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The role of serum proteins in *Staphylococcus aureus* adhesion to ethylene glycol coated surfaces

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ABSTRACT

Bacterial adhesion on implants is a first step in the development of chronic foreign body associated infections. Finding strategies to minimize bacterial adhesion may contribute to minimize such infections. It is known that surfaces with oligo-ethylene-glycol (EG_3OMe) or poly-ethylene-glycol (PEG2k) terminations decrease unspecific protein adsorption and bacterial adhesion. However, little is known about the influence of serum and its components on bacterial adhesion. We therefore prepared two coatings on gold surface with HS-$(CH_2)_{11}EG_3OMe$ (EG_3OMe) and PEG2k-thiol and studied the role of bovine serum albumin (BSA), γ-globulins, and serum on *Staphylococcus aureus* adhesion. While BSA and lysozyme showed no adherence even when applied at very high concentrations (100 mg/ml), γ-globulins adsorbed already from 10 mg/ml on. The adsorption of γ-globulins was, however, significantly decreased when it was mixed with BSA in a ratio of 3:1, as it is in the serum. Pretreatment of EG_3OMe and PEG2k coatings with γ-globulins or serum strongly promoted adherence of *S. aureus* when resuspended in buffer, suggesting that γ-globulins play a pivotal role in promoting *S. aureus* adhesion by its IgG binding proteins; the finding that a *spa*-deletion mutant, lacking the IgG binding protein A, showed decreased adherence corroborated this. Similarly, when *S. aureus* was pretreated with serum or γ-globulins its adherence was also significantly decreased. Our findings show that particularly γ-globulins bind to the coated surfaces thus mediating adherence of *S. aureus* via its protein A. As pretreatment of *S. aureus* with serum or γ-globulins significantly decreased adherence, treatment of patients with γ-globulins before implant surgery might lower the risk of implant-associated infections.

Introduction

Infections related to indwelling medical devices are one of the major causes of persistent clinical infections (Costerton et al., 1999). These infections are frequently complicated by biofilm formation, during which the pathogenic microorganisms adhere to the surfaces of the medical devices and develop a thick, multilayered slimy matrix (Cramton and Götz, 2004; Cramton et al., 1999; Gross et al., 2001; Kropec et al., 2005; Rohde et al., 2005). Once the biofilm is formed, the embedded microorganisms are highly resistant to multiple antibiotics and host immune defense (Gray et al., 1984; Stewart and Costerton, 2001). Often, the only way to eradicate such infections is to surgically remove the infected indwelling device. The nonspecific and reversible adhesion of microorganisms to the surfaces of the biomaterials is the critical step in biofilm infections. Pathogenic microorganisms have employed a variety of factors for successful adhesion. As the most common biofilm associated microorganisms, *Staphylococcus epidermidis* and *Staphylococcus aureus* exploit major autolysin (Heilmann et al., 1997), surface proteins SasG, SasC (Roche et al., 2003; Schroeder et al., 2009) as well as teichoic acids (Gross et al., 2001) to initiate adhesion. Immunoglobulin-, fibrinogen-, fibronectin-, or collagen-binding proteins promote the adhesion of *S. aureus* to a surface if the surface is coated with the corresponding matrix proteins (Götz and Peters, 2000).

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Preventing the initial adhesion of microorganisms to the surfaces of biomaterials is thought to be an efficient way to treat implant-associated infections. Apart from attempts to target bacterial adhesion factors by selected antibiotics (Saising et al., 2012), much attention has been paid in recent years to engineering biomaterial surfaces to become more resistant to bacterial adhesion. Self-assembled monolayer (SAMs) based surface coatings with oligo-ethylene-glycol (OEG) or poly-ethylene-glycol (PEG) terminations render surfaces resistant against non-specific protein adsorption and bacterial adhesion, which appears to be a promising approach (Pale-Grosdemange et al., 1991; Prime and Whitesides, 1993). The mechanisms for the protein resistance property of PEG coated surfaces are proposed to be free energies of steric repulsion, hydrophobic interactions and van-der-Waals interactions (Jeon and Andrade, 1991). Both grafting density and chain length are critical parameters for the efficiency of PEG in preventing protein adsorption and bacterial adhesion (Holmberg et al., 1993). Due to the short chains and highly ordered conformation, the mechanisms of the protein resistant property of OEG coatings are different in many parameters such as the surface coverage, the conformation and the types of end groups (Love et al., 2005; Schreiber, 2004; Ulman, 1996).

As the implant-associated infections often occur in complex biological systems, it is important to assess the influence of environmental factors on the interaction between bacteria and the biomaterial's interfaces. Blood plasma is one of the most relevant environmental factors for medical device related infections. Under clinical conditions, significant protein adsorption from blood plasma has been observed for various modified interfaces (Benesch et al., 2001; Olsson et al., 1992). While previous studies focus either on the resistance against bacterial adhesion in single protein solution (Cheng et al., 2007) or on plasma protein adsorption without testing bacterial adhesion (Zhang et al., 2008), an advanced understanding of how plasma proteins affect the bacterial adhesion to the interfaces is largely missing (Deng et al., 1996; Katsikogianni and Missirlis, 2004).

In the current work, we prepared EG_3OMe (HS-$(CH_2)_{11}EG_3OMe$, one kind of OEG coating) and PEG2k (one kind of PEG coating) coated surfaces that had good physicochemical properties in preventing protein adsorption and *S. aureus* adhesion. We then compared the protein adsorption behavior of bovine serum albumin (BSA), γ-globulins and whole bovine serum at their physiological concentrations. Further, the effects of BSA, γ-globulins and serum on initial adhesion of *S. aureus* to the EG_3OMe and PEG2k coated surfaces were investigated. Our results showed that albumin, γ-globulins and serum had distinct protein adsorption behaviors and consequently different effects on bacterial adhesion. While γ-globulins readily adhered to the coated surfaces, albumin did not adhere and even suppressed γ-globulin adherence in mixed solution. Protein A played a crucial role in adherence when the coatings were pretreated with serum or γ-globulins. However, when *S. aureus* cells were pretreated with serum or γ-globulins to saturate protein A adherence was significantly decreased.

