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Theresa A. Fassel
University of Wisconsin-Madison

James R. Sanger
Medical College of Wisconsin, Milwaukee

Charles E. Edmiston
Medical College of Wisconsin, Milwaukee

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LYSINE EFFECT ON RUTHENIUM RED AND ALCIAN BLUE PRESERVATION AND STAINING OF THE STAPHYLOCOCCAL GLYCOCALYX

Theresa A. Fassel[1,*], James R. Sanger[2], and Charles E. Edmiston[3]

[1]Muscle Biology Laboratory, University of Wisconsin-Madison, Madison, Wisconsin 53706
[2]Dept. Plastic and Reconstructive Surgery, and [3]Surgical Microbiology Research Lab., Dept. Surgery, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

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Abstract

The effect of lysine on ruthenium red (RR) and alcian blue (AB) preservation and/or staining for transmission electron microscopy of glycocalyces was evaluated for three species of the gram-positive, coagulase-negative staphylococci. A RR and an AB procedure were compared with and without lysine included in the glutaraldehyde prefixation. Minimal or no glycocalyx was preserved or stained by glutaraldehyde fixation only. For all species, the inclusion of lysine increased visualization of fibrous material. RR treatment without lysine, results in sparse or limited fibrous material or artifactual condensations which contrasts with enhanced fibrous material seen when lysine is included with RR. The effect of lysine is also to enhance the visualization of staphylococcal glycocalyx by AB. Without lysine, condensed curved structures are consistently seen following AB processing. In contrast, extensive and elaborate glycocalyces are observed with inclusion of lysine with AB. Thus, ultrastructural visualization by RR and AB of the staphylococcal glycocalyx in the species studied was enhanced by use of lysine.

Key Words: Lysine, ruthenium red, alcian blue, bacterial glycocalyx, polysaccharide, staphylococci, staining, transmission electron microscopy.

*Address for correspondence:
C. Edmiston,
FMLH, Department of Surgery,
Medical College of Wisconsin,
9200 W. Wisconsin Ave.,
Milwaukee, Wisconsin 53226,
Phone No. (414)-454-5739
Fax No. (414)-259-9225

Introduction

The gram-positive coagulase-negative staphylococci are widespread potentially opportunistic pathogens and infectious agents. These bacteria are thought to be important in latent infection associated with bioprosthetics and have been recovered from the surface of infected biomaterials. After an initial adhesion event, it is thought the production of polysaccharide slime aids in staphylococci proliferation and persistence (Edmiston *et al.*, 1989). Thus, electron microscopic visualization and understanding of the bacterial extracellular polysaccharide, or glycocalyx (Costerton *et al.*, 1981) is important. The bacterial glycocalyx consists of extracellular polysaccharide of bacterial origin that lies outside the outer membrane of gram-negative cells and outside the peptidoglycan of gram-positive cells (Costerton *et al.*, 1981). There are two major types of bacterial glycocalyx. The first type is the S layer. It is composed of globular glycoprotein subunits, often in regular arrays (Sutherland, 1972; Costerton *et al.*, 1981). The second major type is composed of fibrous polysaccharide and is referred to as a fibrous matrix or a fibrous anionic glycocalyx (Costerton *et al.*, 1981). The staphylococcal glycocalyx of this study are of this fibrous type and fit the definition of Costerton *et al.* (1981) and van Iterson (1984). Therefore, this terminology will be used to describe the bacterial glycocalyx material reported in this study.

The bacterial glycocalyx is highly hydrated (Sutherland, 1972), highly polymeric and highly charged (Costerton *et al.*, 1981; van Iterson, 1984). Its chemical composition is highly variable and diverse (Costerton *et al.*, 1981; van Iterson, 1984). Some small coalesced granules or particles are not excluded from the fibrous matrix, although others, such as, India ink or nigrosin are excluded. The tightness of the association of the glycocalyx with the cell surface is often variable as well (Costerton *et al.*, 1981).

These characteristics make effective visualization of the bacterial glycocalyx by transmission electron microscopy (TEM) difficult. Since aldehydes, as well as osmium tetroxide, have a low affinity for polysaccharide (Hayat, 1981), stabilization by these chemical fixatives

alone may be insufficient. An additional difficulty may involve a lack of innate electron density and an inability to stain with conventional reagents (Roth, 1977). Dehydration, which subjects samples to the removal of water and replacement with dehydrant, may adversely deform, modify or condense a highly hydrated structure such as the glycocalyx (Sutherland, 1972; Springer and Roth, 1973; Roth, 1977; Costerton *et al.*, 1981).

Cationic reagents, ruthenium red (RR) and alcian blue (AB), have been used to improve visualization of the bacterial glycocalyx. Ruthenium red and AB have traditionally been used to improve observation of acidic polysaccharide in light and electron microscopy. Ruthenium red is a low molecular weight compound and a highly positively charged sphere of 1.1 nm size (Luft, 1971a; Hanke and Northcote, 1975; Schrevel *et al.*, 1981). Ruthenium red was used to stain pectin semi-specifically (Jensen, 1962). It has been used extensively to study bacterial outer layers (Pate and Ordal, 1967; Springer and Roth, 1973; Titus *et al*, 1982). However, artifactual condensation and/or collapse of glycocalyx structures continued to be a limitation (Costerton *et al.*, 1981; Molinari *et al.*, 1988). The employment of cross-linking antibodies with RR have aided maintenance of extended fibrillar structures (Patterson *et al.*, 1975; Mackie *et al.*, 1979).

