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Isolation, structural characterization, and immunological evaluation of a high-molecular-weight exopolysaccharide from *Staphylococcus aureus*

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Abstract

Colonization of implanted medical devices by coagulase-negative staphylococci such as *Staphylococcus epidermidis* is mediated by the bacterial polysaccharide intercellular adhesin (PIA), a polymer of β-(1→6)-linked glucosamine substituted with *N*-acetyl and *O*-succinyl constituents. The *icaADBC* locus containing the biosynthetic genes for production of PIA has been identified in both *S. epidermidis* and *S. aureus*. Whereas it is clear that PIA is a constituent that contributes to the virulence of *S. epidermidis*, it is less clear what role PIA plays in infection with *S. aureus*. Recently, identification of a novel polysaccharide antigen from *S. aureus* termed poly *N*-succinyl β-(1→6)-glucosamine (PNSG) has been reported. This polymer was composed of the same glycan backbone as PIA but was reported to contain a high proportion of N-succinylation rather than acetylation. We have isolated a glucosamine-containing exopolysaccharide from the constitutive over-producing MN8m strain of *S. aureus* in order to prepare polysaccharide–protein conjugate vaccines. In this report we demonstrate that MN8m produced a high-molecular-weight (> 300,000 Da) polymer of β-(1→6)-linked glucosamine containing 45–60% *N*-acetyl, and a small amount of *O*-succinyl (approx 10% mole ratio to monosaccharide units). By detailed NMR analyses of polysaccharide preparations, we show that the previous identification of *N*-succinyl was an analytical artifact. The exopolysaccharide we have isolated is active in in vitro hemagglutination assays and is immunogenic in mice when coupled to a protein carrier. We therefore conclude that *S. aureus* strain MN8m produces a polymer that is chemically and biologically closely related to the PIA produced by *S. epidermidis*.

Keywords: High-molecular-weight exopolysaccharide; *Staphylococcus aureus*; Polysaccharide intercellular adhesin

1. Introduction

Gram-positive staphylococci, primarily *Staphylococcus aureus* and *S. epidermidis*, are important facultative human pathogens. Together, they account for the majority of nosocomial sepsis cases reported each year.[1,2] Adherence and accumulation of these organisms to implanted medical devices can subsequently lead to systemic bacteremia and potentially fatal conditions such as endocarditis and ventilator-associated pneumonia.[3–5] Of mounting concern is the number of organisms exhibiting antibiotic resistance, particularly methicillin- and vancomycin-resistant *S. aureus*

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strains.[6,7] The increasing ineffectiveness of antibiotic therapies has led to the search for new avenues of protection. Vaccines that target bacterial exopolysaccharides, known to be important in mediating adherence and accumulation, are particularly appealing as new therapeutics.

One staphylococcal carbohydrate contributing to accumulation of bacterial cells is the polysaccharide intercellular adhesin (PIA) characterized by Mack and co-workers.[8] PIA isolated from *S. epidermidis* is a polymer composed of β-(1→6)-linked glucosamine with approx 85% of the amino groups substituted with *N*-acetyl, and a minor proportion of the sugar hydroxyl groups esterified as phosphates and succinates. PIA is an integral constituent of biofilm, an amorphous exopolysaccharide produced during the active growth phase. It is responsible for mediating cell–cell adhesion,[9,10] and, likely, also functions to shield the growing colony from host immune defenses.[11,12] PIA production is associated with the *icaADBC* gene locus[9] and has been demonstrated in vitro under certain defined growth conditions for several coagulase negative strains. PIA production can be modulated or lost in response to culture parameters such as substrate, medium formulation, temperature, and aeration,[13–15] and it has been difficult to show with certainty that organisms grown in vitro produce the same surface polysaccharides as those present in an in vivo infection. Moreover, accurate structural characterization of polysaccharide preparations has often been hampered by residual media contaminants, and this has occasionally led to the mistaken identification of what were believed to be novel antigens.[16–18]

Recently it has been shown that certain *S. aureus* strains also contain the *icaADBC* locus,[19] and these strains are able to elicit biofilms in vitro. Experiments with isogenic knockouts have demonstrated that biofilm production is lost when the *ica* locus is removed but can be restored when the mutant is complemented with an *ica*-containing plasmid.[19] Subsequent reports described the identification of an antigen similar to PIA, termed poly-*N*-succinyl-β-(1→6)-glucosamine (PNSG) or capsular polysaccharide adhesin (PSA), which consists of the same sugar backbone as PIA but contains primarily *N*-succinyl instead of *N*-acetyl, and is of significantly higher molecular mass.[20] The identification of a constitutively over-producing mutant strain (MN8m) allowed purification of the antigen in sufficient quantities for physiochemical characterization and subsequent immunogenicity studies.[21,22] Based on these claims, we sought to scale up the production of PNSG for evaluation of its immunoprophylactic value in additional animal models. However, we have recently reported that the MN8m strain produces a polymer of variable molecular mass, and that the identification of *N*-succinylation was incorrect.[23] In the current report we describe a thorough biochemical and structural characterization of the high-molecular-mass form of this polymer and unambiguously define it as β-(1→6)-glucosamine substituted with N-linked acetyl and *O*-succinyl. We show that previous NMR-based identification of *N*-succinylation[20,21] was due to a misinterpretation of analytical data resulting from hydrolysis artifacts. The purified polysaccharide was conjugated to carrier protein and used for evaluation of immunogenicity in mice. Since we had not demonstrated a definitive biological role for this polymer in adhesion, we wish to avoid characterizing it with one of the previously used acronyms and so refer to it in this publication as *S. aureus* exopolysaccharide (SAE).

2. Results

2.1. Purification of SAE polysaccharides

Previous reports describing isolation of the putative PNSG antigen from *S. epidermidis* and *S. aureus* indicated the need for harsh extraction conditions utilizing strong acid and processing steps at low pH to produce a soluble product.[20] We found these conditions to be unnecessary and were able to purify large quantities of exopolysaccharide starting from culture supernatant and using a minimal process consisting of membrane filtrations, enzymatic treatment for selective contaminant removal, and a chromatographic polishing step. This protocol resulted in overall process yields of 400 mg/L culture fluid which was significantly higher than the 0.5–2 mg/L reported for *S. epidermidis* strains.[20] Part of this was attributable to use of the overproducing MN8m strain, but optimization of fermentation conditions was more important for achieving high productivity. When MN8m was grown using chemically defined medium and conditions described for *S. epidermidis*, the final OD_{600nm} was approx eightfold lower than those obtained using the conditions described in this paper, and process yields were significantly reduced to levels of approx 1 mg/L. The use of complex seed medium also shortened the required fermentation time from 72 to <24 h. The lyophilized form of native SAE was soluble in DI water at concentrations of 1–5 mg/mL and in HEPPS buffer, pH 7.7 at 2 mg/mL, although a minor amount of insoluble material was detected after overnight dissolution. It was noted during preparation of N-deacetylated SAE that solubility of this minor component increased as the deacetylation reaction proceeded. The product contained only low levels of non-proteinaceous acidic contaminants as discussed below. The final anion-exchange chromatographic (AEC) polishing step reduced these contaminants to non-detectable levels as determined by NMR spectroscopy while providing high mass recoveries.

2.2. Characterization of SAE

Analysis of acid-hydrolyzed SAE by high-performance anion-exchange chromatography (HPAEC) showed glucosamine as the major monosaccharide component, indicating SAE is a homopolymer of glucosamine or its derivatives. Since the strong hydrolysis conditions required by this method could effectively remove any substituents from the component sugars, this method was used primarily to follow the SAE through the purification process.

2.3. Structure elucidation

In contrast to the simple spectral profile of PIA,[8] the one-dimensional (1D) ^{1}H NMR spectrum of native SAE at 600 MHz (Fig. 1(a)) shows a complicated spectrum with broad lines. Removal of all substituted groups by strong alkaline treatment of SAE yielded a much simplified spectrum (Fig. 1(b)). This spectrum shows only a single anomeric proton with a large coupling constant ($J_{HH} = 8.4$ Hz), indicating that the sugar backbone of SAE contains a monosaccharide

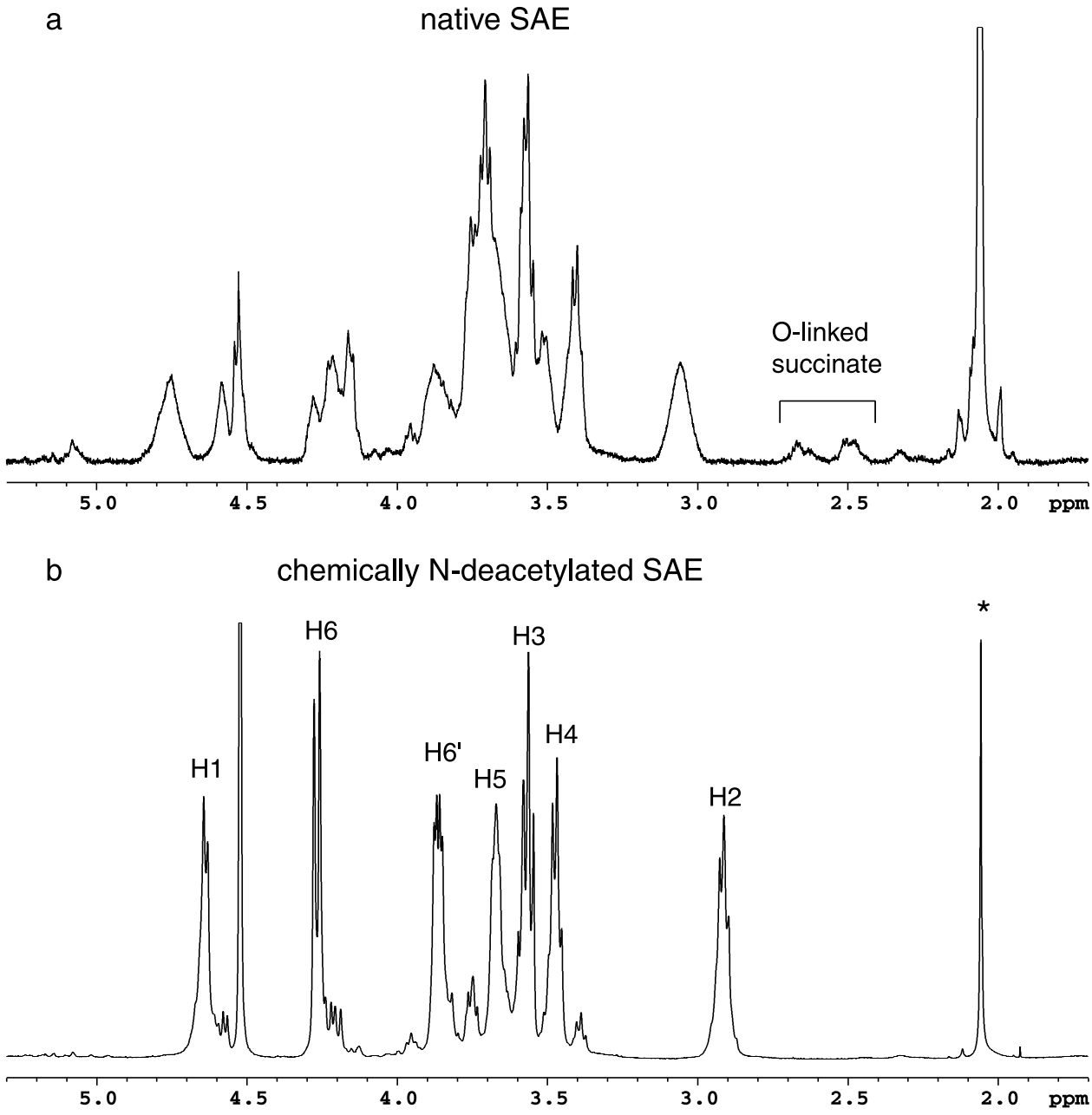

Fig. 1. 600-MHz 1D ^{1}H NMR spectra of: (a) native; and (b) chemically N-deacetylated SAE in D_2O at 50 °C. Peaks are labeled according to attached carbon centers in the β-GlcNH_2 residue. * Represents residual *N*-acetyl signal.

