Tangled up in fibers: How a lytic polysaccharide monooxygenase binds its chitin substrate

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Running title: SANS studies of GbpA-chitin complex

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Abstract

Lytic polysaccharide monooxygenases (LPMOs) are redox enzymes that bind to and oxidize insoluble carbohydrate substrates, such as chitin or cellulose. This class of enzymes has attracted considerable attention due to their ability to convert biomaterials of high abundance into oligosaccharides that can be useful for producing biofuels and bioplastics. However, processes at the interface between solution and insoluble substrates represent a major challenge to biochemical and structural characterization. Here, we investigated the four-domain LPMO from *Vibrio cholerae*, *N*-acetyl glucosamine binding protein A (GbpA), to elucidate how it docks onto its insoluble substrate with its two terminal domains. First, we developed a protocol that allowed GbpA and chitin to form a stable complex in suspension, overcoming incompatibilities of the two binding partners with respect to pH. Using contrast variation small-angle neutron scattering (SANS), we attempted to isolate the scattering signal from the individual components of the chitin-GbpA complex. After determining the neutron scattering contrast match point for chitin (47% D_2O), we characterized the structure of GbpA in complex with chitin by SANS. We found that GbpA binds rapidly to chitin, where it spreads out on the chitin fibers, and smooths them. In some locations, GbpA binding induces the formation of protein-chitin clumps containing hundreds of GbpA molecules. These findings are supported by electron microscopy. Placing our findings into a biological context, we discussed the potential advantages of GbpA secretion and how chitin binding may prepare the ground for microcolony formation by the bacteria.

Introduction

Since the discovery of lytic polysaccharide monooxygenases (LPMOs) in 2010 [1], their ability to degrade crystalline cellulose, xylan or chitin has drawn massive interest for conversion of renewable biomaterials to biofuels. LPMOs are multi-domain proteins found in a wide range of organisms, including bacteria, fungi, algae and insects as well as viruses [2, 3]. The proteins are secreted by human pathogenic bacteria and are employed for survival, both on carbohydrate surfaces outside and inside the host, using different strategies [4-6] including direct interference with the immune system [6]. Given the abundance of these enzymes, and their sequence variation, it is likely that other important functions of these proteins are yet to be revealed. The discovery of LPMOs necessitated a reclassification of enzyme families in the Carbohydrate-Active enZYmes database (CAZY; http://www.cazy.org), where they are listed under 'auxiliary activities' families (AA9-11 and AA13-17). The first crystal structures of LPMO complexes with carbohydrate ligands (cellotriaose, Glc_3 and cellohexaose, Glc_6) were solved in 2016 by Frandsen *et al.* [7], for an AA9 LPMO from *Lentinus similis*. At approximately the same time, Courtade *et al.* probed ligand binding to another AA9 LPMO, from *Neurospora crassa*, by nuclear magnetic resonance (NMR) spectroscopy [8]. These, and subsequent studies, showed how the ligand stretches across the flat surface of the pyramidal LPMO structure, with the +1 sugar unit binding to the N-terminal histidine of the characteristic copper-coordinating histidine-brace-motif present in the active site of LPMOs (Fig 1A). The catalytic mechanism of LPMOs, and current controversies, are summarized in an excellent review by Forsberg *et al.* [9]. Despite increasing structural and functional data, there is scarce structural information on the molecular interaction of LPMOs with their complex, recalcitrant carbohydrate substrates.

We are interested in virulence factors of *Vibrio cholerae*, the causative agent of cholera. Therefore, we selected *N*-acetylglucosamine binding protein A (GbpA) from *Vibrio cholerae* as a model system to study its interaction with chitin (for a review of chitin-active LPMOs, see Courtade & Aachmann [3]). GbpA was discovered in 2005 [4], and found to be important for colonization in the aquatic environment [4, 10, 11] as well as in the human host, binding to (and up-regulating) human mucins [5, 10]. GbpA consists of four domains, of which the first domain, which structurally resembles carbohydrate-binding module CBM33, is a chitin-degrading LPMO [12, 13] with calcium-binding properties [14], and additionally binds to mucins [12]. The fourth domain (classified as a CBM73 in CAZy) also binds chitin [12], whereas domains 2 and 3, which structurally resemble flagellin and pilin-binding proteins,

respectively, mediate attachment to the cell surface of the bacteria [12]. The structure of the first three domains of GbpA has been solved by X-ray crystallography [12], recently complemented with the full-length crystal structure of a GbpA homolog from *Vibrio campbellii* [15]. In addition, the solution-structure of GbpA has been characterized with Small-Angle X-ray Scattering (SAXS) and Small-Angle Neutron Scattering (SANS) [12, 16], revealing a monomeric elongated structure. How the structure of GbpA changes upon binding to chitin, and how the domains align in this state, is, however, unknown.

Here, we set out to reveal how GbpA interacts with chitin fibers with SANS. We characterized the bound and unbound GbpA structures by applying SANS to deuterated and non-deuterated GbpA (alone or in complex with chitin), and by varying the contrast using different D_2O/H_2O-mixtures, as schematically shown in Figure 1B. Additional, complementary insights were obtained from negative-stain electron microscopy (EM).

Results

Development of a protocol for solution studies

A suitable method for structural characterization of individual partners in a protein complex is SANS. This poses a challenge for studying protein complexes of chitin, which is an insoluble homopolymer of *N*-acetyl glucosamine (GlcNAc) residues. To our knowledge, chitin has rarely been used for solution scattering studies, because of its low solubility. It has, however, been shown that fine β-chitin nanofibers can be suspended in slightly acidic buffers (pH ~ 3). For shorter periods of time, chitin can be kept at ~5, however, this is still low for GbpA, which shows some precipitation at pH ≤ 5. Fortunately, we could overcome these challenges by adding small volumes of 10-12 mg/mL GbpA (pH = 8.0) to chitin nanofibers (pH = 5.0), followed by immediate thorough pipetting and—for SANS experiments—sonication for 15-20 sec with a tip sonicator, as described in the *Experimental procedures*. We observed that the mixture immediately became less viscous in this process. The samples treated in this manner were subsequently dialyzed to 20 mM sodium acetate-HAc pH = 5.0 during a 24-hour period. After this procedure, the chitin-GbpA complex could be kept in suspension for 12-24 hours, sufficient for several SANS experiments. For the lengthier USANS measurements, rotating tumbler cells were used to keep the samples in suspension. In the case of delays, the complex was re-sonicated directly before the experiments. We observed that the solubility of the chitin fibers increased after the addition of GbpA, indicating that a complex had indeed formed. It was even possible to adjust the pH of the complex mixture to higher pH values (~7), without immediate precipitation.

Scattering of GbpA and chitin

Before exploring the structure of the chitin-GbpA complex, we investigated the structures of the two individual components. The solution structure of GbpA had already previously been characterized by SAXS and SANS [12, 16], showing an elongated, flexible shape with a radius of gyration (R_g) of 35-40 Å. Indeed, the scattering of GbpA cannot be described by a simple ellipsoid, but requires at least five connected ellipsoids to replicate the scattering data well (Fig 2A). Even though GbpA is a four-domain protein, this is not entirely surprising since the first domain is approximately twice the size of the other domains, and pyramidal in shape (not ellipsoidal). The chitin fibers could be well fitted with a flexible cylinder model including a power-law (Fig 2B) to also fit the slope at low-q, which approximates a decay exponent of 4,

indicating sharp interfaces impenetrable for the solvent. The chitin fibers have a radius of 16.0 +/- 0.1 Å and a Kuhn length of 317 Å (Table S1).

Chitin can be matched out in SANS

To obtain structural information of GbpA in its chitin-bound state with SANS, it is necessary to measure the sample at the chitin D_2O/H_2O match point. Since this match point has not previously been established, we set out to do so. After dispersing the fibers evenly in 20 mM acetic acid (pH 3.2), the suspension was dialyzed into six different levels of D_2O and measured by SANS (Fig S1A). We calculated the chitin match point to be at 47% D_2O (Fig S1B). This is extremely close to the theoretical match point of most globular proteins (≈40-45% D_2O), necessitating the use of deuterated GbpA (D-GbpA) for SANS interaction studies since it has a significantly different match point (>100% D_2O). To confirm that chitin is well matched out in the q-range where D-GbpA scatters and show that D-GbpA can indeed be used for interaction studies with chitin at the match point, we measured chitin, D-GbpA and non-deuterated GbpA (H-GbpA) individually at 47% D_2O (Fig 2C-D). The scattering of 2.5 mg/mL D-GbpA at 47% was much stronger than for chitin, which was well matched out, except at very low q-values, giving us green light regarding the feasibility of the planned SANS experiments with D-GbpA. The results also suggested that using H-GbpA would be impossible for this kind of study, as it scatters poorly at 47% D_2O.

GbpA decorates chitin, thickening and smoothing the fibers

Based on our promising initial contrast matching experiments of chitin, we progressed to study the complex of chitin and GbpA. An indication of complex formation was already obtained upon mixture of the two components, as described above. The chitin suspension is highly viscous, but upon addition of D-GbpA and sonication, the solution became easier to pipet and stuck less to surfaces (images of the chitin suspension and the mixture are shown in Fig 3A, B). Nevertheless, some sedimentation occurred, which affected reproducibility and prevented a more detailed analysis of the scattering data. The SANS curve of the complex at the chitin match point (Fig 3C, D) does not feature the characteristic GbpA form factor bump around q = 0.05 $Å^{-1}$, showing GpbA to be in the chitin-bound state, where an R_g of 35-40 Å does not persist. Interestingly, the SANS curve has features similar to the SANS curve of chitin itself at a good contrast (100% D_2O). In particular, a steep slope at low q-values is present for both chitin and the complex mixture (Fig 3E), indicating that GbpA spreads out along the chitin fibers. The steep slope also indicates that the samples have a protein-protein structural

correlation dictated by the chitin structure. These results were confirmed by negative-stain EM when analyzing GbpA and chitin mixed together. Here, the addition of full-length GbpA ($GbpA_{fl}$) to chitin produces a change in appearance in the chitin fibers (Fig 4A and B). The fibers appear less stiff and with less defined edges, indicating that GbpA coats the fibers uniformly.