Another significant approach is the utilization of the diamine lysine with glutaraldehyde and RR. Boyles (Boyles, 1982, 1984; Boyles *et al.*, 1985) used the capability of lysine to form large cross-linked polymers with glutaraldehyde in fixing actin filaments, extracellular matrix and mammalian glycocalyx. Ruthenium red was included with glutaraldehyde and lysine by Jacques and Graham (1989). It has since been utilized to study ruminal bacteria (Akin and Rigsby, 1990), *Clostridium difficile* (Davies and Borriello, 1990), *Streptococcus suis* (Jacques *et al.*, 1990), methanotrophs (Fassel *et al.*, 1992b), and staphylococci (Fassel *et al.*, 1992b). The mechanisms of the interaction of RR with glutaraldehyde and lysine in this scheme of preservation and/or staining is not yet established. It was thought by Luft (1971a, 1971b, 1976) that RR reacts with polyanions of high charge density, such as acidic polysaccharide or glycosaminoglycans. Neutral polysaccharide, polypeptides or proteins are generally less reactive (Luft, 1971a).

Alcian blue is larger, planar and has a less localized charge distribution on its side chains than RR (Scott, 1972a, b; Ghiggeri *et al.*, 1988). Its mechanism of specificity is not established although it may act through electrostatic or ionic linkages with negatively charge moieties (Scott *et al.*, 1964; Behnke and Zelender, 1970; Luft, 1971a). Alcian blue has been used to stabilize extracellular carbohydrate containing coats of mammalian cells (Behnke and Zelender, 1970) and bacterial cells, *Eikenella* (Progulske and Holt, 1980), *Pseudomonas fragi* (Herald and Zottola, 1988), methanotrophs (Fassel *et al.*, 1992a, b), and staphylococci (Fassel *et al.*, 1992b). In general, AB is thought to act through electrostatic or ionic linkages with negatively charge moieties notably acidic complex polysaccharide and glycoconjugates (Scott *et al.*, 1964; Behnke and Zelander, 1970; Luft, 1972a).

For the gram-positive coagulase-negative staphylococci, we have previously compared RR, AB and RR-lysine procedures. The RR and AB *en bloc* procedures were based on the work of Patterson *et al.* (1975), and Mackie *et al.* (1979). In those experiments, RR or AB were included at 0.15% in the prefixation with 0.2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3 for 30 minutes. Each subsequent stage of processing (fixation in 1% glutaraldehyde, postfixation in 2% OsO_4, all 0.1 M cacodylate buffer pH 7.3 washes, and dehydration through 70% ethanol) included 0.05% RR or AB. After RR processing, limited fibrous material was observed. After AB processing, discrete, often curved, electron dense structures were seen (Fassel *et al.*, 1992b). The use of RR and lysine with glutaraldehyde, based on Jacques and Graham (1989), was also studied. By this approach, RR was included at 0.075% in both the prefixation with lysine and glutaraldehyde and the fixation with glutaraldehyde. For the staphylococci, extensive fibrous and elaborate glycocalyces were observed by this method. This contrasted with the images obtained by the RR or AB procedures above. For the methanotrophs, the fibrous layers were often comparable between AB and RR-lysine. These observations were attributed to genera/species variation (Fassel *et al.*, 1992b).

These observations lead to the questions whether lysine, when added to AB, would result in enhanced stabilization of the staphylococcal glycocalyx, if a fibrous matrix type of glycocalyx would be observed and if the RR-lysine and AB-lysine images would then agree? The answers to these questions are the major purpose of this study. Additionally, we examined the effects of RR and lysine separately by examining the effect of a lysine-glutaraldehyde method without RR.

The RR-lysine method utilized previously (Fassel *et al.*, 1992b) and based on Jacques and Graham (1989), became the model for the methods examined in this study. A glutaraldehyde control has only glutaraldehyde in the prefixation and fixation stages and is called glutaraldehyde prefixation (**method 1**). Lysine is added to the prefixation for glutaraldehyde-lysine prefixation (**method 2**). Ruthenium red only is added to the prefixation and fixation stages for RR prefixation (**method 3**). Lysine and RR are added to the prefixation and RR to the fixation of RR-lysine prefixation (**method 4**). Alcian blue is added to the prefixation and fixation stages for AB prefixation (**method 5**). Lysine and AB are added to the prefixation and AB to the fixation stages for AB-lysine prefixation (**method 6**). These six methods were compared for three staphylococcal species. *Staphylococcus epidermidis* RP62 is a copious slime producer. *Staphylococcus aureus* ATCC 25923 produces considerable amounts of slime. The least relative slime producer of our test bacteria is *Staphylococcus hominis* SP2.

Materials and Methods

Cell culture

Three gram-positive coagulase-negative staphylococcal species, *Staphylococcus epidermidis* RP62, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus hominis* SP2, were recovered from -70°C frozen storage and plated on blood agar plates to test viability. After 24 hours, cells were transferred to trypticase soy broth and incubated at 35°C for 18 hours.

