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Tissue Plasminogen Activator Coating on Implant Surfaces Reduces *Staphylococcus aureus* Biofilm Formation

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Staphylococcus aureus **biofilm infections of indwelling medical devices are a major medical challenge because of their high prevalence and antibiotic resistance. As fibrin plays an important role in *S. aureus* biofilm formation, we hypothesize that coating of the implant surface with fibrinolytic agents can be used as a new method of antibiofilm prophylaxis. The effect of tissue plasminogen activator (tPA) coating on *S. aureus* biofilm formation was tested with *in vitro* microplate biofilm assays and an *in vivo* mouse model of biofilm infection. tPA coating efficiently inhibited biofilm formation by various *S. aureus* strains. The effect was dependent on plasminogen activation by tPA, leading to subsequent local fibrin cleavage. A tPA coating on implant surfaces prevented both early adhesion and later biomass accumulation. Furthermore, tPA coating increased the susceptibility of biofilm infections to antibiotics. *In vivo*, significantly fewer bacteria were detected on the surfaces of implants coated with tPA than on control implants from mice treated with cloxacillin. Fibrinolytic coatings (e.g., with tPA) reduce *S. aureus* biofilm formation both *in vitro* and *in vivo*, suggesting a novel way to prevent bacterial biofilm infections of indwelling medical devices.**

Modern medicine uses increasing numbers of indwelling medical devices. Over 5 million implants and 150 million vascular catheters are used each year in the United States alone (1, 2). This leads to a rising challenge of infections associated with indwelling medical devices, which already constitute 60 to 70% of hospital-acquired infections (2). These infections are typically caused by microorganisms that grow in biofilms, three-dimensional communities of bacteria covered in an extracellular matrix and attached to an implant surface (3). Biofilms damage surrounding tissues, trigger inflammation, interfere with device function, and might further seed other body sites with bacteria (4). Importantly, biofilms are inherently resistant to antibiotics and host immune defenses, and removal of the device is frequently indispensable for successful treatment (4). Efforts to prevent biofilm formation, including the use of antibacterial coating and materials surfaced with nanostructures, have had some success (4–6), but other efficient methods to prevent biofilm formation are still urgently needed.

One of the leading bacteria in biofilm infections is *Staphylococcus aureus*. It forms biofilms on vascular catheters, peritoneal dialysis catheters, joint prostheses, pacemaker/defibrillator leads, prosthetic heart valves, vascular grafts, orthopedic fixation devices, and other implants (5, 7). All of the above-mentioned infections occur inside the human body; therefore, host factors play an essential role in their development. Local coagulation induced by *S. aureus* coagulases promotes bacterial attachment to the surfaces of implants (8–10). After an initial adhesion phase, bacteria divide, deposit an extracellular matrix, and develop complex three-dimensional biofilm structures. Recently, several studies have demonstrated that, at this stage, fibrin deposits act as a central structural component of the *S. aureus* biofilm matrix (11–14). Intriguingly, certain *S. aureus* strains secrete large amounts of staphylokinase, a bacterial plasminogen activator (15–17). Release of staphylokinase induces local activation of fibrinolysis, resulting in less fibrin deposition on the implant surface and reduced bacterial attachment and biofilm formation (11). Therefore, we hypothesize that precoating of the surfaces of indwelling medical devices with plasminogen activators might reduce the risk of attachment of free-floating bacteria with subsequent biofilm formation.

In the present study, we analyzed the anti-*S. aureus* biofilm effect of precoating implants with tissue plasminogen activator (tPA). Our data demonstrate that tPA precoating induced local fibrinolysis at the implant surface and efficiently prevented *S. aureus* biofilm formation both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Mice. Female NMRI mice, 6 to 8 weeks old (Charles River Laboratories), were housed in the animal facility of the Department of Rheumatology and Inflammation Research, University of Gothenburg. Mice were kept under standard temperature and light conditions and fed laboratory chow and water *ad libitum*. The ethics committee of animal research of Gothenburg approved this study.

Bacteria and growth conditions. *S. aureus* strain LS-1 was used in most of the assays. The other laboratory strains used were SH1000, Newman, RN6390, V329 (kindly provided by Pietro Speziale, University of Pavia), and SA113 (= CCUG41582; kindly provided by Edward R. B. Moore, Culture Collection of the University of Gothenburg). Strains LS-1, SH1000, and V329 do not secrete staphylokinase; Newman and RN6390 secrete moderate quantities of staphylokinase; and SA113 se-

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cretes large quantities of staphylokinase. V329 is a typical strain forming protein-dependent biofilms, while SA113 is a typical polysaccharide biofilm former (18). Additionally, three congenic strains differing in staphylokinase secretion, previously described LS-1*EP*, LS-1*sak*, and LS-1*spa-sak* (16, 17), were used. Thirteen randomly selected clinical *S. aureus* isolates from biofilm-related infections were taken from a previously described collection (19).