By analyzing the scattering data of the complex using a unified Beaucage scattering model (Fig 3F, Table 1), we obtained R_g values for the different structural levels within the samples. For most of the samples, an R_g in the range 125-220 Å was obtained (compared to an R_g ~16 Å for undecorated chitin fibers; see Section *Scattering of GbpA and chitin*). Thickening of the fibers was also observed in transmission electron microscopy (TEM) images obtained under similar conditions (Fig 4), although the fiber thickness measurements in negative-stain EM are not very reliable due to variations in staining intensity between samples (even when the samples are stained for similar times).

GbpA alters the chitin network structure and forms clusters

We observed that the overall scattered intensity (Fig 3D) increased about a 100-fold at low-q compared to the sum of chitin and GbpA scattering, indicating aggregation of GbpA on chitin at large length-scales. While the data do not contain a well-defined Guinier-region at low-q, a rough estimate can be made of the features around 0.01 $Å^{-1}$ where the forward scattering is of the order of 200-2000 cm^{-1}, which corresponds to a 100 to 1000-fold increase compared to the monomeric protein. This suggests that the scattering in the complex comes from larger aggregates—comprising hundreds of protein molecules on the chitin network—rather than from GbpA monomers. Such clusters can be clearly observed in the EM micrographs, where protein aggregates create bundles of GbpA-covered chitin fibers (Fig 4D). Interestingly, the R_g is not completely reproducible, and a sample with higher chitin:protein ratio instead exhibits an R_g of 16.0 Å, which is closer to the previously reported radius of the nanofiber chains [17]. This may be because excess chitin enables GbpA to spread more evenly on the chitin network, and clustering occurs mainly when the "empty" space on the chitin fibers is "used up". However, similar results were not obtained at lower concentrations (while retaining the chitin:GbpA ratio), possibly due to a low signal at higher-q. The overall discrepancy in R_g between samples can also be explained by different parts of the chitin network being in the neutron beam at the time of SANS data acquisition. SANS measurements of the D-GbpA–chitin complex at D_2O levels between the match points of the two components (Fig S2) also

yielded an R_g of 220 Å for the protein aggregate; nevertheless, some of the characteristic features of chitin fibers are still present in the SANS data at high-q.

In addition to R_g values, the unified scattering model gives decay exponents for the different levels of structures (results are summarized in Table 1). The decay exponent describes the surface scattering or the fractal dimensionality of the aggregate (Porod-like) if D∼4 [18]. Chitin itself has a decay exponent of 4.0 (Table S1), indicating very large aggregates with a sharply defined surface [18, 19] (as seen in Fig 4A). For the GbpA-chitin complex at the chitin match point, the low-q decay exponent is 2.5-3.6, consistent with aggregates in a state between random and globular. The smaller decay exponent compared to chitin suggests a partial loss of the sharp interfaces of the chitin fibers when decorated with protein, as seen by EM (Fig 4B). This may be because GbpA does not penetrate the fibers, but remains on the surface under the experimental conditions (*i.e.*, after sonication, when fibers are much less tangled).

Since GbpA does not only coat the chitin fibers, as originally anticipated, but additionally forms local clusters of multiple protein molecules, our attempt to increase the ratio of chitin to GbpA in order to spread out the GbpA molecules to analyze the structure of individual GbpA molecules bound to chitin, will not work. To address this problem in a different way, we used mixtures of H-GbpA and D-GbpA bound to chitin at the chitin match point (Fig 3G). Since H-GbpA is well matched out at the chitin match point of 47% D_2O, we expected to still mostly see D-GbpA on the chitin fibers. However, since D-GbpA and H-GbpA compete for the binding sites, the distance between D-GbpA on the fibers should increase, perhaps to the point of scattering independently. As expected, the SANS intensities for the mixtures of D-GbpA and H-GbpA on chitin decreased with higher proportion of H-GbpA (Fig 3G). Using the unified scattering model, an R_g of 43-47 Å was obtained, which is close to that of monomeric GbpA; however, no clear features could be observed in the scattering curve that resemble the GbpA form factor. While the low R_g likely originates from the GbpA-layer on chitin, the actual value may not be indicative of the thickness, as some of it corresponds to matched-out H-GbpA. The decay exponent was 2.8, similar to the other GbpA-chitin samples and thus this approach did not remove the structure factor effect. This finding showed that even when the distance between D-GbpA particles on chitin is increased, D-GbpA molecules do not scatter independently (further increasing the distance between D-GbpA molecules by lowering the D-GbpA to H-GbpA ratio was technically impossible, since the signal became too low).

We also performed Ultra-Small-Angle Neutron Scattering (USANS) experiments, which give structural information in the hundreds of nanometer range, thus providing an estimate of the

size of aggregates. However, we observed that the slope for the chitin-bound GbpA extends to very low angles without flattening out, indicating that GbpA decorates very large areas (or domains) on the chitin fibers (beyond the resolution of several micrometers; Fig 5). EM analysis provided a good explanation for this, with GbpA completely coating chitin fibers and inducing aggregation of the fibers in clumps as observed in Fig 4D. A more complete set of EM micrographs can be found in Figs S3 and S4.

EM analysis of truncated GbpA and *V. cholerae* microcolonies

We were curious about the contributions of the different GbpA domains to chitin binding and complex formation, and therefore extended the EM analysis to truncated constructs of GbpA. Since both terminal domains (D1 and D4) are known to bind chitin [12], we selected constructs consisting of domains 1 to 3 ($GbpA_{D1-3}$) and domain 1 alone ($GbpA_{D1}$) for comparison with full-length GbpA ($GbpA_{fl}$). $GbpA_{D1-3}$ induced chitin aggregates similar to $GbpA_{fl}$ (Fig 6B-D, compared to Fig 4D). However, $GbpA_{D1}$ appears to have a weaker effect on the fibers, as seen in Fig 6B and D. While the fibers are still decorated with the protein, they have much sharper edges compared to $GbpA_{D1-3}$ and $GbpA_{fl}$, and are visually more similar to the free chitin ultrastructure (Fig 4A and Fig S3). A more complete set of EM micrographs can be found in Figs S5 and S6.

Additional EM experiments were performed by adding to chitin fibers an inactivated strain of *V. cholerae*, in which the toxin co-regulated pilus (TCP) was deleted. Interestingly, some of the bacteria were found to create microcolonies (Fig 6E) that were not observed for the bacteria imaged alone.

Discussion

We developed a suspension protocol for the GbpA-chitin complex and carried out SANS, USANS and negative-stain EM experiments to characterize the structure of GbpA upon complex formation. We observed that upon GbpA binding to chitin, the suspension of chitin-fibers immediately becomes less viscous, suggesting that the properties of the GbpA-chitin complex differ significantly from those of both partners alone. In the SANS and USANS experiments, however, we experienced limits regarding the structural modeling. We suspect that it may not be possible to obtain a detailed structural model of independently scattering GbpA particles on β-chitin fibers using SANS, due to persisting structural order along the chitin fibers. This is not surprising, since the fibers are long and likely rigid, inducing a fixed order even over long distances. Much shorter chitin fibers would be required in order to overcome these technical impediments. However, we have obtained other important insights: our study demonstrates that it is possible to use chitin in neutron scattering studies, as the polymer can be well matched out in the mid to high-q range at 47% D_2O. Using both SANS and EM, we could further show that GbpA decorates the chitin fibers with many binding sites over the chitin network, and with a preference for the chitin-bound state over the solution state. This explains why GbpA can effectively degrade insoluble chitin substrates, while glycosidic hydrolases cannot.

GbpA does not adsorb evenly to all parts of the chitin network, possibly because some parts of the fibers are less accessible than others. The regions of chitin with high accessibility for GbpA-binding likely exhibit protein-protein interactions yet to be elucidated. This hypothesis is supported by the weakened effect that shortened forms of GbpA (*i.e.*, $GbpA_{D1}$) have on the chitin ultrastructure (as shown in Fig 6). For example, domain 1 alone does not appear to be able to induce the formation of larger chitin aggregates. This lessened interaction cannot be fully explained by the loss of the chitin-binding fourth domain, however, as the qualitative, *visual* effect of $GbpA_{D1-3}$ on chitin was similar to $GbpA_{fl}$ (Fig 6). Domains 2 and 3 therefore appear to be highly important for the observed clustering effect. These domains have previously been reported to be important for binding to the bacteria [12], thus they may have a dual role. The full implications of these observations remain to be determined.

Implications for GbpA's role as colonization factor

Chitin is the most abundant biopolymer in marine environments, and is found *e.g.*, on zooplankton, mussels and crustaceans. *V. cholerae* would therefore be well served by using a

chitin-binding colonization factor, *i.e.* GbpA, as one of its first anchors when forming microcolonies (as suggested by [20]). Other important factors for microcolony formation are the toxin-co-regulated pilus (TCP) [21, 22], the chitin-regulated pilus (ChiRP) [23] and outer membrane adhesion factor multivalent adhesion molecule 7 (Mam7) [24]. An extracellular matrix consisting of polysaccharides and proteins then encases the microcolonies, completing the protected environment by forming a biofilm [25-28], which can adapt to changing environmental conditions [29, 30] and raises infectivity [31]. Such a complex may be influenced by calcium [14, 29]. A negative-stain TEM image of a microcolony from an inactivated, TCP-deleted strain of *V. cholerae* is presented in Fig 6E (revealing that TCP is not essential for microcolony formation). The halo surrounding the microcolony is presumed to be the beginnings of a biofilm, and a hazy, fibrillar mass in the space in the center of the colony might be chitin fibers. Chitin binding may thus initiate a cascade of events that culminates in biofilm formation and hyper-infectivity. Moreover, chitin binding has been shown to induce natural competence in *V. cholerae* [32], increasing bacterial fitness by exchange of genes. The first step of these events appears to be the secretion of GbpA and chitin binding. – But why would it make sense for the bacteria to throw out anchors without a connecting line, and attaching to them in a second step? The picture emerging from the present study is that GbpA efficiently prepares the ground for microcolony formation by forming aggregates dispersed throughout the fibers (Fig 7), and with this reserves space for *V. cholerae* over competitors, as previously suggested by Kirn *et al.* [4]. Secreting anchors first, and then attaching to them before reproducing, allows the bacteria to most effectively colonize biotic surfaces in the aquatic environment. Additionally – and at least as important –, GbpA ensures abundant food supplies through its lytic polysaccharide mono- (or per-) oxidase activity, giving the bacteria an excellent return on their initial energy investment when producing and secreting GbpA.

