The *Chlamydia pneumoniae* Adhesin Pmp21 forms Oligomers with Adhesive Properties

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**ABSTRACT**

Chlamydiae sp. are obligate intracellular pathogens that cause a variety of diseases in humans. Adhesion of Chlamydiae to the eukaryotic host cell is a pivotal step in pathogenesis. The adhesin family of polymorphic membrane proteins (Pmp) in *Chlamydia pneumoniae* consists of 21 members. Pmp21 binds to the epidermal growth factor receptor (EGFR). Pmps contain large numbers of FxxN and GGA(I, L, V) motifs. At least two of these motifs are crucial for adhesion by certain Pmp21 fragments. Here we describe how the two FxxN motifs in Pmp21-D (D-Wt), a domain of Pmp21, influence its self-interaction, folding and adhesive capacities. Refolded D-Wt molecules form oligomers with high sedimentation values (8 to 85 S). These oligomers take the form of elongated protofibrils which exhibit Thioflavin-T fluorescence, like the amyloid protein fragment β42. A mutant version of Pmp21-D (D-Mt), with FxxN motifs replaced by SxxV, shows a markedly reduced capacity to form oligomers. Secondary-structure assays revealed that monomers of both variants exist predominantly as random coils, whereas the oligomers form predominantly β-sheets. Adhesion studies revealed that oligomers of D-Wt (D-Wt-O) mediated significantly enhanced binding to human epithelial cells relative to D-Mt-O and monomeric protein species. Moreover, D-Wt-O bound EGFR more efficiently than D-Wt monomers. Importantly, pre-treatment of human cells with D-Wt-O reduces infectivity upon subsequent challenge with *C. pneumoniae* more effectively than all other protein species. Hence, the FxxN motif in D-Wt induces the formation of β-sheet-rich oligomeric protofibrils which are important for adhesion to, and subsequent infection of human cells.

*Chlamydiae* are Gram-negative bacteria with compact genomes, and some species represent significant threats to human health. *Chlamydia trachomatis* is the most prevalent sexually transmitted bacterial pathogen worldwide (1). Furthermore, it causes trachoma, a form of ocular conjunctivitis characterized by massive inflammation that leads to scarring of the inner epithelial lining of the eyelid and eventually to blindness (2). *Chlamydia pneumoniae* (*C. pneumoniae*) is an important respiratory pathogen, causing pneumonia, pharyngitis, sinusitis and bronchitis. Moreover, it is associated with several chronic diseases including atherosclerosis, central nervous system disorders and Alzheimer’s disease (3-5). Despite the clinical relevance of *Chlamydia*, no vaccine is available for use in humans (6,7). *Chlamydia* species have a unique biphasic
Pmp21 forms functional Oligomeric Structures

developmental cycle, alternating between the infectious elementary body (EB) and the intracellular, metabolically active, reticulate body (RB) that replicates in eukaryotic cells (8,9). Polymorphic membrane proteins (Pmps) found in different species of the Chlamydiaceae are adhesion-mediating proteins (10-13). Bioinformatic analysis has shown that the Pmp protein family is comprised of nine members (PmpA to PmpI) in C. trachomatis and 21 (Pmp1 to Pmp21) in C. pneumoniae (11,14). The pmp gene family has been subdivided, on phylogenetic grounds, into six subtypes (14). The subtypes from both species have retained a significant degree of sequence similarity across species. Thus, the level of identity between the PmpD subtype members PmpD and Pmp21 of C. trachomatis serovar E and C. pneumoniae CWLO29, respectively, is 33%, indicating some level of functional similarity across chlamydial species (14).

The various Pmp families show a unique overrepresentation of repeats of the motifs GGA(I,L,V) and FxxN (14,15). For example, the FxxN motif is found on average 11.3 times in the Pmps of C. pneumoniae, while its average incidence in the rest of the proteome is 0.84 (14).

Furthermore, Pmp proteins are united by their predicted autotransporter characteristics. Thus all Pmps share an N-terminal Sec-dependent leader sequence, followed by a passenger domain (PD) and a C-terminal β-barrel (14,16). Structure predictions have suggested that a large region of the PD of Pmp6 folds into a parallel β-strand in a helical pattern with three faces that form a β-helix (17,18). Beta-helical structures are a characteristic of autotransporters, and might be required for their efficient translocation across the outer membrane (OM) and for folding (19). Moreover, it has been speculated that Pmp β-helices could associate with each other to generate oligomers (20). Like other autotransporter proteins, many Pmps undergo complex proteolytic processing (13,18,21-24).

Several of the C. pneumoniae and all C. trachomatis Pmps have been shown to be located on the chlamydial surface (10,13,18,21,22,24-26).

All C. trachomatis Pmps, as well as Pmp6, Pmp20 and Pmp21 from C. pneumoniae, have been found to serve as adhesins, mediating the attachment of chlamydial EBs to human epithelial cells. In addition, blocking experiments using recombinant Pmp proteins have provided direct evidence for the critical role of the Pmp proteins in chlamydial pathogenesis (22,27) More recently, the epidermal growth factor receptor (EGFR) was identified as the host receptor for the C. pneumoniae adhesin Pmp21, and binding to EGFR was shown to induce the uptake of the chlamydial EB, thus qualifying Pmp21 as an invasin (28).

Pmp21 occurs on the surface of infectious EBs in various processed forms, referred to as N-M-C-Pmp21, N-M-Pmp21, N-Pmp21 (31 – 670 aa), M-Pmp21 (671 – 1145 aa) and C-Pmp21 (1146 – 1609 aa) (Fig. 1C) (13,18,22,29). Interestingly, Pmp21 possesses multiple adhesion domains (18), and truncation experiments have demonstrated (Fig. 1D + E) that adhesion critically depends on the presence of the repetitive GGA (I,L,V) and FxxN motifs. Thus, targeted mutagenesis has revealed that at least one GGA (I,L,V) and one FxxN motif (present in Pmp21-A) (Fig. 1E) or two FxxN motifs (present in Pmp21-D) (Fig. 1D) are required and sufficient for significant adhesion to HeLa cells (22).

Strikingly, immunoaffinity enrichment of PmpD, the C. trachomatis homologue of Pmp21, from infectious EBs resulted in the isolation of high-molecular-weight structures which included full-length PmpD and two proteolytically processed forms. The functional significance of these structures remains unknown (24).