Bacteria were grown at 37°C in tryptic soy broth (TSB) with shaking. Stock cultures were stored in 10% glycerol at −70°C and checked for purity before experiments by being streaked onto blood agar plates.

tPA coating. Ninety-six-well polystyrene plates with a MaxiSorp surface (Nunc; Thermo Scientific) were coated by being filled to 100 μl/well with 10 μg/ml tPA (Actilyse; Boehringer Ingelheim) in 100 mM carbonate buffer, pH 9.6, and incubated for 18 to 20 h at 4°C. Control wells were coated with buffer only. Afterwards, wells were washed three times with phosphate-buffered saline (PBS) at 200 μl/well and used for assays. An analogous procedure was used to coat eight-well μslides with ibiTreat surface (ibidi), with coating and washing volumes of 300 and 500 μl, respectively, and 13-mm Thermanox coverslips (Nunc) placed in the wells of a 24-well plate with volumes of 500 and 750 μl. In some experiments, coating with human high-molecular-weight urokinase plasminogen activator (uPA; Medac) or bovine serum albumin (BSA; Sigma-Aldrich) was performed analogously.

Biofilm formation assays. A microplate method (20) was used for biofilm formation assays. Bacteria from an overnight TSB culture were diluted 100× in fresh biofilm medium. The biofilm medium used, if not indicated otherwise, was TSB with 50% human plasma (heparinized with Li-heparin, collected from healthy donors, and heat inactivated at 56°C for 30 min) and 0.25% glucose. In some experiments, pure TSB with 0.25% glucose was used instead. In some experiments, aprotinin (125 μg/ml; Sigma-Aldrich) or plasminogen activator inhibitor 1 (PAI-1; 10 μg/ml; Molecular Innovations) was added to the medium. Culture plates were filled with a bacterial suspension (100 μl) and incubated at 37°C for 24 h (or another time, as indicated), the medium was removed, the wells were washed with PBS (200 μl) to remove nonadherent bacteria, and the plates were dried at 60°C for 2 h. The biofilms in the wells were stained for 5 min with 0.5% crystal violet (80 μl), rinsed under running tap water, and dried overnight, and stain bound to the biofilm was dissolved by the addition of 33% acetic acid (80 μl). The resulting solution was diluted 20×, the optical density at 570 nm (OD_{570}) of 100 μl was measured with a SpectraMax 340PC348 microplate reader (Molecular Devices), and the OD_{570} of blanks (wells filled originally with uninfected medium) was subtracted, and the OD_{570} of the undiluted solution was calculated. As crystal violet binds to bacterial cells and the extracellular matrix, this measurement reflected the total biofilm biomass. All assays were done in triplicate in two to four separate experiments. For clinical isolates, the assay was run in triplicate and mean values were calculated for each strain.

Confocal microscopy of biofilms. Biofilms were grown for 24 h in 300 μl of medium in eight-well μslides. The wells were washed with PBS, stained for 15 min with 300 μl of 6.7 μM Syto9 dye (Molecular Probes, Life Technologies), washed, and filled with fluorescence mounting medium (ibidi). Biofilms were visualized with an LSM 700 confocal fluorescence microscope equipped with ZEN2009 software for capturing images (Carl Zeiss Microscopy). Images were acquired from four random sites in the well and analyzed with the ISA-2 software (ISA3D) (21).

Electron microscopy of biofilms. Biofilms grown on 13-mm Thermanox polyester coverslips in 300 μl of medium in the wells of a 24-well plate were visualized with a scanning electron microscope as described previously (11).

Plasmin activity measurement. Biofilm supernatants were centrifuged for 10 min at 3,000 × *g* at 4°C to remove the debris and assayed for plasmin activity with a previously described assay (22). Assays were performed in triplicate in two separate experiments.

MIC and MBEC determination. The MICs and minimal biofilm eradication concentrations (MBECs) of four different antibiotics (vancomycin, ciprofloxacin, rifampin, and cloxacillin) were assayed as described previously (23). MICs were determined by the microdilution method, and MBECs were determined with a metabolic activity assay based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye by viable cells. Determination was performed in duplicate.

Primary adhesion assay. Bacteria from an overnight culture were centrifuged, resuspended in PBS, and diluted in the biofilm medium to a final OD_{600} of 0.2 of 100 μl measured with the microplate reader. One hundred microliters of this mixture per well was added to a 96-well plate and incubated at 37°C for 30 min. Afterwards, the medium was aspirated, the wells were washed three times with PBS, and the amount of adherent material was measured either by drying and staining with crystal violet as for the biofilm assay or by filling the wells with 200 μl of PBS, sonicating them for 3 min at 45 kHz in an ultrasonic bath (USC300TH; VWR International), and counting the CFU by serial dilution on horse blood agar (detection limit, 2×10^3 CFU/well). Assays were performed in triplicate in three separate experiments.