Experimental procedures

Protein production and deuteration

Perdeuteration of GbpA involved expression in deuterated media, according to the protocol described in [16], which was inspired by [33]. This protocol relies on BL21 star (DE3) cells containing the GbpA-encoding gene in the pET22b vector [16]. Growth and expression media contained D_2O and d_8-glycerol (99% D) purchased from ChemSupport AS (Hommelvik, Norway). The production of H-GbpA and the purification of D-GbpA and H-GbpA is also described in [16]. The constructs of the truncated variants of GbpA ($GbpA_{D1}$ and $GbpA_{D1-3}$) for EM analysis are described in [12, 16], and the protocol for protein production and purification is almost the same as that used for the full-length construct, described in [16]. The only difference concerns the purification of $GbpA_{D1-3}$, where a step gradient was introduced during anion-exchange chromatography to improve the yields of pure protein. Specifically, a step corresponding to 180 mM NaCl (8 column volumes) was added to the isocratic gradient elution.

GbpA was saturated with copper to activate the enzyme prior to the SANS experiments, by incubation with $CuCl_2$ in 5-fold molar excess for at least 30 min. Free copper was then removed by passing the protein through a HiTrap Desalting column (GE Healthcare). Prior to the SANS experiments, the protein was dialyzed against 100 mM NaCl, 20 mM Tris-HCl pH 8.0 with 47% of the water being D_2O.

Preparation of chitin, GbpA and chitin-GbpA samples for SANS

β-chitin nanofibers (≈180 micrometer in length) from squid pens (France Chitine, Orange, France) were kindly provided by Jennifer Loose and Gustav Vaaje-Kolstad and prepared according to the protocol described in [13]. The fibers were dissolved in 20 mM acetic acid (pH 3.2) to a concentration of 10 mg/mL and subsequently sonicated at 50-70% power with a Vibra Cell Ultrasonic Processor until they were well dispersed and did not sink to the bottom of the tube for days. Finally, the fiber suspension was dialyzed against 20 mM sodium acetate-HAc pH 5.0 with the desired amount of D_2O for at least 24 hours. At higher pH or in the presence of salts, it was observed that the fibers precipitated. However, at pH 5, GbpA is not very stable. Nevertheless, it was possible to add GbpA to the fibers from a highly concentrated stock solution of the protein (prepared by dialysis against 10 mM NaCl, 20 mM Tris-HCl pH 8.0), if the mixture was immediately treated by thorough pipetting (*i.e.*, pipetting the suspension up and down 7-10 times with a micro pipette, using a cut-off 1 mL pipette tip). To

further stabilize the suspension, we sonicated the mixture for 15-30 sec with a tip sonicator and re-dialyzed the sample for 24 hours against 20 mM sodium acetate-HAc pH 5.0 with relevant amounts of D_2O. After any dilution of the chitin fibers or the mixture, the solutions were re-sonicated for 15-30 seconds to ensure proper suspension, but avoiding a raise in temperature of the sample.

GbpA samples without chitin were dialyzed into 100 mM NaCl, 20 mM Tris-HCl pH 8.0, with the desired level of D_2O prior to SANS analysis.

SANS measurements

Samples were measured with Small-Angle Neutron Scattering (SANS) at beamline D11 at Institut Laue-Langevin (ILL) [34], with wavelength $\lambda = 5.6$ Å and a wavelength spread of $\Delta\lambda/\lambda = 9\%$. Data were acquired for the q-range of 0.0013 – 0.4102 $Å^{-1}$. Ultra Small-Angle Neutron Scattering (USANS) experiments were carried out at beamline BT5 [35] at the National Institute of Standards and Technology (NIST) Center for Neutron Research, using $\lambda = 2.4$ Å and a wavelength spread of 6% $\Delta\lambda/\lambda$, for the q-range 0.00005 $Å^{-1}$ to 0.001 $Å^{-1}$. Tumbling cells were used for USANS measurements. To find the chitin match point, 10 mg/mL chitin was measured in 0%, 20%, 42%, 66%, 80% and 100% D_2O for 15 min per sample at ILL beamline D11. The data were integrated from 0.03 to 0.4 $Å^{-1}$, and the integrated data were fitted with a second degree polynomial function. The chitin match point was estimated from the minimum of this function.

While SANS samples were measured for 2-3 hours, USANS measurements lasted 11 hours per sample. Buffer, empty cell and H_2O measurements were used for background subtraction and calibration to absolute scale. Processing was done with software at the respective beamlines [36, 37]. The scattering data of GbpA were fitted with a random-walk ellipsoidal model [38]. For chitin the scattering was fitted with a flexible-cylinder model including a power-law. For the complex of GbpA and chitin, the data were fitted with the Beaucage unified scattering model [39, 40]:

$$I(q) = c + \sum_{i=1}^{N} \left[G_i \exp\left(-\frac{q^2 R_{gi}^2}{3}\right) + B_i \exp\left(-\frac{q^2 R_{g(i+1)}^2}{3}\right) \left(\frac{1}{q_i^*}\right)^{D_i} \right] \quad (1)$$

Where c is a constant background, G is the Guinier scaling factor, Rg the radius of gyration, B is the Porod constant and D the Porod exponent. N is the number of levels used and is set to two for all fits. q_i^* is given by:

(2) $$q_i^* = q\left[erf\left(\frac{qR_{gi}}{\sqrt{6}}\right)\right]^{-3}$$

Data were plotted with Origin (Version 2017 64bit, OriginLab Corporation), and the modeling was carried out with SASVIEW 5.0.5 [41] and QtiSAS [42]. The SANS data have been submitted to the Small Angle Scattering Biological Data Bank (SASBDB) [43], and the IDs are included in Table 1 and Table S1.

Negative-stain EM

GbpA and chitin samples were prepared, essentially as described above. 10 mL of 3 mg/mL chitin were subjected to sonication in 20 mM acetic acid pH 3.2. Sonication was performed in a Q500 Sonicator (Qsonica), using the thinnest tip at 30% intensity in 3/3 sec on/off pulses until the sample was completely suspended. Chitin was dialyzed overnight against 20 mM acetic acid pH 5.0 and sonicated again to ensure that it remained suspended. Chitin aliquots were then mixed with a few microliters of GbpA (or its truncated variants) at a final concentration of 400 nM and incubated with agitation for 1h at 21 ˚C. Uncharged, carbon-coated 300 hex-mesh Cu TEM grids were exposed to 8 µL of sample, washed in deionized water 10x and stained with 1% uranyl acetate (UAc) for 30-60 seconds. Separate, additional TEM experiments were performed with an inactivated, TCP-deleted *V. cholerae* strain (*V. cholerae*$_{\Delta TCP2}$) and β-chitin. The bacterial samples were prepared by growing 100 mL liquid colonies in LB media overnight in a Multitron Standard shaker at 37 ˚C and 120 rpm. These bacterial samples (OD_{600} = 4) were mounted to 300 hex-mesh Cu TEM grids that were negatively charged in a glow-discharger. Each grid was applied to their respective sample drop (8 µL) for 30 minutes, then washed 5 times in distilled water and stained with 1% UAc for 30-60 seconds. All images were taken with a JEOL 1400Plus Transmission Electron Microscope operating at 120kV. Micrographs were analyzed with ImageJ [44].

Data availability

SANS data are deposited in the Small Angle Scattering Biological Data Bank (SASBDB), with IDs given in Table 1. All other data are published in this manuscript.

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Author contributions

K.B.-A. and U.K. conceived the study. H.V.S. and M.M.-C. produced the protein, and H.V.S. established a protocol for sample preparation and performed SANS experiments, supervised by K.B.-A., R.L. and U.K., and with assistance of S.P. at ILL and S.K. at NIST. M.M.-C. and A.C. prepared the samples for EM and acquired the data at the EM core facilities at UiO. G.V.-K. supported the work with samples and know-how. The manuscript was written by H.V.S., with contributions from M.M.-C. and U.K.. It was revised with input and approval from all authors.

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Conflict of Interest

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

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Abbreviations: CBM, carbohydrate-binding module; GbpA, *N*-acetylglucosamine binding protein A; D-GbpA, deuterated GbpA; EM, electron microscopy; H-GbpA, hydrogenated (= non-deuterated) GbpA; HAc, acetic acid; ILL, Institut Laue Langevin; LPMO, lytic polysaccharide monooxygenase; NIST, National Institute of Standards and Technology; PDB, Protein Data Bank; SANS, Small-Angle Neutron Scattering; SASBDB, Small Angle Scattering Biological Database; SAXS, Small-Angle X-ray Scattering; TEM, Transmission Electron Microscopy; USANS, Ultra-Small-Angle Neutron Scattering; UAc, uranyl acetate.

