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Surface-grafted zwitterionic polymers improve the efficacy of a single antibiotic injection in suppressing *S. aureus* periprosthetic infections

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Abstract

Implant-associated bacterial infections are difficult to treat due to the tendency of biofilm formation on implant surfaces, which protects embedded pathogens from host defense and impedes antibiotic penetration, rendering systemic antibiotic injections ineffective. Here, we test the hypothesis that implant coatings that reduce bacterial colonization would make planktonic bacteria within the periprosthetic environment more susceptible to conventional systemic antibiotic treatment. We covalently grafted zwitterionic polymer brushes poly(sulfobetaine methacrylate) from Ti6Al4V surface to increase the substrate surface hydrophilicity and reduce *staphylococcus aureus* (*S. aureus*) adhesion. Using a mouse femoral intramedullary (IM) canal infection model, we showed that the anti-fouling coating applied to Ti6Al4V IM implants, when combined with a single vancomycin systemic injection, significantly suppressed both bacterial colonization on implant surfaces and the periprosthetic infections, outperforming either treatment alone. This work supports the hypothesis that grafting anti-fouling polymers to implant surfaces improves the efficacy of systemic antibiotic injections to combat periprosthetic infections.

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Author Contributions

B.Z. prepared and characterized Ti-pSBMA substrates and conducted *in vitro* experiments and performed histology staining. J.D.S. conducted *in vivo* experiments and microCT analyses. B.M.B. contributed to *in vivo* experiments. D.C.A. advised *in vivo* model. J.S. designed study and oversaw project execution and data analyses. B.Z., J.D.S. and J.S. wrote the manuscript.

ASSOCIATED CONTENT

Supporting Information: Supporting figures showing *in vivo* study design and key/scavenger organ pathology; supporting table detailing the *p*-values of the quantitative μ CT analysis.

Keywords

anti-fouling; periprosthetic infections; zwitterionic polymer brush; *Staphylococcus aureus*; osteomyelitis; bacterial colonization on surfaces; planktonic bacteria

1. Introduction

Medical implant associated infections present major health threats to patients and impose economic burdens to the healthcare budget^{1–3}. It was reported that in the United States alone, over one million total hip and knee arthroplasties were performed in 2010⁴, and this number is estimated to reach over 4 million by 2030⁵. Prosthetic joint infection (PJI) rates related to hip and knee arthroplasties in the United States were about 2% in 2009, and the cost associated with treating PJI is projected to exceed \$1.62 billion in 2020⁶. Treatments for PJI involves surgical intervention such as debridement and one-stage or two-stage revisions, along with prolonged high-dose/combinatorial antibiotic therapy^{7–12} known for causing adverse side effects and raising risks for bacterial drug resistance¹³. Unsuccessful treatment of PJI could result in amputations or deaths. Thus, preventing periprosthetic infections in the first place remains a top priority in this battle.

High tendencies for bacterial colonization and biofilm formation on medical implant surfaces are major contributing factors to the significantly higher infection rate by a factor of >100,000 in the presence of a foreign material¹⁴. Therefore, great attention has been paid to implant surface modifications aimed at reducing bacterial colonization and biofilm formation^{15–16}. Covalently modifying implant surfaces with antibiotics has been attempted to combat bacterial colonization on orthopedic implants with some success^{17–19}. We recently showed that Ti6Al4V substrates covalently grafted with vancomycin-bearing polymers significantly reduced colonization of *S. aureus* (*S. aureus*) on substrate surfaces *in vitro* and *in vivo*²⁰. However, a common limitation of this approach is the short-range protection provided by the covalently tethered antibiotics, which are unable to diffuse away and protect the surrounding tissue from being infected. This limitation can be overcome by installing a labile linkage between the substrate and the antibiotics that is sensitive to cleavage by bacterial enzymatic activities for on-demand antibiotic release that can exert its bactericidal activity beyond the immediate implant surface²¹. The dense extracellular matrix

envelope of the biofilm, capable of protecting embedded pathogens from host defense and slowing antibiotic penetration^{1, 22–24}, however, presents challenges for even free-diffusing antibiotics. Towards this end, antifouling coatings have been shown effective in reducing bacterial colonization and biofilm formation^{25–27}, but anti-fouling coatings alone do not prevent periprosthetic infections as they exhibit no bactericidal properties.