High-molecular-weight structures with adhesive characteristics have been identified on the surface of a number of pathogenic bacteria. In Enterobacteriaceae, including E. coli, highly aggregative surface fibres called curli have been found (30,31). First observed in 1989 in fibronectin-binding E. coli isolates from bovine faecal samples (32), curli fibres have been shown to mediate interactions between individual bacteria, bacteria and host tissues, and bacteria and inert surfaces like Teflon and stainless steel, which are usually refractory to bacterial colonization (30,33-37). Subsequently, curli fibres were shown to be made up of an amyloid-like protein that binds the amyloid-specific dye Thioflavin T (ThT) (38,39). Amyloids are insoluble protein aggregates derived from the conversion of protofibrils into amyloid fibrils which are formed by proteins characterized by a typical beta-sheet structure. The commercially available human amyloid β42 (Aβ42) is an amyloid-like peptide which can also form amyloid fibrils in vitro.

In this study, we focus on Pmp21-D (D-Wt), a C-terminal fragment derived from the
naturally occurring M-Pmp21. It is the smallest Pmp21 fragment identified thus far which exhibits adhesion and infection blocking capacity (22) (Fig. 1E), and demonstrate that the monomer (D-Wt-M) forms oligomers that adopt an amyloid-like structure. The Pmp21-D oligomers (collectively referred to as D-Wt-O) are comparable in size and shape to protofibrils of αβ42. Comparison of the oligomerization capacity of D-Wt with that of a previously analyzed mutant form (D-Mt) with poor adhesion properties revealed that the FxxN motif in D-Wt contributes significantly to the formation of oligomers. Interestingly, both the adhesion to its host cell receptor EGFR and the neutralization capacity of D-Wt require its oligomerization.

RESULTS

Recombinant Pmp21-D can exist in monomeric and diverse homo-oligomeric forms.—When we performed an automated structural characterization based on the C. pneumoniae Pmp21 protein sequence, we found that the processing product M-Pmp21 is predicted to form a long, right-handed β-helix domain, which could provide a rigid platform on which multiple adhesive sites can be presented, and could self-associate to generate oligomers (Fig.1B). In order to test this hypothesis experimentally, we expressed Pmp21-D (D-Wt; Fig. 1E), an adhesive C-terminal fragment of M-Pmp21 (13,18), in E. coli. We then purified the recombinant protein under denaturing conditions via its N-terminal His-tag, and allowed it to refold (at a concentration of 3.1 mg ml⁻¹) prior to elution from the affinity column (see Materials and Methods).

The eluate was first analyzed by SDS-PAGE, using an anti-His antibody confirmed the identity of the ~30 kDa and ~60 kDa bands as recombinant D-Wt-M (23 kDa). Thus, under non-denaturing conditions, only a minor fraction of recombinant D-Wt exists in the monomeric form, which in turn suggested that the broad first peak was made up of high-molecular weight oligomers (D-Wt-O). Representative fractions of D-Wt-O were chosen from this peak and its descending shoulder and refolded it by dialysis for 36 h in PBS before SEC. In this SEC we observed the same elution profile as for the previously described ‘on column refolding’ procedure (data not shown).

In order to determine the molecular mass of the protein species in the second peak, analytical size-exclusion chromatography coupled to multiangle light scattering detection (SEC-MALS) was performed. The data revealed that this peak (Fig. 3) corresponds to the monomeric species D-Wt-M (23 kDa). Thus, under non-denaturing conditions, only a minor fraction of recombinant D-Wt exists in the monomeric form, which in turn suggested that the broad first peak was made up of high-molecular weight oligomers (D-Wt-O). Representative fractions of D-Wt-O were chosen from this peak and its descending limb (D-Wt-O1,3 with apparent sizes of 839 kDa, 554 kDa and 366 kDa respectively) (Fig. 2B) for further experiments.

Pmp21-D oligomers form rod-like structures—The size distribution of the D-Wt species in these fractions was analysed in solution by analytical ultracentrifugation (AUC). Because the AUC assay has a running time of several hours, we first determined the stability of D-Wt-O1 by analytical SEC on the time scale required for AUC sample preparation and measurement. Even after an incubation period for 20 h in PBS at 4°C, D-Wt-O1 was still stable and had not dissociated into lower-molecular-weight species (Fig. 4A).

The peak fraction of monomeric D-Wt-M (Fig. 3) was analyzed by measuring its sedimentation velocity with AUC at 40,000 rpm and 20°C. Subsequent data analysis by SEDFIT, assuming a continuous c(s) distribution model, indicated particles of 1.6 S with a small side peak at 2.6 S and an overall frictional ratio of 1.9 (Fig. 4B, C). Similarly D-Wt-O1 was analyzed at a sedimentation velocity of 20,000 rpm. The results suggested that D-Wt-O1 accounted for about 87.4% of D-Wt, with a size distribution ranging from 8 S to 85 S, with a mean size of 23.8 S and a frictional ratio of f/f₀ = 2.8 (Fig. 4D, E). This frictional ratio suggests the presence of long rod-
After brief washing with H$_2$O, the grids were centrifuged at 10,000 x g for 10 min and applied to carbon-coated copper grids. After brief washing with H$_2$O, the grids were stained with 1% uranyl acetate. D-Wt-O$_1$, as well as D-Wt-M, were incubated at 37°C for 48 h in the presence of 10 µM ThT, and the change in ThT fluorescence emission at 480 nm was monitored. PBS and BSA both showed only a very low emission during the entire assay with maximally 325 relative fluorescent units (RFU) (Fig. 5E). In contrast, the positive control Aβ$_{42}$ started with an RFU of 831 and ended with 2,898 ± 688 RFU at 48 h. Most strikingly, emission levels with D-Wt-O$_1$ and D-Wt-O$_2$ protofibrils were even higher, starting with 4570 RFU and 3407 RFU respectively at time point 0h, and increasing with time to 7766 RFU and 6441 RFU respectively at the end of the assay. Notably, D-Wt-M exhibited with 234 RFU a very low fluorescence emission at time point 0 h, which increased over time to an RFU of 2505 ± 86 (48 h) indicating slow oligomer formation. Taken together with our EM data, these results confirm that D-Wt has a strong tendency to form ThT-sensitive, amyloid-like structures.