***In vivo* biofilm models.** tPA-coated polyester coverslips (13 mm; Thermanox) were cut in half and placed in TSB with 50% human plasma (500 μl) with *S. aureus* LS-1 in the wells of a 24-well plate to give the bacteria an opportunity to adhere to the surface. After 2 h of incubation at 37°C, the coverslips were washed three times with PBS and inserted into subcutaneous pockets made on the shaved backs of NMRI mice ($n = 8$). A coverslip coated with tPA was inserted into one flank of each mouse, and one coated with buffer was inserted into the other flank. The pockets were closed with metal clips, and a biofilm was allowed to develop for 3 days. The mice were then euthanized; the implants were retrieved, washed with PBS, placed in 1 ml of PBS, and sonicated for 5 min at 45 kHz in an ultrasonic bath; and the CFU in the biofilm were counted by serial dilution on horse blood agar. The results of two experiments were pooled.

Since washing steps before implantation may cause a lower bacterial burden on the tPA-coated coverslips than on the buffer-coated controls prior to introduction into the animals, a modified protocol was used to study the effect of tPA coating on *S. aureus* biofilm infection in more clinically relevant settings. *S. aureus* LS-1 was cultured overnight in TSB medium and then diluted 1:20 in PBS with 50% heat-inactivated mouse plasma. A 25-μl volume of a bacterial solution was added on top of the tPA-coated coverslips and incubated in 37°C for 4 h in a humid chamber. Without a washing step, the infected coverslips were then implanted subcutaneously into the flanks of mice as described above (one side with the tPA coating and the other side with the control). Mice were either not treated ($n = 18$) or treated with an antibiotic ($n = 13$) for 3 days. On day 3 after implantation, the coverslips were collected for analysis of the bacterial loads on the surfaces of implants. Cloxacillin (Cloxacillinnatr; Stragen) dissolved in sterile PBS was used for the antimicrobial treatments. The mice were injected with 0.2 ml of the solution (0.5 mg/g of body weight) intraperitoneally twice a day, starting at 12 h after implantation.

Statistical analysis. Statistical significance was assessed with the Mann-Whitney test for continuous variables between two groups. Differences among biofilms formed by clinical isolates and differences among biofilms formed on implants *in vivo* were analyzed with the Wilcoxon matched-pair signed-rank test. Two-tailed *P* values were used, and values of <0.05 were considered significant. Prism 6.3 software (GraphPad Software) was used for statistical calculations.

RESULTS

tPA coating prevents *S. aureus* biofilm formation on polystyrene material. Coating of polystyrene culture wells with tPA efficiently inhibited biofilm formation by *S. aureus* LS-1 in TSB with 50% human plasma (Fig. 1A). This was confirmed when biofilms were directly visualized with a confocal microscope. Instead of the thick, robust biofilm structure in the control well, only small bacterial clumps appeared in the tPA-coated well (Fig. 1B). Image analysis showed that structures on the tPA-coated surfaces had a smaller total biovolume, were thinner, and were composed of