Cells were handled as pellets until all fixation/wash procedures were completed. Then, cells were enrobed in 4% agar and handled as 1 mm^3 blocks for dehydration, infiltration and embedding procedures. The buffer for all fixation and wash solutions was 0.1 M cacodylate buffer (pH 7.3). All steps were carried out at room temperature.

Glutaraldehyde prefixation (method 1)

Prefixation was in 2.5% glutaraldehyde (GA) in buffer for 20 minutes. Fixation was in 2.5% GA in buffer for 2 hours. This was followed by three 10 minute washes in buffer. Postfixation was in 1% (weight/volume, w/v) OsO_4 in buffer for 2 hours. This step was followed by three 10 minute washes in buffer. Dehydration was in a graded ethanol series: 10%, 25%, 50%, 70%, 95%, 100% anhydrous, and 100% anhydrous. Samples were transferred directly to LR White without use of a transition solvent. Infiltration and embedment for LR White followed processing instructions Data Sheet 305A (Polysciences, Inc.). Thin sections were cut using a diamond knife and post stained in 25% ethanolic 2% uranyl acetate and Reynolds lead citrate (Reynolds, 1963). Samples were observed in a Hitachi H-600 TEM operated at 50 kV.

Glutaraldehyde-lysine prefixation (method 2)

Prefixation was in 75 mM lysine in 2.5% GA in buffer for 20 minutes. Fixation was in 2.5% GA in buffer for 2 hours. Further processing after this step was identical to the GA prefixation (method 1) above.

Ruthenium Red (RR) prefixation (method 3)

Prefixation was in 0.075% RR, 2.5% GA in buffer for 20 minutes. Fixation was in 0.075% RR, 2.5% GA in buffer for 2 hours. Further processing after this step was identical to the GA prefixation (method 1) above.

Ruthenium red-lysine prefixation (method 4)

Prefixation was in 75 mM lysine, 0.075% RR, 2.5% GA in buffer for 20 minutes. Fixation was in 0.075% RR, 2.5% GA in buffer for 2 hours. This method is based on Jacques and Graham (1989) and is identical to the RR-lysine *en bloc* procedure of Fassel *et al.* (1992b). Further processing after this step was identical to the GA prefixation (method 1) above.

Alcian blue (AB) prefixation (method 5)

Prefixation was in 0.075% AB, 2.5% GA in buffer for 20 minutes. Fixation was in 0.075% AB, 2.5% GA in buffer for 2 hours. Further processing after this step was identical to the GA prefixation (method 1) above.

Alcian blue-lysine prefixation (method 6)

Prefixation was in 75 mM lysine, 0.075% AB, 2.5% GA in buffer for 20 minutes. Fixation was in 0.075% AB, 2.5% GA in buffer for 2 hours. Further processing after this step was identical to the GA prefixation (method 1) above.

Results and Discussion

For *Staphylococcus aureus* ATCC 25923 cells, the effect of the absence (Fig. 1A) or presence (Fig. 1B) of lysine in the prefixation were compared. In the GA prefixation (method 1, Fig. 1A), glycocalyx material is absent or extremely limited. In the GA-lysine prefixation (method 2, Fig. 1B), fibrous material (arrow) is observed irregularly around and occasionally between cells. The effect of lysine separate from the effect of RR on the stabilization of the glycocalyx was next evaluated. The RR prefixation (method 3, Fig. 1C) was compared to RR-lysine prefixation (method 4, Fig. 1D). For RR prefixation (method 3, Fig. 1C), some sparse fibrous material (arrow) is present around cells. For RR-lysine prefixation (method 4, Fig. 1D), fibrous material is more abundant and may be found surrounding cells as well as extending from the cell body (arrow). We desired to ascertain whether lysine when added to AB would enhance stabilization of the staphylococcal glycocalyx, over the discrete irregular electron dense structures observed previously. The latter structures were concluded to represent a collapsed and insufficiently stabilized glycocalyx (Fassel *et al.*, 1992b). Thus, AB prefixation (method 5, Fig. 1E) was compared to AB-lysine prefixation (method 6, Fig. 1F). For AB prefixation (method 5, Fig. 1E) discrete curved electron dense structures were observed as expected. Often these structures do not appear well associated with specific cells. These structures agreed with observation of previous AB processing even though the two approaches differed slightly. For the AB procedure of our previous work, 0.15% AB was included in the prefixation with 0.2% GA for 30 minutes. This was followed by inclusion of 0.05% AB at each subsequent stage of processing through 70% ethanol (Fassel *et al.*, 1992b). In the present study, 0.075% AB was included in the prefixation with 2.5% GA for 20 minutes. This was followed by 0.075% AB in the fixation with 2.5% GA. Alcian blue was not included in any subsequent stage of processing. The present approach was chosen here to bring concentrations and times of the experimental stages into better agreement and direct comparison with the lysine methods under study. For AB-lysine prefixation (method 6, Fig. 1F), fibrous material (arrow) is observed.