The FxxN motif strongly promotes the formation of D-Wt oligomers —In previous studies we found that mutating the two FxxN motifs present in D-Wt to SxxV (D-Mt) results in complete loss of adhesive capacity (22). Thus we speculated that these motifs might also be relevant for oligomer formation. Therefore recombinant D-Wt and D-Mt were refolded at different concentrations (0.7, 1.7, 2.4 and 3.1 mg ml$^{-1}$) centrifuged and analyzed by SEC. The area under the oligomer peaks was then expressed as a percentage of the total area under the curve. To get an impression of the ratios easily, the monomer peaks were set to 1 (Fig. 6A). At a concentration of 0.7 mg ml$^{-1}$ 22 % of total D-Wt protein eluted as oligomeric D-Wt-O. The figure for oligomeric mutant D-Mt-O was only 4%. Increasing the total concentration of either monomer in the assay also increased the percentage of oligomers formed (Fig. 6B). However, D-Wt always gave rise to a significantly higher proportion of oligomers than the same concentration of the mutated D-Mt protein. This difference was very pronounced at a concentration of 1.7 mg ml$^{-1}$, at which the mutant form D-Mt produced about 10-fold fewer oligomers (5%) compared to D-Wt (53%). Thus, these data demonstrate that both D-Wt and D-Mt can form oligomers, and that the capacity to form oligomers is significantly enhanced by the two FxxN motifs.

The fact that D-Mt has a strongly reduced propensity to form oligomers points to structural differences between the monomers and possibly their respective oligomeric forms too. Therefore, the secondary structure of wild-type and mutant
Pmp21 forms functional Oligomeric Structures

Monomer and oligomer forms was determined by circular dichroism (CD) spectroscopy (Fig. 6C). Data analysis using the program CONTINLL revealed that D-Wt-O1 consists of ~11.5% β-sheets and ~3.4% α-helices, while D-Wt-M displayed a random-coil structure similar to that of a disordered protein lacking any secondary structure. D-Mt-O1 showed strong similarities to D-Wt-O1 but with some tendency to adopt the random coil pattern of D-Wt-M. Finally D-Mt-M produced a random-coil spectrum comparable to that of D-Wt-M. Hence, oligomers of D-Wt-O1 and D-Mt-O1 differ somewhat in their secondary structures, while both monomers, D-Wt-M and D-Mt-M, show predominantly random-coil structures.

We next analysed representative SEC fractions corresponding to D-Mt-O1 – 3 and D-Mt-M by TEM (Fig. 6 D+E). Interestingly, D-Mt-O1 showed rod-shaped protofibrilar structures, very similar to those found for D-Wt-O1. The smaller D-Mt oligomers (O2 – 3) appeared globular. As for D-Wt-M no oligomeric structures were detectable by EM in the D-Mt-M fraction.

FxxN-induced oligomerisation of Pmp21-D is crucial for its ability to adhere to human cells—In previous studies, Pmp6, 20 and 21 from C. pneumoniae and all nine Pmps from C. trachomatis serovar E, were characterized as bacterial adhesins that are important for the infection of human epithelial cells (22,27). The motifs GGA (I, L, V) and FxxN are characteristic for Pmps and essential for adhesion (22). Our results so far have demonstrated that these motifs are additionally responsible for protein oligomerisation (Fig. 6A, B). In order to analyze which of the different protein species represents the adhesion-competent conformation and whether adhesion is mediated by the FxxN motifs alone, we performed bead-based adhesion assays with all D-Wt and D-Mt protein species (Fig. 7A). Protein-coated green fluorescent latex beads were incubated with human epithelial HEp-2 cells. The coating efficiency estimated by immunoblotting revealed only slight differences in the amount of each protein coupled to the beads (data not shown). The negative control, BSA-coated beads, only bound to 15.9 ±3 % HEp-2 cells. Importantly, D-Wt-O1 showed a significant adhesion capacity with 50 ±4.3 % HEp-2 cells carrying bound beads. In contrast, the monomeric protein species D-Wt-M and D-Mt-M, as well as the oligomeric mutant form D-Mt-O1, mediated comparatively little binding of beads to HEp-2 cells (20.6 ±3.2 %, 20.5 ±2.1 % and 20.3 ±5 % respectively). Hence, the bead assay suggests that only the oligomeric wild-type protein species D-Wt-O1 exhibits strong adhesion capacity. This implies that both the formation of oligomers as well as presence of FxxN motifs is crucial for binding to HEp-2 cells.

The oligomeric form of Pmp21-D is important for the binding to the EGFR—In previous studies the EGF receptor was identified as the receptor for the M-Pmp21 protein domain, which occurs naturally (28). In order to analyze whether the monomeric and oligomeric D-Wt species have identical or different affinities for EGFR, we performed pulldown assays with D-Wt-O1 and D-Wt-M. As positive control we used recombinant M-Pmp21 (28), while other controls were recombinant GST, the C. pneumoniae adhesin OmcB-BD (46), and the C. trachomatis adhesin and invasin Ctad1 (47) (Fig. 8A). After biotinylation the recombinant proteins were incubated with human epithelial HEp-2 cells, cross-linked, and affinity-purified using a streptavidin resin. Interaction partners were eluted after cross-link removal. Western blots of whole cell lysates cross-linked to the recombinant proteins revealed the presence of identical amounts of EGFR (Fig. 8B). After pull-down the EGFR signals were found to be of similar strength for the positive control M-Pmp21 and the oligomeric D-Wt-O1 (1.03 and 1.0 respectively) (Fig. 8C). In contrast the pull-down using the monomeric D-Wt-M brought down only about 50 % of the amount brought down by the oligomeric form (0.5). Recombinant GST as well as OmcB-BD failed to bring down detectable amounts of EGFR. A very weak signal was observed for Ctad1. These results provide evidence that both D-Wt-M and D-Wt-O1 can interact with EGFR; however, the efficiency of the oligomeric species is twice as high as that of monomeric species. This again strongly suggests the relevance of oligomer formation for the infection process.