Here we test the hypothesis that anti-fouling polymer coatings reducing bacterial colonization and biofilm formation on implants could enhance the efficacy of systemic antibiotics injections in preventing periprosthetic infections due to the higher susceptibility of planktonic bacteria to antibiotics. We chose Ti6Al4V as the model implant substrate since this metallic alloy has been frequently used in orthopedic and dental prostheses due to its corrosion resistance, biocompatibility and attractive stiffness (less stress shielding compared to Co/Cr or stainless steel)^{28–29}. We chose the zwitterionic polymer poly(sulfobetaine methacrylate) (pSBMA) as the anti-fouling coating due to the commercial availability of the monomer, mild polymerization conditions, established ability to reduce biofoulant attachment^{30–32}, excellent cytocompatibility, and the ability to template surface mineralization on titanium alloy Ti6Al4V in a pro-mineralization environment³³. Here, we grafted high molecular weight pSBMA from Ti6Al4V substrates via surface-initiated polymerization. Adequate surface coverage by grafted pSBMA on the modified substrate (Ti-pSBMA) was validated by surface elemental analysis (X-ray photoelectron spectroscopy, XPS), increased surface hydrophilicity (water contact angle), and resistance to non-specific protein adsorption and *S. aureus* colonization *in vitro*. Identically modified Ti-pSBMA pins and unmodified Ti6Al4V pins were then implanted into mouse femoral intramedullary (IM) canals inoculated with 40-CFU bioluminescent *S. aureus* with or without a single systemic injection of vancomycin administered at day 7 post-operation. The degree of bacterial colonization of the implants and development of femoral bone infections at 21 days were evaluated by a combination of bioluminescent quantification, bacterial counts on the retrieved pins, and μ -CT and histology analyses of the femurs. Systemic organ pathology was also evaluated to determine the biocompatibility of the coating.

2. Materials and Methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich and used as received unless noted otherwise. Ti6Al4V plates (0.5 mm in thickness, TMS Titanium, Poway, CA) were cut into 1 × 1 cm² square pieces, and Ti6Al4V pins (0.5 mm in diameter, Goodfellow Corporation, Coraopolis, PA) were cut into 1-cm in length. All substrates were polished under water with silicon carbide sandpapers, sequentially washed in organic solvents, annealed and cleaned by air plasma before being immobilized with bromide initiator (Ti-Br) as we previously reported²⁰. Bioluminescent *S. aureus* strain Xen29, susceptible to vancomycin, was purchased from PerkinElmer.

2.2. General instrumentation

¹H NMR (500 MHz) and ¹³C NMR (125 MHz) were recorded on a Bruker spectrometer. Gel permeation chromatography (GPC) was performed on PL Aquagel–OH columns

(Agilent Technologies) with Trisma buffer as detailed previously³³, with weight- and number-averaged molecular weights (M_w and M_n) and polydispersity index (PDI) of the polymers calculated referring to PEO standards. XPS was carried out on a Thermo Scientific K-Alpha XPS at 200 and 50 eV for survey and high-resolution scans, respectively²⁰. Survey scan spectra were obtained from five consecutive scans of a randomly chosen area of interest (spot size of $400 \times 400 \mu\text{m}^2$), while high-resolution scan spectra were obtained from 10 consecutive scans. For scanning electron microscopy (SEM), the Ti6Al4V and Ti-pSBMA plates retrieved from the *S. aureus* culture were fixed with 2% EM-grade glutaraldehyde, sputter-coated with Au, and imaged on a Quanta 200 FEG MKII SEM²⁰.