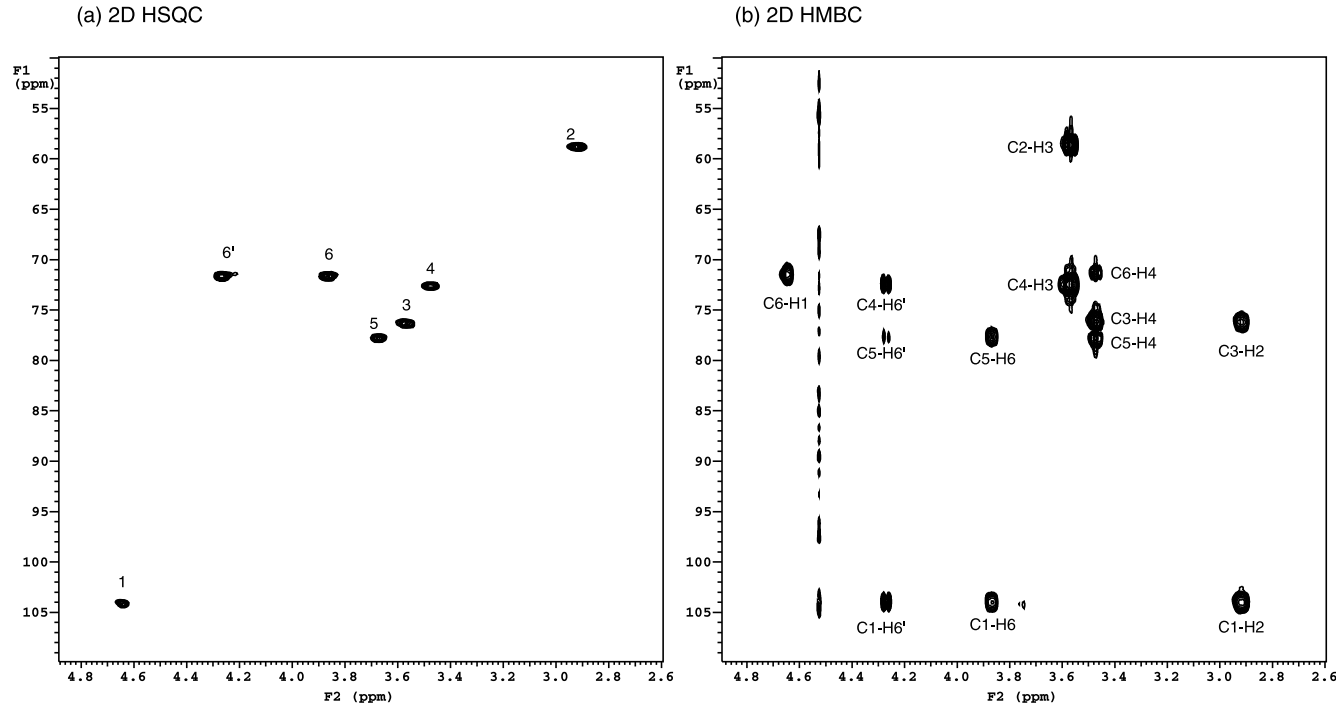

Fig. 2. 2D $^{1}H-^{13}C$ HSQC (a) and HMBC (b) spectra of N-deacetylated SAE in D_2O at 50 °C. Cross peaks are labeled according to carbon centers in the β-$GlcNH_2$ residue.

repeating unit with β anomeric linkages. The proton spin assignments by two-dimensional (2D) homonuclear correlation spectra (data not shown) showed large coupling constants throughout the spin system, indicating a glucopyranose configuration of the monomer units. The $^{1}H-^{13}C$ correlation spectra of N-deacetylated SAE is shown in Fig. 2. The complete ^{13}C assignments of the polymer were obtained by direct correlation to attached protons in the 2D HSQC spectrum (Fig. 2(a)). The glycosidic linkage assignments between monomer units were shown to be β-(1→6) from the observed correlations in the 2D HMBC spectrum (Fig. 2(b)) where long-range $^{1}H-^{13}C$ cross peaks were seen from anomeric protons to C-6 as well as C-6 protons to the anomeric carbon. The remaining correlations in the HMBC spectrum follow the well-established[24] pattern for the β glucopyranose spin system. The complete ^{1}H and ^{13}C assignments of N-deacetylated SAE are summarized in Table 1.

Once the polymer backbone was established, the structural heterogeneity leading to the complicated spectral features seen for the native SAE were examined by 2D NMR analysis in pure D_2O at 50 °C. Proton 2D homonuclear correlation spectra of native SAE (data not shown) clearly indicated the presence of at least four different anomeric resonances. The anomeric signals at 4.59 and 4.53 ppm were mapped to β-GlcNAc residues, the intensities of which were well correlated to those of the *N*-acetyl signal at 2.06 ppm. The remaining signals (broad resonance at 4.76 ppm) were assigned to β-$GlcNH_2$ residues based on the observed downfield shifts of the respective H-2 protons. This was confirmed by the pH sensitivity of H-2 protons (Fig. 1(a and b)). In contrast to N-deacetylated SAE, the 2D HSQC spectra (Fig. 3) show multiple correlations belonging to most carbon centers of both residues. The complete resonance assignments of native SAE reported in Table 1 were obtained by iterative analysis of all 2D correlation data as described previously.[24] The small chemical shift differences observed for each monosaccharide unit could be explained by nearest neighbor effects. For example, one of the spin systems assigned to β-GlcNAc (Table 1) agrees well with the previous assignments (after correcting for systematic shifts due to difference in reference standards) reported for PIA by Mack and co-workers[8] and hence was assigned to be from β-GlcNAc residues flanked by the same monosaccharide. The observed difference (mostly on the anomeric and C-6 carbon centers) of the remaining system assigned to β-GlcNAc could be the result of one or both flanking residues being the $GlcNH_2$. However, the quality of the NMR data precluded assigning those changes to a specific diad or triad. Similarly, the subtle chemical shift changes seen for $GlcNH_2$ could not be assigned to specific sequence pairing.

In addition to the aforementioned differences, the 1D spectrum of the native SAE contains two small signals centered at 2.65 and 2.50 ppm that could not be assigned to sugar spin systems. Two-dimensional correlation spectra show that these signals belong to a single

spin system and have attached carbon chemical shifts at 31.5 and 32.0 ppm. These chemical shifts agree well with the published values of sugar *O*-succinyl groups.[25] The total signal intensity of succinate proton resonances in the 1D spectra indicates that less than 10% of the repeating units are substituted with *O*-succinyl groups. Since only two free hydroxyl groups (O-3 and O-4) exist in the repeating units of the molecule, succinate groups have the potential to occupy either site on both monosaccharides. Detailed examination of 2D correlation data confirmed the existence of two sets of chemical shifts for *O*-succinyl groups (Table 1), both of which were mapped to β-GlcNAc residues based on spin assignments and the expected chemical shift changes due to O-acylation of sugar residues.[24] One minor spin system includes H-3/C-3 chemical shifts with large downfield shifts ($\Delta\delta = 1.48$ and 1.40 ppm for ^{1}H and ^{13}C, respectively) and the corresponding H-2/C-2 and H-4/C-4 resonances at 3.84/56.7 and 3.63/70.8 ppm. The assignment of these systems to a β-GlcNAc residue substituted with O-3 succinyl agrees well with the observed shift changes for adjacent carbon centers ($\Delta\delta = -2.0$ and -1.6 ppm for C-4 and C-2). The remaining spin system, which is somewhat less intense than the one discussed above, contains a similarly downfield shifted proton resonance (5.08 ppm) with the attached carbon at 73.2 ppm. The proton connectivity originating from this signal in 2D DQF-COSY shows overlapping coupling partners at 3.84 ppm. Since no protons attached to C-2 position with 3.84 ppm proton resonance and expected β-shift could be detected in the 2D HSQC spectra, the position of *O*-succinyl was suggestive of an O-4 of β-GlcNAc. While the observed α-shifts ($\Delta\delta = 1.6$ and 0.5 ppm for H-4 and C-4) agree well with this assignment, the respective β-shifts could not be calculated due to the difficulty of finding weak correlation peaks in the crowded region of HSQC spectra. The presence of two distinct *O*-succinyl groups are confirmed by the two sets of resonances (2.51/2.67 and 2.48/2.63 ppm) in the 2D homonuclear spectra. Based on the intensities of respective NMR signals, O-3 of β-GlcNAc appears to be the predominant site of *O*-succinyl attachment ($> 70\%$), while the remainder is on the O-4 of the same residue. The complete agreement between the signal integration of shifted ^{1}H resonances at 5.08 ppm and that of the *O*-succinyl signals indicates that there are no other minor sites of attachments. This data indicates that the weak NMR signals in the spectra are resulting from *O*-succinyl groups occupying both available sites on β-GlcNAc rather than one site being occupied by *O*-succinyl, and the remainder is shifted due to sequence heterogeneity. Since little is known about the specific enzyme(s) responsible for *O*-succinylation in *S. aureus*, it is unclear at this stage whether the distribution of *O*-succinyl is of biosynthetic origin or simply due to the migration of

Table 1
NMR chemical shift assignments at 50 °C for native and stripped SAE

Carbon position	Stripped SAE [a]	Native SAE [a]				
	-[GlcNH$_2$-β-(1→6)-]$_n$-	-[GlcNAc]$_n$-	**-GlcNAc**-GlcNH$_2$-Glc NH$_2$-**GlcNAc**-	GlcNAc-*O*-succinyl		**-[GlcNH$_2$]$_n$**-GlcNAc-**GlcNH$_2$-GlcNH$_2$**-GlcNAc-
1	4.64/104.3	4.53/104.4	4.59/104.5	4.71/104	4.76/104	4.75/102.4; 4.78/102.6
2	2.92 [c]/59.2	3.71/58.3	3.76/58.2	3.84/56.7	3.84 [b]/58.2	3.06/58.57
3	3.56/76.6	3.58/76.5	3.59/76.5	5.06/77.9	3.80 [b]	3.69/75.1
4	3.47/72.9	3.42/72.8	3.42/72.8	3.63/70.8	5.08/73.2	3.51/72.6
5	3.67/77.9	3.58/77.4	3.67/77.3		3.91 [b]	3.72/77.7; 3.64/77.7
6	3.86, 4.25/71.9	3.74, 4.17/71.2	3.76, 4.21/71.3			4.28, 3.89/71.5; 4.23, 3.87/71.3
O-Succinyl			2.48, 2.51/34.6 2.63, 2.67/33.3 177.2, 180.1			
N-Acetyl		2.06/25.10 177.0	2.00/25.0			

[a] Chemical shifts are reported relative to internal DSS-d_6 (0 ppm). Residue identification for the listed assignments are shown in bold letters. Underlined assignments represent significant chemical shift deviations due to the direct succinate substitution at the O-3 or O-4 hydroxyl groups of the β-GlcNAc residue.

[b] Assignments can be interchanged.

[c] The proton chemical shift changes to 2.66 ppm under alkaline conditions.