The oligomeric form of Pmp21-D is important for infection—Next we asked whether the adhesion-competent oligomeric D-Wt-O protein species is also relevant for a chlamydial infection. To this end, soluble recombinant
monomeric or oligomeric D-Wt and D-Mt protein species were preincubated with HEp-2 cells prior to infection. About 48 h post infection the efficiency of infection was measured by counting the numbers of inclusions formed (Fig.9A, B). Pretreatment with the negative control BSA did not significantly reduce the infection upon subsequent exposure to C. pneumoniae EBs, while the positive control heparin reduced infectivity by 99%. Interestingly, D-Wt-O1 had the strongest effect - blocking the subsequent infection by almost 50 % compared to the PBS control (53.5 ±2.3% inclusions). D-Wt-M (82.9 ±5.7% inclusions) and D-Mt-O1 (83 ±1.7% inclusions) had a much weaker effect, reducing infectivity by only about 17%, which is not significantly different from the value for the PBS control. The least effective of the protein species tested was D-Mt-M (92.7 ±2.9%), which inhibited infection to about the same extent as the negative control BSA (96.7 ± 8.8%) (Fig. 9A, B). These data also show that the wild-type and mutant oligomeric forms of Pmp21-D inhibit infection more effectively than the corresponding monomeric forms.

**DISCUSSION**

Successful infection of host cells by C. pneumoniae depends on a variety of virulence factors. These include specialized surface structures which mediate uptake by host cells (48). Chlamydiae enter cells via multiple routes, using mechanisms which are poorly understood (49). In previous studies we identified the Pmp proteins of C. pneumoniae and C. trachomatis as adhesins that are essential for the successful infection of human cells (22,27). Pmps are known to share characteristic features with Type V autotransporters, including proteolytic processing (13,14,16,18,22). Recently, Pmp21 was shown to bind to the host’s EGF receptor and to induce its own uptake; hence Pmp21 acts both as an adhesin and as an invasin (28). Interestingly, the characteristic FxxN and GGA(I, L, V) motifs, which are known to occur in multiple copies exclusively in chlamydial Pmp proteins (14), have been shown to be crucial for Pmp21-mediated adhesion (22). Structure prediction programs have indicated that the passenger domains of Pmps are dominated by parallel β-strands disposed in a helical pattern with three faces that form a β-helix (17,18). It has therefore been speculated that these β-helices could associate with each other to generate Pmp oligomers (20).

Our initial characterization of refolded recombinant Pmp21-D, an adhesion-competent subdomain of Pmp21, by SEC revealed that homooligomeric forms (D-Wt-O) were dominant, while the monomer (D-Wt-M) made up only a relatively small fraction of the whole (Fig.2B+C). Interestingly, we found D-Wt-O to be very stable, as no disaggregation was observed after prolonged incubation in PBS buffer (Fig. 4A). In contrast, with time, the monomeric D-Wt-M (1.6 S) gave rise to a new stable species at 2.6 S, probably a dimer, which may nucleate the formation of higher-order oligomers (Fig. 4C). The *in-vitro* formation of Pmp21-D oligomers is in agreement with earlier findings which indicated that the C. trachomatis homologue of Pmp21, PmpD, is part of a protein complex on the EB cell surface (24). Moreover, it was reported that Pmps of C. psittaci also occur in supramolecular complexes (50). Analysis of the sizes of the oligomeric D-Wt-O yielded remarkable large S-values of up to 85 S with an average of 23.8 S, which may reflect the formation of amorphous aggregates by refolding intermediates. However, determination of the frictional ratio f/f₀ of 2.75 for the D-Wt sample argues that large elongated structures are formed (Fig. 4E). Typical examples for highly elongated proteins are human fibrinogen (MW = 330 kDa) with an S-value of 7.6 S and an f/f₀ = 2.3, and myosin (MW = 570 kDa) with an S-value of 6.4 S and an f/f₀ = 3.6 (51). However, in contrast to those proteins, Pmp21-D is very small (23 kDa) supporting the idea that it might form long homooligomeric protein species.

Indeed, EM analysis revealed that the D-Wt-O oligomers form protofibril-like structures (Fig. 5A and B). Interestingly the three differently sized D-Wt-O species isolated by SEC correspond to protofibrils with almost identical widths (~10 nm) but different lengths. One might speculate that the largest oligomer W-Wt-O₁ (~60 nm) could be the most mature form produced in *vivo*, while the others could represent intermediates. Interestingly, the D-Wt protofibrils exhibit amyloid-like characteristics, as they bind the dye thioflavin T and strongly enhance its fluorescence, as does the prototypical β-sheet-rich Aβ42 (Fig. 5E). Interestingly and in agreement with the theory, the longest Pmp oligomers (D-Wt-O₁) yielded a significantly higher ThT fluorescence emission
than the shorter D-Wt-O2. In contrast monomeric D-Wt at time point 0 only showed background ThT fluorescence, indicating the absence of β-sheet-rich oligomer structures. However, over time D-Wt-M also yielded significant ThT fluorescence, likely due to spontaneous protein oligomerization (as observed also in Fig. 4C). Indeed, the folding of Pmp21-D is remarkably stable, as dimers can be detected by SDS-PAGE (Fig. 2A). Such high stability is characteristic for a number of amyloid-like proteins (31,52-54). However, dimer formation has also been observed for amyloid-like proteins upon sample preparation for SDS-PAGE (55). Moreover, in vitro ThT-induced amyloid aggregation has been described, and this might also contribute to the formation of oligomeric D-Wt (56).

Previous work has demonstrated that the FxxN motifs in D-Wt (Pmp21-D) are essential for adhesion and for the ability of soluble Pmp21 fragments to block infection (22). In the present study, we have now shown that the two FxxN motifs in D-Wt are also crucial for its ability to form protofibrils, as the capacity for oligomerisation is strongly reduced when these motifs are mutated (D-Mt) (Fig. 6B). It is well known that specific protein sequences are involved in the induction of amyloid-like structures (57). Thus the FxxN motif is very probably an essential part of the amyloid-promoting sequence within Pmp21-D. However, it is worth mentioning that the D-Mt-O1 oligomers formed again exhibited a rod-shaped structure (Fig. 6C), similar to those seen for the corresponding Wt oligomers. Thus the structures of Wt and Mt oligomers are identical, yet the tendency to be formed is highly increased for D-Wt.

Interestingly, our CD-analysis show that D-Wt-O1 harbors some β-sheet structure while D-Mt-O1 shows a certain shift towards random coils, suggesting that the FxxN motifs play a role in protein folding. The β-sheets may instead adopt β-helical structures. Amyloids and β-helices in general are suggested to share similar motifs (58). Indeed, β-helical amyloids are already known for other organisms in various contexts (59-62).

