (grey line). (*C*) Their corresponding mean zeta potential values (mV). *Not determined as OligoG clouded this measurement.

Figure 6. AFM images **(**4 µm) of (*A*) untreated *P. aeruginosa* PAO1 cells and (*B*) PAO1 treated with 0.2% OligoG (Z scale of 800 nm). (*C*) AFM images (7 µm) of PAO1 treated with 0.5% OligoG (post-wash) (Z scale of 700 nm).