The structure of the cell wall of *Staphylococcus aureus* studied with neutron scattering and magnetic birefringence

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The cell wall complex was extracted from *Staphylococcus aureus* and characterized in suspension by means of small-angle neutron scattering and magnetically induced birefringence. The neutron scattering measurements show that the complex has a thickness of ~420 Å, a mass per unit area of 93 ± 11 daltons/Å^2 and is ~75% water by volume. The neutron scattering density is higher near the surface than in the interior in accordance with a trilamellar structure. The magnetically induced birefringence measurements demonstrate that a high degree of magnetic orientation is possible due to the anisotropic nature of the wall complex structure.

Staphylococcus aureus *Peptidoglycan* *Cell wall* *Neutron scattering, small angle* *Magnetic orientation*

1. INTRODUCTION

The cytoplasmic membrane of a Gram-positive bacterium is surrounded by a semi-rigid wall which acts to maintain the cell's integrity in different environments. The wall is porous to cellular products, nutrients, and ions and is involved in such phenomena as opsonization, phage binding and pathogenicity [1,2]. The staphylococcal wall is typical of Gram-positive bacteria and has three main constituents: peptidoglycan, ribitol teichoic acid and protein [3]. Peptidoglycan, the major structural component, is a highly cross-linked polysaccharide–peptide polymer whose main function is to maintain the shape and rigidity of the wall. Teichoic acid, which can make up to 20–40% (by wt) of the staphylococcal wall [4] is composed of ribitol, phosphate, sugar residues and D-alanine and is covalently bonded to the peptidoglycan [5]. It is a negatively charged polymer which can bind Mg^{2+} necessary for various bacterial enzyme systems [6], acts as an antigen [7] and is involved in phage attachment [1]. The proteins in the staphylococcal wall make up to 10% by wt of the whole wall and are probably covalently bonded to the peptidoglycan matrix but can be removed by proteolytic enzymes without damaging wall integrity [2].

Here, we report measurements on suspensions of a cell wall complex isolated from *Staphylococcus aureus* type 42D. The mass/unit area and the radius of gyration of thickness is determined by means of neutron scattering. The thickness is found to be ~420 Å which is ~100 Å larger than that seen in electron micrographs [8,9]. It is shown that the neutron scattering density is somewhat higher near the surfaces than in the interior. This supports a trilamellar structure [9] but it is not clear if this is due to a partition of the different chemical components or only to an increase in packing density as suggested in [9]. The wall complex can be highly oriented in a strong magnetic field which suggests a new method for preparing highly oriented samples for structural studies.

2. MATERIALS AND METHODS

2.1. *Sample preparation*

Staphylococcus aureus type 42D was grown aerobically in oxoid CM1 nutrient broth, harvested

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and washed in water then suspended at ~10 mg/ml in 0.1 M barbiturate–acetate buffer at pH 7. Preparations were shaken with an equal volume of glass beads in a Braun homogeniser for ~5 min. Glass beads and whole cells were removed by filtration and centrifugation, and a wall preparation obtained from the supernatant by centrifugation at 10 000 rev./min for 10 min. The thoroughly homogenized walls were suspended in 2–4% sodium lauryl sulphate, heated to 100°C for 5–10 min, then centrifuged at 10 000 rev./min for 10 min. Deposited walls were washed once with water, 3 times with 1 M NaCl, 4 times with water. The method is from H.J. Rogers (communicated to W.A. Hamilton) and is based on methods in [10,11]. No absorption peaks were observed in absorbance-scattering spectra (200–700 nm) from suspensions. As no explicit measures were taken to remove the covalently bound protein it probably remained attached but as a minor component.