Figures

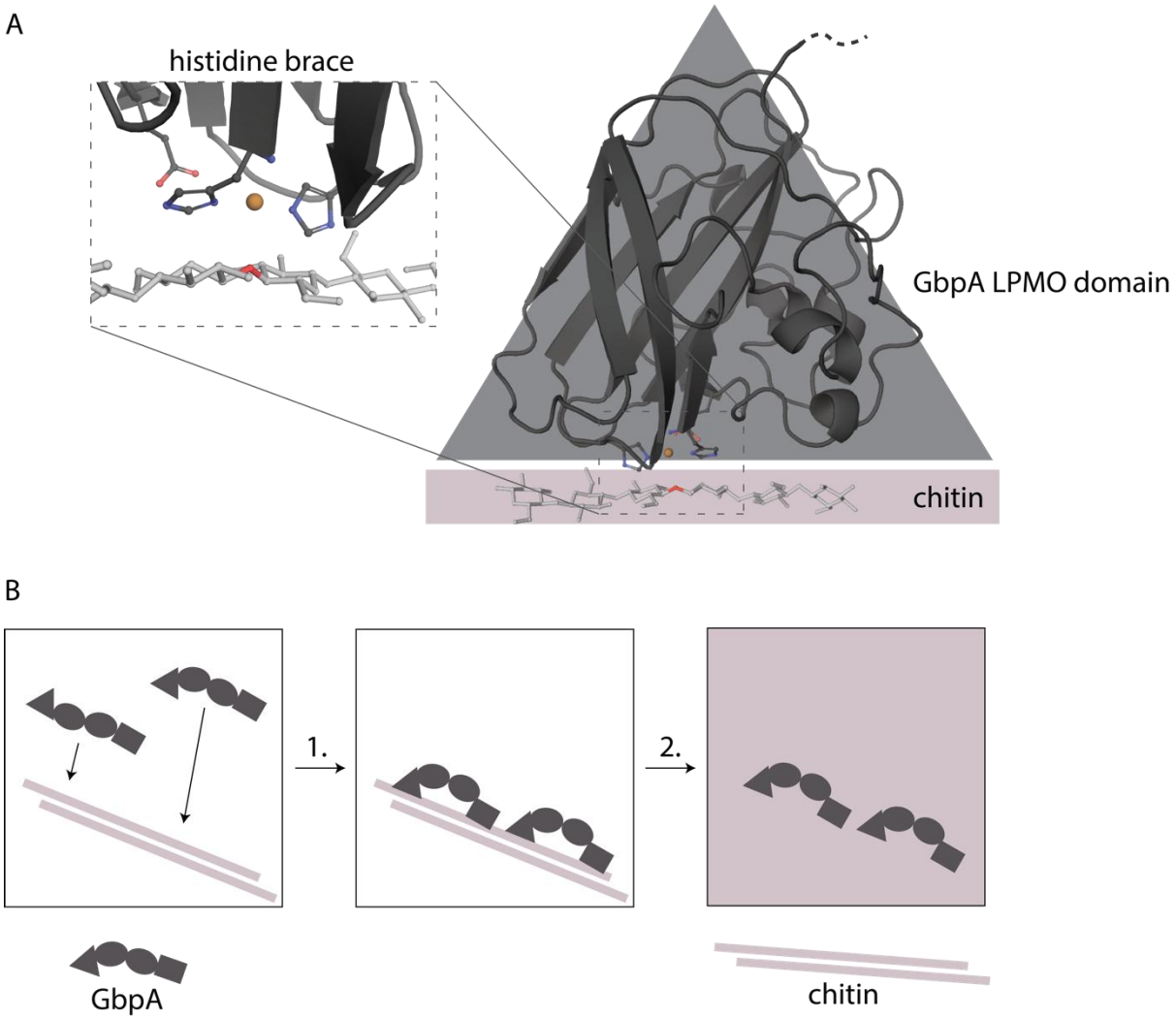

Fig 1 GbpA and schematic representation of SANS experiment. A The LPMO domain of GbpA binds to chitin and produces oxidative breaks in the fiber using its copper-containing histidine brace in the active site. Chitin has been manually modeled in its expected position based on [45], taking into account information from [46] (figure produced based on PDB ID: 2XWX [14]).**B** Representation of SANS experiment with GbpA and chitin using contrast matching: (1) GbpA binds chitin, undergoing a conformational change; (2) by applying mixtures of D_2O and H_2O, chitin can be matched out in the SANS experiment, leaving GbpA as the only visible scatterer.

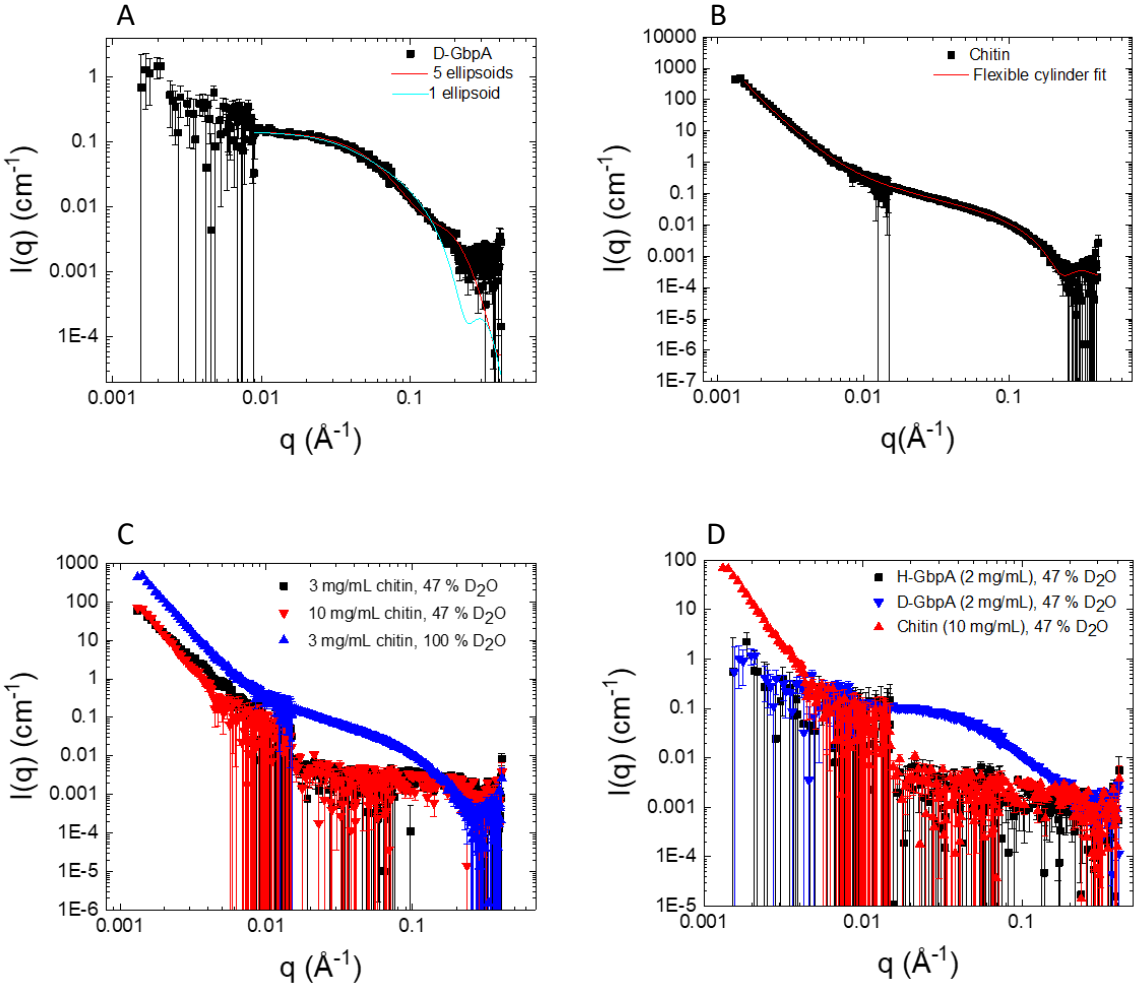

Fig 2 Independent scattering of GbpA and chitin. A SANS of D-GbpA in 47 % D_2O, (the chitin match point). The protein is fairly extended in solution, hence describing the scattering as an ellipsoid (blue curve) is not possible, a better fit can be achieved with 5 random-walk ellipsoids (red curve). **B** 3 mg/mL chitin was measured with SANS in D_2O, the data can be fitted with a flexible cylinder model including a power-law for the steep upturn at low-q. **C** The scattering of chitin in 47% D_2O is significantly weaker than in 100% D_2O, and is matched out in the middle to high q-range. However, at low-q, chitin could not be perfectly matched out. **D** Comparing the scattering of D-GbpA in 47 % D_2O to chitin shows the protein scatters very well in the middle to high q-range, where both chitin and H-GbpA are well matched-out. Deuteration of the protein is hence essential to get any contrast between the two components in an interaction study. Error bars represent one standard deviation.