2.3. Preparation of Ti-pSBMA

The catalyst complex was prepared from 2,2'-bipyridyl (14 mg, 0.09 mmol) and CuBr (5.2 mg, 0.033 mmol) in trifluoroethanol (TFE, 1 mL) under argon protection as previously reported²⁰. Sulfobetaine methacrylate (SBMA) (0.5 g, 1.79 mmol) and TFE (2 mL) were charged in a dry Schlenk flask containing Ti-Br substrates and degassed by ten “freeze–pump–thaw” cycles, followed by the injection of freshly prepared dark brown catalyst complex via a dry syringe. Polymerization continued overnight at RT and was terminated by exposure to air. The solution mixture was precipitated in methanol to obtain non-surface bound pSBMA while the Ti-pSBMA substrates were thoroughly washed with TFE reflux for 24 h to remove any residual unbound pSBMA physically absorbed on the substrates using a Soxhlet extractor.

2.4. Cleavage of grafted pSBMA from Ti-pSBMA substrates

Ti-pSBMA substrates were subjected to acidic treatment (2 N HCl, 0.2 M NaCl, rt) for 3 days, and the cleavage solution was neutralized and desalted via dialysis as detailed previously³³. Cleaved pSBMA recovered from the dialysis tubing was freeze-dried for subsequent analyses.

2.5. Water contact angle measurements

Water contact angles on the substrates before and after surface modifications were recorded on a CAM200 goniometer (KSV Instruments). A droplet (2 μL) of Milli-Q water was placed on the Ti6Al4V or Ti-pSBMA substrate ($n = 3$) and the contact angles (left and right) of the droplet were recorded after 30 s. The reported contact angles were averaged from three randomly selected regions on each substrate.

2.6. Nonspecific protein adsorption on surfaces

Ti6Al4V and Ti-pSBMA plates ($n = 3$) were incubated in 1 mL of bovine serum albumin (BSA)-fluorescein conjugate/Dulbecco's phosphate-buffered saline (PBS) solution (500 $\mu\text{g/mL}$) at 37 °C overnight. All substrates were rinsed with fresh PBS before being imaged by fluorescent microscopy. The fluorescence intensities were quantified by ImageJ.

2.7. *In vitro* bacterial culture on Ti6Al4V and Ti-pSBMA plates

Ti6Al4V and Ti-pSBMA plates were sterilized in 70 % alcohol for 1 h and dried, followed by incubation in 1 mL of bioluminescent Xen29 suspension in Luria-Bertani (LB) broth (2.3

$\times 10^6$ CFU/mL) on a shaking incubator (1 Hz) for 7 h at 37 °C. The retrieved plates were washed with PBS before being fixed for SEM. For imaging by bioluminescence, the retrieved plates, upon gently rinsing with 1 mL of LB three times, were imaged on an IVIS-100 (n = 3; 30 s exposure time).

2.8. *In vivo* studies

The animal study was carried out per procedures approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee (IACUC). Skeletally mature male CL57BL/6 mice 8–12 weeks old (n = 8–13 for each group) were used. To establish *S. aureus* infection and enable longitudinal monitoring of the infection in the mouse femoral canal, bioluminescent Xen29 *S. aureus* (4 μ L of 10^4 CFU/mL stock solution in LB) was injected into the femoral canal immediately prior to surgical insertion of an unmodified Ti6Al4V IM pin or a Ti-pSBMA pin. A single systemic injection of vancomycin was given to a subset of mice at day 7 post-operation to allow adequate time for bacteria colonization and rigorous examination of the efficacy of pSBMA coatings in combating infections with/without a systemic vancomycin injection. Mice receiving Ti-pSBMA pins but no Xen-29 or systemic vancomycin injections served as uninfected controls (n = 8). The *in vivo* study design including experimental and control groups, sample sizes, and outcome measures is detailed in Figure S1.

2.9. Animal surgery

The animal surgery was carried out as detailed previously²⁰. Upon exposure of the intercondylar notch of the femur, a sterile 25-gauge hypodermic needle was used to broach and ream the femur before 4 μ L of either sterile LB solution or Xen29 *S. aureus* solution (10^4 CFU/mL in LB) was injected into the reamed femoral canal. A sterile unmodified Ti6Al4V or Ti-pSBMA pin (~10 mm long and 0.5 mm in diameter) was then inserted into the femoral canal. Post-operative care was given as previously described²⁰. A subset of the mice was given an intraperitoneal (IP) injection of 110 mg/kg vancomycin at day 7 post-operation. No mice died of gross infections during the course of the study.