The results presented here document a functional role for Pmp21-D oligomers during the C. pneumoniae infection. The oligomer Pmp21-D (D-Wt-O1) shows significant adhesion to human epithelial cells, in contrast to monomer and mutant species (Fig. 7). Moreover, D-Wt-O1 interacts significantly more strongly with the Pmp21 receptor EGFR than the monomeric D-Wt (Fig. 8). Finally, recombinant D-Wt-O1 blocks chlamydial infection more efficiently than any other species tested (Fig. 9). These data strongly suggest a functional role of oligomeric Pmp species in the chlamydial infection. Unexpectedly, the pull-down with Ctad1 brought down small amounts of EGFR. We recently reported that Ctad1 binds to Integrin β1 (47). As it is known that the integrin and EGFR receptors exhibit intensive crosstalk (63), it may be speculated that Ctad1-mediated integrin activation results in the association of both receptors in downstream signaling complexes.

In conclusion Pmp21 may belong to the group of functional, oligomeric structures found on microbial cell surfaces, the prototypes of which are CsgA and CsgB, which are secreted by their own apparatus to form the filamentous cell surface structures called curli produced by many Enterobacteriaceae (38). Curli shares all of the biophysical properties of amyloids, including the propensity to form ordered β-sheet-rich fibers with a capacity to bind the dye ThT (31), and produces extracellular proteinaceous fibers that contribute to biofilm formation, host colonization, immune activation and cell invasion (64-66).

So far, Pmp21 oligomers have not been described for extracellular infectious EBs or dividing RBs within the inclusion. Either these structures do not survive the harsh fixation protocols used for sample preparation for immunofluorescence and electron microscopy, or the size of the oligomeric structures is well controlled by the chlamydia, and thus their small in vitro size does not enable their detection. Alternatively, Pmp protofibrils might not form in vivo, because the proteins are physically constrained by their anchorage through their β-barrel in the outer membrane, as has been shown for the Candida Als adhesins which are also anchored in the yeast cell wall. Nevertheless, these fungal adhesins form cell surface amyloid patches of arrayed adhesin molecules (“adhesin nanodomains”) 100 to 1,000 nm in size thus binding ligands with high avidity (67,68). It is tempting to speculate, that the flower-like structures observed by EM in affinity-enriched preparations of endogenous PmpD-enriched protein complexes might possibly represent the in vivo version of the Pmp21 protofibrils detected in vitro in this study (24).
The data presented here are compatible with the idea that oligomeric Pmp complexes might enhance the chlamydial cell's capacity for adhesion to human epithelial cells and be important for the initial step in infection.

Finally, there is a striking correlation between chlamydial infection and amyloid formation in the brains of mice (69-71). However, there is currently no evidence for a direct or indirect connection between amyloid formation in the mouse brain and Pmp proteins.

Future work needs to focus on whether or not the different Pmp family members (21 in *C. pneumoniae* and nine in *C. trachomatis* (11,14)) can interact with each other to form heteromeric protofibrils. If so, this would provide for greater antigenic complexity and enable *Chlamydiae* to adapt to a larger range of cellular niches (14,20).

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and culture conditions**—*Escherichia coli* strain BL21 (DE3) (Agilent Technologies) was used for protein expression and plasmid amplification. *C. pneumoniae* strain GiD was propagated in HEp-2 cells (ATCC No. CCL-23) as described (72). Chlamydial EBs were purified by using a 30% gastrographin gradient (Schering).

**DNA manipulations and plasmid construction**—Plasmids were generated in *S. cerevisiae* as described in (22) and their structures were verified by sequence analysis (GATC).

**Protein expression and affinity purification of His6-tagged proteins**—Growth for protein expression in BL21 (DE3) was performed in LB media + 0.8% glucose at 37°C. Cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an OD${}_{600}$ of 0.6-1 and grown for a further 4 h. The cells were then harvested by centrifugation in a JLA10.500 rotor (Beckman) at 5,000 rpm for 10 min at 4°C.

The cell pellets were lysed with 10 ml lysis buffer (20 mM Tris-HCl pH 8, 6 M guanidine HCl, 0.5 M NaCl, 1 mM β-mercaptoethanol, 5 mM imidazole) per g cell pellet overnight. Cell debris was removed by centrifugation in a Type 45Ti rotor (Beckmann) at 42,000 rpm for 1 h at 4°C.

Purification under denaturing conditions and refolding of the His6-tagged D-Wt and D-Mt was performed with HiTrap Chelating Nickel-IDA columns (5 ml column volume) at 4°C using an AKTA Prime plus (GE Healthcare). The system was equilibrated with lysis buffer. Subsequently the cleared whole cell lysate was applied to the column. After washing with 50 ml of lysis buffer, the column was washed with 10 ml of washing buffer (20 mM Tris-HCl pH 8, 8 M urea, 0.5 M NaCl, 1 mM β-mercaptoethanol, 5 mM imidazole). Thereafter washing buffer was gradually replaced over 30 ml with refolding buffer (20 mM Tris-HCl pH 8, 0.5 M NaCl, 1 mM β-mercaptoethanol, 5 mM imidazole). Proteins were eluted at 4°C by applying a 40-ml imidazole gradient (0.005-0.5 M in the same buffer) and the eluate was collected in 1-ml fractions. The whole process was monitored by absorbance at 280 nm. All subsequent steps were also performed at 4°C. The protein-containing fractions were centrifuged at 10,000 x g for 10 min. The two peak fractions were pooled and protein concentrations were determined with the Bradford assay. Within two hours after elution from the HiTrap column the samples were analyzed by preparative size-exclusion chromatography (SEC). Different concentrations of refolded protein were generated by starting with different amounts of whole cell lysate.

**Size-exclusion chromatography**—SEC was performed on an ÄKTA Prime plus (GE Healthcare) equipped with a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) at 4°C. SEC was performed at a flow rate of 0.5 ml/min in phosphate-buffered saline (PBS) (10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and 1.2-ml fractions were collected. The void volume of the column was determined by using blue dextrane (BD) and the separation range of the column was verified by standard proteins for gel filtration (Sigma-Aldrich). Within six hours after elution from the SEC column, the sample were used for further analysis. In the meantime the samples were kept on 4°C.