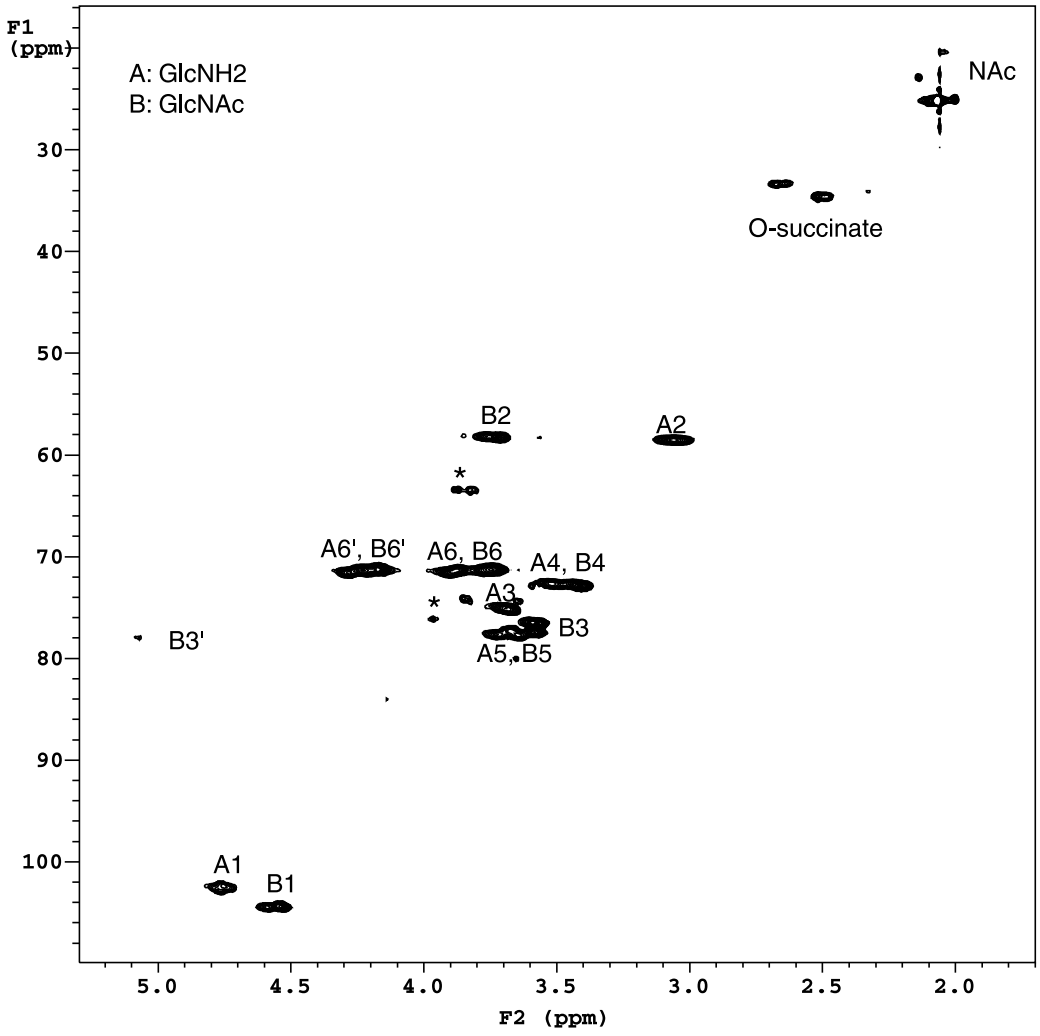

Fig. 3. 2D $^{1}H-^{13}C$ HSQC spectrum of native SAE in D_2O at 50 °C. Correlation peaks are labeled with residue identification of β-GlcNH_2 (A) and β-GlcNAc (B). Peaks marked by the asteric are due to residual polyglycerol phosphate. The minor signal marked B3′ is due to *O*-succinyl substitution at O-3 of β-GlcNAc.

O-succinyl from one hydroxyl group to the other. Such migrations are well documented for O-linked acetates in polysaccharides.[26]

2.4. Composition analysis and impurity profile of SAE polysaccharides

Even though we were able to fully characterize native SAE in aqueous solutions at neutral pH, some difficulty in solubilizing SAE preparations in pure D_2O was encountered throughout this study. Moreover, such observations were also noted for *S. aureus* PNSG where extreme pH conditions were necessary for complete dissolution. Since incomplete dissolution during the NMR analysis could lead to inaccurate characterization of polysaccharide preparations, we have established an alternate method of dissolving SAE for NMR analysis. In most instances, complete dissolution of SAE preparations was possible at high pH (>12.0). Therefore, routine compositional analysis by 1D NMR was performed on SAE samples dissolved in D_2O containing $\sim 5\%$ of 0.5 M NaOD–KCl buffer prepared in D_2O. To minimize alkali-induced degradation, samples were analyzed immediately after dissolution at 20 °C.

During development of the purification process, it was observed that lyophilized crude SAE prepared without the use of a high-salt diafiltration (2.5 M NaCl) step was extremely difficult to solubilize in pure D_2O, but dissolved completely in D_2O buffered at pH >12.0. NMR spectra of SAE prepared in this manner are shown in Fig. 4. In contrast to spectra recorded at neutral pH (Fig. 1(a)), these spectra showed significantly higher quality primarily due to line narrowing. In addition, significant shifts of β-GlcNH_2 resonances were observed due to complete titration of the free amine groups. Most importantly, the quality of the spectra allowed us to accurately quantitate various components associated with SAE directly from the 1D

NMR data. Relative molar ratios of each component were established based on relative integration of NMR signals at 2.05 (β-GlcNAc), 2.65 (H-2 of β-$GlcNH_2$) and 2.40 ppm (*O*-succinyl, detected as free succinic acid) with respect to the total anomeric signals at 4.6–4.3 ppm. Alternatively, anomeric signals centered at 4.53 and 4.40 ppm could be integrated separately to yield a β-GlcNAc to β-$GlcNH_2$ ratio.

Fig. 4 shows that SAE spectra also showed distinct NMR signals arising from the polyglycerolphosphate (PGP) component of teichoic acid, which is a common contaminant in *S. aureus* preparations. The identification of PGP is confirmed by the 1D $^{1}H-^{31}P$ spin echo difference spectra (Fig. 4(d)) where the only proton resonances observed were the four methylene protons of PGP due to 3JPH couplings. The absence of any SAE-related signals in the $^{1}H-^{31}P$ spin echo spectra clearly indicated that SAE does not contain any ester-linked phosphate. The signal at 4.04 ppm (H-2 of glycerol units) in the normal 1D proton spectra (Fig.

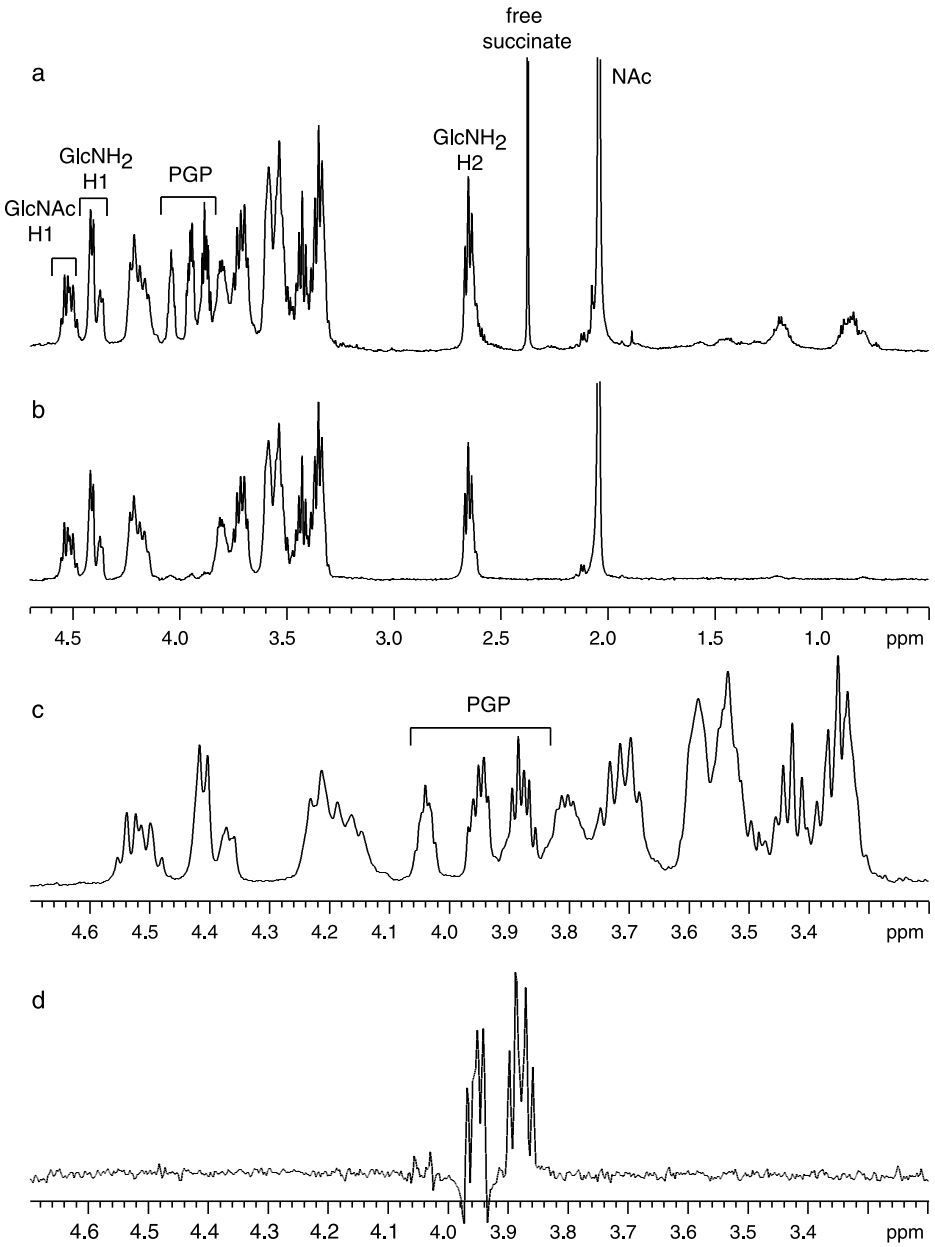

Fig. 4. NMR analysis of the crude SAE for residual components. All spectra were acquired using the crude SAE sample dissolved in D_2O at 20 °C with solution pH of >12. The lack of covalent association between SAE and polyglycerol phosphate is demonstrated by comparison of the 1D ^{1}H spectrum of crude SAE (a) with the corresponding 1D diffusion-edited ^{1}H spectrum (b). The identity of polyglycerol phosphate impurity is confirmed by the comparison of 1D proton spectrum (c) with the ^{31}P edited proton spectrum (d).

Table 2
Compositional analysis of purified SAE preparations

Component	Component mole percent		
	Native SAE	HF–SAE	AEC–SAE (intact)
$GlcNH_2$ [a]			
N-Acetyl	57	53	55
Free amino	43	47	47
O-Succinyl			
HPAEC [b]	9	1.2	8
NMR	8	0.2	17
N-Succinyl [a]	ND [d]	ND	ND
Glycerol phosphate [b]	2	ND	ND
Polyphosphoglycerol [a]	5	ND	ND
DNA [c]	4	NA [e]	ND

[a] Measured by 1D ^{1}H NMR.
[b] Measured by HPAEC. Quantification of the acid-liberated glycerol phosphate and succinate was achieved by comparing area of the glycerol phosphate peak to area of a 4 nmol of hydrolyzed glycerol phosphate and 0.5 nmol of hydrolyzed succinate standard. The linear detection range was 0.016–16 nmol for glycerol phosphate and 0.016–1 nmol for succinate per injection.
[c] Measured by absorption at 260 nm. Value is in weight percent.
[d] Not detected.
[e] Not assayed.

4(c)) can be integrated relative to the anomeric protons to yield the molar ratio of PGP contamination relative to the SAE. The complete attenuation of PGP resonances in the 1D NMR spectrum recorded under diffusion weighing conditions (Fig. 4(b)) indicated a relatively smaller molecular size of PGP relative to the SAE. More importantly, this data shows the absence of any covalent linkage of PGP to SAE. This indicates co-purification of PGP may be due to ionic interactions with charged SAE (net positive charge due to high degree of β-$GlcNH_2$) and that levels of PGP might be reduced by diafiltration against high salt concentrations. When the purification scheme was modified to include a high-salt diafiltration step, the resultant product (native SAE) showed a marked reduction ($>$ 10-fold) of PGP compared to early process material. In order to reduce residual contaminant levels further, native SAE was processed by either HF or AEC treatment. The spectra of these products compared with native SAE are shown in Fig. 5. As previously reported,[20] HF treatment of the product was effective at removing acidic contaminants. However, AEC provided a chemically mild alternative and was highly effective in reducing teichoic acid and DNA (discussed below) to non-detectable levels while yielding high product recovery. Comparison of the products obtained from each spectra showed that except for the reduction of PGP and *O*-succinyl in HF-treated material, the two samples were the same as native SAE. The apparently higher succinate value observed for AEC–SAE likely resulted from incomplete solubilization of the D_2O-exchanged sample after lyophilization. Since the succinate was completely released by base treatment, this resulted in a higher succinate-to-$GlcNH_2$ ratio by mass.