2.10. μ CT

Mice were scanned the day after operation (day 1) and at 21 days post-operation on a Scanco vivaCT 75 system (Scanco Medical, Switzerland) at an effective voxel size of $20.5 \times 20.5 \times 20.5 \mu\text{m}^3$. The identification of femoral region of interest (ROI) and the application of global thresholds for quantitative analyses of bone volume fraction (BVF), bone mineral density (BMD) and cortical thickness (C. Th.) follow previously published methods²⁰.

2.11. *In vivo* bioluminescence imaging

The bioluminescence of Xen29 *S. aureus* within the infected mouse femoral canals was visualized and quantified using the IVIS-100 imaging system (Perkin-Elmer) on day 21 post-operation. Mice were anesthetized with 5% isoflurane-oxygen, placed on the imaging platform, and imaged with a 5 min overall exposure time with open emission filter. Background subtraction was performed with an uninfected femoral region of interest.

2.12. Quantification of Xen29 *S. aureus* on retrieved IM pins

At the 21-day endpoint, the mice were sacrificed following the IVIS scans. The metal pins were retrieved from the explanted femur and vortexed in 1 mL of cold LB (4 °C) for 5 min. The vortexed solution was diluted and loaded on agar plate for 24-h culture to determine the bacterial CFU counts as previously described²⁰.

2.13. Femoral histology and key/scavenger organ pathology

After the IM pin removal, the femoral explants retrieved at day 21 were fixed in periodate-lysine-paraformaldehyde (PLP) fixative,³⁴ decalcified, paraffin embedded, and longitudinal sectioning (6 μ m) as previously described²⁰. The sections were stained with hematoxylin and eosin (H&E) for cellularity and tartrate-resistant acid phosphatase (red)/alkaline phosphatase (blue) (TRAP/ALP) for osteoblast/osteoclast activity.

Heart, lung, kidney, liver, pancreas, spleen, and ribs were retrieved at 21 days post-operation in a subset of animals and fixed and stained with H&E for comparison with the key/scavenger organs retrieved from healthy age matched controls. All histology images were taken at 50 $\times$ or 100 $\times$ magnifications.

2.14. Statistical analysis

All statistical analysis was performed using Prism 7.0. Shapiro–Wilk normality testing was used to evaluate data distribution. Pair-wise comparisons passing normality test were analyzed with Student's *t*-test, otherwise Mann-Whitney test was used. Parametric multiple-group comparisons were analyzed using one-way analysis of variance (ANOVA) with Tukey's posthoc while nonparametric multiple-group comparisons were carried out with the Kruskal-Wallis test. P-values of <0.05 were considered significant. All data was presented as mean $\pm$ standard error of the mean (SEM).

3. Results

3.1. Grafting Ti-pSBMA with high molecular weight pSBMA

Ti-pSBMA was prepared via surface-initiated polymerization of SBMA in TFE (Figure 1A). Using well-controlled surface-initiated ATRP carried out with the addition of ionic liquid (slowing polymerization) and sacrificial initiators (lowering grafted polymer molecular weight), we previously demonstrated a direct positive correlation between longer pSBMA brushes grafted to Ti6Al4V substrates (e.g. degree of polymerization of 200, 100 vs. 50) and higher surface hydrophilicity and reduced non-specific surface protein adsorptions.³³ Accordingly, to enable the grafting of higher molecular weight pSBMA for maximal surface polymer coverage in this study, the reaction was allowed to proceed in TFE without the addition of ionic liquid or sacrificial initiator. GPC results showed that free pSBMA formed had a M_n of ~179 kDa and a PDI of 2.32 (Figure 1B). Following a previously reported method to cleave the grafted pSBMA from Ti-pSBMA substrates³³, a higher M_n of ~271 kDa was observed for the grafted pSBMA. To examine whether the acidic cleavage condition could have hydrolyzed the side chains of pSBMA, the 179-kDa pSBMA formed in the reaction solution was subjected to the same treatment, which did not reveal obvious loss of SBMA side chains based on the retention of the characteristic signals in ¹H NMR results