The D_2O content and pH of suspensions of freshly prepared complex (non-freeze-dried) were adjusted by repeatedly forming very loose pellets on a bench centrifuge, the supernatant was removed on each occasion and the sample resuspended. Thus any small fragments which might have complicated the interpretation of the results were removed with the supernatant. The buffer was sodium acetate 7.5 mM, sodium barbitone 7.5 mM, and 1 mM NaCl adjusted to pH 7 with a solution of 1 M HCl and 0.02 M NaCl. Samples that had been freeze-dried and resuspended proved to be highly aggregated.

The concentration which varied between 2–30 mg/ml was measured by weighing a known volume then drying it to constant weight in vacuum. Phosphate content was determined as in [12] and assuming it is only present in the teichoic acid the complex was 20% teichoic acid, the remainder being mostly peptidoglycan and counter ions. Experiments were conducted at 22°C.

2.2. *Neutron scattering*

The neutron measurements were made with the small-angle scattering camera D11 [14] at the Institut Laue-Langevin. The sample to detector distance was 10 m and the wavelength was 9.0 Å. $I(Q)$ was obtained as in [34].

The coherently scattered intensity $I(Q)$ from sheet-like monodispersed particles (without correlations in position and orientation) can be, over a limited range of Q ($Q < 1/R_t$), approximated by the following equation [16]:

$$\frac{I(Q)Q^2}{c} = 2\pi N_A \mu \left(\frac{\Sigma b}{M_r} - \frac{V_F}{M_r}\rho_s\right)^2 \exp(-R_t^2 Q^2) \quad (1)$$

where:

- $I(Q)$ = intensity of the scattered neutrons divided by the thickness and transmission of the solution and by the incident neutron flux;
- Q = $(4\pi \sin\theta)/\lambda$; λ is the wavelength and θ is half the scattering angle;
- c = concentration;
- N_A = Avogadro's number;
- μ = mass/unit area, in daltons/Å^2;
- $\Sigma b/M_r$ = scattering length/unit mass;
- V_F/M_r = the solvent excluded volume/unit mass and is equal to $\bar{v}/N_A$ where $\bar{v}$ is the partial specific volume;
- ρ_s = solvent scattering density;
- R_t = radius of gyration of thickness.

As only the ratios $\Sigma b/M_r$ and V_F/M_r appear it is not necessary to know the number of subunits per particle, and polydispersity in size has no effect provided the sheet-like approximation is valid for all the particles. The equation is a satisfactory approximation for values of $Q \lesssim 1.0/R_t$; the actual extent of its validity is determined experimentally. At a given contrast the plot of $\ln[I(Q)Q^2]$ against Q^2 yields both R_t^2 and, by extrapolation, the value of $I(Q)Q^2$ at $Q = 0$,$[I(Q)Q^2]_0$. Usually Σb varies linearly with solvent D_2O content (i.e., with ρ_s) and thus so does the square-root of $[I(Q)Q^2]_0$. In the zero contrast condition $[I(Q)Q^2] = 0$, $V_F = \Sigma b/\rho_s$ and $\bar{v} = N_A \Sigma b/\rho_s M_r$ where Σb and ρ_s are the match point values. The value of μ is estimated most accurately in H_2O solvent when the particle is fully protonated and the effect of an error in V_F or $\bar{v}$ is reduced. There are two definitions of contrast in the literature $\bar{\rho}$ and $\bar{\rho}'$. $\bar{\rho} = \rho_M - \rho_s$ and is the difference between the average scattering density when the particle is in the zero-contrast condition, ρ_M, and the actual scattering density of the solvent. $\bar{\rho}' = \Sigma b/V_F - \rho_s$ and takes account of the H/D exchange (i.e., change in Σb) that occurs when the H_2O/D_2O ratio of the solvent is altered. $\bar{\rho}'$ is the actual contrast but does require the value of $\Sigma b/V_F$ to be known for each H_2O/D_2O ratio of

the solvent. At zero contrast $\bar{\rho} = \bar{\rho}' = 0$, otherwise $|\bar{\rho}| \geq |\bar{\rho}'|$.

