FbsA-Driven Fibrinogen Polymerization: A Bacterial "Deceiving Strategy"

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We show that FbsA, a cell wall protein of the bacterium *Streptococcus agalactiae*, promotes large-scale aggregation of human plasma fibrinogen, leading to the formation of a semiflexible polymerlike network. This extensive aggregation process takes place not only in solution, but also on FbsA-functionalized colloidal particles, and leads to the formation of a thick layer on the bacterial cell wall itself, which becomes an efficient mask against phagocytosis.

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Protein aggregation processes, either due to structural mutations or to metabolic dysfunctions, are increasingly seen as the main source of serious pathologies that include for instance Alzheimer, Parkinson, and prionic diseases [1]. Yet, extensive protein association effects can also be brought in by external microbial agents. Pathogenic bacteria have indeed developed a number of strategies to overcome the protective barriers of their hosts, which range from hampering recognition by the immune system, to hedging against antibacterial effectors and escaping from phagocyte attack [2]. The latter goal may be pursued by inhibiting phagocyte chemotaxis, killing phagocytes before or after ingestion through secreted toxins, or simply resisting engulfment through hard polysaccharide capsules. But phagocytosis can also be hindered by more subtle strategies, aimed at deceiving phagocyte recognition. The aim of this Letter is show that this is one of the *modi operandi* of *Streptococcus agalactiae* (also named GBS, group B *Streptococcus*), the main microbial cause of bacterial sepsis and meningitis in neonates. The "deceiving strategy" we shall describe exploits the aggregation of fibrinogen (Fbg), a 340 kDa plasma glycoprotein playing a key role in hemostasis. Under the catalytic action of thrombin, a protease enzyme, Fbg polymerizes to form fibrin gels, the main constituent of blood clots [3]: In the present case, Fbg aggregation is conversely induced by FbsA, a GBS cell wall protein driving the buildup of a Fbg layer on bacterial cells, possibly seen by phagocyte receptors as "harmless."

FbsA proteins expressed from different GBS strains vary in molecular weight and composition: however, all different isoforms of FbsA contain, as a distinctive feature, a variable number of tandem repeats composed of 16 amino acids, which protrude outside the GBS cell wall. Recently [4], FbsA expression has been assuredly identified as the main source of GBS affinity for human Fbg [5,6]. In addition, survival assays yield clear evidence that GBS bacterial strains lacking the gene coding for FbsA (ΔfbsA GBS) are more prone to phagocytosis than wild-type (WT) GBS [4]. Yet, neither the microscopic interaction mechanism, nor the specific action of FbsA on Fbg are so far fully understood. In this Letter, we first prove that FbsA/Fbg interaction cannot be envisaged as a simple binding of the two proteins: rather, small amounts of FbsA are sufficient to induce a *huge scale aggregation* of fibrinogen leading to the formation of a semiflexible, polymerlike network. Then, we show that similar aggregation processes take place both on the cell wall of GBS and on the surface of model colloidal particles laden with FbsA. Finally, we show that phagocytosis of "Fbg-laden" bacteria is severely hindered, while no similar reduction is seen for ΔfbsA GBS.

Recombinant FbsA, carrying 19 internal repeat units (MW 33 kDa), was produced and purified as described previously [4] and ΔfbsA GBS was obtained by deletion of the FbsA gene in the chromosome of the GBS 6313 strain according to the method described in [4]. Human Fbg (Calbiochem, CA) was purified from fibronectin and other contaminants using a gelatin-Sepharose ffinity column. FbsA was added to 0.3 μM Fbg solutions in Tris-HCl 20 mM (pH 7.4) at variable molar ratio, and the kinetics of aggregation followed by dynamic light scattering (DLS) at 25 °C as a function of the time t_{agg} after mixing. We first discuss DLS results obtained at a FbsA:Fbg molar ratio of 1:10 and fixed scattering angle $\vartheta = 90°$ corresponding, at the wavelength $\lambda = 532$ nm of the laser source, to a scattering vector $q = 3.15 \times 10^5$ cm^{-1}. As shown in the main body of Fig. 1, the addition of FbsA yields a rapid and substantial increase of the decay time of the correlation functions $g(t)$, which grows by 2 orders of magnitude in less than two hours. For $t_{\mathrm{agg}} \gtrsim 10^3$ s, all correlation functions are well fitted, except at very short times, by a stretched exponential $g(t) = \exp[-(t/\tau)^{\alpha}]$, where the stretch exponent $\alpha = 0.70 \pm 0.05$ does not appreciably depend on t_{agg}. Yet, close inspection shows that, at variance with a pure stretched exponential, $g(t)$ retains a finite slope for $t \to 0$, behaving at short time as a simple exponential