(Figure 1C). Thus, the M_n of ~271 kDa corresponded to an estimated average of 971 repeating units grafted to the Ti-pSBMA. These surface polymers possessed significantly higher averaged molecular weight but broader polydispersity than those obtained with the help of ionic liquid and sacrificial initiators (e.g. M_n of 25 kDa and PDI of 1.17)³³, a trade-off for achieving good surface coverage by the anti-fouling polymer.

3.2. XPS confirmation of surface coverage of grafted pSBMA on Ti6Al4V substrates

Adequate surface coverage of the grafted pSBMA on the Ti6Al4V substrate was confirmed by XPS (Figure 2). Compared to unmodified Ti6Al4V, signal intensity for Ti was drastically reduced on the Ti-pSBMA in the survey scan (Figure 2A) due to masking by surface polymers. In addition, new N1s (402.5 eV) and S2p (168 eV) signals characteristic to the sulfobetaine group were expectedly detected from the Ti-pSBMA surfaces but not the Ti6Al4V (Figure 2A and B). XPS elemental mapping of these substrates (Figure 2C) confirmed excellent coverage by the grafted pSBMA as shown by the high-intensity and uniformity of N1s and S2p signals across the randomly chosen areas of analysis ($400 \times 400 \mu\text{m}^2$) on the Ti-pSBMA.

3.3. *In vitro* anti-fouling properties of Ti-pSBMA

As pSBMA is a hydrophilic antifouling polymer, the successful grafting of pSBMA to the Ti6Al4V substrate was further validated by changes in surface hydrophilicity and surface fouling. Water contact angle was reduced from 60° on Ti6Al4V to 10° on Ti-pSBMA, supporting an increased surface hydrophilicity upon pSBMA grafting (Figure 3A). To examine the antifouling capacity, non-specific absorption of fluorescein isothiocyanate (FITC)-labeled BSA on Ti-pSBMA and Ti6Al4V surfaces was quantified. As shown in Figure 3B, a large amount of fluorescent BSA was observed on the Ti6Al4V plate after incubation overnight, while few were detected on the Ti-pSBMA plate (with minimal signal detected above the gap background). Ti-pSBMA also significantly reduced the adhesion and colonization of Xen-29 *S. aureus in vitro* as shown by SEM micrographs (Figure 3C) and IVIS bioluminescence quantification (Figure 3D). Within hours of shaking incubation, *S. aureus* colonized throughout the unmodified Ti6Al4V substrate, consistent with the characteristic aggregations leading towards the formation of biofilm matrix^{1, 24}. On the contrary, only sparse bacterial adhesions were observed on isolated regions of the Ti-pSBMA substrates. These results support that the surface-grafted pSBMA rendered the desired antifouling property to the Ti-pSBMA.

3.4. Inhibition of *S. aureus* colonization on IM pins *in vivo* by pSBMA coating

To evaluate the efficacy of pSBMA coatings, with and without a single systemic injection of vancomycin, in combating periprosthetic infections *in vivo*, we inserted Ti-pSBMA and uncoated Ti6Al4V IM pins into mouse femoral canals inoculated with 40-CFU Xen-29. We have shown previously that this inoculation dose of Xen-29 was adequate in establishing periprosthetic infection in this murine femoral IM pin infection model, as evidenced by the detection of bioluminescent signals within the ROI, bacterial counts on retrieved unmodified pins, femoral cortical thickening, and reduced BV and BMD by 3 weeks^{20–21}. Given the low 40-CFU inoculation dose of *S. aureus*, we chose to deliver the systemic injection of vancomycin at day 7 post-operation to allow time for bacterial colonization and growth on