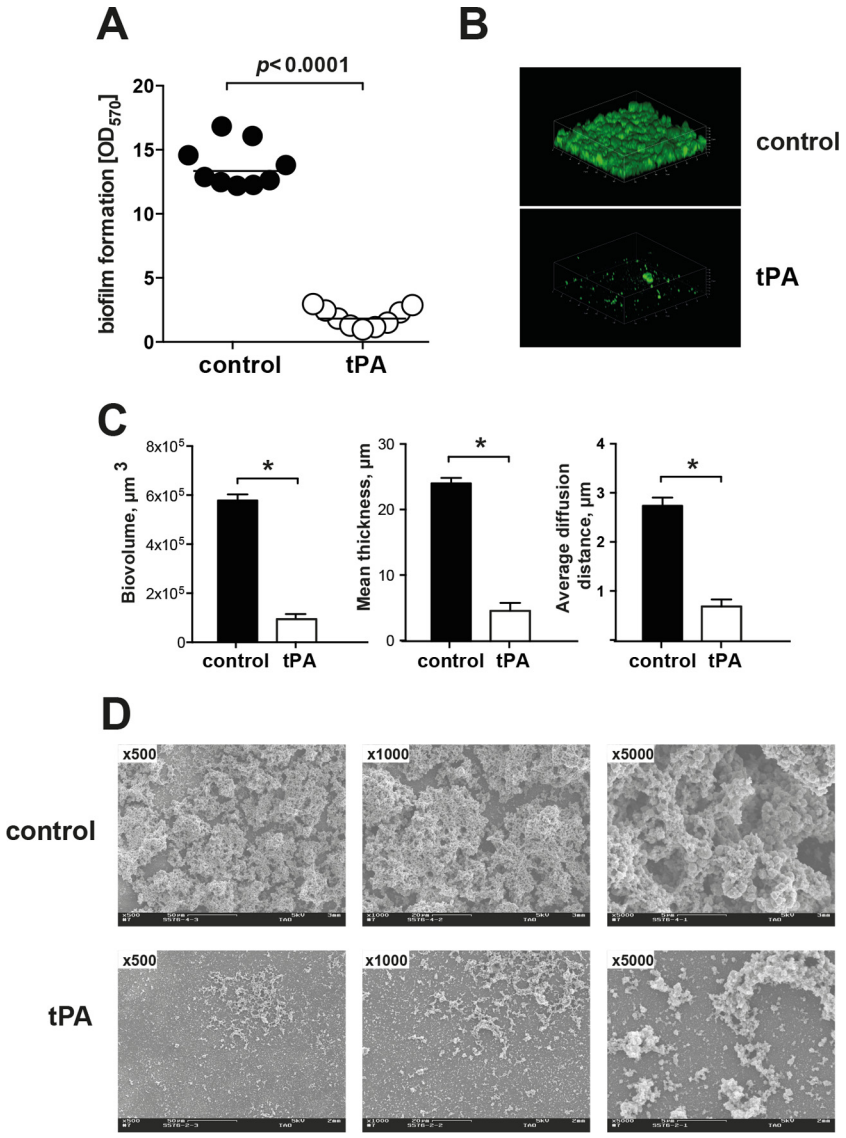

FIG 1 Coating of polystyrene surfaces with tPA reduces *S. aureus* biofilm formation. (A) Biofilm formation on tPA-coated or buffer-treated polystyrene surfaces by *S. aureus* LS-1 after overnight culture in TSB with 50% heparinized human plasma. A microplate colorimetric assay was used. (B) Confocal microscopy images of *S. aureus* LS-1 biofilms on buffer-treated and tPA-coated polystyrene surfaces after overnight culture in TSB with 50% heparinized human plasma. (C) Biovolume, thickness, and diffusion distance (proxy for clump size) of biofilm masses formed by *S. aureus* LS-1 on buffer-treated or tPA-coated polystyrene surfaces after overnight culture in TSB with 50% heparinized human plasma. (D) Representative scanning electron microscopy images of *S. aureus* LS-1 biofilms on buffer-treated (upper row) or tPA-coated (lower row) polyester surfaces after overnight culture in TSB with 50% heparinized human plasma. Magnifications: ×500, ×1,000, and ×5,000. Data are presented as the mean and the standard error of the mean. *, $P < 0.05$.

much smaller cell clumps (Fig. 1C) than those of the control group. Scanning electron microscopy images also showed much less biofilm formation on the tPA-coated surface than on the surface without pretreatment (Fig. 1D).

The same antibiofilm effect was achieved when coating with other plasminogen activators, e.g., urokinase, was tested (see Fig. S1 in the supplemental material). A similar difference was also observed when tPA-coated wells were compared to BSA-coated wells (see Fig. S2 in the supplemental material), suggesting that the antibiofilm activity of tPA was not due to unspecific effects of protein surface coating.

Local plasminogen activation by tPA coating mediates antibiofilm effects. When TSB-glucose medium without plasma was used for biofilm culture, no inhibition by tPA coating was observed (Fig. 2A). This indicates that certain plasma components are crucial for the inhibitory effect of tPA coating. Indeed, supernatant in the wells coated with tPA had markedly increased plasmin activity (Fig. 2B), showing that surface-bound tPA activates plasminogen from plasma to induce local fibrinolysis. Importantly, when PAI-1 (an efficient tPA inhibitor) or aprotinin (a plasmin inhibitor) were added at concentrations resulting in complete inhibition of fibrinolysis, the antibiofilm effect of tPA coating was abrogated and biofilms were successfully formed (Fig. 2C).

tPA coating prevents primary adhesion and delays biofilm formation. To study whether tPA coating affects the early stage of biofilm formation, the mass and the number of attached *S. aureus*