By analogy with the radius of gyration of a whole particle [15] the measured radius of gyration of thickness can be split into 3 components:

$$R_t^2 = R_{t,0}^2 + (\alpha/\bar{\rho}) - (\beta/\bar{\rho}^2 \quad (2)$$

$R_{t,0}$ is the radius of gyration of thickness at infinite contrast and is equal to the radius of gyration of thickness of a homogeneous particle with the same thickness. If such a particle has a thickness d then $12R_{t,0}^2 = d^2$. α and β are given by the one-dimensional form of the equations in [15].

The plot of R_t^2 against $1/\bar{\rho}$ has an intercept and slope at infinite contrast which give $R_{t,0}$ and α, respectively. A positive α means that material with high scattering density is preferentially on the outside and vice versa if α is negative. If the line given by the above plot is curved β is non-zero and the center of scattering mass moves with contrast. If a straight line is obtained $\beta = 0$ which means in terms of scattering density the sheets are centrosymmetric.

2.3. *Magnetically induced birefringence*

The magnetic birefringence (Δn) was measured as in [17]. $\Delta n = n_{\parallel} - n_{\perp}$ where $n_{\parallel}$ and $n_{\perp}$ are respectively the refractive indices ($\lambda = 6328$ Å) parallel and perpendicular to the field.

As the magnetic orientation of diamagnetically anisotropic particles is the analogue of induced electric dipole orientation the theory of the latter in [24,25] can be easily adapted for the former. Consider a dilute suspension of non-interacting rigid particles which have an axis of rotational symmetry in their diamagnetic and optical properties. (Let $\Delta\chi$ = diamagnetic anisotropy/particle; H = magnetic field; k = Boltzmann constant; T = absolute temperature; Φ = orientation factor.) In a magnetic field these particles experience a torque (maximum value $\Delta\chi H^2/2$) which is opposed by the randomizing effects of Brownian motion and so Φ is a function of the ratio $\Delta\chi H^2/2kT$. If $\Delta\chi$ is positive the particle symmetry axis moves nearer to the field direction and at full alignment parallel to the field $\Phi = 1$. Whereas if $\Delta\chi$ is negative the particles rotate to bring their symmetry axis in a plane perpendicular to the field and with all the particles so oriented $\Phi = -0.5$ (thus $1 \geq \Phi \geq -0.5$). The variation of Φ as a function of $\Delta\chi H^2/2kT$ is different for positive or negative $\Delta\chi$ [24,25]. We have used the magnetically induced birefringence Δn to monitor orientation, $\Delta n = \Delta n_s \Phi$ where Δn_s = birefringence when $\Phi = 1$. As Δn_s and Φ can be positive or negative the sign of $\Delta\chi$ cannot be directly deduced from that of Δn. By fitting the measured Δn curve, if it covers a sufficient range, with the theoretical plot for Φ, $\Delta\chi$ can be determined in sign and magnitude [24,25].

3. RESULTS AND DISCUSSION

3.1. *Neutron scattering*

The plots of $I(Q)Q^2$ against Q^2 gave a straight line within a range $0.6 < QR_t < 1.2$ (example in fig.1). Fig.2 shows that the plot of $([I(Q)Q^2]_0/c)^{1/2}$ against D_2O content is linear in accordance with the Guinier approximation for monodispersed sheets.

The chemical composition of the peptidoglycan and teichoic acid of *Staphylococcus aureus* type 42D has not been published to our knowledge. We have therefore taken the average subunit composition of the peptidoglycan and teichoic acid from [13] which is very similar to that given in [2], the slight differences have negligible effect on the fol-