Analytical size-exclusion chromatography was performed on an AKTA Prime plus (GE Healthcare) equipped with a Superdex 200 HR 10/30 column (GE Healthcare) at 4°C. Analytical SEC was performed at a flow rate of 0.3 ml/min in PBS, and 0.3-ml fractions were collected. In both cases, the elution profiles were analyzed with the PrimeView software package (GE Healthcare).

Combined analytical SEC and multi-angle light scattering (SEC-MALS)—Analytical size-exclusion chromatography coupled to multi-angle
light scattering detection (SEC-MALS) was performed on a HPLC system from Agilent Technologies, using a Superdex 200 10/300 GL column (GE Healthcare), equilibrated and run at a flow rate of 0.3 ml/min in PBS at 4°C. For MALS analysis, the eluate was monitored with a miniDAWN TREOS triple-angle light-scattering detector in combination with an OPTILab T-rEX differential refractive index detector (both from Wyatt Technology). Typically, 100 µl of purified D-Wt-M (0.5 mg/ml) was loaded onto the Superdex 200 10/300 GL column, and the elution data were analyzed with the ASTRA software package (Wyatt Technology).

**Analytical ultracentrifugation (AUC)**—AUC was performed in an Optima XL-A analytical ultracentrifuge (Beckman Coulter) with absorbance optics, using an An-50 Ti for D-Wt and an An-60 Ti rotor for D-Wt-M. Sedimentation velocity centrifugation was done at 20,000 rpm and 20°C for D-Wt and at 40,000 rpm and 20°C for D-Wt-M. The signal intensity for D-Wt was recorded at 280 nm and for D-Wt-M at 230 nm over the 7-h duration of the analysis. Data were fitted to the continuous distribution ($c(s)$) Lamm equation model with a partial specific volume of 0.7286 cm$^3$/g (based on the D-Wt sequence) using the software package Sedfit. The density and viscosity of the buffer were 1.0053 and 0.01019 respectively, according to SEDNTERP (Sedimentation Interpretation Program, Version: 20120828, University of New Hampshire; (73)). A resolution of 200 was chosen for the S value. The relative amounts of the different species in the D-Wt-M sample were derived from the $c(s)$ distribution exclusive of the area below 0.6 S, which contains a baseline deconvolution artifact. The S values determined were corrected for PBS at 20°C.

**Transmission electron microscopy (TEM)**—D-Wt samples were diluted to 1 µM and 10-µl aliquots were incubated for 5 min at room temperature (RT) on freshly glow-discharged S162 Formvar/carbon-coated copper grids (Plano). The grids were then washed three times with 10 µl H$_2$O and subsequently incubated for 1 min with 10 µl of 1% aqueous uranyl acetate for negative staining, then air-dried for 5 min at RT. The samples were examined with an E902 electron microscope (Zeiss) operating at 80 kV.

**Thioflavin-T (ThT) fluorescence assay**—For fluorescence measurements, ThT (Sigma Aldrich) was added at a final concentration of 10 µM to protein samples containing 10 µM D-Wt or BSA in PBS or 48 h preincubated Aβ$_{25}$ (Bachem) in a final volume of 100 µl of 10 mM sodium phosphate, 50 mM NaCl, pH 7.4. ThT was allowed to bind at 37°C in a (stationary) round-bottomed 96-well black plate (Nunc) for 48 h. Fluorescence was excited at 442 and measured at an emission wavelength of 484 nm in an Infinite 200pro plate reader (Tecan). The slit width was 10 nm.

**Circular dichroism spectroscopy (CD)**—Far-UV CD spectra were measured on a JASCO J-815 spectropolarimeter equipped with a 1-mm Suprasil quartz cuvette (Hellma) at 20°C. Protein species (D-Wt-O1, D-Wt-M, D-Mt-M) were analyzed at a concentration of 10 µM in 20 mM NaPi, 75 mM NaF, pH 7.4 or D-Mt-O$_1$ in PBS. The D-Wt-O$_1$ spectrum was analyzed on the Dichroweb server using the program CONTINLL (74-78).

**Adhesion assays with protein-coated latex beads**—Adhesion assays with protein-coated latex beads were performed as described (27). Coating efficiency with 100 µg/ml His6-tagged proteins was estimated by immunoblotting prior to use. Confluent monolayers of HEp-2, HeLa, or HUVE cells were grown in 24-well plates and incubated with a 10-fold excess of protein-coated latex beads (Polyscience) (diameter 1 µm, green fluorescent) for 1 h at 37°C. Cells were washed twice with PBS, detached with cell dissociation solution, fixed with 3% formaldehyde for 20 min at room temperature, and analyzed by flow cytometry using a FACSAria instrument (BD Biosciences, San Jose, California, USA).

**EGFR Pull-down assay**—The EGFR pulldown assay was performed with 200 µg/ml D-Wt, M-Pmp21, GST, Ctad1 or the binding domain of OmcB (OmcB-BD) in a volume of 2 ml. All proteins were biotinylated according to the manufacturer’s instructions (Thermo Scientific). Afterwards the biotinylated proteins were incubated with HEp2 cells to allow adhesion. After 1 h incubation at 37°C the unbound protein was washed away and the bound proteins were crosslinked with DTSSP (Thermo scientific). The HEp2 cells were lysed and the soluble lysate was incubated for 12 h at 4°C with streptavidin resin to allow binding of biotinylated proteins. The streptavidin resin was washed with PBS (3 x 0.5 ml) and afterwards the recombinant proteins and
their interaction partners were eluted with 100 mM DTT. The elution fractions were analyzed by western blot with anti EGFR antibodies (Thermo scientific) and quantified by ImageJ.

*Infection inhibition assay*—These assays were performed as previously described (Moelleken and Hegemann 2008). Briefly, HEp-2 cells were grown on glass coverslips (12-mm diameter) for 48 h and incubated (as confluent monolayers) with 250 μL of medium containing recombinant protein (100 μg/mL in PBS) for 2 h at 37°C. Purified chlamydial elementary bodies (EBs) (multiplicity of infection (moi) 20) were added to the protein suspension and incubated for 2 h at 37°C without centrifugation in order to avoid any influence of the centrifugation procedure on the adhesion/infection process (46). The cells were subsequently washed three times, covered with chlamydial growth medium and incubated for 48 h at 37°C for *C. pneumoniae* infection. Subsequently the cells were fixed with 96% methanol for 2 min. Each monolayer was then washed three times with PBS. For detection of chlamydial inclusions, a monoclonal fluorescein isothiocyanate (FITC)-conjugated antibody directed against chlamydial LPS (Bio-Rad) was used. Cells were viewed using a C2 confocal microscope (Nikon). Inclusions were counted and the results were expressed as percentages of the number found in PBS-treated control samples.