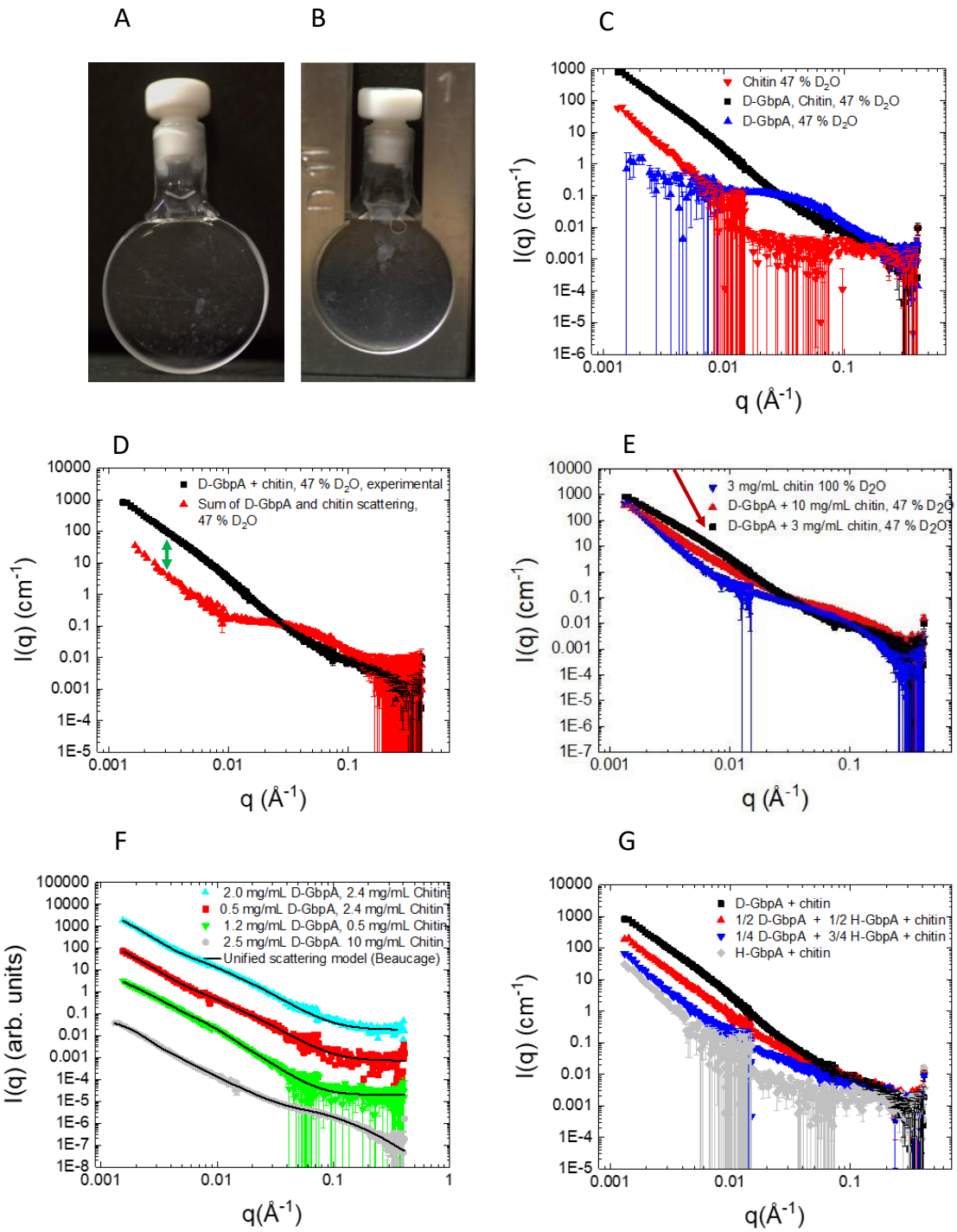

Fig 3 Sample preparation and SANS experiments. A, B Images of 3 mg/mL chitin samples, without GbpA (A) or with 2.5 mg/mL GbpA (B). **C-G** SANS data of GbpA, chitin or GbpA-chitin mixtures, plotted as intensities (I(q)) vs. the scattering vector (q) on double-logarithmic scale. Unless stated otherwise, 3 mg/mL chitin (except samples of GbpA alone), 2.5 mg/mL GbpA (combined concentration of H-GbpA and D-GbpA; except for samples of chitin alone) was used and measurements were done at 47% D_2O. **C, D** Chitin and D-GbpA clearly form a complex, since the SANS data of the mixture is significantly different from the sum of the scattering of the individual biomolecules. GbpA may bridge multiple chitin fibers, together creating thicker layers. If the scattering mainly originates from regions of bundled fibers, many GbpA molecules might be clustered together, whereas in single-chain regions, GbpA may form a more uniform layer on the fibers. In these regions, a radius similar to that of a single chitin fiber would be expected to dominate in the scattering data. **E** Increasing the amount of chitin relative to GbpA changes the overall structure of the GbpA aggregate, but the steep slope at low-q persists, suggesting a structural correlation between the proteins. The bump at mid-q (indicated by red arrow), is related to changes in fiber thickness upon GbpA binding. **F** The SANS data of the GbpA-chitin complex can be fitted with

a unified scattering model, see parameters in Table 1. **G** Varying H-GbpA to D-GbpA ratios affected the structure factor (intensities were lower for higher fractions of H-GbpA, since H-GbpA is almost matched at 47% D_2O), but the steep slope at low-q persisted.

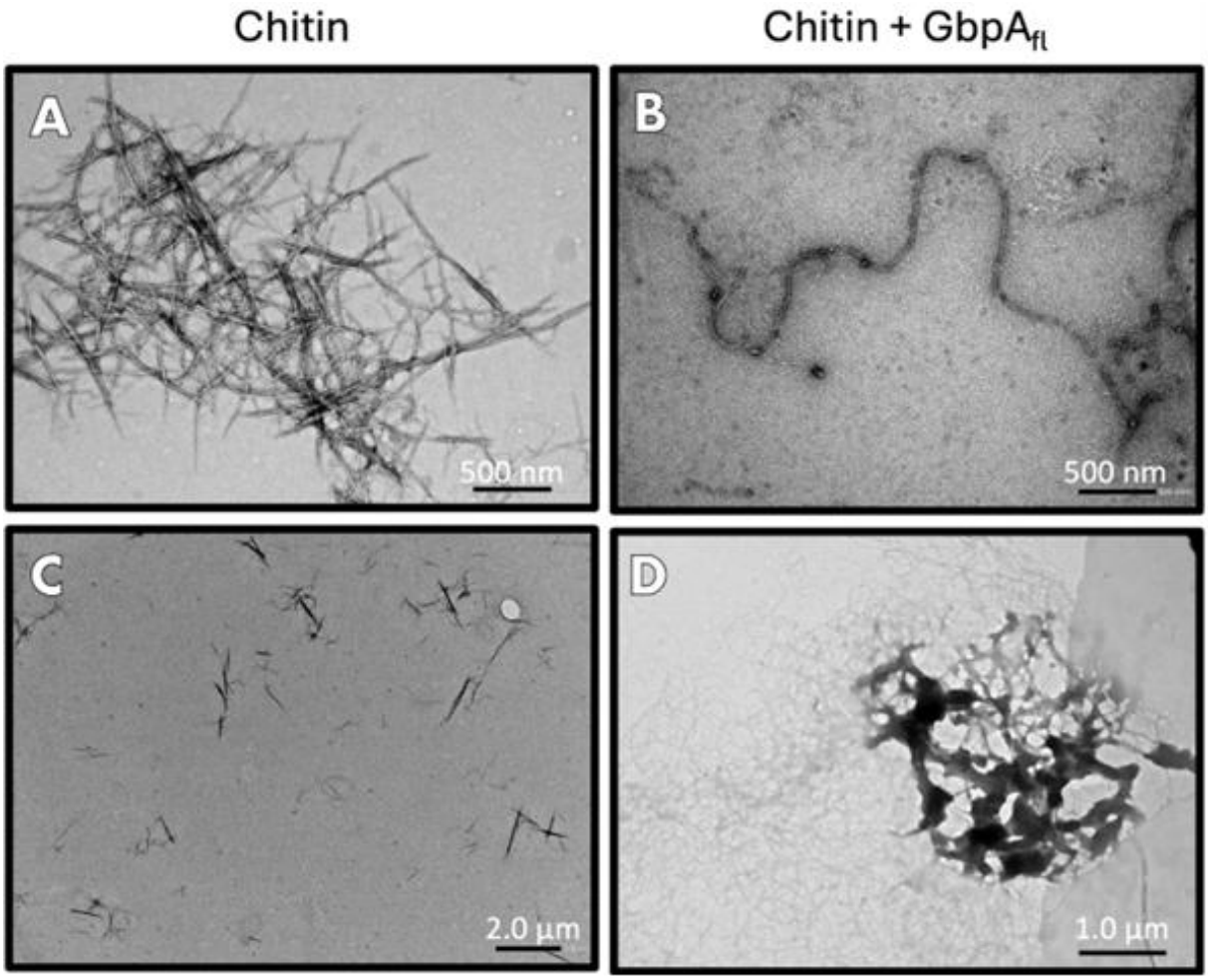

Fig 4 Negative-stain EM of chitin and GbpA$_{fl}$. A Cluster of chitin fibers. **B** Individual chitin fiber coated with GbpA$_{fl}$. The structure is longer and smoother, like a string. **C** Diffuse spread of chitin fibers (uncoated). **D** Representative dense aggregate of chitin fibers caused by GbpA$_{fl}$. Individual fibers spread out from the cluster. Extended TEM micrographs are shown in Fig S3-6.

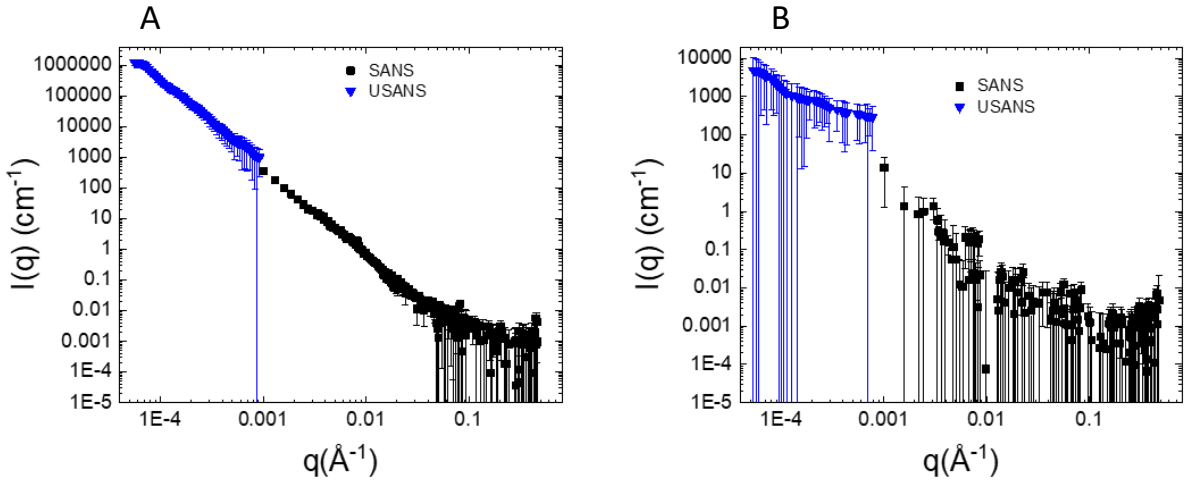

Fig 5 SANS and USANS data. A GbpA bound to chitin and **B** Chitin alone. Data were collected at 47% D_2O. USANS data confirmed a structural order in the micrometer regime. Error bars represent one standard deviation.