implant surface, enabling more rigorous evaluation of the effect of the pSBMA coating. A single intraperitoneal injection of 110 mg/kg vancomycin in mice³⁵, comparable to ~550 mg/60-kg human dose based on mouse-human allometric scaling³⁶, was chosen to approximate the lower-end of the 500-mg to multi-gram a day intravenous injection dose applied to adult humans³⁷. It should be noted that the intensity of detected bioluminescence reflects the overall metabolic activity of Xen-29 within the entire ROI including those colonized on the implant (note that bacteria within established biofilm are metabolically less active), planktonic bacteria within the IM canal, and those invading the surrounding bone. By contrast, quantification of bacteria on retrieved implants more accurately accounts for the number of bacteria bound to the implant surface at the endpoint while μ -CT and histology analyses at the ROI shine light on the extent of bacteria invasion into bone and the resulting structural/morphological changes in the infected bone.

As shown in Figure 4A, without the systemic vancomycin injection, *S. aureus* caused infections in both Ti-pSBMA and Ti6Al4V groups. At day 21 post-operation, both groups showed high bioluminescence levels and significant bacteria were detected on the retrieved pins. Although there was an overall reduction in adherent bacteria on Ti-pSBMA, the difference is not statistically significant compared to the untreated pin group ($p = 0.29$). Similarly, in the absence of the pSBMA surface coating, the systemic injection of vancomycin at day 7 failed to eliminate *S. aureus* from the unmodified Ti6Al4V pin surface or within the femoral canal, as evidenced by high IVIS intensities and bacterial counts on retrieved pins at day 21 post-operation (Figure 4B, black bars). However, when the pSBMA coating was combined with the vancomycin injection at day 7 post-operation, adherent bacteria on the retrieved Ti-pSBMA pins was significantly reduced by 2 orders of magnitude, with only a minimal count detected in some and none in others. We also observed a reduction in IVIS bioluminescent signals in the Ti-pSBMA+vancomycin group compared to the Ti6Al4V+vancomycin group, although the difference was not statistically significant ($p = 0.28$).

3.5. Suppression of periprosthetic infections by pSBMA coating in combination with a single systemic injection of vancomycin

Consistent with end-point bacterial counts on the retrieved pins, μ -CT images of the femurs (Figure 5A) revealed pronounced cortical bone thickening, characteristic of osteomyelitis³⁸, in both of the pin groups without vancomycin injection. Compared to uninfected controls, a significantly lower bone volume fraction (BVF) and bone mineral density (BMD) were also observed in these infected groups (Figure 5B & Table S1). However, vancomycin injection, when combined with pSBMA coating, helped to keep the C. Th, BMD and BVF at the same levels as uninfected mice (Figure 5A and B). Of note, vancomycin treatment failed to prevent femoral bone changes in the unmodified Ti6Al4V pin group, although the extent of cortical thickening was reduced compared to the infected group without vancomycin. H&E staining of the explanted femurs at 21 days showed a highly cellularized IM canal and significant cortical bone thickening consistent with μ -CT findings in both infected pin groups without vancomycin injection and the infected Ti6Al4V group with vancomycin injection (Figure 5C, top). Only the Ti-pSBMA+vancomycin injection group exhibited normal femoral cortical thickness compared to the uninfected control. TRAP/ALP staining

(Figure 5C, bottom) also showed enhanced osteoblast/osteoclast bone remodeling activities within the thickened/infected cortical bone, drawing stark contrast to the uninfected and the pSBMA+vancomycin groups. Finally, H&E staining of key/scavenger organs retrieved at 21 days in both infected and uninfected groups did not reveal abnormal findings compared to normal controls (Figure S2), supporting the biocompatibility of the pSBMA coatings and the localized nature of the periprosthetic infection in this murine model.

