Electronic Supplementary Information (ESI) for

Smart Zwitterionic Coatings with Precise pH-Responsive Antibacterial Functions for Bone Implants to Combat Bacterial Infections

Qinsheng Hu,[a, b] Yangrui Du,[c] Yangjing Bai,[d] Dandan Xing,[c] Chengcheng Wu,[c] Kaijun Li,[c] Shiying Lang,[c] Xiaoyan Liu [*e] Gongyan Liu[*c].

a Department of Orthopedic Surgery and Orthopedic Research Institute, West China Hospital, Sichuan University, Chengdu 610041, China.

b Department of Orthopedic Surgery, Ya'an People's Hospital, Ya'an 625000, China.

c College of Biomass Science and Engineering, Sichuan University, Chengdu 610065, China.

d West China School of Nursing, Sichuan University/Department of Cardiovascular Surgery, West China Hospital, Sichuan University. Chengdu, 610041, China.

e Department of Orthopedic Surgery, West China Hospital, Sichuan University/West China School of Nursing, Sichuan University. Chengdu, 610041, China.

*Corresponding Author: Xiaoyan Liu (E-mail: lxy711010@163.com), Gongyan Liu (E-mail: lgy3506@scu.edu.cn).

This supporting information includes:

(1) Methods: 1 to 10

(2) Supplementary Figures: Figure. S1 to S5.

Methods:

1 characterization of the PSB/GS-loaded coating onto the Titanium Surface

The surface morphology as well as the elemental compositions of pristine titanium, PSB coating and PSB/GS coating were investigated using a Scanning electron microscopy. X-ray photoelectron spectroscopy was performed using a monochromatic Al Kα (1486.6 eV) X-ray source. Since the macroscopic shape of the titanium foils were found to be too irregular for determining the coating thickness and surface wettability, regular shaped (1 × 1 cm) monocrystalline silicon wafers were used to deposit PSB coating and PSB/GS coating instead of titanium foils. The changes in surface wettability of PSB and PSB/GS coatings were investigated using an OCA20 contact angle measurement device. The thickness of the PSB coating and PSB/GS coating were then examined by a spectroscopic ellipsometer.

2 Drug-release behavior of PSB/GS coating

The PSB/GS coating was placed in a well plate and PBS solutions of different pH (pH 7.4; pH 5.5) were added dropwise. At regular intervals, 500 μL of drug release solution was removed from the well plates and 500 μL of PBS solution at pH 7.4 or 5.5 was added. Subsequently, the establishment of the standard curve required the use of a UV-visible spectrophotometer to determine the concentration of GS and the use of acetyl acetone–formaldehyde as a derivatizing reagent and boric acid–acetic acid as a buffer. Following this, the cumulative release curve of GS over a 30-day period was calculated.

3 Zone of inhibition assay

Bacterial suspensions (10^5 CFU mL^{-1}) were added to agar medium, mixed well and spread flat in Petri dishes. The two samples (PSB coating and PSB/GS coating) were carefully placed in the center of the petri dish and the dish was incubated at 37°C for 24 h. The diameter of the zone of inhibition was recorded.

4 Bacterial anti-adhesion ability

The bacterial suspension (2×10^8 CFU mL^{-1}) was co-cultured with the pristine Ti, PSB coating, and PSB/GS coating for 24 h, and then the pristine titanium, PSB coatings, and PSB/GS coatings were rinsed with PBS to remove loose bacteria. The samples were then immersed in PBS and sonicated, which was repeated 5 times. After staining, the bacterial status of the sample surface was observed by CLSM. In addition, after diluting to the appropriate concentration, the bacteria were cultured onto the NA plates and incubated for 24 h. the bacterial antiadhesion rate was calculated using a plate counting method as following formula:

$$Bactericidal\ anti-adhension\ rate\ (\%) = \frac{cell\ count\ of\ control - cell\ count\ of\ samples}{cell\ count\ of\ control} \times 100\%$$