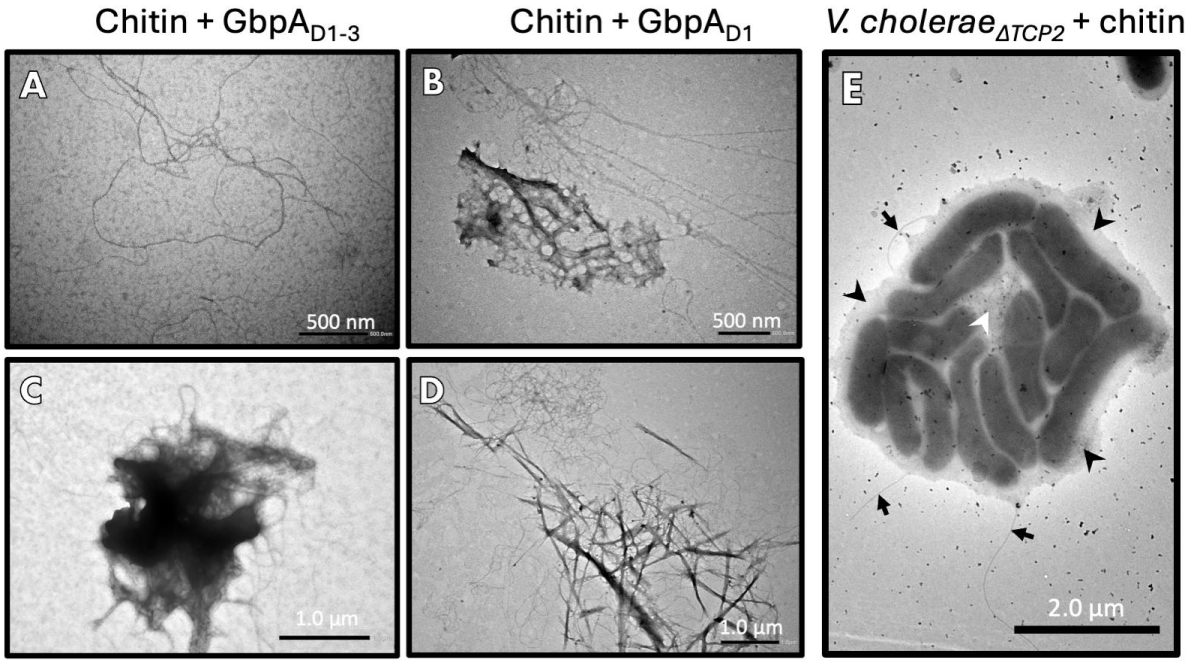

Fig 6 Extended negative-stain EM experiments including GbpA truncations and *V. cholerae* cells. **A** Individual chitin fibers coated by the GbpA$_{D1-3}$ truncation. **B** Individual GbpA$_{D1}$-coated chitin fibers extending from an aggregation of fibers and protein. **C** Cluster of aggregated GbpA$_{D1-3}$-coated chitin and individual, coated chitin-fibers. **D** Cluster of chitin bound to GbpA$_{D1}$. **E** Microcolony of V. *cholerae* observed only for bacteria incubated with chitin. A matrix of extracellular polysaccharides is indicated with black arrowheads, while the space in the center with potential chitin fibers is indicated with a white arrowhead. Flagella are indicated with smaller black arrows.

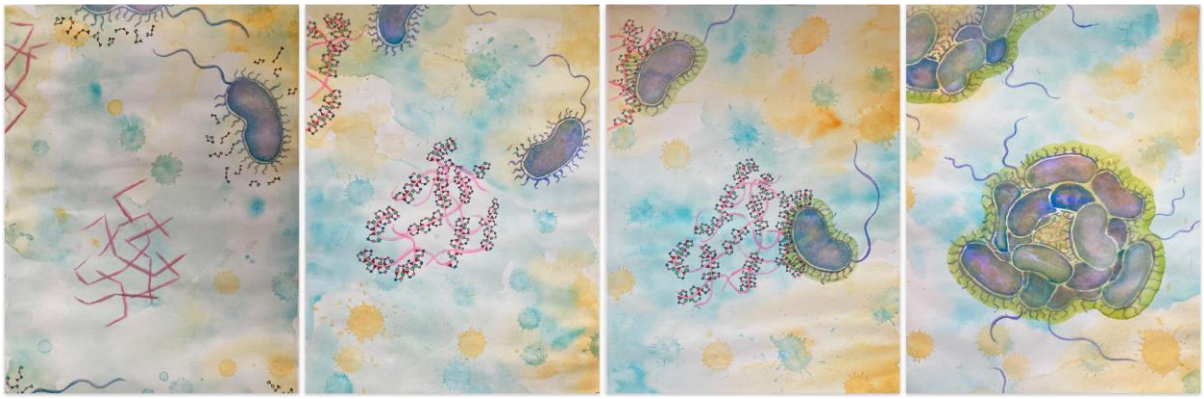

Fig 7 Water-color paintings of *V. cholerae* sketching microcolony formation on chitin aided by GbpA. *From left to right*: 1. *V. cholerae* bacterium secretes the four-domain protein GbpA (domains shown in different colors) close to a cluster of chitin fibers (pink, ragged appearance); 2. GbpA decorates chitin by attaching with its two terminal domains (LPMO-domain 1 (red triangle) and domain 4 (green)), smoothing the fibers' appearance; 3. Bacteria attach via GbpA domains 2 and 3 (blue, pink) to GbpA-coated chitin fibers and produce a hazy, fibrillar mass (green halo around the bacteria), initiating biofilm formation; 4. Formation of *V. cholerae* microcolony attached to chitin via GbpA. (Credit: Ayla Coder)

Tables

Table 1: Fitting parameters, unified scattering model

Sample*	**Decay exponent 1**	***R_g* 1 (Å)**	**Decay exponent 2**	***R_g* 2 (Å)**	**χ^2**	**SASBDB ID**
2.5 mg/mL D-GbpA, 10 mg/mL chitin	2.69 ± 0.01	1278 ± 11	3.88 ± 1.1	16.2 ± 0.3	2.66	SASDW92
2.5 mg/mL D-GbpA, 3.0 mg/mL chitin	2.64 ± 0.01	1859 ± 71	2.63 ± 0.02	125.2 ± 0.2	4.18	SASDWA2
2 mg/mL D-GbpA, 2.4 mg/mL chitin	3.64 ± 0.05	1600 ± 169	2.98 ± 0.01	202.5 ± 0.3	2.86	SASDWC2
0.5 mg/mL D-GbpA, 2.4 mg/mL chitin	3.40 ± 0.17	1633 ± 387	2.97 ± 0.07	183.1 ± 12.0	0.98	SASDWD2
1.2 mg/mL D-GbpA, 0.48 mg/mL chitin	3.16 ± 0.14	1484 ± 210	3.40 ± 0.06	221.8 ± 6.9	1.60	SASDWF2
1.0 mg/mL D-GbpA, 1.2 mg/mL chitin, 72 % D_2O	3.03 ± 0.06	1797 ± 223	2.36 ± 0.60	220.3 ± 5.3	1.03	SASDWE2
1.25 mg/mL D-GbpA, 1.25 mg/mL H-GbpA, 3.0 mg/mL chitin	2.81 ± 0.01	1845 ± 131	0.94 ± 0.11	43.4 ± 1.8	1.99	SASDWH2
0.625 mg/mL D-GbpA, 1.875 mg/mL H-GbpA, 3.0 mg/mL chitin	2.78 ± 0.03	2140 ± 165	0.43 ± 0.11	47.2 ± 1.7	1.95	SASDWJ2

The data were fitted with Beaucage unified scattering model with two levels, yielding two R_gs and two decay exponents. The first R_g is not reliable, as the Guinier region is outside of the q-range, the first decay exponent relates to the fractal dimensionality of the aggregate. The second R_g and decay exponent relates to structural features of the samples. An R_g of 125-220 suggests several protein molecules contribute to the scattering. All datasets are available in the SASBDB, with the accession codes in the last column.
*Samples are in 47% D_2O unless stated otherwise.

SUPPORTING INFORMATION

Tangled up in fibers: How a lytic polysaccharide monooxygenase binds its chitin substrate

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Content:

Fig S1: Matching out chitin in SANS experiments
Fig S2: SANS data
Fig S3: Extended TEM micrographs of chitin fibers
Fig S4: Extended TEM micrographs of chitin–GbpA complex
Fig S5: Extended TEM micrographs of chitin fibers incubated with $GbpA_{D1-3}$
Fig S6: Extended TEM micrographs of chitin fibers incubated with $GbpA_{D1}$
Table S1: Fitting parameters chitin cylinder model with power law

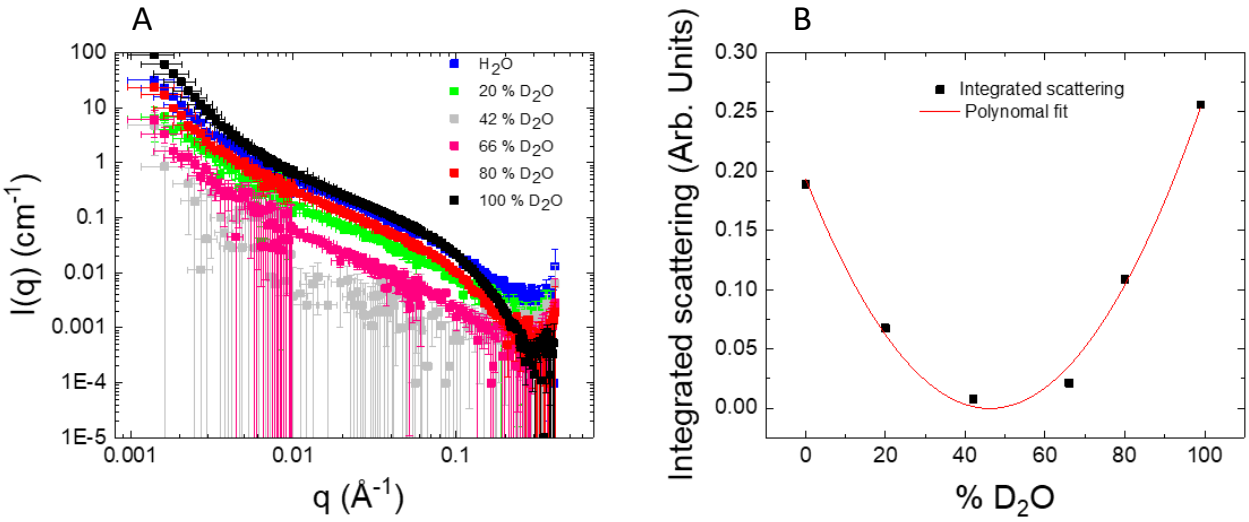

Fig S1 Matching out chitin in SANS experiments. A 10 mg/mL chitin was subjected to SANS at six different ratios of D_2O to H_2O, and the data were plotted as intensities (I(q)) vs. the scattering vector (q) on double-logarithmic scale. **B** The scattering for each of the six conditions was integrated, plotted and fitted with a polynomial function, to find the chitin match point at minimal scattering intensity (determined to be at 47% D_2O).