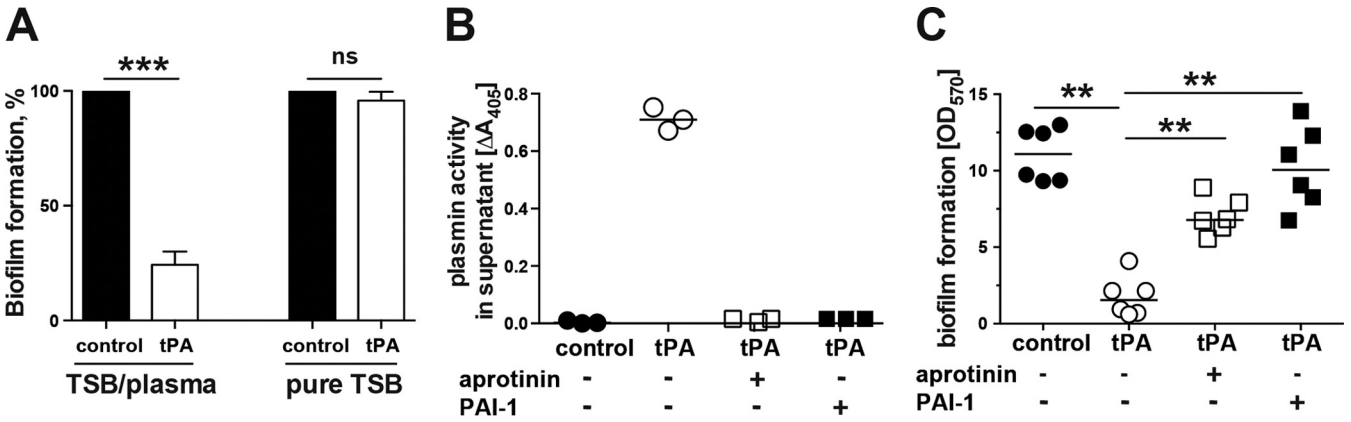

FIG 2 The preventive effect of tPA coating against *S. aureus* biofilm formation was mediated by plasminogen activation. (A) Biofilm formation (percent) by *S. aureus* LS-1 on tPA-coated polystyrene surfaces after overnight culture in TSB with 50% human plasma or pure TSB. A microplate colorimetric assay was used. Biofilm formation on a buffer-treated polystyrene surface (control) was assigned a value of 100%. (B) Plasmin activity in biofilm culture supernatants from control and tPA-coated surfaces. (C) Effect of inhibition of tPA (PAI-1) and plasmin (aprotinin) on biofilm formation on tPA-coated versus buffer-treated (control) surfaces. Data are presented as the mean and the standard error of the mean or as a scatter dot plot with the mean indicated by a horizontal bar. ns, not significant; **, $P < 0.01$; ***, $P < 0.001$.

bacteria on the surface were analyzed after 30 min of exposure of culture wells to a bacterial suspension (Fig. 3). Both the biomass (Fig. 3A) and the number of bacteria (Fig. 3B) attached to the tPA-treated surface after this initial short exposure were greatly reduced.

Also, a striking difference in biofilm formation kinetics was found (Fig. 3C). Biofilm development on the tPA-coated surface was delayed up to 32 h after bacteria contacted the surface, confirming that bacterial adhesion was already blocked by the tPA coating in the initial phase.

tPA coating is effective against biofilm formation by various *S. aureus* strains. To assess whether the results observed are applicable to other *S. aureus* isolates, we tested tPA coating on various laboratory *S. aureus* strains and clinical isolates from biofilm-associated infections (Fig. 4). tPA coating reduced biofilm formation by nearly all of the laboratory strains tested (Fig. 4A). Interestingly, tPA coating had a weak effect on strain SA113, a poor biofilm former that secretes vast quantities of staphylokinase.

To further study how staphylokinase secretion interacts with the antibiofilm effect of tPA coating, three congenic strains secreting different amounts of staphylokinase were used (Fig. 4B). Coating with tPA efficiently blocked biofilm formation by a strain secreting no staphylokinase (LS-1*EP*) or a moderate amount (LS-1*sak*), while it had no additional effect on a strain overexpressing staphylokinase (LS-1*spa-sak*), which failed to form a biofilm because of staphylokinase hypersecretion (Fig. 4B).

The efficacy of tPA coating was not limited to laboratory strains. When clinical *S. aureus* isolates from biofilm-related infections were tested, all of them showed inhibited biofilm formation on tPA-coated surfaces (Fig. 4C).

tPA coating exposes *S. aureus* to antibiotics. Bacteria in biofilms are known to be protected against antibiotics because of various mechanisms, e.g., poor penetration by antibiotics. Indeed, all four of the antibiotics tested in this study (vancomycin, ciprofloxacin, rifampin, and cloxacillin) lost their efficacy when *S. aureus* LS-1 was grown as a biofilm (Table 1). The MICs of the anti-