4. Discussion

pSBMA, a zwitterionic polymer containing both positive and negative charges on each sidechain, is known for its antifouling properties resulting from the formation of thick hydration shells around the hydrophilic polymer³⁹. Here we chose to graft high molecular weight pSBMA from the Ti6Al4V surface to maximize surface coverage by the anti-fouling polymer³³ at the expense of compromised control over molecular weight distribution. With an averaged $M_n \sim 270$ kDa of grafted pSBMA, a PDI of 1.88 was obtained, which was higher than those (e.g. PDI ~ 1.2) achievable with lower molecular weight pSBMA grafted using similar surface-initiated polymerization process³³. The grafted pSBMA brushes resulted in excellent surface coverage of the Ti-6Al4V substrate as revealed by XPS analyses (Figure 2C) and significant reduction in water contact angle (Figure 3A). As expected, Ti-pSBMA showed excellent antifouling capacity compared to the unmodified Ti6Al4V substrates, resulting in only residual non-specific protein adsorption (Figure 3B) and significantly reduced adherence and colonization of *S. aureus in vitro* (Figure 3C and D).

As pSBMA coating is not bactericidal, insertion of Ti-pSBMA pins into the mouse femoral canals pre-inoculated with 40-CFU Xen-29 did not inhibit the bacterial growth within the marrow cavity as supported by the bioluminescence at the ROI comparable to those inserted with uncoated Ti6Al4V pins by 21 days (Figure 4A, left). With an uninhibited bacterial proliferation within the ROI, the reduction in *S. aureus* adhesion on the anti-fouling surface of Ti-pSBMA pins, retrieved at 21 days, was insignificant compared to uncoated Ti6Al4V pins (Figure 4A, right). Periprosthetic infections, as evidenced by significant cortical bone thickening, decreased cortical BVF and BMD (Figure 5A and B), and enhanced osteoblast (ALP)/osteoclast (TRAP) remodeling activities (Figure 5C), was developed in both Ti-pSBMA and Ti6Al4V groups. These results clearly show that the antifouling coating on the metallic implant alone is unable to prevent periprosthetic infections *in vivo*.

Meanwhile, a single dose of systemic injection of vancomycin administered 7 days after 40-CFU Xen-29 inoculation and unmodified Ti6Al4V pin insertion failed to prevent periprosthetic infections. Substantial bioluminescence, significant bacterial counts on retrieved pins (Figure 4B), as well as cortical thickening, reduction in BVF/BMD, and enhanced bone remodeling at the ROI consistent with periprosthetic infections (Figure 5) were observed in this group. The timing of the systemic vancomycin injection was chosen to allow the very low dose of inoculated *S. aureus* to proliferate within the marrow cavity and adhere to implant surfaces if able. The development of periprosthetic infections with the systemic injection of vancomycin but in the absence of anti-fouling implant coating highlights the difficulty in eradicating bacteria once they have had a chance to colonize on conventional implant surfaces. The tendency for *S. aureus* colonization/biofilm formation on

conventional Ti6Al4V substrates has likely contributed to the poor susceptibility of the bacteria to the systemic antibiotic treatment and/or clearance by host defense. The persistent presence of the bacteria within the periprosthetic microenvironment also facilitated their invasion into the surrounding bony tissue.

Consistent with our hypothesis, the combination of the anti-fouling pSBMA coating and a single systemic injection of vancomycin at day 7 effectively inhibited periprosthetic infections in the femoral canal inoculated with 40-CFU Xen-29. Near-zero bacterial counts on retrieved Ti-pSBMA pins (Figure 4B, right) and normal femoral bone structures revealed by μ -CT analyses (Figure 5A and B) and femoral histology (Figure 5C) were observed following this combinatorial treatment. These data collectively validate the ability of the antifouling pSBMA coating to inhibit bacterial colonization/biofilm formation on implant surfaces, which in turn more effectively protects the surrounding bone from infection when combined with a single systemic vancomycin injection.