5 Antibacterial assay

Sterilized pristine Ti, PSB coating and PSB/GS coating were placed into 12-well culture plates, and 100 μL of bacterial suspension (10^5 CFU mL^{-1}) was added to each well. The samples were incubated at 37°C for 24 h at a humidity of not less than 90%, then removed, washed with 5 mL of PBS, and the solution was diluted 10-fold. Finally, 20 μL of the diluted solution was spread on the plate and incubated at 37°C for 24 h. The number of bacterial colonies on the plate was recorded and the antibacterial rate was calculated according to the following formula:

$$Antibacterial\ rate\ (\%) = \frac{CFU{\cdot}mL_{0h}^{-1} - CFU{\cdot}mL_{24h}^{-1}}{CFU{\cdot}mL_{0h}^{-1}} \times 100\ \%$$

where CFU represents the number of colonies counted per plate at the indicated times, and bactericidal rate is taken as the average of the data from six parallel samples.

6 Antibiofilm assay

Pristine Ti, PSB coating and PSB/GS coating were immersed in a bacterial suspension at a concentration of 10^7 CFU mL^{-1} and incubated at 37°C for 24 h before the samples were removed and rinsed three times with PBS. This process was repeated daily for 7 days. Finally, images of pristine titanium, GS-coated and PSB/GS-coated titanium foils were observed by scanning electron microscopy (SEM). Biofilm biomass and bacterial counts on the samples surface were measured using crystal violet staining and well plate counting methods, respectively.

7 Hemocompatibility

After centrifugation (1300 rpm, 10 min) of fresh anticoagulated rabbit blood, 1.2 mL of red blood cells from the bottom of the centrifuge tube is diluted to 50 mL (2.4 v/v%) with saline. Pristine Ti and PSB/GS coating were submerged in diluted erythrocyte solution to detect hemolysis. The red blood cell suspensions treated with deionized water and saline were used as positive and negative controls, respectively. All samples were shaken at 37°C for 1 h and then centrifuged (8000 rpm, 5 min). The supernatants after centrifugation were taken and the absorbance of hemoglobin was measured at 540 nm with a spectrophotometer (UV-2700, Shimadzu instruments). The hemolysis ratio of the sample was calculated using the following formula:

$$Hemolysis\ ratio\ (\%) = \frac{A_{Sample} - A_{Negative\ control}}{A_{Positive\ control} - A_{Negative\ control}} \times 100\ \%$$

8 Biocompatibility

To evaluate the biocompatibility of the pristine Ti and PSB/GS coating i*n vitro*, the toxicity of the sample extracts to L929 cells was assessed using the CCK-8 method, and PBS was served as the control. L929 cells were inoculated into 96-well plates and incubated for 24 h (37°C, 5% CO_2). The cell culture medium was then replaced with the sample extract for another 24 h of incubation. At last, CCK-8 reagents were added to the 96-well plates and their absorbance at 450 nm was detected using a microplate reader. The morphology of L929 cells was observed using fluorescent staining.

9 *In vivo* host response

Before the experiment, the samples (5 × 5 mm) were sterilized by UV irradiation for a period of 2 h. Next, the samples were implanted into the skin of the dorsal surface of the mice and the relevant areas were labeled. At days 3 and 7 of sample implantation, the marked skin was picked up with forceps and the skin tissue surrounding the center point that was in touch with the sample was cut with sterile surgical scissors. The sampled skin tissues were fixed using 4 % paraformaldehyde. Finally, H&E staining was performed for microscopic examination and picture collection for analysis.

10 Antifouling assay

Bovine serum albumin (FITC-BSA) was diluted to 0.1 mg/ml in PBS, and the prepared layer samples were immersed in the protein solution and incubated under dark conditions at 37°C for 1 h. The sample surface was washed with PBS solution to remove non-adhered proteins on the surface, and the procedure was repeated three times. The

protein adhesion on the sample surface was then observed using confocal laser scanning microscope and the expression was quantified by relative fluorescence intensity.