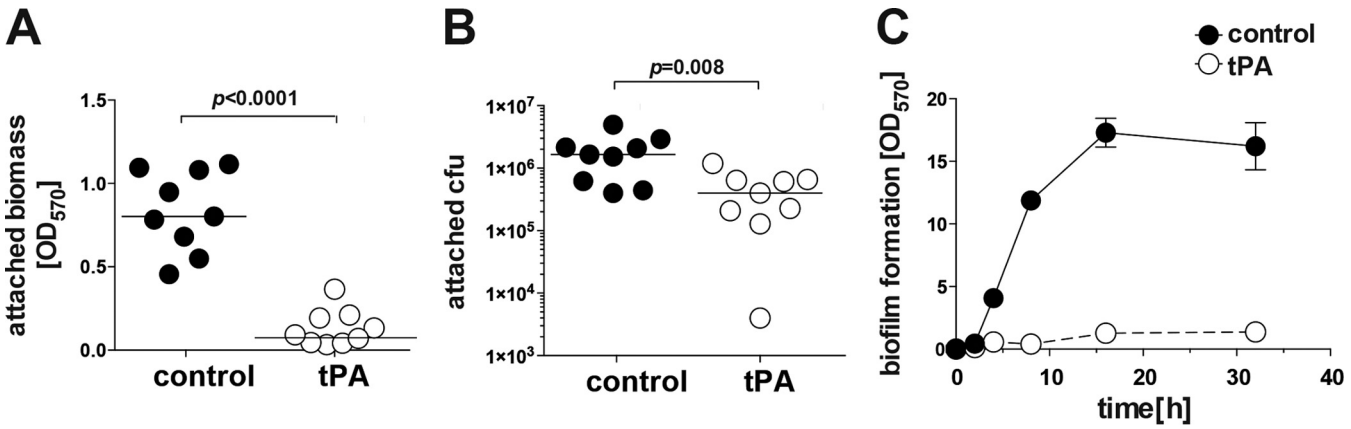

FIG 3 tPA coating prevents *S. aureus* attachment to polystyrene surfaces. Shown are the attached biomass (A) and CFU counts of attached bacteria (B) on buffer-treated or tPA-coated polystyrene surfaces after 30 min of incubation with *S. aureus* LS-1 in TSB with 50% heparinized human plasma. (C) Differences in the kinetics of biofilm formation on buffer-treated or tPA-coated polystyrene surfaces after 32 h of culture of *S. aureus* LS-1 in TSB with 50% heparinized human plasma.

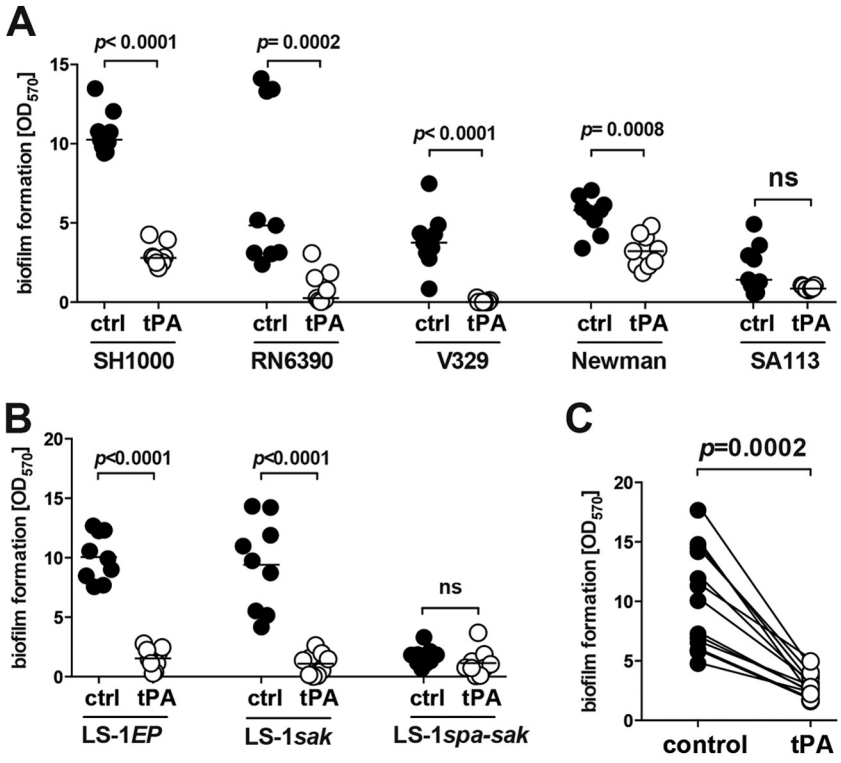

FIG 4 tPA coating reduces biofilm formation by both laboratory strains and clinical *S. aureus* isolates on polystyrene surfaces. Biofilm formation on tPA-coated and buffer-treated (control [ctrl]) polystyrene surfaces by laboratory *S. aureus* strains (SH1000, RN6390, V329, Newman, and SA113) (A), three congenic *S. aureus* strains differing in the level of expressed Sak (*EP*, no expression; *sak*, moderate expression; *spa-sak*, high expression) (B), and 13 *S. aureus* clinical isolates from biofilm-related infections (C) after overnight culture in TSB with 50% heparinized human plasma. A microplate colorimetric assay was used. ns, not significant.

biotics tested were low (≤1 mg/liter). However, when a biofilm was formed, all four antibiotics failed to eradicate the bacteria inside the biofilm matrix even at a concentration of 1,024 μg/ml. In contrast, bacteria on a tPA-coated surface were eradicated by significantly lower concentrations of antibiotics (4 to 128 μg/ml).

tPA coating prevents biofilm-related infection in a mouse model. To examine the effect of tPA coating *in vivo*, *S. aureus*-inoculated coverslips were washed and then implanted subcutaneously into mice and the bacterial loads on the coverslips were analyzed after 3 days. This led to biofilm formation on the control (buffer-treated) implant surface, which could be seen by the naked eye during the surgical procedure, but bacteria failed to form a biofilm on tPA-coated implants. In line with this, the count of viable bacteria attached to the implant was reduced more than 20-fold by tPA coating (Fig. 5A).

Since washing before implantation may cause a lighter bacterial burden on the tPA-coated coverslips prior to their introduction into the animals, unwashed *S. aureus*-infected coverslips were implanted into mice and the CFU counts on the coverslips were analyzed after 3 days (Fig. 5B). Twelve of 18 tPA-coated implants had fewer bacteria attached to their surfaces than the control coverslips ($P = 0.09$). No significant difference in the CFU counts on the coverslips was observed between the groups ($P = 0.284$).