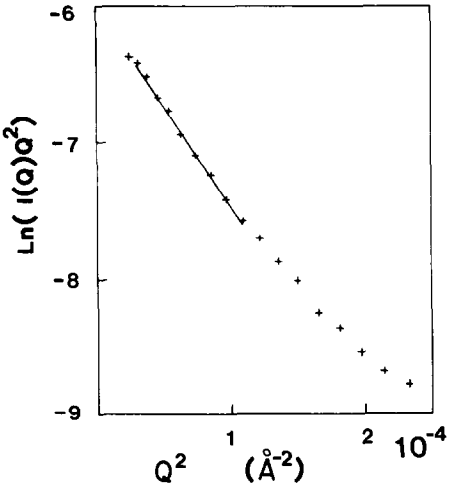

Fig.1. The logarithm of the scattering intensity, $I(Q)$, times Q^2 plotted against Q^2. The slope of the least squares-fitted straight line is equal to the square of the radius of gyration of thickness, R_t, and the intercept at $Q = 0$ gives $[I(Q)Q^2]_0$. The sample was in H_2O buffer and $c = 8.4$ mg/ml.

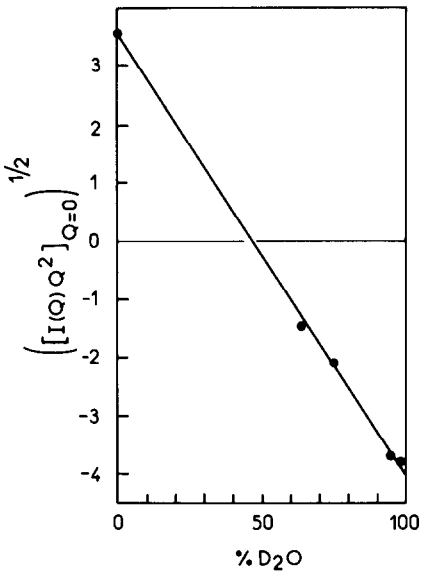

Fig.2. The variation of the square root of the intensity $I(Q)Q^2$ (divided by concentration) extrapolated to Q = 0 as function of D_2O content. From the intercept the zero contrast point of wall complex is 45.5 ± 0.5%. D_2O which corresponds to an average scattering density of 2.68×10^{10} cm^2.

lowing analysis. (Wyke et al. [22] found no major differences in the composition of the various strains of *S. aureus* they examined.) Hence the residues in an average subunit of peptidoglycan are: isoglutamine, 1; lysine, 1; alanine, 2.25; glycine, 4.9; serine 0.1; *O*-acetyl, 0.6; *N*-acetyl-glucosamine, 1; and *N*-acetylmuramic acid, 1 (M_r = 1201; $\Sigma b(H_2O) = 28.19 \times 10^{-12}$ cm; $\Sigma b(D_2O) = 48.37 \times 10^{-12}$ cm). Also the average subunit of teichoic acid contains 1 mol of the sodium salt of *N*-acetylglucosaminylribitol phosphate and 0.4 mol of alanine (M_r = 467.5; $\Sigma b(H_2O) = 9.49 \times 10^{-12}$ cm; $\Sigma b(D_2O) = 16.5 \times 10^{-12}$ cm). The complex used in these experiments contained 80% peptidoglycan and 20% teichoic acid by weight (the influence of the presence of counter ions on the results will be discussed later). As the average protein [20] has a $\Sigma b/M_r$ very similar to that of peptidoglycan the small amount of protein that may be associated can be considered along with the peptidoglycan. By summing the volumes taken from [18–21] of the components the partial specific volume $\bar{v}$ = 0.71 cm$^2 \cdot$ g^{-1}. In the zero contrast condition (i.e., in 45.5%, D_2O) $\bar{v} = N_A \Sigma b/\rho_S M_r$ which gives a measured value for $\bar{v}$ (= 0.70 ± 0.01 cm$^2 \cdot$ g^{-1}) very close to the above estimate. It is assumed that the D substitution of exchangeable hydrogens is equal to the fraction of D_2O in the solvent which is reasonable as the wall is an open structure and therefore easily accessible to solvent.