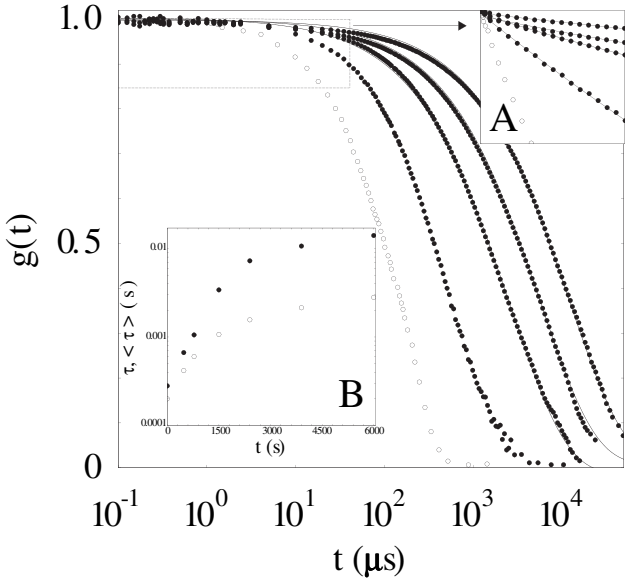

FIG. 1. Main body: $g(t)$ for a Fbg + FbsA solution at molar ratio 1:10 taken 8, 25, 40, and 100 min after mixing (●, from left to right) and for pure Fbg (○). Full lines are stretched exponential fits to $g(t)$ for $t_{agg} \gtrsim 10^3$ s. Inset A: Semilog plot of the short-time behavior for the same data. Inset B: Short-time (○) and average (●) decay time of $g(t)$.

$g(t \to 0) \sim \exp(-t/\tau)$. Both τ and the average decay time $\langle\tau\rangle = \int_0^\infty g(t)dt$ [which for a stretched exponential is given by $\langle\tau\rangle = \alpha^{-1}\Gamma(\alpha^{-1})$, where $\Gamma(x)$ is the Euler gamma function], shown in the lower left inset of Fig. 1, display a fast initial exponential growth, while they tend to saturate at longer t_{agg}.

Substantial Fbg aggregation takes place even at much lower FbsA molar fraction. Table I gives the apparent diffusion coefficients $D = (q^2\tau)^{-1}$ obtained 20 min after mixing FbsA to Fbg in molar ratio R, scaled to the diffusion coefficient D_0 for pure Fbg. A noticeable decrease of D/D_0 is already evident for $R = 1/300$.

At a first inspection, the peculiar shape of the data shown in Fig. 1 is akin to what is expected for semiflexible polymer solutions [7]. Beyond a crossover time τ_q, which can be interpreted as the characteristic relaxation time of a chain bending mode of wavelength $2\pi/q$, the dynamic structure factor of the latter is indeed predicted to decay as a stretched exponential with $\alpha = 3/4$. This behavior becomes dominant in the limit $ql_p \ll 1$ (where l_p is the polymer persistence length), which also sets the limit of the approximations made in the derivation. A further distinctive prediction is that the rate of the initial exponential decay, which still dominates for $t \ll \tau_q$, scales as the cube of the scattering wave vector, an additional factor of q, besides the usual q^2 diffusive relaxation, arising since q also sets the probed length scale [8].

TABLE I. Reduction of the apparent diffusion coefficient as a function of FbsA : Fbg molar ratio R.