*Immunoblot analysis*—SDS-PAGE and immunoblot analysis were performed as described (79). The PageRuler™ (Thermo Fisher Scientific) set of prestained markers was employed as a molecular weight standard. The His₆-tagged recombinant proteins purified from *E. coli* were detected with an anti-His₆ antibody (Qiagen) and visualized with AP-conjugated anti-mouse antibody (Promega).

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**Conflict of interest:**
The authors declare that they have no conflicts of interest with the contents of this article.

**Author contributions:**
Johannes H Hegemann conceived and coordinated the study. Johannes H Hegeman and Soeren ET Luczak designed, performed and analyzed the experiments. Sander HJ Smits and Lutz Schmitz gave general biochemical advise and support. Christina Decker and Luigard Nagel-Steger gave advise and support concerning AUC and ThT-assay. Johannes H Hegemann and Soeren ET Luczak wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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**FIGURE LEGENDS**

**FIGURE 1.** (A) Schematic representation of Pmp21. The full-length protein is depicted with the N-terminal signal sequence (SS) and the C-terminal β-barrel domain (β-barrel). Each of the tetrapeptide motifs GGA(I, L, V) (in yellow) and FxxN (in red) in the central passenger domain is marked. Two known proteolytic cleavage sites in Pmp21 are indicated by arrows (13,14,18,22). (B) Structure of M-Pmp21 as predicted by I-Tasser (C-score: -1.58) (80-82). The β-sheets are displayed in yellow, random coils in grey.

(C) Predominant forms of Pmp21 in vivo, as detected by proteome analysis (13,18,22,29). (D+E) The recombinant full-length Pmp21 passenger domain (PD-Pmp21), the processed forms N-Pmp21 and M-Pmp21, and the truncated subdomains Pmp21-A to Pmp21-D (D-Wt) mediate adhesion. A mutant form of Pmp21-D (D-Mt) in which each FxxN motif is replaced by SxxV was found to be incapable of mediating adhesion (22).

**FIGURE 2.** (A) Analysis of an aliquot of the recombinant D-Wt by SDS-PAGE prior to SEC revealed monomers (*: ~30 kDa) and SDS-resistant dimers (**: ~60 kDa) (independent replicates n=3). (B) Preparative SEC (HiLoad 16/600 Superdex 200 pg) of D-Wt refolded at a concentration of 3.1 mg ml⁻¹ and dissolved in PBS. The flow rate used for separation of diverse oligomeric forms (D-Wt-O1-3) from the monomer (D-Wt-M) was 0.5 mL min⁻¹ (independent replicates n=3) Blue dextrane (BD) as well as the molecular weights of the standard proteins are indicated above the chromatogram. (C) The SEC eluate was collected in 1.2-ml fractions. Every second fraction was analyzed by SDS-PAGE (independent replicates n=3).

**FIGURE 3.** Analytical SEC (Superdex 200 10/300 GL; flow rate 0.3 mL min⁻¹ in PBS) (solid line) of the D-Wt-M peak isolated by preparative SEC (Fig. 2B). The eluate was monitored by multi-angle light scattering (broken line) and a monomer of 23 kDa was identified.

**FIGURE 4.** (A) Analytical SEC (Superdex 200 HR 10/30; flow rate 0.3 ml min⁻¹) of the D-Wt-O₁ fraction isolated by preparative SEC (Fig. 2B) was used to monitor the dissociation of D-Wt-O₁ into D-Wt-M over time (independent replicates n=2). (B-E) Size distribution of D-Wt monitored by AUC. (B) Subset of the raw data for velocity sedimentation of D-Wt-M in a Beckman An-60 Ti rotor at 20°C, 230 nm and 40,000 rpm, recorded over a period of 280 scans (at 1 scan per 1.5 min). (C) Distribution of sedimentation coefficients for D-Wt-M analyzed using a continuous c(s) distribution model (independent replicates n=2). (D) Subset of the raw sedimentation data obtained for the D-Wt SEC input sample (D-Wt refolded at a concentration of 3.1 mg ml⁻¹). (Fig. 2A) under the same conditions as in B. (E) Distribution of sedimentation coefficients for D-Wt, analyzed as in C. input analyzed using a continuous c(s) distribution model. Residuals after data fitting are shown, together with the calculated S values (independent replicates n=2).

**FIGURE 5.** Transmission electron microscopy (TEM) (80 kV) of samples (1 µM) of D-Wt (D-Wt-O₁, and D-Wt-M) isolated by SEC (Fig. 2B). Samples were applied to carbon-coated copper grids, negatively stained with 1% uranyl acetate and imaged at 50,000 x magnification (A) and 85.000 x magnification (B, scale bar= 100 nm). The images shown are representative for the whole grids (independent replicates n=2). The widths (C) and lengths (D) of 300 D-Wt particles from each sample were determined with ImageJ. The data are displayed in boxplots. Maximum and minimum values are indicated by the whiskers; 1st and 3rd quantiles represent 50% of the total data. The means are represented by the red dots within the boxes. (E) Relative Thioflavin-T (ThT) fluorescence assay. The indicated protein samples were incubated (at a final concentration of 10 µM) with 10 µM ThT for 48 h. BSA and preincubated amyloid
Pmp21 forms functional Oligomeric Structures

$\beta_42$ (A$\beta_42$) were used as negative and positive controls, respectively (independent replicates n=3). At the 0 h time point all protein samples except for A$\beta_42$ exhibited low relative fluorescent units (RFU). Data shown are means ± standard deviations. Statistical significance was assessed with Student’s t-test (*$P=0.05$, **$P=0.01$, ***$P=0.001$).