The mass/unit area, μ, can be found using eq. (1). The value of $[I(Q)Q^2]_0/c$ in H_2O buffer and the measured partial specific volume are used. The ratio $\Sigma b/M_r$ and $\bar{v}$ are only weakly influenced by the uncertainty in the chemical composition of the cell wall of *Staphylococcus aureus* type 42D. We find that μ = 91 ± 9 daltons/Å^2. Counter ions may make up as much as 8.5% of the dry weight of the cell wall [2]. To investigate how these would influence the results the calculations were redone assuming 6% by wt of sodium counter ions in the complex in addition to the 1% included above with the teichoic acid. Now the cell wall is considered to be composed by weight of 74% peptidoglycan, 20% teichoic acid (sodium salt) and 6% Na^+. For this composition the measured $\bar{v}$ is 0.68 ± 0.01 cm$^2 \cdot$ g^{-1} and the mass/unit area, μ, is 95 ± 9 daltons/Å^2. As sodium contributes an additional 6% to the mass the value of μ for the peptidoglycan–teichoic acid complex is almost unchanged.

The plot of the square of the radius of gyration of thickness, R_t, against inverse contrast, $1/\bar{\rho}$, gives a straight line (fig.3). The intercept at infinite contrast ($1/\bar{\rho}$ = 0) corresponds to R_t = 121 ± 6 Å and when multiplied by $12^{1/2}$ gives the thickness of the complex to be 420 ± 20 Å which is significantly larger than that seen in electron micrographs ~300 Å [9,27]. This supports the long held suspicion that the preparation for electron microscopy can result in wall contraction. However, ~400 Å is near to half that estimated for living bacteria by means of light scattering [26]. The latter samples are very complex and the difference may be in part due to the presence of the cytoplasmic membrane. The method we used to extract the wall complex is similar to that used by others [9–11,27] but nevertheless could give rise to changes of structure.

The slope of the line in fig.3, is positive which indicates that the scattering density is somewhat higher near the surfaces than in the interior which is in harmony with the trilamellar stain distribution seen in electron micrographs [9]. By calculation the neutron scattering densities of pep-

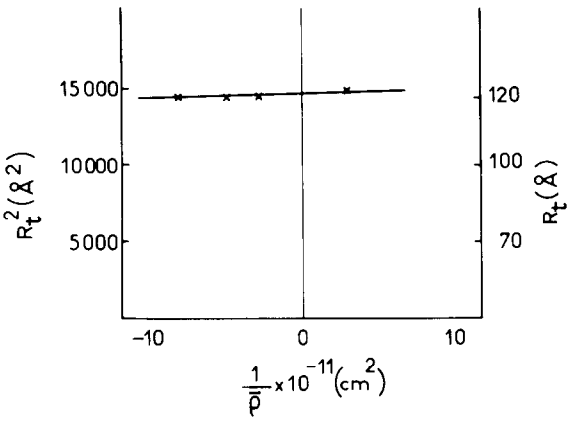

Fig.3. The square of the radius of gyration of thickness, R_t, versus the reciprocal of the contrast $1/\bar{\rho}$. The intercept at infinite contrast ($1/\bar{\rho} = 0$) is 121 ± 6 Å and the slope, α, is 1.0 ± 0.2 × 10^{-3}.

tidoglycan and teichoic acid are rather similar (although the latter is slightly higher and will be increased if it associates preferentially with counter ions). Thus it is not possible to deduce the structural origins of the positive slope (fig.3); if may arise due to an increase in anhydrous packing density near the sufaces which may or may not be [9] related to a change in the relative proportions of the components. We calculate that a sheet of thickness 420 Å with an outer 20 Å layer on the surfaces which have a scattering density 10% higher than the interior would give the observed slope in fig.3. But there are many possible models. The line in fig.3 does not show curvature (although careful measurements at low contrast are necessary to be sure), therefore β in eq. (2) is approximately zero and the complex is close to symmetric in neutron scattering density. However, as pointed out above peptidoglycan and teichoic acid may have similar scattering densities so there could be asymmetry and yet $\beta = 0$.