In addition to PGP, UV absorption spectroscopy revealed the presence of a 260-nm-absorbing impurity suggestive of nucleic acids. Treatment of SAE with deacetylation conditions resulted in removal of the peak at 260 nm, which is consistent with the known lability of nucleic acids to alkali. Results from agarose gel electrophoresis with specific stain detection (M. Yeager, personal communication) identified the contaminant as DNA. The level of presumptive DNA in the powder was 4% (w/w, uncorrected for moisture content of powder) based on its absorbance at 260 nm. The compositional analysis of various SAE preparations used for this study are summarized in Table 2.

2.5. NMR analysis of derivatized polysaccharides

The fact that previous reports had suggested the protective efficacy of PNSG was dependent on *N*-succinylation led us to closely investigate the nature of the substituted $GlcNH_2$ residues. In order to prepare standards for NMR analysis, native SAE was stripped of its N- and O-linked substituents by treatment with base. Complete N- and partial *O*-succinylation was obtained by reaction with succinic anhydride. The selective removal of *O*-succinyl groups was achieved by mild alkali treatment. Under these conditions amino group substitution was preserved as determined by lack of ninhydrin activity (data not shown). Fig. 6 shows the ^{1}H NMR spectra of *N*-succinylated and stripped SAE and demonstrates that N-substituted succinate, unlike *O*-succinyl, survives the alkaline solubilization conditions used for NMR analysis. The NMR spectrum of chemically *N*-succinylated SAE appears to be similar to that of the fully acetylated polymer (data not shown), with the exception that it contains methylene protons of succinate at 2.50 ppm. At high pH, proton signals of the succinate group appear to be strongly coupled, yielding a complex pattern. To the best of our knowledge this is the first time that NMR assignments of N-linked succinate are reported for an amino sugar in a polysaccharide. Moreover, NMR spectra recorded for all SAE preparations show a complete absence of the 2.50 ppm signal, indicating no evidence of *N*-succinylation.

The final confirmation that previous reports describing the presence of *N*-succinylation[20–22] were indeed

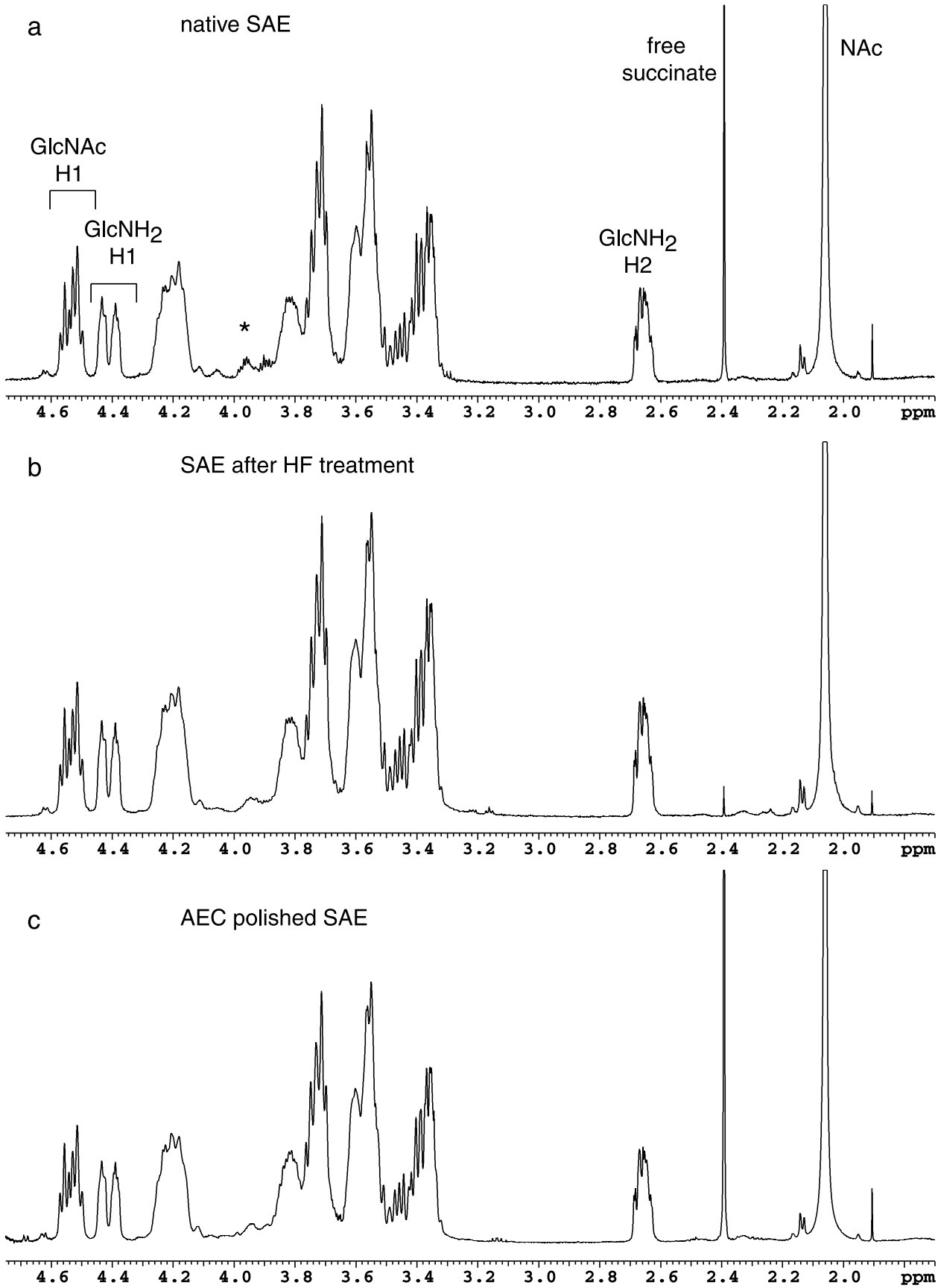

Fig. 5. 600-MHz 1D ^{1}H NMR spectra of purified SAE forms: (a) native; (b) HF-treated; and (c) intact SAE after AEC purification. All spectra were acquired in D_2O at pH $\sim$12 and 20 °C. The peak labeled 'free succinate' is due to hydrolysis of *O*-succinyl groups in the polysaccharide. Signal due to trace levels of PGP is labeled with the (*).

due to misinterpretation of NMR data was obtained from the analysis of acid-hydrolyzed SAE. For this experiment, native SAE was completely hydrolyzed with 6 N HCl at 100 °C for 14 h. The sample was evaporated to dryness and reconstituted with D_2O for the NMR analysis. In addition to expected resonances from the monosaccharide products (α- and β-$GlcNH_2$), the 1D NMR spectrum (Fig. 7(a)) shows several unidentified peaks including two well-defined triplets at 2.80 and 2.60 ppm. In the report by McKenney and co-workers,[21] these signals were interpreted as resulting from *N*-succinylated glucosamine monomers. However, at least in our spectra, the intensities of these signals, including the singlet at 2.22 ppm, did not show any correlation to monosaccharide resonances, indicating that these signals are not related to substituted sugars. It is well known that under the hydrolysis conditions with mineral acids, sugar substrates decompose to a variety of small molecules,[27–30] one of which is levulinic acid ($CH_3COCH_2CH_2COOH$). The 1D ^1H spectrum of authentic levulinic acid (Sigma) is shown in Fig. 7(b). When spiked to the acid-hydrolyzed SAE sample (Fig. 7(c)), levulinic acid resonances showed identical chemical shift with the aforementioned peaks, confirming the identity of the byproduct.

2.6. Sizing analysis of SAE

To judge the effect of different treatments on polymer size, high-performance size-exclusion chromatography (HPSEC) was performed, and molecular mass was estimated either by refractive index detection with multi-angle laser light scattering (MALLS) or by extrapolation from a pullulan standard calibration curve. Table 3 shows the results generated by each method. In all cases the MALLS values were roughly twice as high as those determined by elution time relative to standards. Light-scattering measurements are sensitive to low-abundance contaminants, and even very low concentrations of a high-molecular-mass spe-

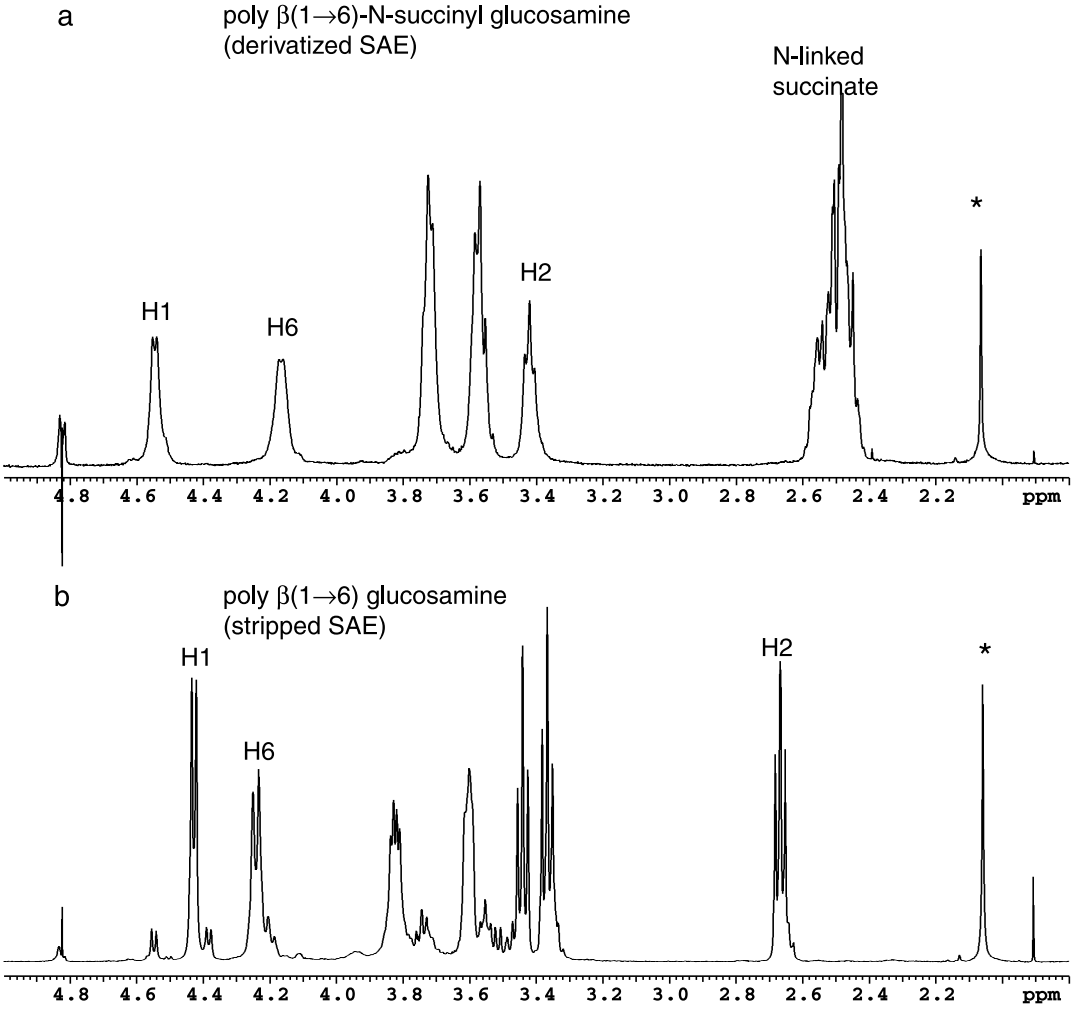

Fig. 6. 600-MHz 1D ^1H NMR spectra of chemically modified SAE: (a) *N*-succinyl form; and (b) N-deacetylated form. All spectra were acquired in D_2O at pH ~12 and 20 °C. The residual HDO peak was suppressed with a soft proton pre-saturation.