**FIGURE 6.** The FxxN motif is essential for oligomer formation by D-Wt-M. (A) D-Wt and D-Mt were refolded at 3.1 mg ml$^{-1}$ and analysed by SEC for monomer and oligomer formation. To get an impression of the ratios easily, the monomer peaks were set to 1 (independent replicates n=2). (B) Percentage of oligomers found in the total input after refolding of D-Wt and D-Mt at the indicated concentrations (independent replicates n=2). (C) Circular dichroism (CD) spectroscopy (at 20°C) of D-Wt-O$_1$ (solid line), D-Wt-M (dashed line) and D-Mt-M (dashed-dotted line). Samples were dissolved at 10 µM in 10 mM sodium phosphate buffer, 75 mM sodium fluoride, pH 7.5 (independent replicates n=2). Because very little D-Mt-O$_1$ was formed (dotted line), these measurements were performed in the running buffer PBS directly after SEC. (D+E) Transmission electron microscopy (TEM) (80 kV) of samples (1 µM) of D-Mt (D-Mt-O$_1$, and D-Mt-M) isolated by SEC (SEC data not shown). The analyzed fractions O$_{1,3}$ and M correspond to the elution volumes taken for the corresponding D-Wt species (see Fig. 2B). Samples were applied to carbon-coated copper grids, negatively stained with 1% uranyl acetate and imaged at 50,000 x magnification (D) and 85,000 x magnification (E, scale bar= 100 nm). The images shown are representative for the whole grids (independent replicates n=2).

**FIGURE 7:** Adhesion of D-Wt to human cells depends on the presence of FxxN motifs and oligomerisation. Binding of fluorescent latex beads coated with BSA or the indicated recombinant protein species isolated by preparative SEC (100 µg mL$^{-1}$) to HEp-2 cells was analysed as described in Materials and Methods. Fluorescent latex beads were coated with BSA or the recombinant proteins after preparative SEC (100 µg mL$^{-1}$), and 1 x 10$^6$ beads were incubated with confluent HEp-2 cells at 37°C for 1 h. The number of HEp-2 cells with bound beads was determined by flow cytometry. Results were derived from three independent experiments (independent replicates n=3). Data shown are means ± standard deviation. Statistical significance was assessed with Student’s t-test (**$P=0.01$, ***$P=0.001$).

**FIGURE 8.** (A) Coomassie-stained SDS-PAGE of the recombinant proteins GST, Ctd1, M-Pmp21, D-Wt-O$_1$, C-Wt-M and OmcB-BD used in the pull-down experiment. Western blot of the whole cell lysate after crosslinking (B) and of the eluate after pull-down (C) using an anti-EGFR antibody. (B) All HEp2 cell lysates harbored similar amounts of EGFR. (C) After pull-down and elution from the streptavidin resin different amounts of EGFR were detected, quantified by Image J and normalized against the EGFR signal obtained for D-Wt-O$_1$ which was set to 1. Relative EGFR amounts are indicated at the bottom of each lane (independent replicates n=2).

**FIGURE 9.** The ability of Pmp21-D to inhibit infection of HEp-2 cells by *C. pneumoniae* depends on the presence of FxxN motifs and protein oligomerisation. HEp-2 cells were incubated with PBS, BSA, heparin or the indicated recombinant proteins (100 µg mL$^{-1}$) prior to infection with *C. pneumoniae* EBs (moi 20). Cells were fixed 48 h post infection and the number of inclusions formed was determined by microscopy. The number of inclusions per 1 x 10$^3$ human cells was determined, and expressed as percentage relative to the number of inclusions found in PBS-treated samples. (A) Results are derived from three independent experiments (independent replicates n=3). Data shown are means ± standard deviations. Statistical significance was assessed with Student’s t-test (*$P=0.05$, **$P=0.01$, ***$P=0.001$, n.s. = not significant). (B) Chlamydial infectivity as visualized by immunofluorescence microscopy. Methanol-fixed HEp-2 cells were stained with DAPI (blue). Chlamydial inclusions were detected with an antibody directed against chlamydial LPS (green).
Figure 1

A

Pmp21

SS

30 → 670 → 1145

1609

β-barrel

B

M-Pmp21

top view

side view

C

N / M / C-Pmp21

N / M-Pmp21

N-Pmp21

M-Pmp21

C-Pmp21

D

PD-Pmp21

N-Pmp21

M-Pmp21

Pmp21-A

Pmp21-B

Pmp21-C

E

Pmp21-D (D-Wt)

GGA (I,L,V)

FxxN

SxxV

Pmp21-D (D-Mt)
Figure 2

A  

B  

C  

D  

E  

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Figure 3

![Graph showing absorption at 280 nm vs. elution volume and molecular mass.]

- Absorption at 280 nm [a.u.]
- Elution volume [ml]
- Molecular mass [kDa]

D-Wt-M
Pmp21 forms functional Oligomeric Structures

Figure 4

A

absorption at 280 nm

D-Wt-O_1

V_0

D-Wt-M

elution volume [ml]

B

signal (AU)

residuals

radius (cm)

C

c(s) (AU/S)

sedimentation coefficient (S)

D

signal (AU)

residuals

radius (cm)

E

sedimentation coefficient (S)
Figure 5

A

B

C

D

E

PBS-Puffer

D-Wt-O1

D-Wt-O2

D-Wt-O3

D-Wt-M

Pmp21 forms functional Oligomeric Structures
Pmp21 forms functional Oligomeric Structures

**Figure 6**

**A**

![Graph showing elution volume versus absorption at 280 nm for D-Wt-O, D-Wt-M/D-Mt-M, and D-Mt-O.]

**B**

| mg ml⁻¹ | D-Wt-O | D-Mt-O |
|----------|--------|--------|
| 0.7      | 22 %   | 4 %    |
| 1.7      | 53 %   | 5 %    |
| 2.4      | 57 %   | 21 %   |
| 3.1      | 62 %   | 28 %   |

**C**

![Graph showing Molar Residue Ellipticity (MRE) versus wavelength for D-Wt-M, D-Wt-O₁, D-Mt-M, and D-Mt-O₁.]

**D**

![Images of D-Mt-O₁, D-Mt-O₂, D-Mt-O₃, and D-Mt-M samples.]

**E**

![Images of D-Mt-O₁, D-Mt-O₂, D-Mt-O₃, and D-Mt-M samples.]

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Figure 7

Bead coated HEp-2 cells [%]

BSA  D-Wt-M  D-Wt-O₁  D-Mt-M  D-Mt-O₁

***  **  **  **
Figure 8

A

B

C
Figure 9

A

B

Pmp21 forms functional Oligomeric Structures
The Chlamydia pneumoniae Adhesin Pmp21 forms Oligomers with Adhesive Properties
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