To assess the effect of tPA coating combined with antibiotic treatment on *S. aureus* biofilm infection, mice implanted with *S. aureus*-infected coverslips with a tPA coating were treated with cloxacillin for 3 days and the bacterial loads on the coverslips were analyzed (Fig. 5C). Viable *S. aureus* bacteria were found on both tPA-coated and control coverslips 3 days after cloxacillin treatment. Twelve of 13 tPA-coated implants had fewer bacteria attached to their surfaces than the control coverslips on the collateral side ($P < 0.0001$). Also, the CFU counts were four times as low in the tPA-coated group than in the control group ($P = 0.0007$).

TABLE 1 Impact of tPA coating on the MICs and MBECs of different antibiotics for planktonic cells and biofilms of *S. aureus* LS-1

Antibiotic	Planktonic cell MIC (mg/liter)	Biofilm MBEC (mg/liter)	
		Control	tPA coated
Vancomycin	1	>1,024	8
Ciprofloxacin	1	>1,024	16
Rifampin	0.008	>1,024	4
Cloxacillin	0.5	>1,024	128

DISCUSSION

In this study, we demonstrated that a tPA coating on the surfaces of implants prevents *S. aureus* biofilm formation under culture conditions containing plasma. This preventive effect was mediated through plasminogen activation by surface-bound tPA. tPA coating also significantly enhanced the susceptibility of *S. aureus* biofilms to various antibiotics. Importantly, *in vivo* tPA coating combined with antibiotic treatment significantly reduced the amount of bacteria attached to the implant surface.

Growth medium with human plasma added is a more physiological setting in which to study biofilm formation than a pure bacterial culture medium is, since it more closely resembles real-

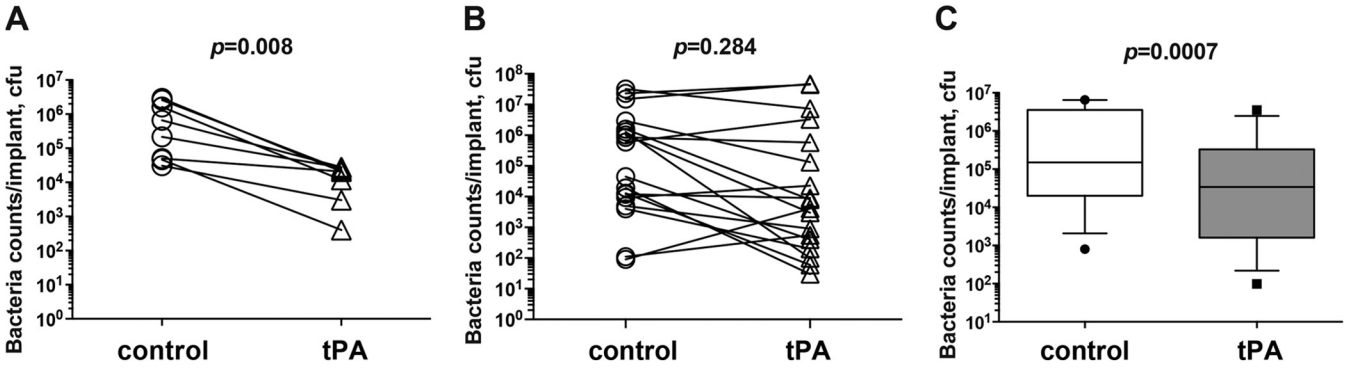

FIG 5 tPA coating reduces *S. aureus* biofilm formation *in vivo*. (A) The buffer-treated and tPA-coated coverslips were infected with *S. aureus* LS-1 for 2 h and washed with PBS and then implanted subcutaneously into both flanks of NMRI mice ($n = 8$). The coverslips were collected 3 days after implantation. The bacterial loads on the coverslips were analyzed as CFU counts. The buffer-treated and tPA-coated coverslips were infected with *S. aureus* LS-1 for 4 h and implanted subcutaneously into both flanks of NMRI mice (B and C). Mice were either not treated ($n = 18$) (B) or treated with cloxacillin ($n = 13$) twice a day starting at 12 h after implantation (C). On day 3, the coverslips were collected to analyze the bacterial loads (CFU counts) on the implant surfaces. Data are presented as dot plots (panels A and B) or medians (center lines), interquartile ranges (boxes), and 80% central ranges (whiskers) (C). The Wilcoxon matched-pair signed-rank test was used.