Materials and methods

Preparation of OEG and PEG coatings on gold (Au) surfaces

Silicon wafers (Si⟨1 1 1⟩) coated with a 5 nm Ti layer and a 200 nm evaporated Au layer were used as substrates for coating. The wafers were cleaned by rinsing in Milli-Q water (18.2 MΩ cm, Millipore) and ethanol (99.9%, Riedel de Haen), dried in an argon stream, treated with ozone producing UV-light for 20 min and rinsed with Milli-Q water again. The coating procedure was performed directly after the cleaning. For the OEG coating preparation, a 500 μM solution of HS-$(CH_2)_{11}EG_3OMe$ (EG_3OMe) or EG6OMe in ethanol was used with an immersion time of 24 h (Skoda et al., 2007; Zorn et al., 2011). For the PEG coatings, a 50 μM solution of PEG2k or PEG5 K in N,N-dimethylformamide (DMF) was used with an immersion time of 48 h (Schilp et al., 2009). After removal of the surfaces from the thiol stock solutions, the coated surfaces were rinsed with pure ethanol and dried with a nitrogen stream (Skoda et al., 2007; Zorn et al., 2010). The coated samples were stored in the dark in a nitrogen environment. The structures of EG_3OMe and PEG2k are shown in Fig. 2.

Protein adsorption experiment

The EG_3OMe and PEG2k coated surfaces were rinsed briefly in Milli-Q water and then dried in an Argon stream. Directly after this procedure the surfaces were incubated in a protein solution for 15 min, washed briefly with Milli-Q water and dried with an Argon stream. Proteins tested were lysozyme, fibrinogen, bovine serum albumin (BSA) and bovine γ-globulins (Sigma–Aldrich, Germany). In the first experiment, BSA, lysozyme and fibrinogen were tested at a concentration of 1 mg/ml. In the second experiment, individual proteins of different concentrations (1, 10, 20, 50, 100 mg/ml) were tested. Lastly, protein adsorption of a mixture of BSA (end concentration 30 mg/ml) and γ-globulins (end concentration 10 mg/ml) in a ratio of 3:1 and the whole bovine serum were studied.

Protein desorption and analysis by SDS-PAGE

Gold wafer samples coated with each of the proteins BSA, γ-globulins and mixture of both were washed with 1 M Tris/HCl pH 8.4 buffer containing 0.1% SDS. After washing the surface with the buffer repeatedly, the wafers were further shaken in the same buffer in a sonication bath to enhance desorption of the coated proteins. The solution was then mixed with laemmli buffer and analyzed by SDS-PAGE.

Bacterial adhesion assay

S. aureus SA113 (pC-tuf-ppmch) constitutively expresses red fluorescent protein mCherry (Mauthe et al., 2012) was used in this work. In addition *S. aureus* SA113 WT (pC-tuf-gfp) and the protein A deficient mutant *S. aureus* SA113 Δ spa (pCX-pp-sfgfp), both expressing the green fluorescent protein (gfp) were used as well. Bacteria were cultivated overnight at 37 °C and 120 rpm in basic medium (BM) composed of 1% peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose and 0.1% K_2HPO_4. The overnight culture was inoculated into fresh medium with an adjusted initial OD_{578} of 0.1 and grown to a density of 1.0. Bacterial cells were then centrifuged and washed three times with sterile phosphate buffered saline (PBS). Subsequently the bacteria were resuspended in PBS or bovine serum to OD_{578} of 1.0. Sterile 12-well cell culture plates (Greiner bio-one), onto which EG_3OMe and PEG2k coated samples were placed, were each filled with 4 ml bacteria suspensions. The plates were incubated at 37 °C on a rotary shaker at 100 rpm. After 1 h incubation, the samples were washed three times for 5 min in fresh PBS buffer and degassed Milli-Q water and finally dried with an Argon stream. In the pre-incubation assay, the EG_3OMe and PEG2k coated samples were each pre-incubated for 1 h with PBS, BSA (20 mg/ml), γ-globulins (60 mg/ml) and bovine serum (100%), in turn, before incubating with the bacteria suspension.

Atomic force microscopy (AFM)

Experiments were performed with a NanoWizard 3 AFM (JPK) in the tapping mode with a line scan rate of 1 Hz and a resolution of 512 × 512 pixels. Scans were performed with a size of 1 μm × 1 μm,

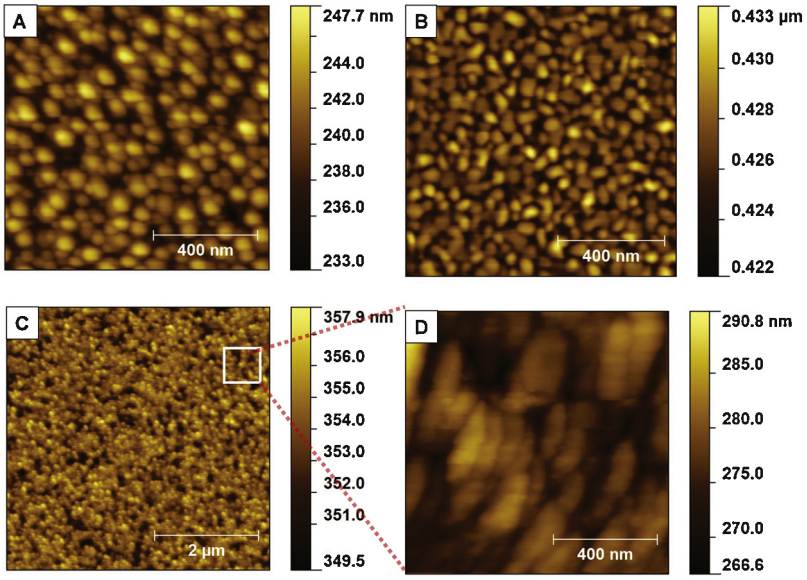

Fig. 1. AFM height images (1 µm × 1 µm) of gold surface alone (A), gold surface coated with EG_3OMe (B) or PEG2k (C and D). The images show the surface morphology. The mean squared surface roughness was deduced from the AFM images using software "Gwyddion". By coating the gold surface with EG_3OMe and PEG2k the mean-squared roughness (MSR) decreased more and more, becoming smoother. The yellow to brown color scales beside the images indicate the vertical scale.