R	D/D_0
1:300	0.82
1:100	0.62
1:10	0.32
1:3	0.14

The former model has been successfully applied to account for DLS results on solutions of biopolymers like actin [9], desmin [10], and, most significantly, fibrin itself [11]. Careful tests of its theoretical predictions require, however, some caution [12]. First of all, the model rigorously applies only to *dilute* polymer solutions. In semidilute polymer networks, chain entanglement has a minor effect only when λ is much shorter than the mesh size, but in the opposite limit a pure exponential decay over the whole time range is recovered. In view of these limitations, it is rather surprising that the model works reasonably well even for *crosslinked* polymer networks. More dangerously, chain length polydispersity easily leads to a long-time stretched exponential decay. We have nonetheless performed a preliminary analysis of the angular dependence of the DLS correlation function. In order to limit kinetic effects, measurements were started about 1 h after mixing when, according to Fig. 1, aggregate growth has considerably slowed down, and data were accumulated only for the minimum amount of time needed to obtain a reasonable statistics. Figure 2 shows that, at variance with what has been found for instance for desmin [10], scaling $g(t)$ to the short-time decay rate $\Gamma = 1/\tau$ does not yield a single master curve. Indeed (see Inset A), the exponent α mono-

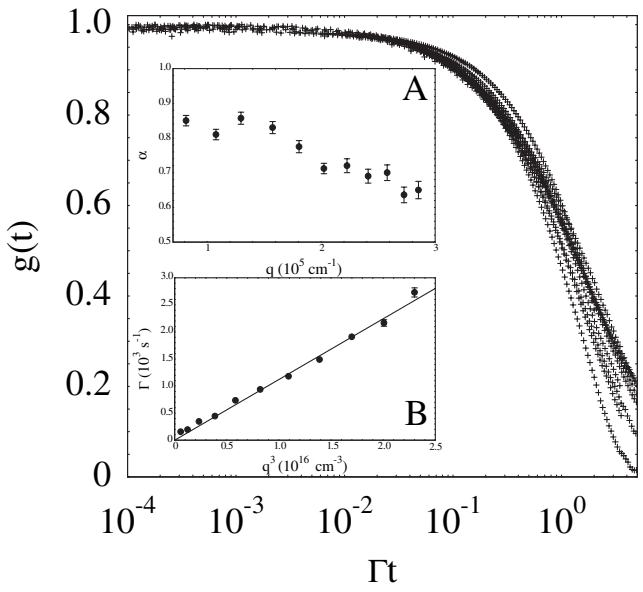

FIG. 2. Main body: Data obtained or FbsA + Fbg solutions at molar ratios 1:10 at various scattering angles 1 h after mixing as a function of Γt, where $\Gamma = 1/\tau$ is the short-time decay rate. The q dependence of the stretch exponent α and of the short-time decay Γ are shown in the Insets A and B.

tonically decreases by increasing q. This might be simply related to the variable ration between the network and the probed length scales ($0.2\ \mu\text{m} < 2\pi/q < 0.6\ \mu\text{m}$). Notice, however, that at large q, α falls *below* the value predicted in [7], suggesting a possible role of polydispersity. Nonetheless, the short-time decay rate Γ *does* scale rather well with q^3. The short-time dynamics of Fbg/FbsA aggregates is therefore consistent with that of polymerlike structures, but, in view of the former observations, we refrain from pushing this parallel further.

The striking effect of FbsA on Fbg solutions is visually confirmed by scanning electron microscope (SEM) images of dried solutions. Albeit probably bearing weak resemblance to the actual structure in solution, the SEM picture in Fig. 3(a) nonetheless shows the presence of a ''polymerlike'' network characterized by ''cages'' on a scale of a few μm. Notice that a fibrin network (upper inset) looks much more filamentous and interconnected.