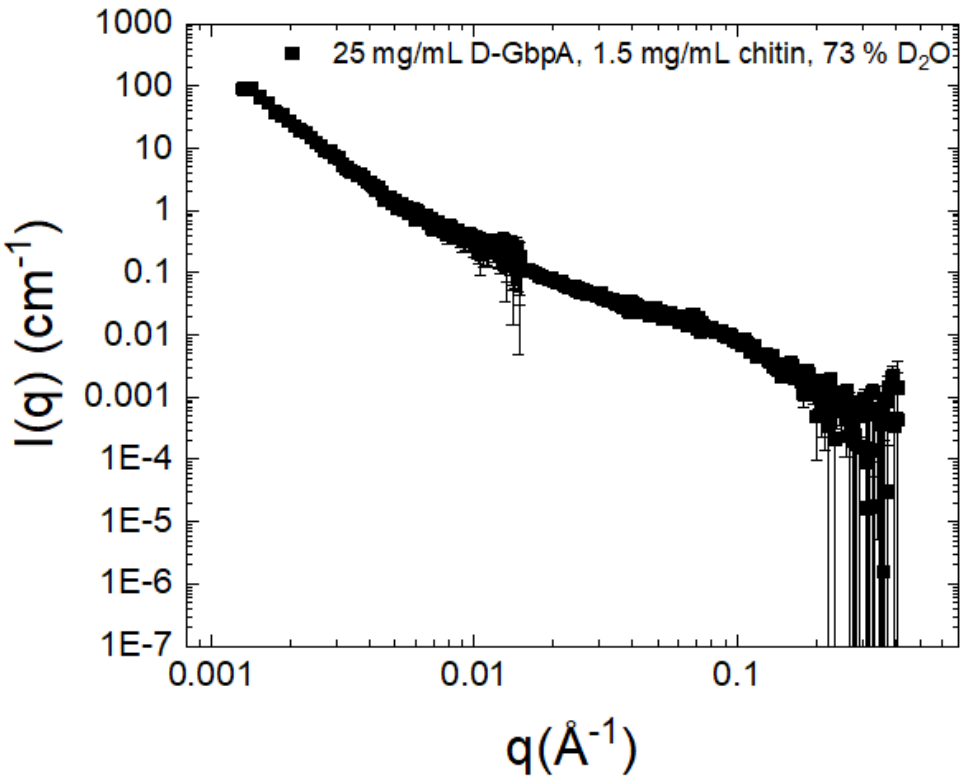

Fig S2 SANS data. Measurement in the middle of the chitin and D-GbpA match point yielded a slope close to the average of the slope for chitin and the complex at 47% D_2O. Error bars represent one standard deviation.

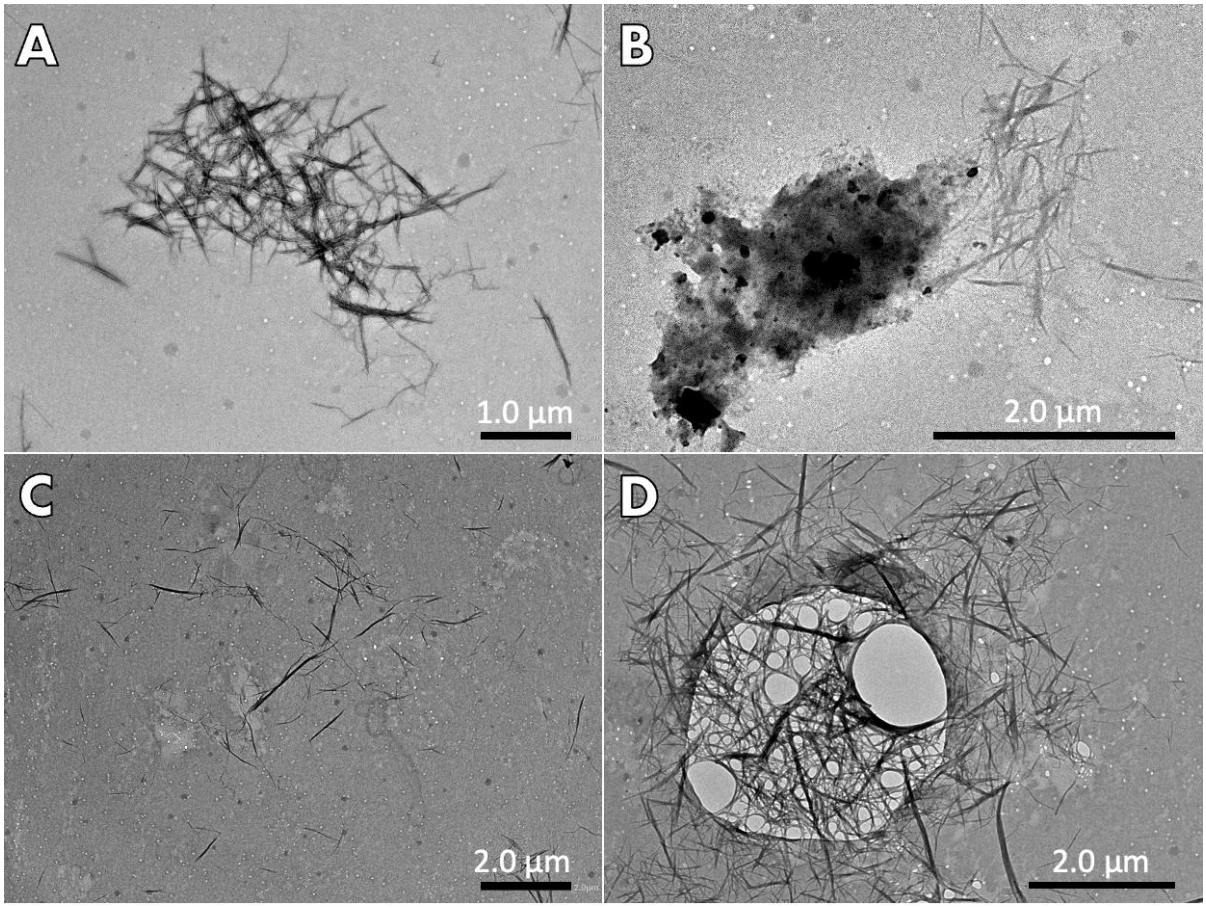

Fig S3 Extended TEM micrographs of chitin fibers. **A** A cluster of short fibers. **B** An unknown contamination or crystallization next to a cluster of short chitin fibers. **C** Several chitin fibers spread out more evenly. **D** A cluster of chitin fibers that have been trapped in a hole in the formvar film.

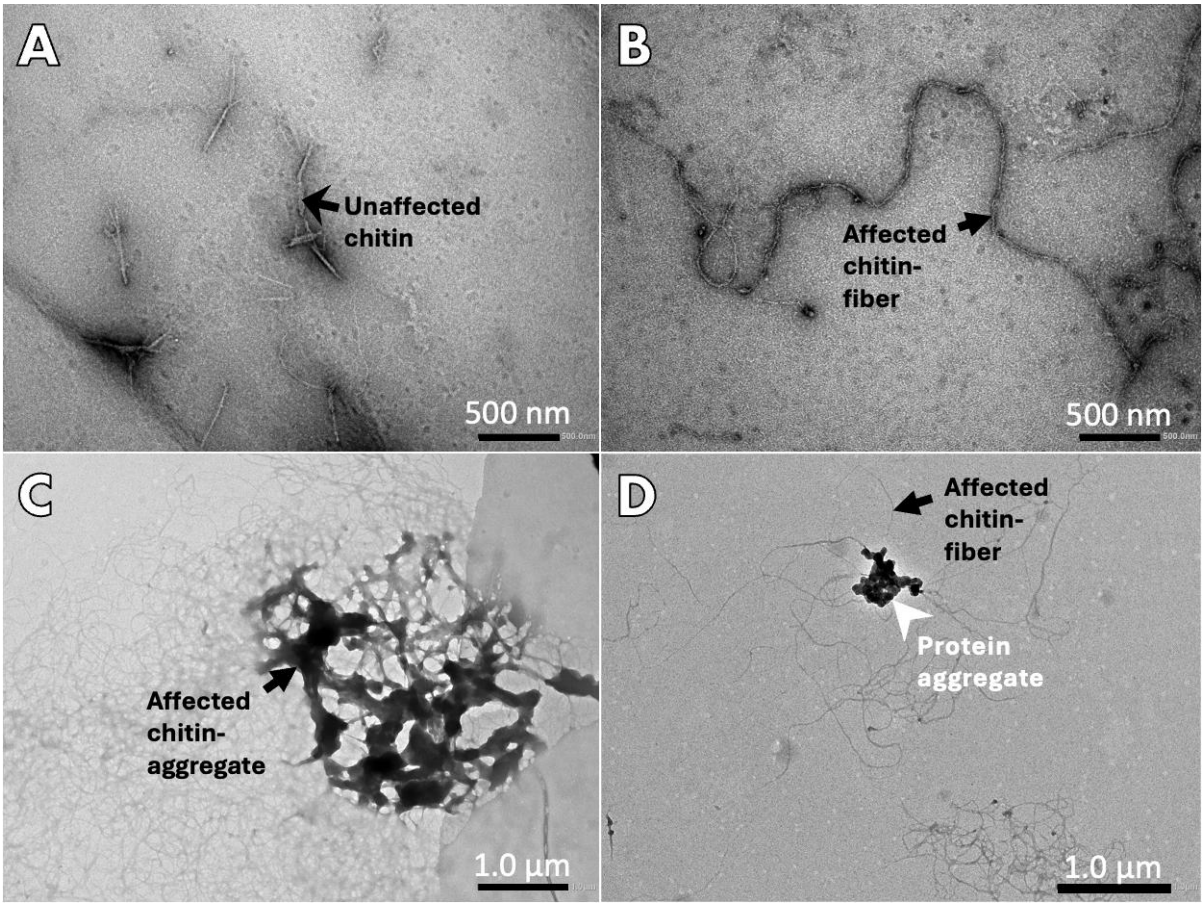

Fig S4 Extended TEM micrographs of chitin–GbpA complex. **A** A few relatively unaffected chitin fibers were present in this sample. **B** Close-up image of elongated fibers created by $GbpA_{fl}$, presumably coating (and potentially linking) the chitin fibers. Note the smoother appearance compared to uncoated fibers (Fig S3). **C** Image of one of the many dense aggregates of chitin observed after complex formation with $GbpA_{fl}$. From the cluster, a plethora of elongated fibers spread out from this denser aggregation, representing "classic" $GbpA_{fl}$-coated chitin fibers shown in B. The hard line at the right side of this image is presumably an artifact. **D** Another GbpA-chitin cluster that is ultrastructurally characteristic of a protein aggregate, with several elongated chitin-$GbpA_{fl}$ fibers extending from it. Additional elongated fibers are found at the bottom of the image.