2 µm × 2 µm, 5 µm × 5 µm and 10 µm × 10 µm to determine the average surface roughness. The AFM images were treated with "Gwyddion" and the surface roughness and height distribution were obtained. The mean-squared roughness (MSR) is given by the standard deviation of the *z*-values for the surface height. It is quantified by the vertical deviations of a real surface from its ideal form; if these deviations are large, the surface is rough, and if they are small the surface is smooth.

Polarization modulation infrared reflection absorption spectroscopy (PMIRRAS)

PMIRRAS measurements were performed on a Vertex70 Spectrometer (Bruker, Ettlingen, Germany) equipped with a PMA50 extension (Bruker) featuring a photoelastic modulator, purged with dry air. The spectra were recorded with a resolution of 4 cm^{-1} and 1024 scans per measurement. The spectra were exported from the Bruker data acquisition program OPUS and baseline corrected in Igor Pro (WaveMetrics, USA). The area, amplitude, width and position of the modes in the fingerprint region were determined by fitting with a Gaussian function (Roosen-Runge et al., 2010). Details of setup and data analysis have been described elsewhere (Skoda et al., 2009). The detector was fixed at an angle of 75.8°. The sample holder position was rotated to an angle of 82.9° to have the maximum interferogram signal (Skoda et al., 2007). These settings were used for all measurements. Surface-bound proteins were identified by the characteristic amide I (CO and N—H groups) absorption (the stretching vibrations of the peptide carbonyl group), which lies in the range of 1600–1700 cm^{-1}.

Fluorescence microscopy

Experiments were performed using a Leica DM5500 B microscope. Images were captured with the Leica DFC360 FX high sensitivity monochrome digital camera. The fluorescence coverage from each photo was calculated with the software "ImageJ", using a size of 1 μm^2 as an input parameter.

Results

Morphology and structure of EG3OMe and PEG2k coated surfaces

Both uncoated as well as EG_3OMe and PEG2k coated gold (Au) surfaces were analyzed by AFM with respect to morphology and mean-squared roughness (MSR) (Fig. 1). The sputtered Au surfaces on silicon wafers had a grain-like morphology with a domain size in the range of 50–70 nm in diameter, MSR of 2.19 ± 0.01 nm (Fig. 1A). These Au surfaces on silicon wafers were coated with EG_3OMe and PEG2k. The EG_3OMe coated surfaces showed a similar grain-like morphology with a similar domain size of ~60 nm in diameter (Fig. 1B). However, the surface became more flattened and more smooth as indicated by a decrease of the MSR from 2.19 ± 0.01 nm (Fig. 1A) to 1.58 ± 0.02 nm. When the gold surface was coated with PEG2k the surface became even smother as indicated by a drop of the MSR to 0.82 ± 0.01 nm (Fig. 1C). An enlargement of the PEG2k coated surface is shown in Fig. 1D, which indicates the formation of larger domains on PEG2k coated surface.

EG_3OMe and PEG2k coated surfaces did not adsorb lysozyme, fibrinogen and BSA at low concentration

The internal structures of EG_3OMe and PEG2k coatings were characterized by PMIRRAS (Fig. 2). The spectrum of EG_3OMe coatings in air showed a single peak at ~1130 cm^{-1} (C—O—C stretching mode) (Fig. 2A, black spectrum, red arrow), indicating that the dominant conformation was the ordered helical conformation. The spectrum of PEG2k coatings had a dominant peak at ~1130 cm^{-1} (Fig. 2B, black spectrum, red arrow) with a shoulder at ~1144 cm^{-1} (Fig. 2B, black spectrum, blue arrow), suggesting a mixture of the ordered helical and the less ordered all-trans conformations, but dominated by helical conformation in the long polymer chain (Matsuura and Miyazawa, 1969; Miyazawa et al., 1962).

To test the protein adsorption properties of EG_3OMe and PEG2k coated surfaces they were incubated with selected blood proteins at a concentration of 1 mg/ml. The proteins vary in molecular weight (M_w) and isoelectric point (p*I*). Lysozyme is a

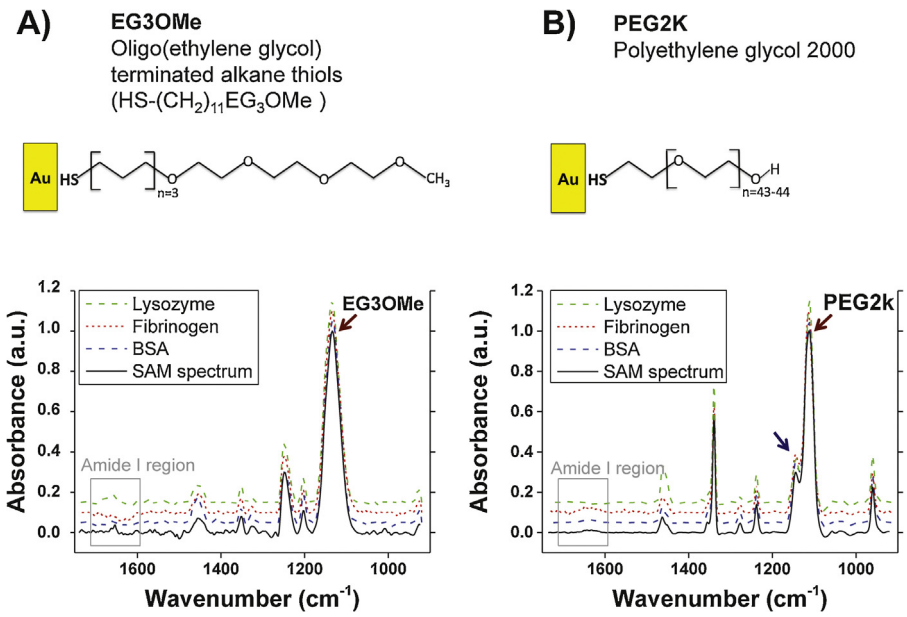

Fig. 2. Structure and determination of protein adherence to EG_3OMe (A) and PEG2k (B) coatings by PMIRRAS spectral analysis. The peaks seen in the range between 1500 and 900 nm were specific for the coated material EG_3OMe and PEG2k (black spectrum); arrows indicate the main peaks. After incubation with selected blood proteins (1 mg/ml) spectral analysis with potentially adhered proteins was carried out again: lysozyme (green-), fibrinogen (red-) and BSA (blue spectrum). The boxed square indicates the amide I region 1600–1700 cm^{-1}, which is characteristic for protein absorption; there is little adherence of these proteins to EG_3OMe or PEG2k coated gold (Au) surface.