11 Blood adhesion assay

Hematocrit adhesion test was performed according to a previously reported study. Fresh rabbit blood was separated into platelet-rich plasma and RBCs suspensions by centrifugation at 1500 rpm for 15 min. The prepared samples were then co-cultured with platelets and RBCs and incubated at 37°C for 1 h. At the end of the incubation, saline rinses were used at least three times to remove non-adhered blood cells. The samples were fixed using 2.5 wt% glutaraldehyde, followed by gradient dehydration of the samples using ethanol. SEM was then used for observation. Quantitative expression of adhesion was performed.

12 *Ex vivo* antithrombogenicity assay

An arteriovenous shunt model was established in three adult New Zealand rabbits (2.5 kg ~ 3 kg) for an *in vitro* antithrombotic test. First, anesthesia was performed, and then the carotid artery and external vein were separated to establish the *in vitro* circuit. The prepared coated samples were assembled with the *in vitro* circuit, and after blood circulation for 2h, the samples were collected and simply washed with saline. Photographs were taken to record cross-sectional and surface images of thrombus formation. Thrombus weight and duct closure rate were quantitatively expressed. In addition, the internal morphology was observed by SEM.

13 *In vivo* antibacterial assay in a rat model

The rats were anesthetized intraperitoneally, and a 2 cm long skin incision was made

on the back of the rats to create a subcutaneous pocket. Two animal models were established in this work. For the first one, 30 μL of *S. aureus* suspension (10^8 CFU mL^{-1}) was first injected into the implantation area, and 30 min later the Ti samples were implanted and the incisions were sutured. For the second one, the implanted Ti samples were first immersed in *S. aureus* solution (10^9 CFU mL^{-1}) for 30 min, and then the samples were continued to be implanted and sutured as mentioned above. In each of these two models, six rats were used in each parallel group. The Titanium foils were kept for 3 days after implantation, and then the SD rats were sacrificed. The implanted Titanium foils and the surrounding subcutaneous tissue were removed from the corresponding areas. In order to observe the colonization of bacteria on the surface of the implanted Titanium foils, CLSM was used to observe the bacteria on the surface of the implanted Titanium foils, meanwhile, plate counting method was used for quantitative counting. In addition, CD68 antibody and CD3 antibody were used to label macrophages cells and T lymphocytes, respectively.

Figures:

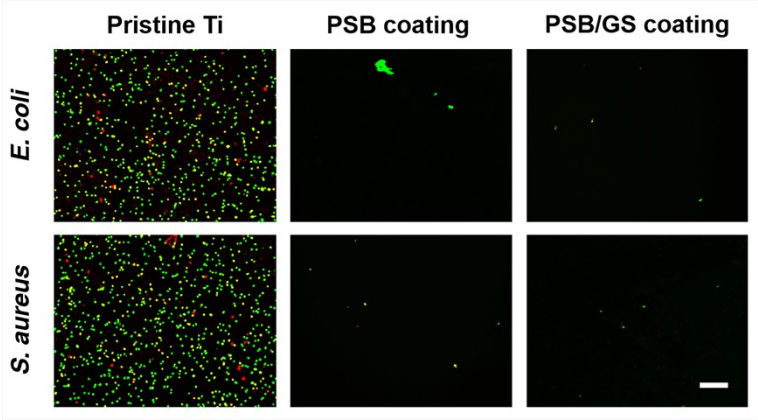

Figure. S1 Fluorescence microscopic images of Live/Dead labeled bacteria showing adhesion on the surface of pristine Ti, PSB coating and PSB/GS coating. Scale bar: 20 μm.

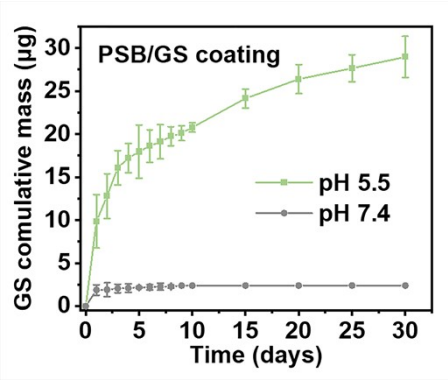

Figure. S2 GS cumulative release mass of the PSB/GS coating.