life conditions. The preventive effect of a tPA coating on biofilm formation became apparent only when human plasma was used, suggesting that plasma proteins are crucial for both biofilm matrix formation and the preventive effect of a tPA coating. Indeed, human plasma proteins are known to immediately coat implant surfaces and mediate bacterial attachment when medical devices are implanted (5, 7). Among those proteins are host fibrinogen and fibrin, to which *S. aureus* binds with bacterial surface proteins (24). Moreover, *S. aureus* induces local coagulation by secreting two coagulases that convert fibrinogen into fibrin nets as an anchoring place for staphylococci and thus enhance bacterial adhesion to the implant surface (8, 9, 25, 26). A tPA coating on implants activated host plasminogen into plasmin and created fibrinolytic activity directly on the implant surface, where it can cleave the fibrin deposited by infecting bacteria. Plasmin is a broad-spectrum protease, so it potentially also cleaves other proteins involved in biofilm formation, further strengthening the antibiofilm effect. As tPA has increased activity in the presence of fibrin (27), tPA coating might selectively induce increased fibrinolysis in response to abundant fibrin, which is one of the essential structural components of the *S. aureus* biofilm matrix (11). tPA coating displayed a robust ability to reduce biofilm formation by clinical isolates from biofilm-related infections, whereas it had no effect on staphylokinase-overexpressing strains, which are usually not associated with biofilm infections (11). This is conceivably due to the fact that staphylokinase-overexpressing strains have already exerted their full effect of local plasminogen activation, which is similar to the mechanism of action of tPA coating. An alternative explanation is that staphylokinase inhibits the activity of a tPA coating on an implant surface. Indeed, staphylokinase was shown to reduce plasmin formation by tPA or uPA (28). Theoretically, coagulation inhibitors might achieve an antibiofilm effect partly similar to that of tPA, but classic anticoagulants (vitamin K antagonists, heparin, hirudin) cannot inhibit fibrin deposition induced by *S. aureus* (9), and heparin even increases its biofilm formation (29).

The first hours after implantation are crucial for biofilm infection and its prevention, as they represent the window of opportunity for the immune system and antibiotics to eradicate free-floating bacteria before they develop a resistant biofilm (4). Unfortunately, fibrin-rich *S. aureus* biofilms appear rapidly, which might decrease the efficacy of antibiotic prophylaxis (30) and blunt phagocytosis (9, 11, 31, 32). Importantly, tPA coating prevents the first step of biofilm formation, the adhesion of *S. aureus* to the implant surface, thereby expanding the window of opportunity and allowing more efficient pathogen clearance. Because of poor biofilm formation, *S. aureus* on a tPA-coated surface remained vulnerable to immune system attack and susceptible to antibiotics, which was apparent in the mouse implant infection model, where tPA coating and antibiotics displayed a synergistic effect against biofilm infection.

The first animal model used in this study was a simple replication of our *in vitro* system, since the free-floating bacteria were washed away and there were already fewer bacteria on a tPA-coated plastic surface than on control coverslips before implantation. In contrast, in the later animal experiments, the same dose of *S. aureus* (irrespective of free-floating bacteria or biofilm-forming bacteria) on tPA-coated and control coverslips were implanted into mice, which ensures the clinical relevance of this study. Interestingly, no significant difference in CFU counts was observed in the absence of antibiotic treatment (Fig. 5B). In this case, only a trend toward lower CFU counts on tPA-coated implants than on contralateral buffer-treated implants was seen. In animal experiments, large inocula are typically used to infect implants, which might overwhelm the bacterial killing capacity of the host immune system. Therefore, the effect of tPA coating on biofilm formation can be masked.

In the presence of antibiotics, however, representative of the clinical situation, tPA coating did result in lighter bacterial loads on the implants. We failed to show a full preventive effect, i.e., eradication of the bacteria on implants, of combining tPA coating and antibiotic treatment, which might be due to the late start of the antibiotic treatment (12 h after implantation) or to the slow inactivation of tPA *in vivo*. Nevertheless, the current study presents proof of the concept that a plasminogen activator coating on the implant surface might be a novel concept in the prevention of biofilm formation. Many practical details, e.g., efficient coating techniques, the type of plasminogen activator, and the starting time of antibiotic treatment, need to be addressed in future studies.

As a new treatment concept, tPA coating might provide efficient protection during the early, most crucial phase of bacterial attachment after implantation. There are, however, some foreseeable challenges. Endogenous PAI-1 in the physiological nanogram quantities found in plasma did not interfere with the efficacy of tPA coating in our experiments (data not shown). However, inside a human body, implants are continuously exposed to PAI-1, which might eventually lead to loss of tPA activity. This notion is supported by our observation that larger (microgram) PAI-1 quantities totally abrogated the protective effect of tPA coating on biofilm formation *in vitro*. Indeed, we have previously shown that PAI-1 levels are greatly increased in both blood and local infected organs in *S. aureus* infections (33). There is, however, the possibility of bypassing the inhibitory effect of PAI-1 by using other fibrinolytic agents, e.g., a PAI-1-resistant variant of tPA (tenecteplase), bacterial plasminogen activators (staphylokinase, streptokinase), or other mammalian activators (desmoteplase) (27). The good availability of various plasminogen activators in combination with different coating methods might lead to the fast development of new prophylactic methods for biofilm-related infections on indwelling medical devices.

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