For *Staphylococcus hominis* SP2, these methods are also evaluated. By GA prefixation (method 1, Fig. 2A), no glycocalyx material is preserved. By GA-lysine prefixation (method 2, Fig. 2B), considerable fibrous material (arrow) surrounds cells. By RR prefixation

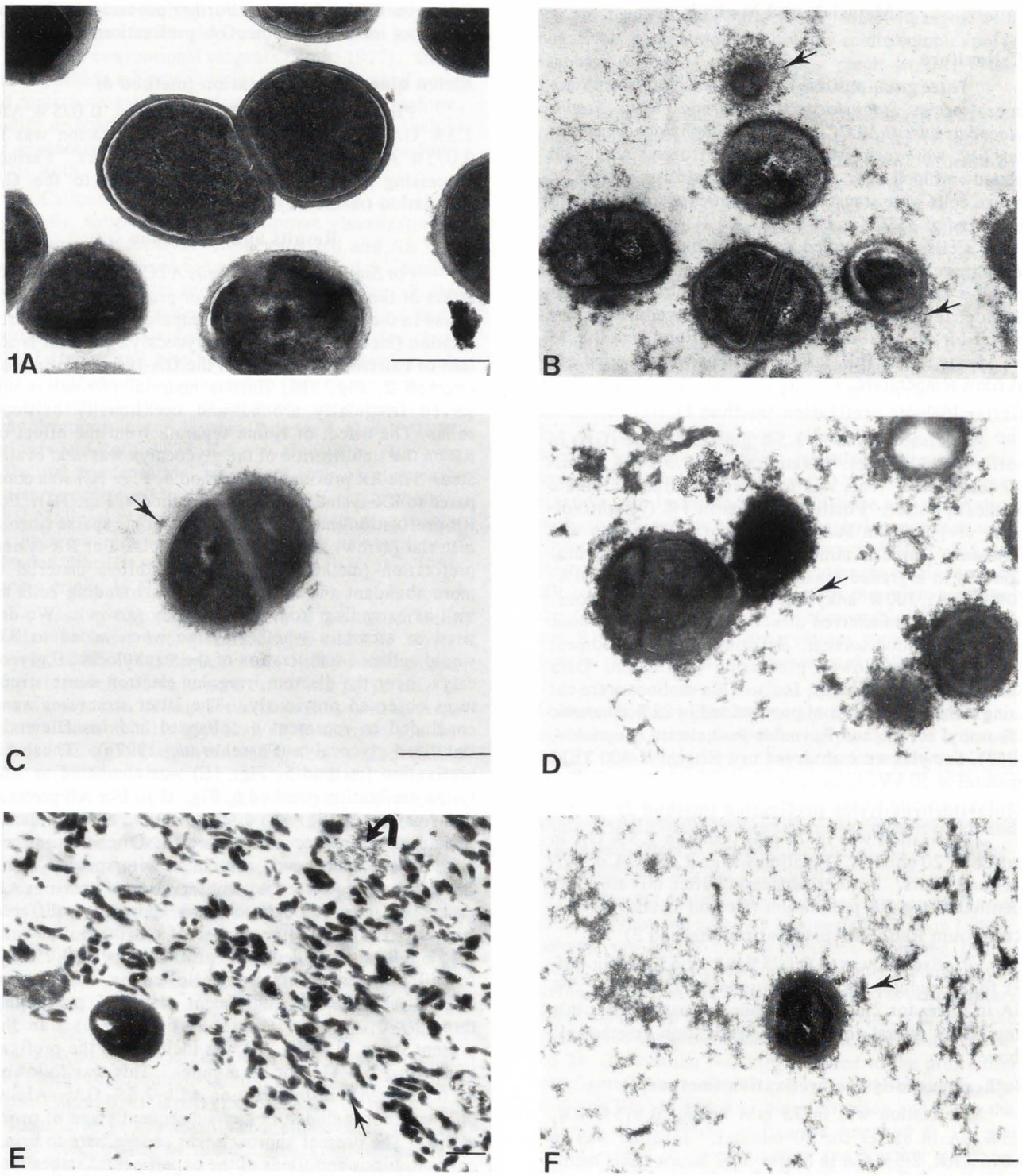

Figure 1. Transmission electron micrographs for *Staphylococcus aureus* ATCC 25923 are shown for (**A**) GA prefixation (nethod 1); (**B**) GA-lysine prefixation (method 2); (**C**) RR prefixation (method 3); (**D**) RR-lysine prefixation (method 4); (**E**) AB prefixation (method 5); and (**F**) AB-lysine prefixation (method 6). (**A**) GA prefixation (method 1) shows no glycocalyx material. (**B**) For GA-lysine prefixation (method 2), fibrous material (arrow) is seen irregularly around and occasionally between cells. (**C**) By the RR prefixation (method 3), fibrous material (arrow) is seen sparsely around cells. (**D**) For the RR-lysine prefixation (method 4), fibrous material is more abundant and extends further out from the cell body (arrow). (**E**) For the AB prefixation (method 5), discrete irregular electron dense structures (arrow) are observed. Condensations of a more fine texture are also occasionally seen (curved arrow). (**F**) For the AB-lysine prefixation (method 6), some fibrous material (arrow) is observed. Bars = 0.5 μm.