The crucial difference between thrombin- and FbsA-induced Fbg aggregation is that, in the latter case, no sign of gelation is observed: up to an equimolar FbsA:Fbg ratio the speckle field is indeed always fully fluctuating and the correlation functions completely ergodic. Therefore, not only the solution macroscopic elastic modulus is negligible, but also Fbg microscopic dynamics is not arrested. At variance with gelation triggered by thrombin, no molecular cleavage yielding self-associating Fbg fragments can be easily envisaged: therefore, the presence of an extended network of chemical cross-links can be excluded. The former evidence points out that, up to an Fbg concentration of 0.3 μM, the solution is not even a *physical* gel, suggesting [at variance with the appearance of dried samples in Fig. 3(a)] a rather loose structure.

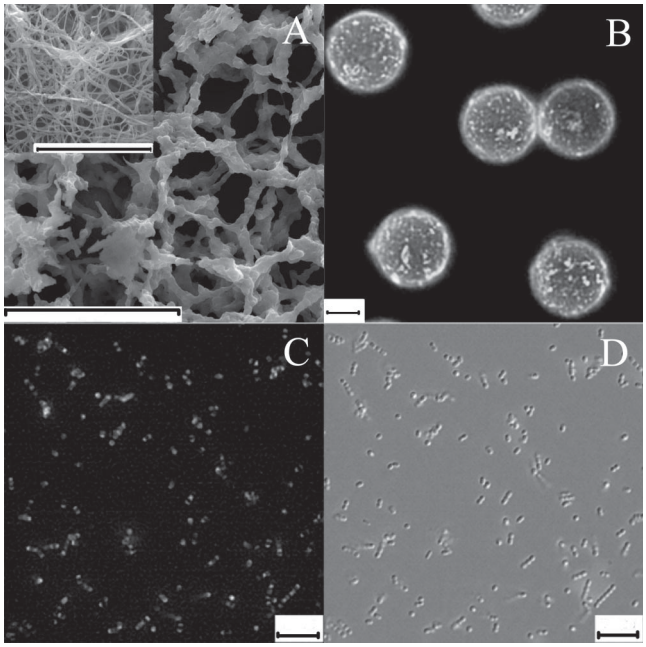

FIG. 3. (a) SEM image of a dried FbsA + Fbg solution at 1:10 molar ratio. A SEM image of a fibrin gel is shown for comparison in the small upper inset. (b) Fluorescence image of Fbg aggregation of FbsA-laden colloids. (c) Fluorescence image of Fbg localization on GBS cells. The same field is shown in (d) as a DIC image. Scale bars correspond to 10 μm.

Although the specific FbsA/Fbg molecular recognition process is still to be unraveled, we believe that charge-coupling effects may play a significant role in FbsA binding to Fbg. In preliminary DLS measurements as a function of the solution ionic strength I, we have indeed observed that Fbg aggregation is consistently *quenched* by the addition of salts. For instance, by adding 0.15, 0.5, and 1 M NaCl, the decay rate at fixed t_{agg} increases of a factor of 1.1, 1.7, and 2.2, respectively. Similar results are obtained by adding KCl or (for the same value of I) a divalent salt like $CaCl_2$. Since FbsA, with an isoelectric point $p\text{I} \simeq 11.3$, is strongly positively charged at $p\text{H} = 7.4$, while Fbg ($p\text{I} = 5.5$) has a net negative charge, FbsA/Fbg coupling implies charge complexation effects that may reduce Fbg hydrophilicity and increase its propensity to aggregation. Yet, specific ion effects (hardly accounted for by a simple electrostatic model) are present: in particular, F^- seems to be much less efficient than Cl^- in quenching aggregation. A detailed study of the addition of salts or disrupting agents will be presented in a forthcoming paper.