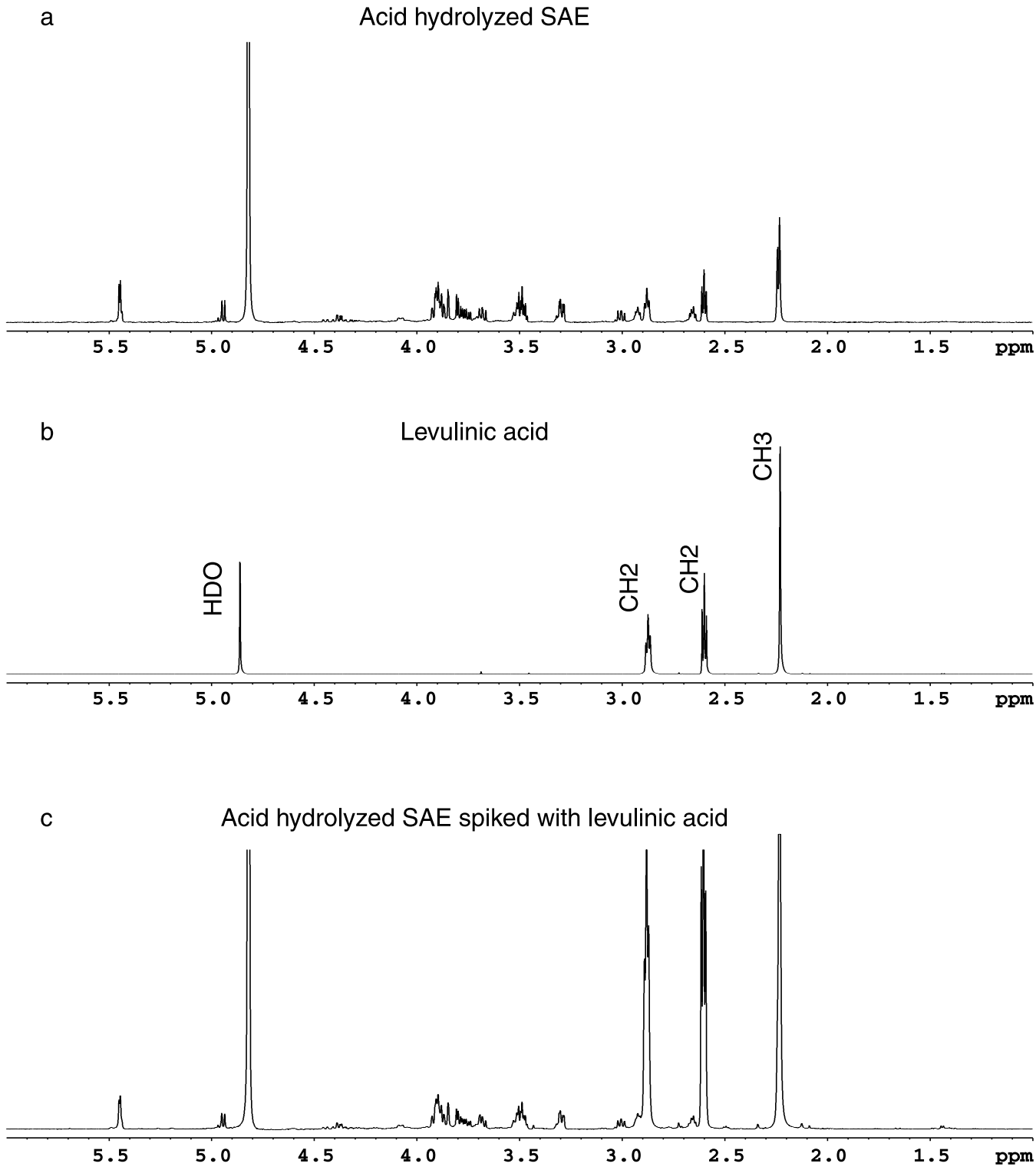

Fig. 7. 1D ^{1}H NMR identification of levulinic acid in the acid hydrolysate of SAE. (a) Acid hydrolysate of native SAE dissolved in D_2O; (b) levulinic acid standard in D_2O; and (c) sample (a) spiked with the levulinic acid.

cies can seriously skew results toward higher apparent molecular weights. To minimize these effects, sharp cuts were taken at the apex of the refractive index peak to obtain data for MALLS size calculations, but contributions from higher mass components was still likely. This explanation is supported by the observation that the mass of a minor leading-edge component present in the AEC-sized SAE was estimated at 2,500,000 by calibra-

Table 3
HPSEC analysis of purified SAE preparations

Quantitative method	Molecular mass (Da)			
	Native SAE	HF–SAE	AEC–SAE (intact)	AEC–SAE (sized)
Pullulan standard calibration [a]	346,000	68,000	259,000	73,000
MALLS/RI [b]	640,000	135,000	445,000	167,000

[a] For calibration, a 50-μL injection of pullulan standards (4–7 mg/mL) covering the molecular mass range 47,300–788,000 Da was made, and the average retention time (RT) was calculated. A linear plot of $\log_{10}$ mass versus $\log_{10}$ RT was constructed, and the best fit line was calculated by least squares regression. For SAE samples, a 75-μL injection of sample (1–3 mg/mL) was made, and the estimated molecular mass was determined by inputing the $\log_{10}$ RT into the standard curve equation.

[b] Standard and sample injections were done as for a. Estimated molecular mass was determined by selecting a narrow data set from the center of the refractive index peak and assuming a dn/dc value of 0.133.

tion standards and 19,000,000 when quantitated by MALLS. Another potential factor contributing to the discrepancy between the MALLS and calibration curve generated size data involves the dn/dc term used to calculate molecular mass from the scatter data. The light-scattering detector was calibrated relative to a 47,300 Da pullulan using a value of 0.133 for dn/dc.[31] This term reflects, in part, molecular shape of the chromatographic analyte, and it is conceivable that the shapes of SAE and the pullulan standard are significantly different. If the analytes were of different shapes, the calibration standard data could be affected in a similar manner. Furthermore, retention behavior can also be affected by non-specific interactions between analyte and column matrix. These possibilities suggested that the MALLS-generated values were likely more accurate. Native SAE was isolated as a high-molecular-mass polymer of $>300{,}000$ Da. Following AEC, the mass was reduced by 25–30%, most likely as a result of removing non-covalently associated acidic contaminants. Treatment of native SAE with HF resulted in considerable hydrolysis with a concomitant size reduction of 5–10-fold. Physical sizing of intact AEC–SAE by sonication was employed in order to control the total reduction obtained and to avoid chemical alteration of the antigen. The conditions employed resulted in a preparation with molecular mass comparable to HF-treated material. Based on analytical HPSEC of the intermediate sonication time points, the reduction process was fairly linear (data not shown). It is unclear why AEC-sized SAE contained a low-abundance peak of very high mass. It is conceivable that under the input energy conditions a small amount of radical-induced polymerization may have proceeded as a minor side reaction.

2.7. Hemagglutination assays

The PIA expressed by *S. epidermidis* strains has been shown to be responsible for their ability to agglutinate red blood cells.[32,33] Thus, the hemagglutination assay can be used as an in vitro functional assay for the different SAE polysaccharide preparations. Table 4 shows the assay results and summarizes the minimum concentration of each SAE form required to agglutinate red blood cells. Hemagglutination with live *S. epidermidis* RP62A cells were run in the same assay as a positive control. All SAE forms were able to agglutinate sheep red blood cells (SRB), just as the carbohydrate present on the surface of *S. epidermidis* cells is able to do. There appeared to be little difference in hemagglutination between the various preparations, with the exception of HF–SAE which showed the lowest agglutination titer and which was both more positively charged than the other forms and of lower molecular mass.

2.8. Immunogenicity of SAE

The AEC-processed intact and sized forms of SAE were activated to introduce a sulfhydryl-reactive maleimide group and subsequently conjugated to thiolated OMPC protein. Table 5 summarizes the analytical characteriza-

Table 4
Hemagglutination by SAE preparations

SAE preparation	Molecular mass (Da) [a]	HA (μg/mL) [b]
Native SAE	640,000	0.625
HF–SAE	135,000	2.500
AEC–SAE (intact)	445,000	0.312
AEC–SAE (sized)	167,000	0.625

[a] Molecular mass determined by HPSEC/MALLS/RI as described in Table 2.

[b] Hemagglutination defined as the minimal concentration of polysaccharide required to agglutinate a 1% suspension of SRB cells after 2 h at ambient temperature.

Table 5
Characterization of activated components and OMPC–SAE conjugates used for vaccine preparation

Property	Activated OMPC	AEC–SAE (intact)	AEC–SAE (sized)
Thiol–lysine (mol/mol) [a]	0.44	NA [d]	NA
Side chain loading (% w/w) [b]	NA	4.8	9.9
$GlcNH_2$–protein (% w/w) [c]	1	11	13
N-Acetylglucosamine	NA	50.5	50.0
Free glucosamine	NA	49.5	50.0
O-Succinylation	NA	1.1	1.0

[a] Thiol content determined by reaction with DTNB; protein concentration determined by modified Lowry assay. Lysine content was 0.4 μmol lys/mg protein.
[b] Determined by 1D ^1H NMR spectroscopy using pulsed field gradient diffusion filtration as described.
[c] Glucosamine determined by HPAEC. Values for conjugates were corrected for residual free polysaccharide as determined by physical mixing controls using activated SAE and non-activated OMPC (data not shown).
[d] Not assayed.

tion of conjugates and activated intermediates. The large, particulate nature of OMPC allowed the use of ultracentrifugation for removal of excess reagents and unconjugated polysaccharide. Polysaccharide activation resulted in side chain loadings of 4.8 and 9.9% for the intact and sized forms, respectively. NMR revealed significant loss of *O*-succinyl groups, but subsequent analyses showed that the pre-activated aqueous bulks had lost comparable levels as a result of extended aqueous storage at 2–8 °C (data not shown). Activated polysaccharide and protein were mixed at a defined maleimide to thiol ratio of 0.5–1, which preliminary experiments had shown yielded 10–20% SAE by weight. Compositional analysis indicated that these targets were achieved for both intact (11%) and sized (13%) preparations. Stringent washing conditions using high salt and detergent were found to be necessary to effect removal of $>95\%$ of unconjugated polysaccharide. The conjugates were adsorbed to alum adjuvant at 0.5 mg/mL at which concentration they were $>99\%$ adsorbed. Routine stability monitoring of the bulk alum product was performed by pelleting an aliquot and measuring the protein in the supernatant fraction. No detectable protein was observed after 45 days at 4 °C storage.

BALB/c mice were immunized with the OMPC conjugates of AEC-intact and -sized SAE, and the anti-SAE ELISA titers of mice from both experiments are shown in Table 6. The conjugate was immunogenic in a dose-related manner, and three injections resulted in a higher titer than two injections; however, with either two or three injections, a robust anti-Ps response was observed. The animals immunized with Merck alum only or OMPC on Merck alum only did not show a significant titer to the AEC–SAE antigen in ELISA.

3. Discussion

The definitive role of PIA in mediating cell–cell attachment and subsequent biofilm accumulation has been well established for *S. epidermidis*, which is the organism responsible for the majority of medical device infections.[8–10] The evidence for involvement of PIA in infection with *S. aureus* is less certain, but biofilm production and identification of the *icaADBC* locus has been confirmed in numerous strains isolated from medical implant infections.[34,35] It is unclear, however, if it plays any role in non-implant *S. aureus* infections, such as those leading to systemic bacteremia. The reported identification of PNSG in *S. aureus* was intriguing in that *N*-succinylated polysaccharides have not been previously reported and because initial studies suggested that this molecule's protective efficacy in a mouse renal infectivity model was a result of this purported structural feature. In addition, a number of studies had previously reported the isolation of novel polysaccharide antigens from a variety of staphylococcal strains, but in many cases structural misidentifications were made, often resulting from contaminants carried through the purification. The problem is further compounded by the fact that bacteria can regulate expression of specific biomolecules as a function of selective growth conditions, a characteristic which has been documented for *S. aureus* capsular polysaccharide.[36] We found the same to be true for isolation of high-molecular-mass SAE. In the interest of generating large amounts of purified polysaccharide we chose to work with the constitutively producing mutant MN8m strain. When employing fermentation conditions described for production of antigen from wild type strains,[20] we saw similar low expression levels despite use of the mutant. However, by careful adjustment of medium composition and growth conditions, we were able to produce

large amounts of polysaccharide that were easily extracted into the culture medium using mild procedures.