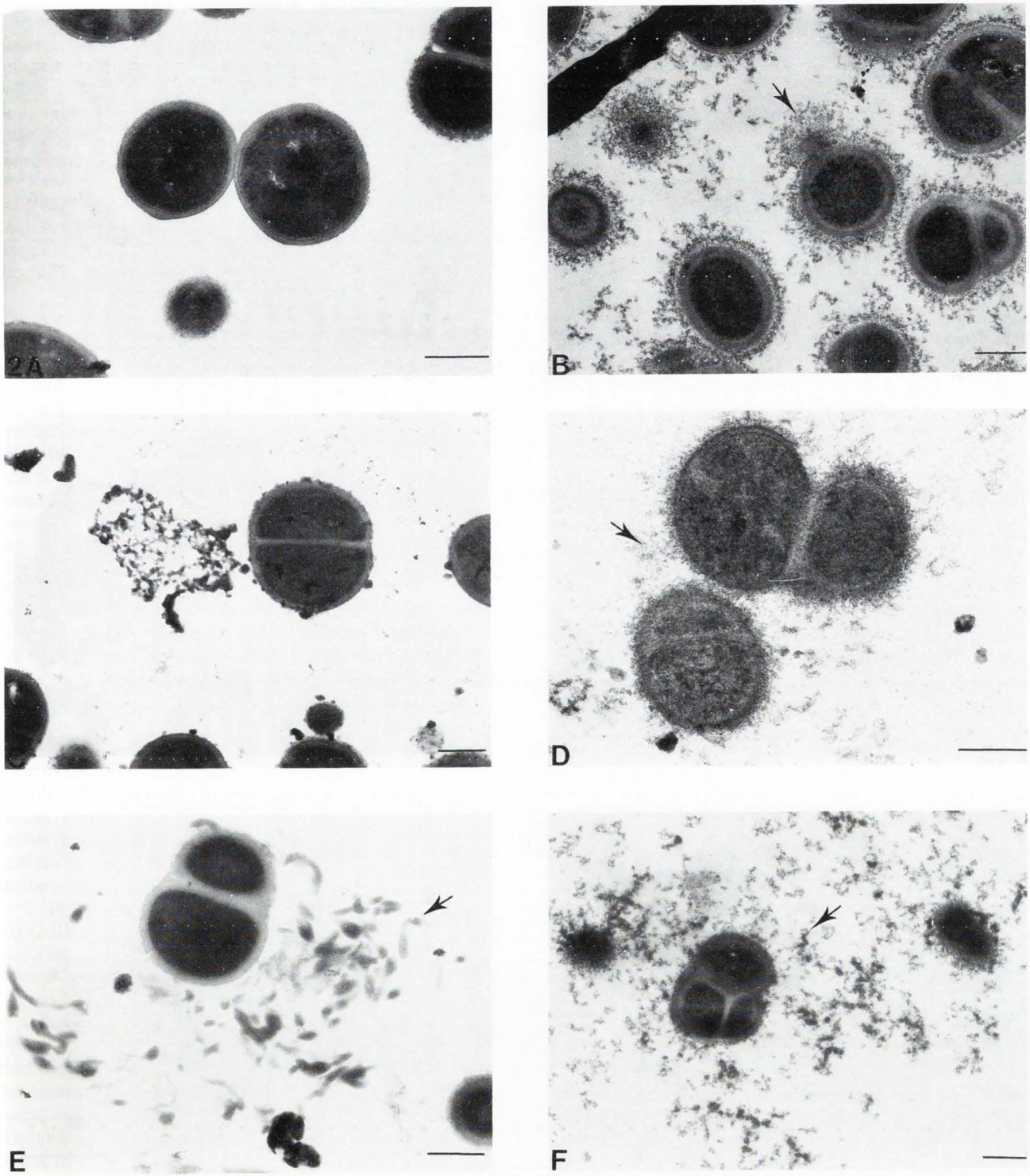

Figure 2. Transmission electron micrographs for *Staphylococcus hominis* SP2 are shown for (**A**) GA prefixation (method 1); (**B**) GA-lysine prefixation (method 2); (**C**) RR prefixation (method 3); (**D**) RR-lysine prefixation (method 4); (**E**) AB prefixation (method 5); and (**F**) AB-lysine prefixation (method 6). (**A**) GA prefixation (method 1), no glycocalyx material is preserved. (**B**) For GA-lysine prefixation (method 2), considerable fibrous material (arrow) surrounds cells. (**C**) By the RR prefixation (method 3), external material is infrequent. (**D**) By the RR-lysine prefixation (method 4), fibrous material surrounding cells is enhanced. (**E**) For the AB prefixation (method 5), curved electron dense material (arrow) are seen. (**F**) For the AB-lysine prefixation (method 6), fibrous material (arrow) is more abundant. Bars = 0.5 μm.