small protein (M_w = 14.3 kDa, pI = 12) that is positively charged under physiological pH (Holmlin et al., 2001). Fibrinogen is a large protein (M_w = 340 kDa) with negative charge (pI = 6.0). BSA has an intermediate protein size (M_w = 68 kDa) and a pI of 4.6. PMIRRAS spectra of freshly prepared EG_3OMe and PEG2k coatings before and after incubating with protein solutions were shown in Fig. 2. In all cases, no significant amide I band (1600–1700 cm^{-1}) was visible after incubating with proteins (Fig. 2, boxed square). The slight deviation from the baseline might be due to the background subtraction. All other features of the absorption bands from the thiols remained identical, indicating no conformation change of thiol molecules in the coatings after exposure to proteins. The amide I band is associated with the stretching vibrations of the peptide carbonyl group, which has been widely used as an indicator to monitor protein adsorption at interfaces (Barth and Zscherp, 2002). Our results showed that both EG_3OMe and PEG2k coatings are largely inert to protein binding, which is consistent with a previous report (Zhu et al., 2001).

At high concentration (physiological) only γ-globulins and serum showed strong adsorption to the coated surfaces but not albumin

As shown above (Fig. 2), lysozyme, fibrinogen and BSA hardly adsorbed to EG_3OMe and PEG2k coated surfaces when applied at a concentration of 1 mg/ml. However, in blood serum the concentration is much higher. In blood serum, the overall protein concentration is about 70 mg/ml. The two most abundant proteins are albumin ~60% (35–50 mg/ml) and γ-globulins ~18% (10–15 mg/ml) (Burtis and Ashwood, 1999). We thus extended our experiments by increasing the protein concentrations (Fig. 3A) and also using a combination of BSA and γ-globulin.

PMIRRAS spectra indicated that the adsorption of BSA and lysozyme was very low for both coatings, even at 100 mg/ml (Fig. 3B, purple and green lines). In contrast, the adsorption of γ-globulins quickly reached a maximum at concentrations of 20–50 mg/ml, the adsorption on PEG2k coatings was lower but continuously increased with protein concentration (data not shown).

When combinations of BSA and γ-globulins with a ratio of 3:1 were tested, protein adsorption was significantly suppressed at all concentrations, indicating that BSA suppressed the adsorption of γ-globulins (Fig. 3B, red line). The suppressive effect of BSA on γ-globulin coating is also illustrated in SDS PAGE (Fig. 3C and D).

However, when whole bovine serum was tested the EG_3OMe and PEG2k coating showed different results (Fig. 3D). With EG_3OMe coatings, the total protein adsorption of serum was low and similar to that of BSA and γ-globulins mixture. With PEG2k coatings, the protein adsorption of serum was significantly increased compared to BSA and γ-globulins mixture (Fig. 3D, yellow bars), indicating that PEG2k coatings adsorbed some other proteins in the serum apart from BSA and γ-globulins.

Our results show that at physiological blood protein concentrations, the adsorption behavior of BSA, γ-globulins and whole bovine serum varies with the coating. BSA showed little adsorption on both coatings, whereas γ-globulins showed strong adsorption. BSA could suppress γ-globulins adsorption when it was mixed with γ-globulins in a ratio of 3:1, which represents serum conditions. Whole serum had strong adsorption on PEG2k coatings, but not on EG_3OMe coatings, suggesting that some other serum proteins were adsorbed on the PEG2k coatings.

Bacteria resuspended in buffer did not adhere EG3OMe and PEG2k coated surfaces

The EG_3OMe and PEG2k coated surfaces were also tested for bacterial adhesion. *S. aureus* (pC-tuf-ppmch) that constitutively expressed red fluorescent protein mCherry was suspended in PBS to an OD_{578} of 1.0 and incubated with coated and uncoated surfaces for 1 h. The uncoated gold surface was heavily covered with cells, while the EG_3OMe and PEG2k coated surfaces were hardly covered with bacteria (Fig. 4A1, 2 and 3). This result was confirmed by the quantitative analysis of the fluorescence coverage of the visual field (Fig. 6, first block, 'PBS + PBS'). Our results show that EG_3OMe