Since we were concerned with isolating the molecule in its native state in order to confirm the structural reports, we developed a purification protocol that employed the mildest conditions compatible with the chemistry of the antigen. Bacterial polysaccharide isolation procedures often employ harsh conditions including pH extremes, solvent precipitations, and chemical hydrolyses, all of which can result in structural modification. The process described in the current study employed an aqueous regimen of membrane filtration steps designed to selectively isolate high-molecular-mass biopolymers. The product obtained from this protocol was highly enriched, with the only detectable contaminants being *S. aureus* teichoic acid and residual DNA, both of which could subsequently be removed by anion-exchange chromatography (AEC) at near neutral pH. Both NMR and HPAEC analyses confirmed high purity levels, while NMR unambiguously identified the repeating unit to be β-(1→6)-glucosamine randomly substituted with *N*-acetyl groups. The presence of *O*-succinylation on the O-3 and O-4 hydroxyl groups of β-GlcNAc residues was demonstrated by NMR spectroscopy. Furthermore, the level of succinate present (10%) was in good agreement with the 6% reported for PIA.[8] Based on these observations we conclude that SAE is very similar in composition to *S. epidermidis* PIA.

The solubility characteristics of SAE were considerably different from those reported for PNSG in that native SAE was soluble in water or aqueous buffer during purification and following lyophilization, whereas PNSG required a pH < 4 to solubilize. This discrepancy might be explained by the higher amount of β-$GlcNH_2$ residues present in SAE that would impart a higher degree of positive charge on the polymer. Alternatively, it might be argued that the presence of acidic contaminants led to a higher solubility, an assumption supported by the observation that AEC- or HF-processed antigen showed decreased water solubility following lyophilization. The process described by McKenney and co-workers[20] incorporated methanol precipitation steps, including one just prior to lyophilization of the final product. It may be that these steps resulted in the removal of more polymer-associ-

Table 6
ELISA results from mice immunized with OMPC–SAE conjugate vaccines; anti-polysaccharide titers

Immunogen	Experiment 1 [a]			Experiment 2 [b]		
	GMT [c]			GMT		
	Day 2	Day 21	Day 28	Day 1	Day 21	Day 35
8 μg Native	229 (*100–400*)	102,479 (*51,200–409,600*)	204,800 (*102,400–409,600*)	373 (*200–400*)	89,144 (*51,200–204,800*)	713,155 (*409,600–819,200*)
0.8 μg Native	114 (*100–200*)	117,626 (*51,200–409,600*)	135,117 (*102,400–409,600*)			
0.08 μg Native	131 (*100–200*)	22,286 (*12,800–51,200*)	58,813 (*51,200–102,400*)			
0.008 μg Native	174 (*100–400*)	2111 (*800–6400*)	9700 (*3200–25,600*)			
8 μg Sized	174 (*100–200*)	204,800 (*51,200–409,600*)	270,235 (*102,400–819,200*)	400 (*400*)	54,875 (*25,600–102,400*)	764,341 (*409,600–1,638,400*)
0.8 μg Sized	151 (*100–400*)	67,558 (*51,200–102,400*)	155,209 (*102,400–409,600*)			
0.08 μg Sized	174 (*100–400*)	16,889 (*6400–25,600*)	77,604 (*51,200–102,400*)			
0.008 μg Sized	263 (*100–400*)	2785 (*1600–6400*)	8444 (*6400–12,800*)			
1× Alum	151 (*100–400*)	303 (*200–400*)	263 (*200–400*)			
OMPC on 1× alum				400 (*400*)	528 (*400–800*)	857 (*400–1600*)

[a] Experiment 1: $N = 5$ mice, immunized days 0 and 14.
[b] Experiment 2: $N = 10$ mice, immunized days 0, 14, and 28.
[c] Geometric mean titer with end-point titer range in italic parentheses.

ated water, thus significantly reducing its re-solubilization potential. Interestingly, the 80–85% acetylation reported for PIA[8] was comparable to the substitution percentages on PNSG, and yet PIA was not reported to be insoluble at neutral pH. It should be noted, however, that PNSG was of significantly higher mass than PIA, and the combined contribution of high molecular mass and charge may have reduced its solubility.

The lack of N-succinylation in our product led us to investigate the conditions under which PNSG NMR analyses were performed. Due to the aforementioned solubility characteristics, PNSG was routinely subjected to strong acid hydrolysis at elevated temperatures prior to analysis, whereas SAE samples were prepared under mildly alkaline conditions. When native SAE was treated using the same hydrolysis conditions detailed for PNSG, the appearance of non-carbohydrate signals was observed. Although the position of these peaks was consistent with a substituted succinate species, their height ratios appeared aberrant. Furthermore, these peaks were also consistent with the spectrum of levulinic acid, a known degradation product of acid-hydrolyzed monosaccharides. When hydrolyzed SAE was spiked with levulinic acid, these peaks increased in proportional intensity, confirming their identification. Finally, to prove that the spectra observed for native SAE were inconsistent with an N-succinylated structure, we chemically prepared succinylated versions by first optimizing conditions for complete removal of N-acetylated substituents and then succinylating the stripped polymer. The spectra of the N- and N- + *O*-succinylated SAE forms under mild alkaline conditions were clearly distinguishable from both native and acid-hydrolyzed SAE, as well as from published spectra for PNSG.[21,23] Together, these findings definitively demonstrated that *N*-succinylation was not present in the native antigen.

SAE also differed from the published description of PIA in several respects: (1) SAE contained a lower overall level of N-acetylation; (2) an absence of detectable O-phosphorylation in SAE; and (3) SAE had a high relative molecular mass compared with PIA. To address the first point, regulation of the *icaADBC* operon appears to be sensitive to environmental conditions,[13,14] and so it is not surprising that levels of polysaccharide substitution might vary from one preparation to another, particularly when comparing material isolated from different strains under different culture conditions, and employing different purification methods. Second, since O-phosphorylation had been reported for PIA, we performed $^{1}H-^{31}P$ NMR experiments to determine if the phosphate observed in our SAE preparations was covalently linked to the polymer. The results showed that all phosphate was present as glycerol phosphate, indicative of teichoic acid impurities. The determination of phosphate in PIA was performed by colorimetric assay on product that was purified in the presence of phosphate-containing buffers.[8] The low ($< 5\%$) amount observed using this non-specific assay most likely represented purification carryover. Lastly, a definitive molecular mass was not reported for PIA, but a value of approx 30,000 Da was inferred from a reported average chain length of 130 residues.[8] The elution characteristics of SAE on high-performance sizing resins, coupled with the fact that it was isolated by high MWCO membranes, indicate that its size is significantly larger. The discrepancy between the MALLS and calibrated HPSEC data has already been discussed, but even at the lower limit the polymer is approx 10-fold higher in mass than PIA. Both sonication and HF-treatment could reduce the mass by about fivefold. Cellular extraction of PIA from *S. epidermidis* utilized sonication, although the experimental conditions were not given.[8] If sufficiently energetic, this may have led to early polymer fractionation. Alternatively, the high molecular mass we observe may be a characteristic of the MN8m mutant. In our hands, large quantities of the polysaccharide were extruded into the culture medium. Mack and co-workers[8] did not state whether they evaluated the culture supernate after separation of the cell mass.

The ability of SAE to mediate erythrocyte hemagglutination provides indirect evidence of its biological similarity to PIA. Biofilm-producing *S. epidermidis* strains show hemagglutination positive phenotypes, while isogenic *icaADBC* knockouts are negative.[33] Surprisingly, purified PIA did not hemagglutinate, although it could competitively reduce hemagglutination mediated by *icaADBC*$^{+}$ strains.[33] In contrast, all forms of SAE were able to hemagglutinate. The native, AEC-intact, and AEC-sized forms showed equivalent titers, while HF-treated SAE was markedly reduced in effectiveness. While our data suggest that overall polymer charge appears to be important for mediating agglutination, it is likely true that a certain minimal chain length is also critical. It is conceivable that a further two- to threefold mass reduction of sized SAE, which would make it more comparable to PIA, might result in loss of hemagglutination ability.

Conjugate vaccines prepared from AEC-processed intact and sized SAE produced a clear, dose-dependent immunological response in mice following a two-dose regimen. The titers achieved at the highest dose were very good for a polysaccharide-induced response and comparable to those seen for other polysaccharide conjugate vaccines such as PRP-OMPC,[37,38] pneumococcal capsular-OMPC,[39,40] and meningococcal type C-OMV.[41] The polysaccharide incorporation achieved (10–20% by mass) in the SAE conjugates was similar to these previously reported preparations. The fact that very similar values were observed for intact and sized materials at a given dosage level implies that size reduc-

tion does not result in destruction of any significant quantity of epitopes, and supports the analytical results showing comparable polysaccharide to protein ratios for both vaccines. The results for intact versus sized material are in contrast to those reported using unconjugated exopolysaccharide preparations where only the high-molecular-mass polymer demonstrated significant immunogenicity.[23] Our current work demonstrates that, when using covalently coupled polysaccharide, high mass is not a requisite for obtaining high serum titers. Encoragingly, these experiments demonstrate that polysaccharide-specific responses continue to increase even 14 days after the final boost. Since no response plateau was achieved after two injections of vaccine, the study was repeated using a single dose level and a three-injection regimen. The absolute magnitude of the responses, as well as the fold-increase following each successive boost in these two experiments, were comparable to those observed in similar studies.[38,39,42] The 8-μg dose used in the current study is within the optimal range reported for previous polysaccharide–protein conjugate vaccines.

The results of the current study definitively show that SAE, PNSG, and PIA are chemically very similar. The previous identification of *icaADBC* in adherent strains of both *S. epidermidis* and *aureus* suggests that these polymers are the product of the same locus with minor structural variations arising from growth or strain-dependent factors. Hemagglutination results show SAE to behave in a manner similar to *S. epidermidis* cultured cells, suggesting that the polymer may mediate similar adhesion properties in those *S. aureus* strains that produce biofilm. While this is insufficient to prove antigenic identity, it certainly shows that strong cross-reactivity exists between SAE and RP62A surface polysaccharide. It must again be emphasized that SAE was isolated from a mutant strain, and it is very possible that its distinctive features, predominantly high molecular mass, are related to source of origin. This fact notwithstanding, the putative *N*-succinylation reported for PNSG has definitively been shown to be an analytical artifact and thus cannot account for any protective efficacy observed with this antigen. Given the chemical near-identity of SAE and PIA, it is not unreasonable to speculate that high polysaccharide-specific titers generated using an SAE-carrier conjugate may be capable of cross-protecting mice from a heterologous *S. epidermidis* challenge. If so, SAE would be an attractive vaccine candidate for prevention of implant-based infections caused by coagulase-negative staphylococci. Further studies are necessary to demonstrate if SAE is characteristic of *S. aureus* strains other than MN8m and to show definitively that it is the product of the *icaADBC* locus. The question of whether biofilm production by *S. aureus* has any in vivo significance remains an open one, but if it does, then SAE may be an important target for prevention of implant-based infections.

4. Experimental

4.1. Bacteria strains and media

The *S. aureus* spontaneous constitutive PIA/PSA overproducing mutant strain MN8m has been described previously.[21] *S. epidermidis* strain RP62A was obtained from the ATCC (Manassas,Va) as ATCC 35984. All staphylococcal strains were routinely cultured in Trypticase Soy broth (TSB) and grown on Trypticase Soy agar (TSA) plates (Becton Dickinson, Cockeysville, MD) at 37 °C unless stated otherwise in the methods.