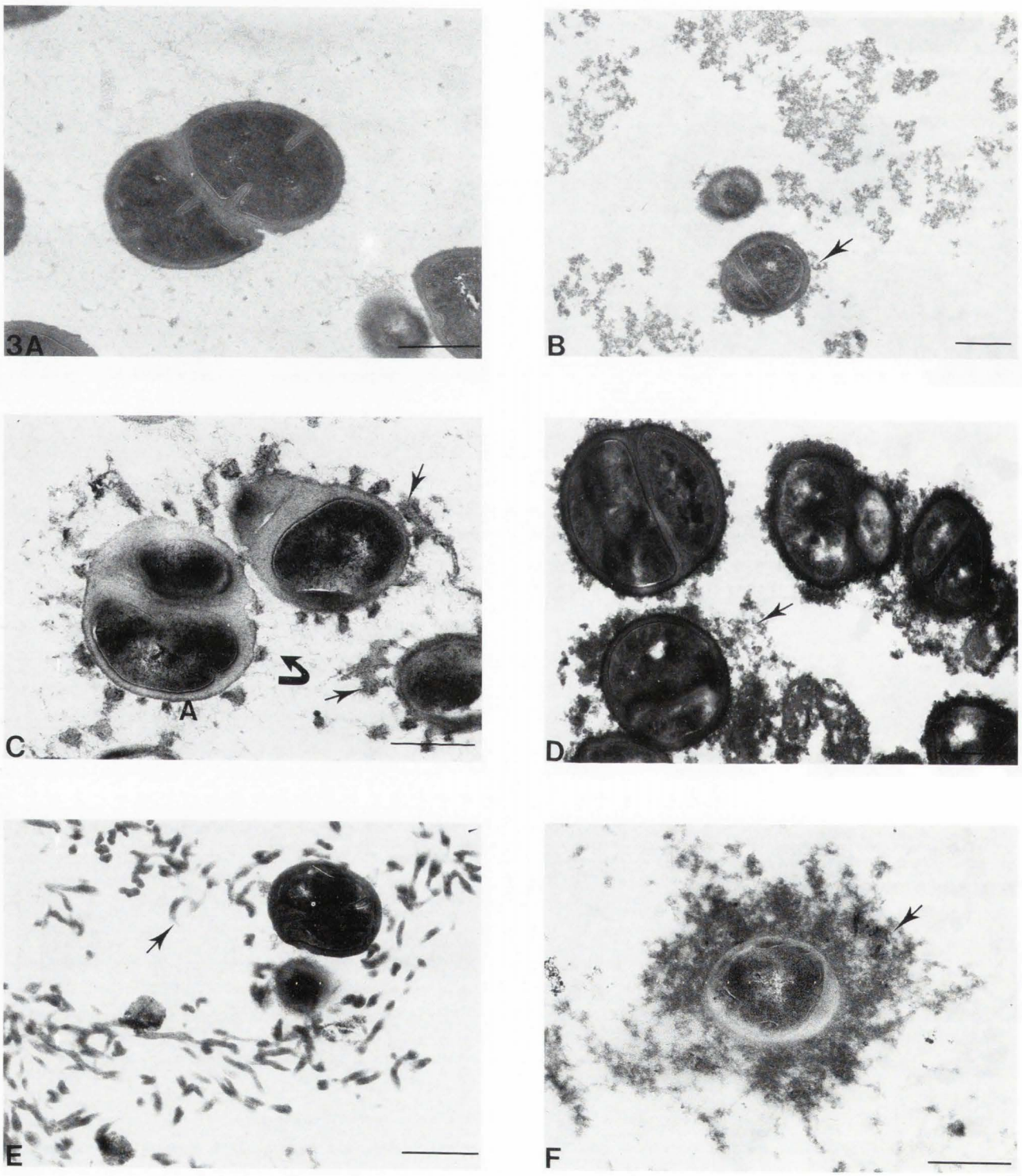

Figure 3. Transmission electron micrographs for *Staphylococcus epidermidis* RP62, are shown for (**A**) GA prefixation (method 1); (**B**) GA-lysine prefixation (method 2); (**C**) RR prefixation (method 3); (**D**) RR-lysine prefixation (method 4); (**E**) AB prefixation (method 5); and (**F**) AB-lysine prefixation (method 6). (**A**) The GA prefixation (method 1) does not preserve glycocalyx material. (**B**) For GA-lysine prefixation (method 2), fibrous material (arrows) is observed. (**C**) By the RR prefixation (method 3), external material appears as condensed in bead-like structures that occasionally merge with material of similar electron density (arrows). Thin thread-like material is also seen (curved arrows). (**D**) By the RR-lysine prefixation (method 4), fibrous material (arrow) is seen around and between cells. (**E**) For AB prefixation (method 5), discrete irregular electron dense material (arrow) is seen. (**F**) For the AB-lysine prefixation (method 6), elaborate and extensive fibrous material (arrow) is observed. Bars = 0.5 μm.

(method 3, Fig. 2C), external material is infrequent. Some coalesced appearing material occasionally occurs (curved arrow Fig. 2C). We consider this material as well as the infrequent round features close to the cell body (arrow) to be artifact which may be due to inadequate stabilization and subsequent collapse of any RR-GA-glycocalyx complex. By RR-lysine prefixation (method 4, Fig. 2D), fibrous material that surrounds cells is enhanced. After AB prefixation (method 5, Fig. 2E), curved electron dense condensations (arrow), are seen. In contrast, by AB-lysine prefixation (method 6, Fig. 2F), fibrous material occurs more extensively.

We also sought to evaluate these methods using a copious slime producer, *Staphylococcus epidermidis* RP62. Glycocalyx material is lacking by GA prefixation (method 1, Fig. 3A). Glutaraldehyde-lysine prefixation (method 2, Fig. 3B) resulted in some fibrous material (arrows) occurring irregularly around cells. For the RR prefixation (method 3, Fig. 3C), external material appears in bead-like structures that occasionally merge with material of similar electron density (arrows). Thin thread-like material is also seen (curved arrows). By the RR-lysine prefixation (method 4, Fig. 3D), fibrous material is observed around and between cells. For the AB prefixation (method 5, Fig. 3E), discrete curved or irregular electron dense condensations (arrow) appear. By the AB-lysine prefixation (method 6, Fig. 3F), elaborate and extensive glycocalyx material is maintained.