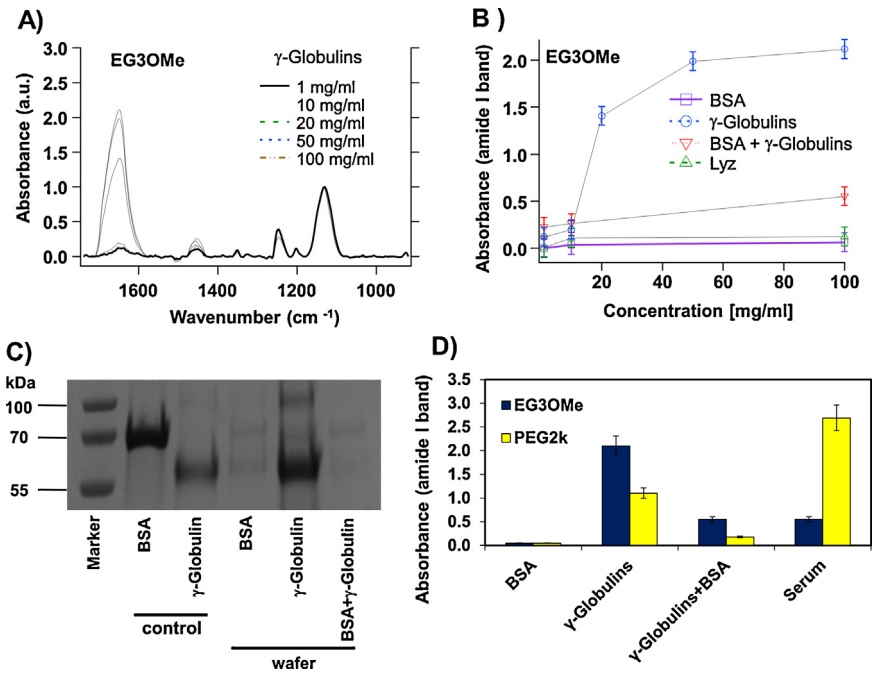

Fig. 3. γ-Globulin binding to EG_3OMe and PEG2k coated surfaces. (A) PMIRRAS spectra of EG3OMe coated surfaces incubated with different concentrations of γ-globulins. (B) Serum protein adherence was determined by amide I absorbance peak intensity from PMIRRAS spectra: BSA (purple line), γ-globulins (blue line), mixtures of BSA and γ-globulins with a concentration ratio of 3:1 (red line) and lysozyme (Lyz, green line). The concentrations used were 1, 10, 20, 50 and 100 mg/ml. (C) Analysis of serum proteins eluted from EG_3OMe coated surface by SDS-PAGE and Coomassie protein staining. (D) Graphical representation of the adherence capacity of BSA (bovine serum albumin), γ-globulins, γ-globulins together with BSA and bovine serum on EG_3OMe and PEG2k surfaces.

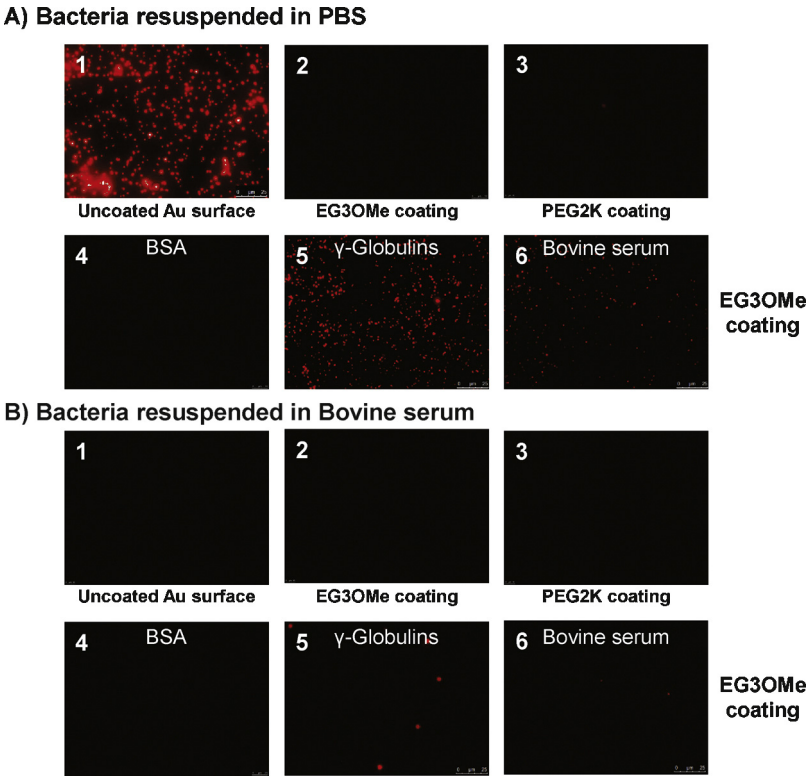

Fig. 4. Effect of *S. aureus* resuspended in PBS buffer (A) or serum (B) on adherence to EG_3OMe and PEG2k coated surfaces. Fluorescence microscopy images of *S. aureus* (pC-tuf-ppmch) adhered on uncoated Au surface (1), EG_3OMe coated surface (2), PEG2k coated surface (3) and EG_3OMe coated surfaces pre-incubated with 20 mg/ml BSA (4), 50 mg/ml γ-globulins (5) or 100% bovine serum (6).

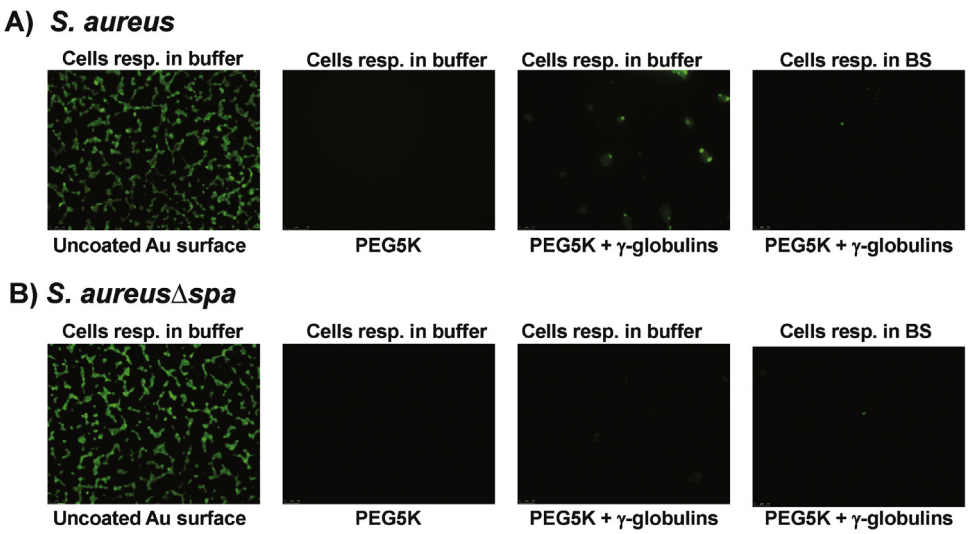

Fig. 5. Comparison of *S. aureus* WT (A) and protein A mutant (B) on adherence. Fluorescence microscope images of *S. aureus* adhesion on uncoated gold (Au) surfaces (1), PEG5k coated (2) and PEG5k coated surfaces pre-incubated with 50 mg/ml γ-globulins (3 and 4). Wafers were incubated with *S. aureus* SA113 WT (pC-tuf-gfp) or SA113Δspa (pCX-pp-sfgfp); cells were either resuspended in PBS (buffer) or bovine serum (BS).

and PEG2k coated surfaces were largely inert to bacterial adhesion. We also tested other coating material with greater length, EG6OMe and PEG5K, and they showed similar results as EG_3OMe and PEG2k (data not shown).