Conventional vancomycin systemic injection doses for treating infections in a 60-kg adult human can be as high as 1.8 g every 8–12 h^{37, 40–41}. For periprosthetic joint infection patients⁴², aggressive intravenous infusion of 1-g vancomycin every 12 h, 500 mg meropenem every 8 h, and prolonged follow-up antibiotic therapy with a β -lactam/glycopeptide for 6 weeks or an oral fluoroquinolone/rifampicin combination for 12 months could still result in recurrent infections. Here, a single dose of 110 mg/kg vancomycin (comparable to 550 mg human dose) intraperitoneally injected 7 days after receiving 40-CFU *S. aureus* inoculation and Ti-pSBMA IM pin insertion achieved significant reduction in bacterial counts on implants by $\sim$ 100 fold (from an average of 1818 CFU to 18.8 CFU). This outcome is superior to the 47-fold reduction in implant bacterial colonization in a mouse joint infection model (10^4 -CFU *S. aureus* was inoculated into the knee joint after metallic implant insertion) following far more frequent vancomycin subcutaneous injections (110 mg/kg twice a day from day 7 to 49)⁴³. The minimal systemic injection dose and frequency applied here, far lower than those applied in literature animal murine orthopedic infection models or human orthopedic infection studies, may be further optimized to achieve complete eradication of periprosthetic infections in combination with the antifouling surface coating.

5. Conclusion

In summary, high molecular weight pSBMA was covalently grafted to the Ti6Al4V substrates, affording Ti6Al4V implants high hydrophilicity and excellent antifouling properties. Compared to unmodified substrates, the pSBMA-modified Ti6Al4V suppressed *S. aureus* colonization *in vitro*, and, when combined with a single low-dose (110-mg/kg) systemic injection of vancomycin at 7 days post-operation, suppressed periprosthetic *S. aureus* infections *in vivo* using a murine femoral infection model. This combinatorial treatment represents a significant improvement in efficacy for combating periprosthetic infections compared to either systemic vancomycin injection alone (same timing and dose) or pSBMA coating alone. Given the ease of pSBMA coating or bulk polymer preparations and the convenience and safety of a single systemic antibiotic injection, this combinatorial strategy may be explored in combating periprosthetic infections with improved efficacy and enhanced safety.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Synthetic scheme, GPC and NMR characterizations of surface-grafted pSBMA. (A) Preparation of Ti-pSBMA by surface-initiated polymerization of SBMA. (B) GPC traces of pSBMA cleaved from Ti-pSBMA substrates (red) and the free pSBMA recovered from polymerization solution (black). (C) ^1H NMR spectra of the free pSBMA formed in solution before (black) and after being treated with the acidic cleavage (blue).

Figure 2. XPS surface characterizations of Ti6Al4V and Ti-pSBMA substrates.

(A) Survey scans of XPS. (B) High-resolution XPS spectra for N1s and S2p. (C)

Representative XPS surface elemental color mapping over randomly chosen areas of 400 μm × 400 μm on Ti6Al4V and Ti-pSBMA.

Figure 3. Surface hydrophilicity and *in vitro* anti-fouling properties of Ti6Al4V vs. Ti-pSBMA.

(A) Water contact angles on Ti6Al4V and Ti-pSBMA surfaces ($n = 3$). (B) Fluorescent micrograph (top) and corresponding intensities (bottom) of FITC-conjugated BSA non-specifically absorbed on Ti6Al4V and Ti-pSBMA substrates after overnight incubation with the protein ($500 \mu\text{g/mL}$) at 37°C . (C) SEM and (D) IVIS bioluminescent signals ($n = 3$) of unmodified Ti6Al4V vs Ti-pSBMA plates after 7-h shaking incubation with Xen29 *S. aureus* ($2.3 \times 10^6 \text{ CFU/mL}$; 1 Hz) at 37°C . Insets in (D): Representative bioluminescent images. * $P < 0.05$, **** $P < 0.0001$ (student's *t*-test).

Figure 4. Ti-pSBMA pins suppressed *S. aureus* colonization in infected mouse femoral canal with a systemic vancomycin injection at day 7.

(A) Bioluminescence of mouse femurs (left) and bacterial counts on retrieved pins (right) on day 21 in the Ti6Al4V vs. Ti-pSBMA pin groups without vancomycin treatment (-VAN).

(B) Bioluminescence of mouse femurs (left) and bacterial counts on retrieved pins (right) on day 21 in the Ti6Al4V vs. Ti-pSBMA pin groups with vancomycin treatment (+VAN). * $P < 0.05$ (Mann-Whitney test). All femurs were inoculated with 40-CFU Xen 29 prior to pin insertion.