For all cells studied, lysine improved observation of the staphylococcal glycocalyx. The effect of lysine alone, as determined by the GA prefixation (method 2), was to stabilize sufficient glycocalyx material to make its observation possible. This was achieved in the absence of the cationic reagents, RR or AB. For *S. hominis* SP2, this stabilization was of particular note. The fibrous material was observed surrounding cells in a manner consistent with the definition of a fibrous/filamentous type of glycocalyx (Costerton *et al.*, 1981; van Iterson, 1984).

In our previous work on the staphylococcal glycocalyx, RR was used at 0.15% in the prefixation and at 0.05% at all subsequent stages of processing thereafter through 70% ethanol. By that procedure, fibrous material was observed that ranged from sparse to more regular around cells (Fassel *et al.*, 1992b). In the present approach, where RR was included in prefixation and fixation stages at 0.075%, fibrous material was more rare. The occasional coalesced material or rare round features around cells of *S. hominis* SP2 as well as bead-like and thin thread like structures of *S. epidermidis* RP62 also are not in agreement with previous observations and the image of the glycocalyx by the RR-lysine prefixation (method 4). Thus, we conclude RR by the present RR prefixation method (method 3), and without lysine, is not adequate to stabilize the staphylococcal glycocalyx. Also, the images obtained by RR alone by the present method (RR prefixation method 3) contain artifactualy collapsed or condensed structures.

Alcian blue in our previous work, was included at 0.15% in the prefixation with 0.2% GA for a 30 minute prefixation and at 0.05% at all subsequent stages though 70% ethanol. For the methanotrophic bacteria, elaborate fibrous layers surrounded cells processed by this method. There was good agreement with images of the fibrous material seen by RR-lysine processing (Fassel *et al.*, 1992b) as well as freeze-etch obtained images (Fassel *et al.*, 1992a). In contrast, when the staphylococci were processed by the previous AB procedure (Fassel *et al.*, 1992b) condensed curved structural features were observed. These disagreed with the elaborate fibrous glycocalyces obtained by previous RR-lysine processing and RR-lysine (method 4). The present AB method (method 5) differs from the previous AB method by changing the percentage of AB to 0.075% for both the prefixation and fixation with glutaraldehyde and eliminating AB from subsequent stages. The purpose of this approach was to make comparison more direct and conditions of concentration and time more uniform for method 1 through method 6. Again, the images of the staphylococcal glycocalyx by AB processing (AB method 5) are of curved electron dense structures. These features agree well with the condensations or distortions of the glycocalyx seen with AB alone previously. Therefore, neither method 5 of the present study, nor the previous AB method are sufficient to stabilize the staphylococcal glycocalyx.

We sought to determine if lysine is capable of improving the interaction of AB with glutaraldehyde in the stabilization of the glycocalyx. Our results indicate lysine does enhance the effect of AB. Lysine with AB appears to make the utility of AB for the staphylococci more feasible. For all species studied, elaborate fibrous material was observed. For *S. aureus* and *S. hominis* SP2, this enhancement is similar to that seen for RR-lysine. For *S. epidermidis* RP62, AB lysine results in visualization of more extensive material than seen by RR-lysine. Thus, lysine appears to be critical in improving visualization of the staphylococcal glycocalyx and enhancing the capability of RR and AB. Additionally, lysine alone gives visualization of glycocalyx material when none is observable in its absence. However, we feel the presence of either RR or AB with lysine yields interesting and valuable information. Presently, we hesitate to conclude that one cationic dye is superior to the other in the current application. In our previous observation of methanotrophic bacteria, AB alone or RR-lysine gave a view of the glycocalyx that was reasonably representative (Fassel *et al.*, 1992a, 1992b). For the staphylococci, the case is more ambiguous with some genera/species variability.

In our previous paper (Fassel *et al.*, 1992b), we speculated that the nature of the staphylococcal glycocalyx, i.e., its biochemical and/or biophysical charge characteristic, may allow a more effective interaction with and stabilization by glutaraldehyde, lysine and RR. This was observed as enhanced fibrous material associated with cells. The secreted polysaccharide of the bacterial glycocalyx is highly negatively charged (van Iterson,

1984), while both RR and AB are highly positively charged. However, the distribution of charge differs. Ruthenium red is a 1.1 nm sphere with a uniform charge distribution over its surface (Luft, 1971a; Hanke and Northcote, 1975; Schrevel *et al.*, 1981). For AB, the charge distribution is less localized and distributed on quarternary amino group side chains (Scott, 1972a, b; Ghiggeri *et al.*, 1988). Lysine is also positively charged at physiological pH and after interaction with glutaraldehyde (Boyles, 1984). At this time, we can only consider and speculate on the possibility that the cross-linking interactions of GA and lysine with secreted anionic polysaccharide may be enhanced by the addition of the cationic reagents RR or AB. Improved stabilization of the entire complex may result. Perhaps, differences in our observations between RR and AB, where they occur, are due to the difference in amount and/or distribution of charge between these reagents as well as differences in the secreted polysaccharide composition between species.