Pre-incubation of EG_3OMe and PEG2k coated surfaces with γ-globulins or serum promoted S. aureus adhesion

As implant material usually comes in contact with plasma components it is important to know what influence they have on bacterial adhesion. To address this question, we used two experimental settings. In the first experiment, the coated surfaces were pre-incubated with buffer (PBS control), BSA (20 mg/ml), γ-globulins (50 mg/ml) and bovine serum for 1 h. Subsequently the surfaces were incubated with *S. aureus* (pC-tuf-ppmch) suspended in PBS (OD_{578} of 1.0) for another hour. After removal of unbound bacteria by PBS-washings, bacterial adhesion was visualized with fluorescence microscopy. With this experiment, we aimed to investigate whether there is a correlation between protein adsorption and bacterial adhesion. When the coated surfaces were pre-incubated with BSA, which showed almost no adsorption itself but significantly prevents adsorption of γ-globulins in mixed BSA-γ-globulins samples (Fig. 3B), there was no adherence of *S. aureus* observed compared to the PBS control (Fig. 4A4). The results were comparable to untreated coatings as shown in Fig. 4A2 and 3. However, if the coated surfaces were pre-incubated with γ-globulins, which readily adsorbs to the coated surfaces (Fig. 3), the adhesion of *S. aureus* was significantly promoted (Fig. 4A5). The adherence of *S. aureus* to EG_3OMe coating was higher than to PEG2k coating, which correlates with the higher adsorption of γ-globulins to EG_3OMe coating (Fig. 3). In the quantitative fluorescence assay the amount of bacteria attached to EG_3OMe coatings was five-fold higher than that on PEG2k coatings (Fig. 6: γ-Globulin + PBS). If the coated surfaces were pre-incubated with bovine serum, *S. aureus* adhesion was also enhanced on both coatings (Fig. 4A6).

As shown in Fig. 6 (block 'serum + PBS'), twice as much bacteria were adhered to the EG_3OMe coatings than to PEG2k coatings, which correlated with the protein adsorption assay (Fig. 3). There was a clear correlation between the amount of γ-globulins bound to the surfaces and the amount of bacterial adhesion. As shown in Fig. 6 (blocks 'γ-Globulins + PBS' and 'serum + PBS'), the bacteria attached after pre-incubating with 60 mg/ml γ-globulins were six-fold higher than that after pre-incubating with serum where the γ-globulins were about 10 mg/ml. We assume that the adherence of *S. aureus* to serum-coated surfaces was essentially due to the relatively high content of γ-globulins in the serum (approximately 20% of total proteins are γ-globulins). We also tested human serum, which had the same effect as bovine serum (data not shown).

Pre-incubation of S. aureus with serum remarkably inhibited bacterial adhesion at EG_3OMe and PEG2k coated surfaces pretreated with γ-globulins or bovine serum

In the above (Fig. 4A) experiment we resuspended *S. aureus* (pC-tuf-ppmch) in PBS, which represents an artificial situation, because under in vivo conditions *S. aureus* comes in close contact with serum. In order to better mimic the in vivo situation we resuspended *S. aureus* in bovine and human serum and carried out the same adherence assay with EG_3OMe and PEG2k coatings pretreated with BSA, γ-globulins or bovine serum as described above (Fig. 4B).

Interestingly, resuspending *S. aureus* with bovine serum remarkably reduced *S. aureus* adhesion to EG_3OMe and PEG2k coatings pre-treated with γ-globulins or bovine serum (Fig. 4B5 and 6). Apparently serum masks the *S. aureus* cell surface with serum components, most likely γ-globulins, thus reducing its binding capacity to coatings pre-treated with γ-globulins or serum. No binding of serum resuspended *S. aureus* was observed with coatings pre-treated with BSA (Fig. 4B4), or with un-pretreated EG_3OMe and PEG2k coatings (Fig. 4B2 and 3). Even with the uncoated Au surface *S. aureus* (pC-tuf-ppmch) adherence was significantly decreased (Fig. 4B1). The same effect was observed when *S. aureus* (pC-tuf-ppmch) was resuspended with human serum (not shown). The results are summarized in the quantitative analysis diagram (Fig. 6).

The pretreatment of EG_3OMe and PEG2k coatings with γ-globulins or bovine serum caused massive adhesion of *S. aureus*. We assume that might be due to the fact that *S. aureus* harbors high immunoglobulin-binding activity by its cell-wall bound protein A (Spa) (Löfdahl et al., 1983; Uhlen et al., 1984). The 'second immunoglobulin-binding protein' (Sbi) is secreted (Zhang et al., 1998) and should not play a major role in adherence. To prove the hypothesis, *S. aureus* SA113Δspa (pCX-pp-sfgfp), which is a Protein A deficient mutant strain, was used for the tests on uncoated Au or PEG5K coated surfaces. When the bacteria were resuspended in