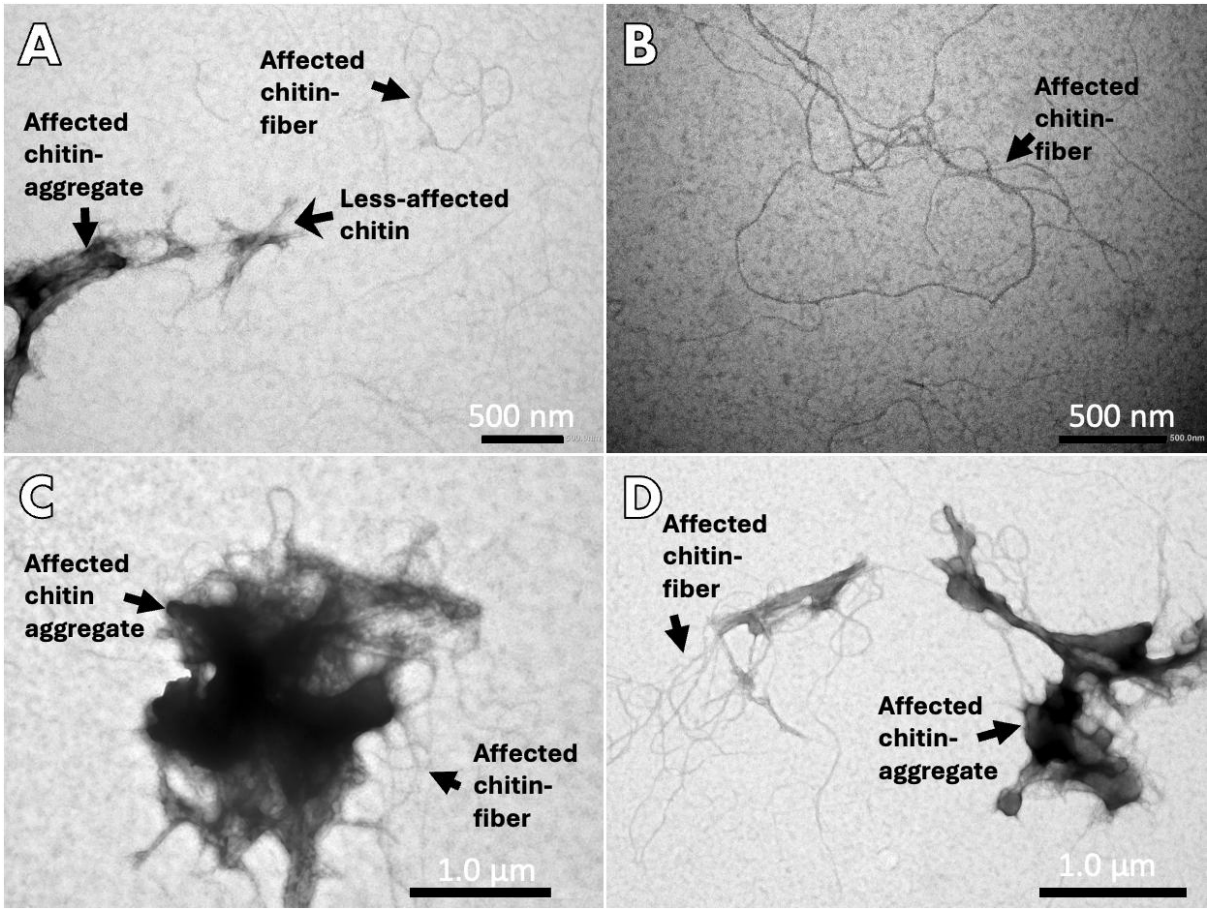

Fig S5 Extended TEM micrographs of chitin fibers incubated with $GbpA_{D1-3}$. A A cluster of aggregated chitin affected by $GbpA_{D1-3}$ is visible on the left, with sharper, more defined chitin fibers right next to it in the center of the image. Classic, individual, elongated looping fibers of chitin affected by $GbpA_{D1-3}$ are also present. **B** Close-up view of individual chitin fibers coated with $GbpA_{D1-3}$. **C** Cluster of $GbpA_{D1-3}$-chitin aggregate with surrounding individual fibers. **D** Two smaller clusters of $GbpA_{D1-3}$- chitin aggregates with individual fibers extending from them.

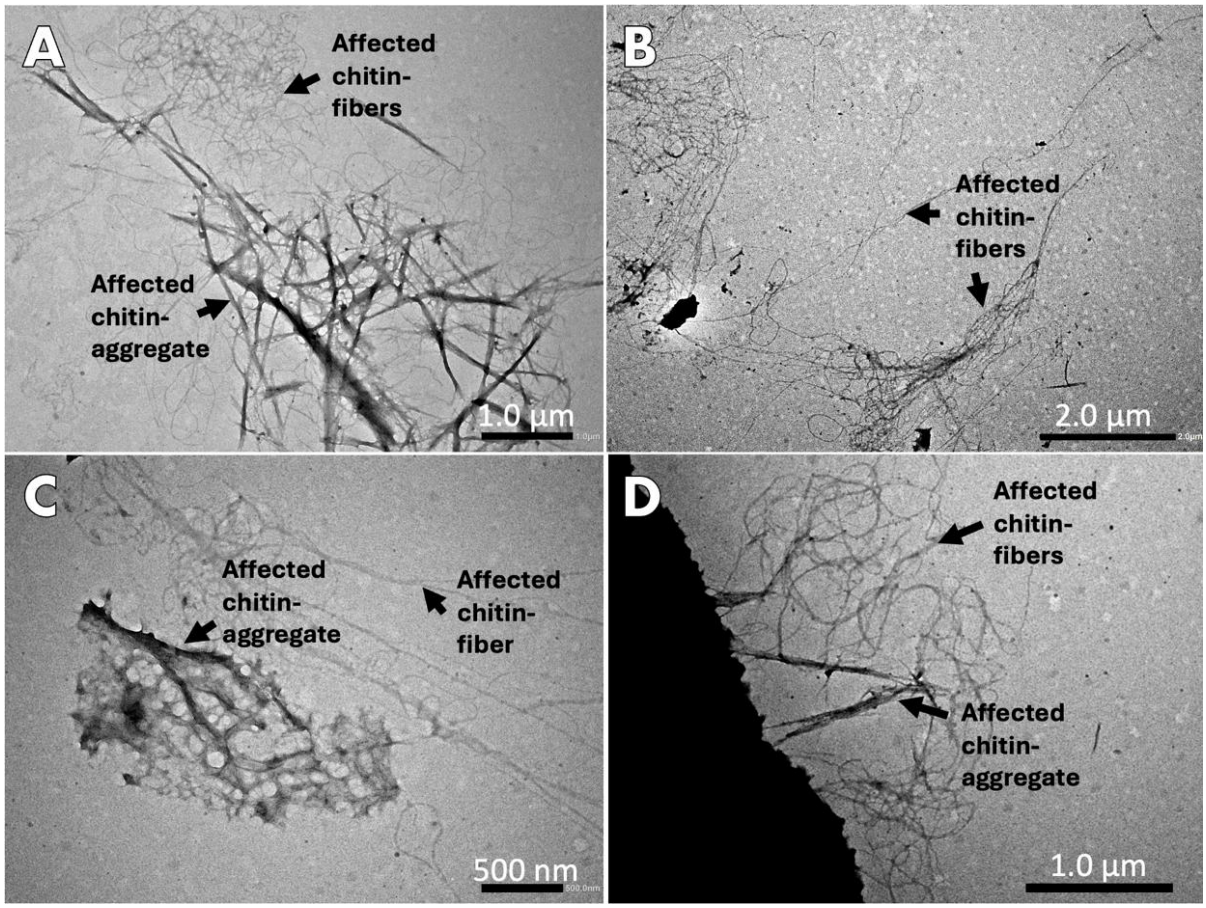

Fig S6 Extended TEM micrographs of chitin fibers incubated with $GbpA_{D1}$. A Cluster of aggregated chitin fibers incubated with $GbpA_{D1}$, with individual fiber loops extending above from the aggregate. Compared to chitin complexes with $GbpA_{fl}$ (Fig S4) or $GbpA_{D1-3}$ (Fig S5), the chitin fibers appear sharper and more defined. **B** Close-up view at individual $GbpA_{D1}$-coated chitin fibers. **C** Cluster of $GbpA_{D1}$-chitin aggregate with surrounding individual fibers. **D** Two smaller clusters of $GbpA_{D1}$-chitin aggregates with individual fibers extending from them. The aggregates clearly have more jagged edges than for the longer GbpA constructs (Fig S4-5).

Table S1*:* Fitting parameters chitin cylinder model with power law

Sample	Length (Å)	Kuhn length (Å)	Radius (Å)	Decay exponent	χ^2	SASBDB ID
Chitin (3 mg/mL), 100% D_2O	5000	318 +/- 12	15.99 +/- 0.02*	3.99 +/- 0.01	2.78	SASDW42

** Note that the statistical error from the modeling is likely much lower than the actual uncertainty of the value.*