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Discussion with Reviewers

M. Jacques: All preparations exposed to alcian blue (Figs. 1E, 2E, and 3E) showed the presence of curved electron dense structures. Could this be a background due to the presence of the stain?

Authors: The curved electron dense structures are present only in alcian blue (method 5) preparation which does not include lysine. In previous work (Fassel *et al.*, 1992b), where alcian blue preparations included a prefixation with 0.15% AB for 30 minutes and 0.05% AB in fixations, wash and dehydration stages to 70% ethanol thereafter, and again no lysine, these curved electron dense structures were also observed. We concluded at that time that these structures were the result of condensation or distortion of the staphylococcal glycocalyx. This seemed likely due to insufficient stabilization and subsequent collapse. These condensed structures also did not agree with the extended configurations seen with the RR-lysine method. In contrast, for the methanotrophs (Fassel *et al.*, 1992b), an elaborate and fibrous glycocalyx image agreed between RR-lysine and AB procedures. It was one of the purposes to this study to clarify whether lysine added to AB would result in enhanced stabilization of the staphylococcal glycocalyx, and further, if the RR-lysine and AB-lysine images would then agree. The answer is affirmative. In the presence of lysine, the curved electron dense structures are absent in AB preparations. We concluded again that the curved electron dense structures are due to the presence of AB and are artifactual representations of the staphylococcal glycocalyx. The staphylococcal glycocalyx appears elaborate and fibrous in both RR-lysine (method 4) and AB-lysine (method 6) images. Thus, lysine with AB improves the stabilization of the glycocalyx for these staphylococci.

M. Jacques: Bacterial cells were embedded in LR White, an acrylic resin. Do you know if this type of resin offers advantages over an epoxy resin concerning bacterial glycocalyx preservation.

Authors: In our previous work (Fassel *et al.*, 1990) on the globular glycoprotein, or S-layer, type of glycocalyx, a difference due to the resin was crucial for Methylomonas albus BG8. We observed that the external regular array of cup-like structures was absent in Spurr (Fassel *et al.*, 1990) as well as epon-araldite (unpublished observations). In LR White, it was present, although lacking strong definition as individual structures and often not in a complete layer around the outer membrane. The differential effect on preservation of the cup layer by embedding resins was overcome by use of RR (Fassel *et al.*, 1990) or AB (Fassel *et al.*, 1992a). These results suggest we could use an epoxy resin in methods utilizing RR and AB, and feel confident of our observations. However, we are also interested in using GA and GA-lysine methods in our comparisons to illuminate the separate effect of lysine from effects of RR and AB on stabilization of the glycocalyx. Therefore, LR White was the resin of choice for our experiments.

M. Jacques: Have you tried to optimize, furthermore, one protocol by varying different parameters such as: lysine concentration, ruthenium red or alcian blue concentration, or duration of fixation.

Authors: Variation and optimization of these parameters constitutes ongoing and future work. We look forward to submitting our results for publication soon.

M. Jacques: In order to better evaluate the effect of the various protocols, a negative control (i.e., a non-capsulated strain or a non-capsulated mutant) should be included.

Authors: Work on slime negative mutants has just been initiated in our laboratory. We are looking forward to increasing our understanding of these organisms and subsequently comparing them with the current test strains.

E. Zottola: What effect does centrifugation have on the cells? Is it changing what is there and how it is there?

Authors: The staphylococcal glycocalyx seems to be of the fibrous/filamentous matrix type of the bacterial glycocalyx, fitting the definitions of Costerton *et al.*

(1981) and van Iterson (1984). This type of bacterial glycocalyx may be more susceptible due to their elaborate and extended nature than the S layer type, discussed above. Previous work comparing the fibrous/filamentous matrix of methanotrophic bacteria between freeze etch and AB processed cells (Fassel *et al.* 1992a) indicated a good correspondence. This suggested the effect of centrifugation is minimal. Thus, although we cannot conclusively eliminate any effect of centrifugation on the staphylococci cells, we have confidence in our methods.

E. Zottola: Is AB staining always different than GA and RR + GA?
Authors: For the staphylococci processed by any method without lysine, AB staining is not in agreement with GA and GA + RR staining. We think that the curved electron dense structures, seen by AB without lysine, are artifacts of the interaction of AB with the staphylococcal glycocalyx, its inadequate stabilization, and subsequent collapse. Ruthenium red without lysine gives images with the most variation between species. These included bead-like structures, thin fibrillar threads, and limited fibrous material. With GA, none of this material is observed. Lysine improves the stabilization of the staphylococcal glycocalyx for each of these methods. The resultant images of these fibrous glycocalyces are in good agreement and therefore, we feel are better representations of the staphylococcal glycocalyx.

E. Zottola: What effect does the OsO_4 post-fix have on the lysine? It could be that the osmium and the lysine are reacting.
Authors: We have not investigated the interaction of lysine and OsO_4 specifically. We feel the critical interaction of lysine occurs in its initial exposure to the glycocalyx during prefixation. Inclusion of lysine in later stages has not been studied.

E. Zottola: Polylysine has been used to stick cells to glass slides.
Authors: Polylysine has been used for that application. In these studies, we are dealing with L-lysine, or L-2,6-diaminohexanoic acid.