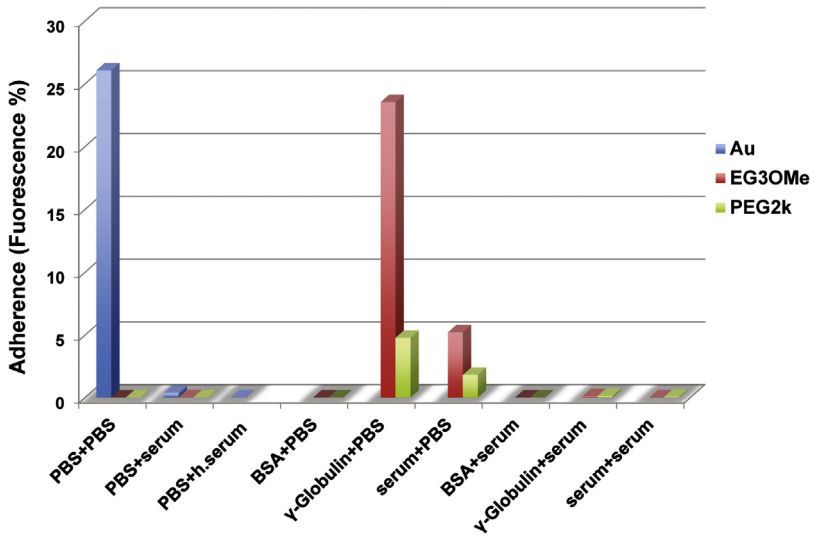

Fig. 6. Quantitative analysis of *S. aureus* (pC-tuf-ppmch) adhesion under different conditions. The coverage of red fluorescent *S. aureus* was calculated by software 'ImageJ', using a size of 1 μm^2 as an input parameter. The analyzed square seen in Fig. 3 was approx. 200 $\mu m \times 120$ μm. The name for each block indicates the experimental conditions as 'surface pre-incubation vs. bacteria suspension'; for example: 'BSA + serum' means that the surfaces were pre-incubated in BSA and the bacteria were resuspended in serum. Blue bars, uncoated Au surfaces; red bars, EG_3OMe coated surfaces; green bars, PEG2k coated surfaces.

PBS, the uncoated Au surface was heavily covered by both strains (Fig. 5A1 and B1). If WT and Δspa mutant were resuspended in buffer there was no adherence to PEG5K coated surface (Fig. 5A2 and B2). On the PEG5K coated surface pretreated with γ-globulins the WT showed good adherence, while the Δspa mutant did not adhere (Fig. 5A3 and B3). When the bacteria were resuspended in bovine serum, the wafers were adhered by neither WT *S. aureus* nor Δspa mutant (Fig. 5A4 and B4).

We showed the different adsorption behavior of albumin, γ-globulins and serum on EG_3OMe and PEG2k coatings and the effect on bacterial adhesion. Although, albumin showed little adsorption to the surfaces, it still caused a decrease of *S. aureus* adhesion. γ-Globulins strongly adsorbed to the surfaces and promoted *S. aureus* adhesion. But most important was the observation that pre-incubation of *S. aureus* with γ-globulins or serum significantly decreased the adherence to coatings that came in contact with γ-globulins or serum – that is the natural situation of implanted material.

Pretreatment of Pseudomonas aeruginosa with serum significantly decreased its adherence to the coatings

We also carried out similar adherence studies with GFP expressing *Pseudomonas aeruginosa* and obtained similar results as with *S. aureus*. *P. aeruginosa* showed massive adherence to uncoated gold (Au) surfaces (Fig. 7A and B); if the cells were however resuspended with bovine serum adherence to uncoated gold (Au) surfaces was decreased (Fig. 7B). Like *S. aureus*, *P. aeruginosa* hardly adhered to EG_3OMe and PEG2k coated surfaces even when resuspended in buffer (Fig. 7B).

Discussion

The aim of this study was to systematically analyze the adherence behavior of gold (Au) as well as EG_3OMe or PEG2k coated Au surfaces to plasma proteins and pathogenic bacteria such as *S. aureus*, which plays a crucial role in implant-associated infections. The coating altered the physical appearance of the surface. The sputtered Au surfaces on silicon wafers had a rough and grain-like morphology, which became smoother and flattened by EG_3OMe and even more smooth by PEG2k coating (Fig. 1).

The EG_3OMe and PEG2k coated surfaces were inert to adsorption of the blood proteins lysozyme, fibrinogen and albumin (BSA) if applied at low concentration (1 mg/ml) (Fig. 2) and they were inert to adherence of *S. aureus* (Fig. 4). BSA did not adsorb to the coated surfaces even when applied at very high concentrations (100 mg/ml), which is in line with the literature (Holmlin et al., 2001; Lokanathan et al., 2011; Prime and Whitesides, 1993). However, γ-globulin adsorbed to the EG_3OMe and PEG2k coatings if applied at concentrations higher than 10 mg/ml (Fig. 3); its adsorption could be, however, significantly suppressed if it was mixed with BSA in a ratio of 1:3 as it also occurs in serum. As BSA itself did not adsorb to the EG_3OMe and PEG2k coatings we assume that BSA is neutralizing γ-globulin's binding domains already in solution. Indeed, it has been shown recently that serum albumin has a moderate attraction to IgGs, which may forestall undesirable protein condensation in antibody solutions (Wang et al., 2011). We also found that PEG2k coatings adsorbed four times less γ-globulins than the EG_3OMe coatings. One reason could be that the long polymer chains of PEG2k cover the coating Au substrate more efficiently then EG_3OMe, with the effect that steric repulsion prevents γ-globulin adsorption more efficiently (Jeon and Andrade, 1991). On the other hand, adherence of whole serum was five times higher with the PEG2k coated surface than with the EG_3OMe coated one (Fig. 3D). A possible reason for this observation might be that PEG2k has a much longer thread-like structure than EG_3OMe and revealed a lower surface coverage than the EG_3OMe coating. We assume that small proteins (other than γ-globulins) can more easily penetrate the PEG2k polymer brush thus enhancing the adsorption on PEG2k coatings (Benesch et al., 2001).