FbsA-induced fibrinogen aggregation takes place not only in solution, but also directly on the bacterial cell wall. GBS cultures (strain 6313, serotype III, see [13]), were exposed to fibrinogen tagged by Fluorescein isothiocyanate (FITC) for 2 h at 22 °C in an end-over-end mixer, and then centrifuged/resuspended to get rid of unbound FITC-Fbg. The microscope image in Fig. 3(c) shows that fluorescent fibrinogen is fully concentrated around the GBS cells, shown by the differential interferometry contrast (DIC) image of the same field in Fig. 3(d). Notice that GBS cells tend to be arranged in chains or lumps, which are typical of *Streptococcus* colonies, but seem to be enhanced by the presence of Fbg. Vice versa, no similar effect is found for ΔfbsA GBS. In order to show that Fbg surface accumulation is due to specific binding to FbsA, polystyrene colloids with a radius $R = 20\ \mu$m were laden with FbsA, which strongly adsorbs on the carboxylated particle surface due to its positive charge. Figure 3(b) shows that Fbg strongly binds to the particles, generating a ''granular'' surface pattern. Conversely, no Fbg binding is detected either on the bare particles or when other serum proteins like albumin are formerly adsorbed on the particle surface.

We now plan to show that the expression of FbsA by GBS, leading to extensive cell coverage by an abundant serum protein like Fbg, may be an adaptive strategy of GBS to survive macrophage attack. To this aim, we have compared macrophage phagocytosis for (a) GBS cells formerly exposed or not to Fbg, and (b) GBS cells expressing or not FbsA, by means of a standard invasion essay [14]. The method consists in counting the number of GBS

TABLE II. Thousands of colony forming units per 10^5 macrophage cells (averages of 6 invasion assays).

GBS strain	Fbg	No Fbg
WT	7.2 ± 0.8	69 ± 1
ΔfbsA	67 ± 1	67 ± 1

cells that, having being ingested by macrophages, resist to the antibiotics (surviving within the macrophage cells) and, once plated, form new colonies. Briefly, human macrophages (J111 cell line) were seeded at a concentration of approximately 5×10^5 cells into wells of a tissue culture plate, where they were incubated for 24 h. Approximately 5×10^7 cells of WT and ΔfbsA GBS were pretreated with or without 3 mg/ml of purified Fbg and added to the culture plates, which were further incubated for 2 h at 37 °C. Extracellular bacteria were killed by adding penicillin (5 mg/l) and gentamicin (100 mg/l) and, after washing away the residual antibiotics, the phagocyte monolayers were lysed with Triton X-100. Appropriate bacterial dilutions were plated to determine the number of viable internalized bacteria. Table II, summarizing the results of 6 independent invasion essays, shows that exposure to Fbg reduces phagocytosis of almost a factor of 10, while no similar effects are detected for the ΔfbsA mutant. Although these preliminary observations need to be supported by further studies aimed to better unravel GBS deceiving strategy, they strongly suggest that Fbg surface aggregation may be a relevant GBS adaptive strategy.

Protection against phagocytosis is probably not the only adaptive advantage of FbsA action on Fbg. Colonization and penetration of epithelial and endothelial tissues, which is an essential prerequisite for bacterial invasion, is certainly favored by the presence on the cell wall of a thick polymerized Fbg layer. FbsA-induced polymerization of Fbg is also very likely involved in favoring thrombus formation and endocarditis. Recently, Pietrocola *et al.* [15] have indeed shown that FbsA elicits a Fbg-dependent aggregation of platelets. In view of the present findings, this effect might be envisaged as a platelet immobilization within the polymer network of the aggregated fibrinogen, to which the platelets are known to adhere by the action of integrins. Albeit a full analysis of the clinical consequences of the evidence we have presented must be deferred to a forthcoming extended manuscript, we want to anticipate here that fibrinogen polymerization does not necessarily require the *actual presence* of GBS bacteria: adding to Fbg solutions a culture medium, where GBS colonies had been grown and then carefully filtered out, still leads to consistent and rather similar aggregation effects. This means that FbsA, or FbsA fragments, are probably released in the surrounding medium, directly acting as an aggregating agent. Finally, we want to point out that the Fbg aggregation phenomena we described are probably not exclusive of *Streptococcus agalactiae*. Several strains of group G streptococci [16] or different bacteria like *Staphylococcus aureus* [17], causing severe septic conditions and infective endocarditis, and *Helicobacter pylori* [18], the causative agent of peptic ulcera and gastric carcinoma, are known to express other Fbg-binding proteins, which may also promote large-scale Fbg association.

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