4.2. Fermentation of *S. aureus* strain MN8m and culture inactivation

Culture broth (15 L) containing 20 g/L soy peptone, 5 g/L NaCl, 2.5 g/L K_2HPO_4, 31.5 g/L yeast extract, ultrafiltrate grade (YEU), and 0.05% UCON LB625 antifoam was supplemented with 1 g/L succinic acid and 1 g/L sodium succinate. The broth was sterilized, and sterile dextrose was added to a final concentration of 1%. A 300-mL Tunair flask containing seed medium (20 g/L soy peptone, 5.8 g/L NaCl, 2.9 g/L K_2HPO_4, 31.5 g/L YEU, 0.46 g/L $NaHCO_3$, 55.5 g/L HEPES buffer, pH 7.0, 1% dextrose) was inoculated with a single colony of *S. aureus* MN8m grown on TSA plates and incubated overnight with agitation at 37 °C. The soy peptone–YEU media was inoculated with 40 mL MN8m inoculum. The culture was agitated and sparged with air to maintain dissolved oxygen at 30%. Temperature was maintained at 37 °C, and pH was maintained at 7.0 by addition of sterile 30% NaOH. Growth was terminated when NaOH ceased to be required to maintain pH 7.0. For harvest, the pH was adjusted to 5.0 with 50% AcOH, and $MgCl_2$ was added to 0.1 M. The culture was heated to 60 °C and held for 90 min. The inactivated culture was centrifuged at 13,600*g* for 10 min, and the supernatant was retained for further processing.

4.3. Purification of SAE antigen

Conditioned supernatant (11.2 L) was clarified by filtration using Suporcap-100 0.8/0.2-μm cartridges (Pall Gelman, Ann Arbor, MI) and then concentrated by tangential flow filtration (TFF) using a 500-kDa molecular weight cut-off (MWCO) hollow-fiber membrane cartridge (A/G Technologies, Needham, MA) to a volume of 700 mL. Following concentration, the retentate fraction was diafiltered against 8 vol of distilled deionized (DI) water. The retentate was adjusted to 5 mM

Tris–HCl, pH 8, 2 mM $CaCl_2$, and 2 mM $MgCl_2$. Proteinase K (14 mg) was added, and the mixture was allowed to incubate for 16 h at 20 °C. The digestion reaction was adjusted to 2 M NaCl and concentrated to 700 mL by TFF as previously described. The retentate was successively diafiltered against 8 vol of 2 M NaCl, 8 vol of low pH buffer (25 mM sodium phosphate, pH 2.5, 0.1 M NaCl), and 10 vol of DI water, after which it was concentrated to a final volume of 340 mL. The concentrate was shell-frozen in pre-weighed bottles and lyophilized to dryness to generate native SAE antigen.

4.4. Polishing and sizing of native SAE

Native SAE (1 g) was dissolved at 2 mg/mL in 10 mM HEPPS, 0.4 M NaCl, pH 7.7 buffer by stirring overnight at ambient temperature. Residual insolubles were removed by centrifugation at 13,000*g* for 30 min at 20 °C. AEC was performed by applying the supernatant fraction at 40 mL/min to a column (11.3 cm i.d. × 9 cm) of Fractogel® EMD TMAE(M) resin (E.M. Sciences, Gibbstown, NJ) equilibrated in 10 mM HEPPS, 0.4 M NaCl pH 7.7 buffer. The column was washed with 3 vol of equilibration buffer and eluted with 10 mM HEPPS, 2 M NaCl, pH 7.8 buffer. Fractions of 100 mL were collected and scanned over the wavelength range 340–240 nm. Flow-through fractions that showed no absorbance maximum at 260 nm were pooled and diafiltered against 18 vol of DI water at ambient temperature using a Biomax™ 50-kDa MWCO membrane (Millipore, Bedford, MA). The diafiltered material was passed through a 0.8-μm filter and stored for short term at 2–8 °C. Size reduction was effected using a cup and horn sonication apparatus (Misonix, Farmingdale, NY) employing a power output of 6.5, a 2 s cycle rate, and a duty cycle of 50% for 30 min, followed by a series of 15-min sonications at an output of 9 out to 110 min. The sample was kept cool by a constant flow of cold water through the apparatus. Time-dependent molecular weight reduction was monitored by HPSEC at 30, 45, 60, 75, 90, and 110 min at which point sonication was terminated.

4.5. HF treatment of native SAE

Native SAE (101 mg) was dissolved in DI water at 5 mg/mL and treated with HF as previously described.[20] Following neutralization and dialysis, the reaction mixture was shell-frozen in pre-weighed bottles and lyophilized to dryness.

4.6. Preparation of *N*-succinylated SAE

In order to effect removal of N- and O-linked substituents, native SAE was dissolved at 4 mg/mL in argon-sparged 5 N NaOH and incubated under Ar for 18 h at 37 °C. The reaction mixture was cooled to ≤ 10 °C in an ice-water slurry and neutralized by slow addition of ice cold 5 N HCl. The reaction was concentrated threefold using a Biomax™ 50-kDa MWCO membrane and successively diafiltered against 5 vol of 2.5 M NaCl and 10 vol of DI water. The product was passed through a 0.45-μm filter, and the free glucosamine content was determined by manual ninhydrin assay. Succinylation of stripped polysaccharide was performed in a pH-stat set to maintain the pH at 8.5 by addition of 5 N NaOH. Succinic anhydride (30 mg/mL in anhyd 1,4-dioxane) was added at a 10-fold molar excess over total glucosamine at a rate that allowed the pH stat to maintain a pH of 8.2–8.5. The reaction was incubated for 4 h at room temperature (rt). To obtain a product with total *N*-succinylation and partial *O*-succinylation (*N*- + *O*-succinylated), the reaction was made 2.5 M in NaCl and successively diafiltered against 6 vol 2.5 M NaCl and 10 vol DI water. For *N*-succinylated-only product, the reaction was adjusted to pH 12 and incubated for 2 h at ambient temperature. It was then diafiltered as was done for the N- + O-product.

4.7. Compositional analyses

Chemical compositional analyses were performed by HPAEC using an alkaline elution protocol combined with pulsed amperometric detection for monosaccharide analysis or suppressed conductivity detection for organic anion analysis. SAE samples were hydrolyzed in 6 N HCl at 95 °C for 72 h, after which time the hydrolysate was evaporated to dryness and reconstituted in 200 μL of DI water. Monosaccharide compositional analysis was performed on a BioLC GP50 system equipped with an ED40 Electrochemical Detector and EG40 eluent generator (Dionex, Sunneyvale, CA). Chromatography was performed at rt on a CarboPac PA1 column (4 mm i.d. × 250 mm) at a flow rate of 1 mL/min using the elution scheme: (a) isocratic at 18 mM NaOH for 19 min; (b) wash at 100 mM NaOH for11 min; (c) restoring of the isocratic condition at 18 mM NaOH for 15 min. Glycerol phosphate and succinate analyses were performed at rt on a IonPac AS11 column (4 mm i.d. × 250 mm) at a flow rate of 1 mL/min using the elution scheme: (a) isocratic at 4 mM NaOH for 5 min; (b) gradient from 4 to 10 mM NaOH in 10 min; (c) step gradient from 10 to 20 mM NaOH in 0.1 min; (d) wash at 50 mM NaOH for14 min; (e) restoring of the isocratic condition at 4 mM NaOH for 10 min.

4.8. High-performance size-exclusion chromatography (HPSEC)

Relative molecular weights of polysaccharide preparations were determined on a Alliance 2690 HPLC system

(Waters, Milford, MA) using two Waters Ultrahydrogel™ Linear columns (7.8 mm i.d. × 300 mm) linked in series behind an Ultrahydrogel™ guard column and a 250-psi flow restrictor. Chromatography was performed in 0.1 M sodium phosphate pH 7.2 buffer containing 0.05% sodium azide at a flow rate of 0.5 mL/min. Detection was by refractive index using a Waters 410 differential refractometer. Pullulan molecular weight markers (Polymer Laboratories, Amherst, MA) were used to calibrate the system. MALLS data was generated using a Precision Detectors PD2000DLS system.

4.9. NMR analysis

All NMR spectra were recorded on a 600-MHz Varian Unity Inova NMR spectrometer using 5-mm heteronuclear indirect probes with z-axis pulse field gradient capabilities. The observed chemical shifts (^{1}H and ^{13}C) are reported relative to internal reference signal of DSS-d_6. All NMR data sets were processed with Varian VNMR software. Polysaccharides with various degrees of purity and compositions were first analyzed by 1D ^{1}H NMR in D_2O. Since some samples showed incomplete solubilization in D_2O at neutral pH, all samples for 1D NMR were prepared by dissolving 1–3 mg of dried polysaccharide in 700 μL of D_2O containing a trace amount of ($\sim$ 50 μg/mL of DSS-d_6) and 40 μL of 0.5 M NaOD–KCl buffer in D_2O. Under these conditions, all samples were fully dissolved to yield clear solutions with measured pH > 12.

Unless otherwise mentioned, 1D ^{1}H NMR spectra were recorded at 20 °C with the standard one-pulse sequence. A residual water signal suppression was achieved by applying a weak pre-saturation during the 30 s relaxation delay. For a typical spectrum, 64 scans were co-added, and data were processed with 0.2-Hz Lorentzian broadening. One-dimensional diffusion-filtered spectra were recorded with the pulse sequence of BPP-LED[43] using a 0.5-s longitudinal delay, 2-ms gradient pulses and 0–60 G/cm gradient strength array. One-dimensional ^{1}H–^{31}P spin-echo spectra for crude polysaccharide samples were recorded with a 2D HMQC sequence with delay optimized for $^nJ_{PH} = 6$ Hz. Resonance assignments of polysaccharides were carried out at 50 °C using samples dissolved directly in D_2O. All 2D correlation data sets were acquired with standard pulse sequences. The parameters used for 2D DQF-COSY[44] and 2D TOCSY[45,46] include 3600 Hz sweep width in both dimensions, carrier frequency set at 3.0 ppm, 1.0-s relaxation delay, 0.52- and 0.142-s acquisition time in t_2 and t_1 dimensions, and 100-ms spin lock time. ^{1}H–^{13}C correlation spectra were collected using 2D HSQC[47] and 2D HMBC[48] sequences. Spectra were recorded in phase-sensitive mode using States-TPPI mode with sweep widths of 3600 and 18,000 Hz in ^{1}H and ^{13}C dimensions, respectively. A low-power ^{13}C decoupling using the GARP sequence was employed during the acquisition time (142 ms) in the HSQC experiments. The ^{13}C dimension in all heteronuclear 2D spectra were processed with backward linear prediction prior to shifted sine bell (90°) apodization.

4.10. Hemagglutination assay

For assay of whole bacteria, *S. epidermidis* strain RP62A cells were grown overnight on TSA plates. A 30-mL TSB culture, inoculated with cells from the plate culture, to a starting $OD_{650\ nm}$ of 0.1, were grown in a 125-mL Erlenmeyer flask for 3–5 h at 250 rpm, 37 °C. Cells were harvested, washed once in PBS (6.25 mM phosphate, pH 7.3, 0.15 M NaCl), and suspended in PBS to an $OD_{650\ nm}$ of 2.0. A 1% sheep red blood cells (SRB) suspension was made by reconstituting lyophilized SRB (Sigma, St. Louis, MO) in PBS, 1% BSA. First, the bacterial suspension (50 μL) was added to each well in the top row of a 96-well plate, and subsequent twofold dilutions of bacteria were made in PBS for the length of the plate. Next, a 50-μL aliquot of 1% SRB was added to each well, and the plate was incubated without mixing at rt for 2 h before visual examination. For analysis of SAE preparations, 50 μL of polysaccharide at a concentration of 10 μg/mL was added to each well in the top row of the plate, diluted, and assayed in a similar manner as the bacterial cells. A positive result was defined as the dilution of bacteria or polysaccharide that produced diffuse red blood cells with no red blood cells pelleting at the bottom of the well. All samples were assessed in duplicate wells, and the assay was performed at least two times on each sample.