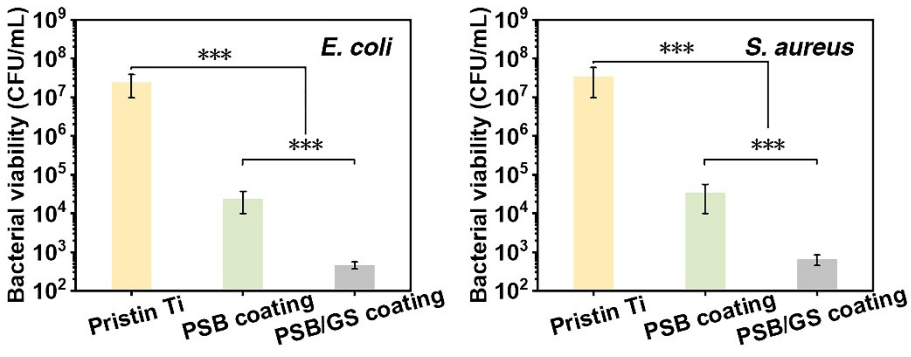

Figure. S3 The bacterial number on the pristine Ti, PSB coating and PSB/GS coating after 7 days.

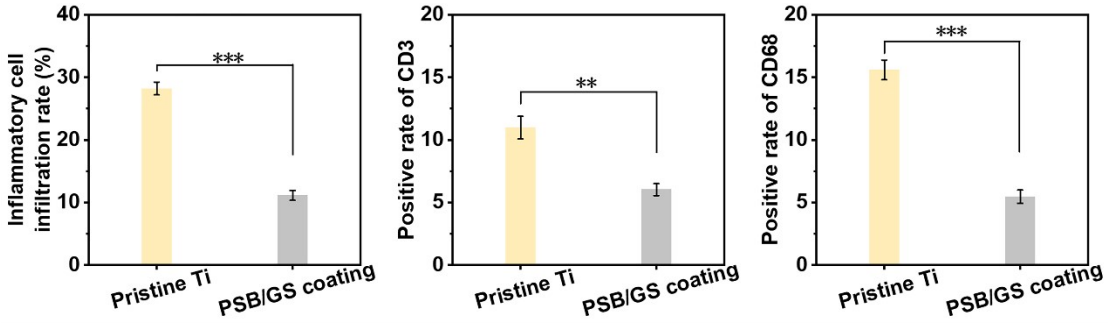

Figure. S4 *On vitro* model of infection, quantitative analysis of the H&E-, CD68-, and CD3-stained tissues surrounding the sample 3 days after implantation.

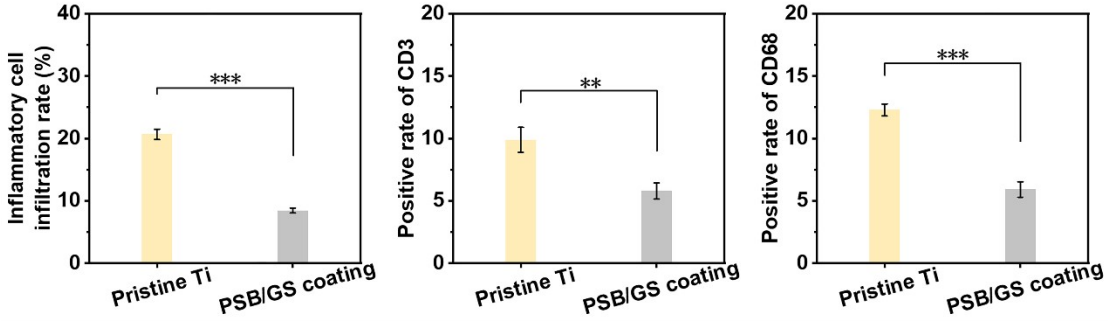

Figure. S5 *On vivo* model of infection, quantitative analysis of the H&E-, CD68-, and CD3-stained tissues surrounding the sample 3 days after implantation.