Making use of the measured thickness, mass per unit area and partial specific volume we find that the solvent occupies 74.5 ± 3% of the cell wall volume if there is 1% by wt of sodium. The density of the complex is 1.11 ± 0.01 g/ml (taking the solvent density as 1 g/ml) which is similar to the wet density (1.07 ± 0.02 g/ml) found in [23] for *S. aureus* Duncan. If the complex has a total of 7% of Na^+ the volume taken up by the solvent is changed little but the density is somewhat larger (1.125 ± 0.012 g/ml). As has been pointed out in [27] these densities are much smaller than those used in [29].

3.2. *Magnetic orientation of cell wall*

The curvature of the magnetically induced birefringence (fig.4) demonstrates that the cell wall complex orients to a high degree. The particles rotate to bring the axis of smallest numeric diamagnetism nearer to the field direction and as the induced birefringence is negative, the direction of minimum diamagnetic susceptibility and minimum optical polarizability are the same [35]. With a negative $\Delta\chi$ the theoretical curve can be adjusted to superimpose almost exactly on the experimental data whereas with a positive $\Delta\chi$ a close fit could not be obtained (fig.4). This is good support for a negative $\Delta\chi$ but is inconclusive because the particles are probably not monodisperse either in size or shape.

The molecular origin of the anisotropy is not clear. As there are no aromatic groups present, which have a relatively large diamagnetic anisotropy, the important groups are probably the pep-

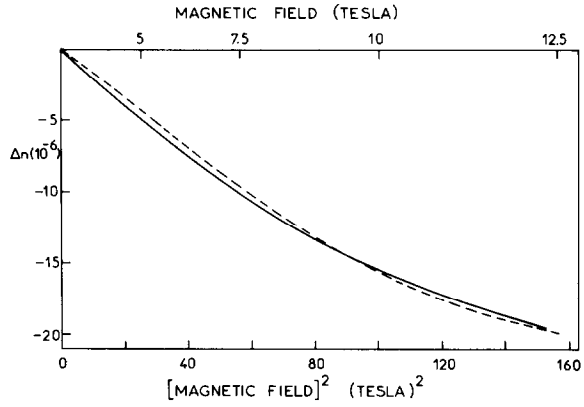

Fig.4. The variation of the magnetically induced birefringence, Δn, as a function of the square of the field strength, H (continuous line). The field varied linearly with time and was swept up (10 min) and down again (10 min). With faster sweep times hysteresis is observed because the large particles rotate slowly and are not in equilibrium with the field. The dashed line shows the best fit obtained using a positive $\Delta\chi$, $\Delta\chi = 3.7 \times 10^{-22}$ JT^{-2} and in maximum field $\Phi = 0.8$. With $\Delta\chi = -2.2 \times 10^{-22}$ JT^{-2}, $\Phi = -0.35$ in maximum field and the theoretical curve superimposes almost exactly on the measured curve.

tide bonds [31] and the polysaccharide chains (including their *N*-acetyl groups). If, as is claimed in [27,28], the peptide component has little preferred orientation the diamagnetic anisotropy must come largely from the polysaccharide which is oriented parallel to the wall surface [27–30]. However, our measurements were made on suspensions while in [27,28] partly dehydrated layers, which may have suffered some distortion to their structure, were used. Therefore, although it is not possible to be sure without more reliable information about the peptide structure the evidence suggests that the magnetic orientation is due to the parallel polysaccharide chains. Pure polysaccharide in solution can be highly oriented in a magnetic field (J.T. in preparation). A limited degree of orientation of some cell walls but not *S. aureus* has been produced by partly drying [27]. Perhaps better orientation at higher hydration will result if samples are formed in a strong magnetic field. This technique has been successful with cell membranes [31–34] which are also sheet-like.

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