4.11. Preparation of SAE conjugates

The outer membrane protein complex (OMPC) of *Neisseria meningitidis* type B was activated by thiolation of lysine residues as previously described[49] with minor modifications that consisted of alternative but equivalent centrifugation conditions, and replacing the sodium phosphate, pH 8.5 buffer used for final re-suspension of the activated OMPC with HEPES–EDTA buffer (20 mM HEPES, 2 mM EDTA, 150 mM NaCl, pH 7.3). Thiol content of activated OMPC was determined using 5,5′-dithionitrobenzoic acid (DTNB).[50] AEC-purified intact and sized SAE were activated by introduction of a maleimide group using the heterobifunctional reagent sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sSMCC, Pierce, Rockford, IL). Solid sSMCC was added to the polysaccharide dissolved in water at a fivefold molar excess over SAE-free $GlcNH_2$ (as determined by NMR spectroscopy). The reaction

was adjusted to pH 7.8 by addition of HEPPS to a final concentration of 35 mM. The reaction was incubated in the dark at rt for 3 h after which time it was concentrated and diafiltered against HBS (20 mM HEPES, pH 7.3, 0.15 M NaCl) using a 10-kDa Pellicon™ (Millipore) flat sheet membrane. Maleimide incorporation was determined by quantitation of thiol consumption, and the activated polysaccharides were snap frozen and stored at −70 °C. For conjugation, polysaccharides were thawed and mixed with thiolated OMPC at a 0.5–1 molar ratio of polysaccharide maleimide to OMPC sulfhydryl. The pH was adjusted to 7.3 by addition of HBS to a final concentration of 20 mM, and reactions were incubated for 15–20 h at rt in the dark. Residual thiols were capped with iodoacetamide, and residual maleimides were capped with *N*-acetylcysteamine. To remove excess reagents and residual free polysaccharide, conjugates were pelleted by centrifugation at 289,000*g* for 60 min, resuspended in TED buffer (0.5% w/v deoxycholate in 0.1 M Tris–HCl, 0.01 M EDTA, pH 8.5) using a dounce homogenizer, and incubated for 15–20 h at rt. The conjugates were pelleted and re-suspended twice in 20 mM HEPES, pH 7.3 buffer containing 0.5 M NaCl. After a final pelleting, the conjugates were re-suspended in HBS and centrifuged at 1000*g* for 10 min to remove any aggregated material. Protein was determined by a modified Lowry assay,[51] and polysaccharide glucosamine content was determined by HPAEC-PAD. Conjugates were adsorbed onto Merck aluminum hydroxyphosphate adjuvant (alum) at a final concentration of 0.5 mg/mL of OMPC protein. The alum-adsorbed bulk was formulated for animal studies by dilution to appropriate polysaccharide concentrations in sterile saline.

4.12. Immunizations

Four-to-five-week-old BALB/c mice were obtained from Taconic (Germantown, NY). All animals were housed in an on-site animal facility, and all experimental procedures adhered to protocols approved by an Institutional Animal Care and Use Committee. For the first experiment, five mice per group were injected i.m. on days 0 and 14 with either 8, 0.8, 0.08, or 0.008 μg of conjugated polysaccharide using sized or intact SAE–OMPC conjugates. Five mice in a control group were immunized with Merck alum equivalent to the amount contained in the conjugates. A serum sample was obtained from these animals at days 2, 21 and 28. For the second experiment, ten mice per group were injected i.m. at days 0, 14 and 28 with 8 μg of conjugated polysaccharide using sized or intact SAE–OMPC conjugates. Ten mice in a control group were immunized with OMPC on Merck alum, equivalent to the amount contained in the conjugates. A serum sample was obtained from these animals at days 1, 21 and 35.

4.13. ELISA

The titer of antibodies specific for the SAE carbohydrate were determined by ELISA. Plates were coated with 0.5 μg/mL of AEC-intact SAE polysaccharide in 100 mM Na_2CO_3 buffer, pH 9.2 overnight at rt, and ELISA performed as previously described.[39] For titer endpoint determination, sera were initially diluted 1:100 in PBS and then in serial twofold steps for assay. Endpoints were assessed as the highest titer that produced a OD_{450} value that was three times higher than the background.

References

1. Na'Was, T.; Hawwari, A.; Hendrix, E.; Hebden, J.; Edelman, R.; Martin, M.; Campbell, W.; Naso, R.; Schwalbe, R.; Fattom, A. I. *J. Clin. Microbiol.* **1998**, *36*, 414–420.
2. Richards, M. J.; Edwards, J. R.; Culver, D. H.; Gaynes, R. P. *Infect. Control Hosp. Epidemiol.* **2000**, *21*, 510–515.
3. Richards, M. J.; Edwards, J. R.; Culver, D. H.; Gaynes, R. P. *Crit. Care Med.* **1999**, *27*, 887–892.
4. Rupp, M. E.; Archer, G. L. *Clin. Infect. Dis.* **1994**, *19*, 231–243.
5. Van Wijngaerden, E.; Peetermans, W. E.; Van Lierde, S.; Eldere, J. *Clin. Infect. Dis.* **1997**, *25*, 69–71.
6. Marchese, A.; Schito, G. C.; Debbia, E. A. *J. Chemother.* **2000**, *12*, 459–462.
7. Swartz, M. N. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 2420–2427.
8. Mack, D.; Fischer, W.; Krokotsch, A.; Leopold, K.; Hartmann, R.; Egge, H.; Laufs, R. *J. Bacteriol.* **1996**, *178*, 175–183.
9. Heilmann, C.; Schweitzer, O.; Gerke, C.; Vanittanakom, N.; Mack, D.; Gotz, F. *Mol. Microbiol.* **1996**, *20*, 1083–1091.
10. Mack, D.; Haeder, M.; Siemssen, N.; Laufs, R. *J. Infect. Dis.* **1996**, *174*, 881–884.
11. Deighton, M. A.; Borland, R.; Capstick, J. A. *Epidemiol. Infect.* **1996**, *117*, 267–280.
12. Rodgers, J.; Phillips, F.; Olliff, C. *FEMS Immunol. Med. Microbiol.* **1994**, *9*, 109–115.
13. Cramton, S. E.; Ulrich, M.; Gotz, F.; Doring, G. *Infect. Immun.* **2001**, *69*, 4079–4085.
14. Deighton, M. A.; Borland, R. *Infect. Immun.* **1993**, *61*, 4473–4479.
15. Hussain, M.; Hastings, J. G. M.; White, P. J. *J. Med. Microbiol.* **1991**, *34*, 143–147.
16. Baldassarri, L.; Donelli, G.; Gelosia, A.; Voglino, M. C.; Simpson, A. W.; Christensen, G. D. *Infect. Immun.* **1996**, *64*, 3410–3415.
17. Drewry, D. T.; Galbraith, L.; Wilkinson, B. J.; Wilkinson, S. G. *J. Clin. Microbiol.* **1990**, *28*, 1292–1296.
18. Tojo, M.; Yamashita, N.; Goldmann, D. A.; Pier, G. B. *J. Infect. Dis.* **1988**, *157*, 713–722.
19. Cramton, S. E.; Gerke, C.; Schnell, N. F.; Nichols, W. W.; Gotz, F. *Infect. Immun.* **1999**, *67*, 5427–5433.
20. McKenney, D.; Hubner, J.; Muller, E.; Wang, Y.; Goldman, D. A.; Pier, G. B. *Infect. Immun.* **1998**, *66*, 4711–4720.
21. McKenney, D.; Pouliot, K. L.; Wang, Y.; Murthy, V.; Ulrich, M.; Doring, G.; Lee, J. C.; Goldmann, D. A.; Pier, G. B. *Science* **1999**, *284*, 1523–1527.

22. McKenney, D.; Pouliot, K. L.; Wang, Y.; Murthy, V.; Ulrich, M.; Doring, G.; Lee, J. C.; Goldmann, D. A.; Pier, G. B. *J. Biotechnol.* **2000**, *83*, 37–44.
23. Maira-Litran, T.; Kropec, A.; Abeygunawardana, C.; Joyce, J.; Mark, G.; Goldmann, D. A.; Pier, G. B. *Infect. Immun.* **2002**, *70*, 4433–4440.
24. Abeygunawardana, C.; Bush, C. A. *Adv. Biophys. Chem.* **1993**, *3*, 199–249.
25. Chouly, C.; Colquhoun, I. J.; Jodelet, A.; York, G.; Walker, G. *Int. J. Biol. Macromol.* **1995**, *17*, 357–363.
26. Ravenscrodt, N.; D'Ascenzi, S.; Proietti, D.; Costentino, P. *Dev. Biol.* **2000**, *103*, 35–47.
27. Mulder, G. J. *J. Prakt. Chem.* **1840**, *21*, 229.
28. Newth, F. H. *Adv. Carbohydr. Chem.* **1951**, *6*, 83–106.
29. Von Grote, A.; Tollens, B. *Ber. Dtsch. Chem. Ges.* **1874**, *7*, 1375.
30. Von Grote, A.; Tollens, B. *Liebigs Ann.* **1881**, *206*, 226.
31. Bednar, B.; Hennessey, J. *Carbohydr. Res.* **1993**, *243*, 115–130.
32. Fey, P. D.; Ulphani, J. S.; Gotz, F.; Heilmann, C.; Mack, D.; Rupp, M. E. *J. Infect. Dis.* **1999**, *179*, 1561–1564.
33. Mack, D.; Riedewald, J.; Rohde, H.; Magnus, T.; Feucht, H. H.; Elsner, H.-A.; Laufs, R.; Rupp, M. E. *Infect. Immun.* **1999**, *67*, 1004–1008.
34. Arciola, C. R.; Baldassarri, L.; Montanaro, L. *J. Clin. Microbiol.* **2001**, *39*, 2151–2156.
35. Fowler, V. G.; Fey, P. D.; Reller, L. B.; Chamis, A. L.; Corey, G. R.; Rupp, M. E. *Med. Microbiol. Immunol.* **2001**, *189*, 127–131.
36. Lee, J. C.; Takeda, S.; Livolsi, P. J.; Paoletti, L. C. *Infect. Immun.* **1993**, *61*, 1853–1858.
37. Donnelly, J. J.; Deck, R. R.; Liu, M. A. *J. Immunol.* **1990**, *145*, 3071–3079.
38. Sturgess, A. W.; Rush, K.; Charbonneau, R. J.; Lee, J. I.; West, D. J.; Sitrin, R. D.; Hennesey, J. P. *Vaccine* **1999**, *17*, 1169–1178.
39. McNeely, T. B.; Liu, X.; Bringman, T.; Donnelly, J. J. *Vaccine* **2000**, *18*, 2808–2816.
40. Vella, P. P.; Marburg, S.; Staub, J. M.; Kniskern, P. J.; Miller, W.; Hagopian, A.; Ip, C.; Tolman, R. L.; Rusk, C. M.; Chupak, L. S.; Ellis, R. W. *Infect. Immun.* **1992**, *60*, 4977–4983.
41. Fukasawa, L. O.; Gorla, M. C.; Schenkman, R. P.; Garcia, L. R.; Carneiro, S. M.; Raw, I.; Tanizaki, M. M. *Vaccine* **1999**, *17*, 2951–2958.
42. Tai, J. Y.; Vella, P. P.; McLean, A. A.; Woodhour, A. F.; McAleer, W. J.; Sha, A.; Dennis-Sykes, C.; Hilleman, M. R. *Proc. Soc. Exp. Biol. Med.* **1987**, *184*, 154–161.
43. Wu, D.; Chen, A. J.; Johnson, C. S., Jr. *J. Magn. Reson. A* **1995**, *115*, 260.
44. Rance, M.; Sørensen, O. W.; Bodenhausen, G.; Wagner, G.; Ernst, R. R.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479–485.
45. Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355–360.
46. Braubschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521–528.
47. Bodenhausen, G.; Ruben, D. J. *Chem. Phys. Lett.* **1980**, *69*, 185–188.
48. Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093–2094.
49. Marburg, S.; Jorn, D.; Tolman, R. L.; Arison, B.; McCauley, J.; Kniskern, P. J.; Hagopian, A.; Vella, P. P. *J. Am. Chem. Soc.* **1986**, *108*, 5282–5287.
50. Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.
51. Markwell, M. A.; Haas, S. M.; Bieber, L. L.; Tolbert, N. E. *Anal. Biochem.* **1978**, *87*, 206–210.