The question is why BSA and lysozyme do not bind to the coatings, even at very high concentrations, while γ-globulins do. Serum albumin is the most abundant plasma protein in mammals with an extraordinary ligand binding capacity; these proteins are relatively large (66 kDa) and negatively charged (Majorek et al., 2012). Lysozymes also have a positive net charge that is thought to play an important role in guiding lysozyme to the negatively charged surface of bacteria. The majority of γ-globulins are immunoglobulins (150 kDa), which are also positively charged. Apparently, the charge of the proteins does not play a crucial role in binding to the coatings, as the positive charged lysozyme and γ-globulins show opposing binding effect. Therefore, we assume that

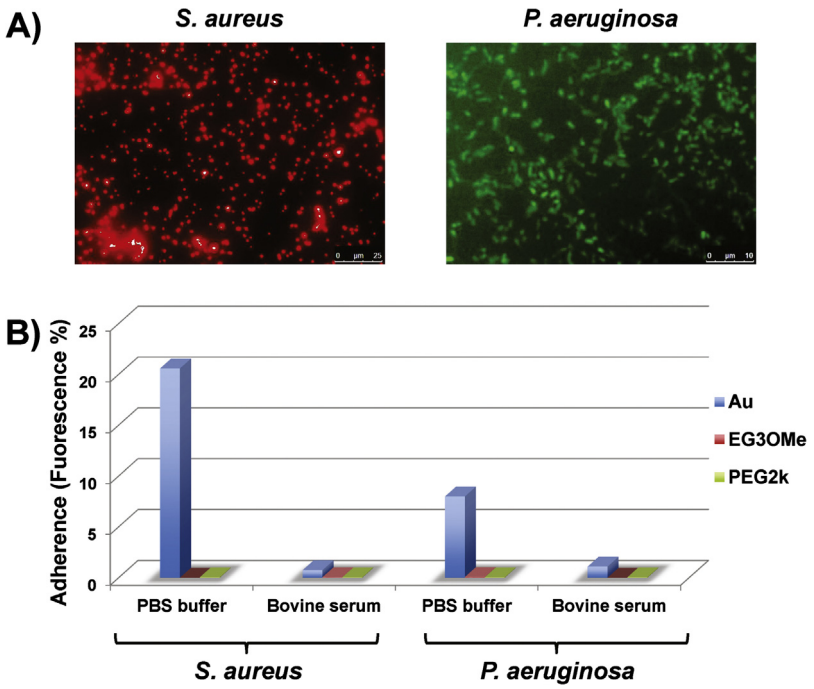

Fig. 7. Adherence comparison between *S. aureus* and *P. aeruginosa*. (A) Fluorescence images on uncoated gold (Au) surfaces after incubation with *S. aureus* or *P. aeruginosa* in PBS buffer. (B) Comparison of the cell counts on uncoated gold (Au) surfaces, EG_3OMe coated and PEG2k coated surfaces for *S. aureus* and *P. aeruginosa* resuspended in PBS buffer or bovine serum.

specific binding domains in γ-globulins interact with the PEG2k and EG_3OMe coatings. It would be interesting to know whether it is the Fc or the Fab part of IgG that mediates binding to PEG2k and EG_3OMe.

The pretreatment of EG_3OMe and PEG2k coatings with γ-globulins or bovine serum caused massive adhesion of *S. aureus* (Fig. 4A). This is not so surprising as it is well-known that *S. aureus* is distinguished by its high immunoglobulin-binding activity via its cell-wall bound protein A (Spa) (Löfdahl et al., 1983; Uhlen et al., 1984). The 'second immunoglobulin-binding protein' (Sbi) is secreted and it contributes to complement evasion (Burman et al., 2008; Zhang et al., 1998); but it should not play major role in adherence of cells to the coatings (Burman et al., 2008; Zhang et al., 1998). In this experiment *S. aureus* cells were resuspended in buffer (PBS). However, if we resuspended *S. aureus* cells with bovine or human serum the adherence to the γ-globulins or serum treated coatings was significantly decreased (Fig. 4B). Besides, *S. aureus* Δspa was not detectable on any PEG surfaces, even on the γ-globulins pre-incubated surfaces (Fig. 5). This decreased adherence is due to the saturation of Spa by IgGs present in the serum. Based on this observation implant-associated infections might be minimized by allowing bacterial pathogens to become saturated with the patient's serum.

The function of serum in preventing bacterial adhesion is not restricted to *S. aureus*, we also see this effect with *P. aeruginosa*. One possible explanation could be that the abundant albumin in the serum neutralizes γ-globulins via non-specific attraction as mentioned above (Wang et al., 2011), thereby decreasing the bacterial adhesion. With respect to Gram-negative bacteria, like *P. aeruginosa*, one must also consider the complement factors present in serum that might play a role in decreasing bacterial adhesion. Since the composition of serum is complex, many factors might play a role. For instance, it has been reported that apo-transferrin prevented bacterial adhesion to the Tecoflex polyurethane surfaces (Elofsson et al., 1997). Kuroda et al. observed that serum globulins could reduce the adhesion of *Prevotella nigrescens* to hydroxyapatite (Kuroda et al., 2003). Future investigations should focus on identifying more inhibitory components of serum.

On the other hand, pre-incubation of the surfaces with albumin did not promote *S. aureus* adhesion, even when the cells were resuspended with serum. It has been known for a while that surface coatings with albumin have an inhibitory effect on bacterial adhesion. It has been reported that albumin adsorbs to the interface and creates a thin film that prevents bacterial adhesion (Ardehali et al., 2003; Katsikogianni and Missirlis, 2004; Ribeiro et al., 2012). However, the role of albumin is not fully understood. In our experiments, the 'protective' role of albumin on bacterial adhesion can be simply explained by the fact that it is not adsorbed on the EG_3OMe and PEG2k surfaces and has therefore no further influence on bacterial adhesion. It would be interesting to test in future experiments whether albumin in the solution will inhibit bacterial adhesion onto a surface, especially when the surfaces are pre-incubated with γ-globulins.

In conclusion, our study contributes to a better understanding about the functions of the major serum components on protein adsorption and *S. aureus* adhesion on EG_3OMe and PEG2k coated surfaces. In particular, we found that serum can significantly inhibit the bacteria adhesion on different surfaces, which might have some therapeutic implications in minimizing implant-associated infections.

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