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Supporting Information

Antibacterial and Osteoinductive Capability on Orthopedic Materials *via* Cation-π Interaction Mediated Positive Charge

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1. EXPERIMENTAL SECTION

1.1 Materials

Dopamine hydrochloride (DA) was bought from Aladdin. Tris (hydroxymethyl) aminomethane (Tris-base) was provided from Solarbio and polyhexamethylene biguanidine hydrochloride (PHMB 20% w/w) was purchased from Qingdao Kedake Biochemical Technology Co,. Ltd. The cell culture reagents, including Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin, trypsin/EDTA, Dulbecco's phosphate buffer saline and Annexin V-FITC/PI kit, were obtained from Solarbio. The fetal bovine serum (FBS) was purchased from Gibco BRL (Carlsbad, CA, USA) and Phalloidin-TRITC, Hoechst 33342 and 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) were bought from Sigma-Aldrich.

1.2 Preparation of polydopamine mediated cationic bio-interface

The glass substrates were dipped into freshly prepared DA solution (2 mg mL^{-1}, pH = 8.5, 10 mM Tris-base buffer) with and without different concentrations (2 mg mL^{-1} and 20 mg mL^{-1}) of PHMB under flowing oxygen (40 mL min^{-1}) for 24 h in 25 °C. Afterwards, the samples were treated ultrasonically in distilled water for 10 min three times, washed again with ethanol using the same protocol to eliminate all free PHMB, and finally dried in air. In this study, three composites were made: polydopamine (demoted as PDA), mass ratios of PHMB/DA of 1:1 (denoted as PD1) and 10:1 (denoted as PD10).

1.3 Physical and Chemical Characterization

The surface topography of PDA, PD1, and PD10 was characterized by atomic force microscopy (AFM, Multimode 8, Bruker, USA) in the tapping mode using a RTESPA (Bruker, USA) probe as well as scanning electron microscopy (SEM, S-4800, Hitachi, Japan). The AFM images were acquired from a scanned area of 4 × 2 μm^2. The height of the films was measured on the half-coated

substrate by the Step command and the roughness of sample surface was determined by the Roughness command in Nanoscope Analysis Software Version 1.40.

The relative surface potential on the samples in the air was measured by Kelvin probe force microscopy-amplitude modulation (KPFM-AM, Multimode 8, Buker, USA) using the tapping mode on a Multi75E-G (budget sensors) probe. The KPFM-AM images were collected from a scanned area of 4 × 2 μm^2. The lift scan height was set as 50 nm on all the samples. The relative surface potential value was determined by the Roughness command in Nanoscope Analysis Software Version 1.40.

The chemical structure was determined by Fourier transform infrared spectroscopy (FTIR, Excalibur 3100, Varian, USA), laser scanning Raman microscopy (Raman-11, Nanophoton Corporation, Japan), solid-state ^{1}H-NMR (AVANCE III 400MHz WB solid-state NMR spectrometer, Bruker, Switzerland), and liquid-state ^{1}H-NMR (AVANCE-400 NMR spectrometer, Bruker, Switzerland). The infrared spectra of the samples scratched from the glass substrates and pressed into KBr disks were obtained between 3500 cm^{-1} and 500 cm^{-1}. The Raman spectra were recorded between 1200 cm^{-1} and 1700 cm^{-1}. The excitation wavelength was 532 nm. The solid-state 1H-NMR determination was conducted on the PHMB, PDA, PD1, and PD10 powders. The PHMB powders were prepared from a 20% PHMB solution by freeze drying and the PDA, PD1, and PD10 powders were scratched from the corresponding samples. Liquid-state 1H-NMR was performed on the PHMB, PDA, PDA+PHMB solutions. The PDA+PHMB solution was obtained from the mixture of PDA and DMSO-d_6 solution of PHMB. The chemical shifts were monitored relative to external tetramethylsilane (TMS = 0 ppm).

1.4 Antibacterial activity evaluation

1.4.1 Antibacterial assays

Commercial strains of S. aureus (ATCC No. 6538) were bought from China General Microbiological Culture Collection Center (CGMCC) as the model of pathogen. S. aureus was cultured on Nutrient Agar (NA) at 37 °C. Twenty-four hours were regarded as the culture passage and two passages (2 × 24 h) were needed to keep the bacterial activity before use. The bacteria were inoculated into 10 mL of normal saline (pH = 7.2~7.4) with 2.5% nutrient broth at the initial concentration of 10^8 cfu mL^{-1} determined by turbidimetry. The bacterial suspension was diluted with normal saline to an appropriate concentration and 0.1 mL of the suspension was dripped on the sample and covered by a 2 × 2 cm^2 polyethylene film. For the free PHMB solution, 0.1 mL of the mixture, bacteria suspension, and PHMB (5 ppm) were prepared instead. After 24 h in an incubator at a constant temperature (37 °C) and humidity (90%), 10 mL of Soya Casiein Digest Lecithin Polysorbate Broth (SCDLP) is added into the culture dish and both the sample surface and polyethylene film were flushed with SCDLP for several times to insure that all the bacteria were suspended in SCDLP. The SCDLP suspension was further diluted with normal saline to an appropriate concentration to be counted. The diluted bacterial suspensions were studied using the Plate Count Ager after incubation for 24 h and all the assays were repeated three times.

1.4.2 Bacterial resistance assays

The bacteria surviving after 24 h on the cationic bio-interfaces were regarded as “resistant passage 1” and they were inoculated on new NA plates and cultured for two passages to recover the activity. They were incubated on the newly prepared cationic bio-interfaces and the surviving ones were regarded as “resistant passage 2”. Three resistant passages of the bacteria were incubated. The antibacterial percentages of the all three passages were calculated to evaluate the pathogen resistance of the samples. The initial bacteria amount for each passage was maintained at about 2.5 × 10^5 cfu cm^{-2} and all the assays were repeated three times.

1.5 Cytocompatible Characterization

1.5.1 Cell culture

Bone mesenchymal stem cells (BMSCs) from two-week aged SD rats were provided by the Stomatology Department of the General Hospital of Chinese PLA. The mouse fibroblast cell line L929 was derived from an immortalized mouse fibroblast cell line. The cells were seeded in a 75 cm^2 culture flask (Corning, Lowell, MA, USA) and cultured in a humidified atmosphere (95% air, 5% CO_2) at 37 °C in DMEM containing 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The cells were fed every 2 days and maintained in the primary culture for 5 to 6 days. After the cells reached confluence, they were trypsinized and placed on new culture plates.

1.5.2 Laser scanning confocal microscopy (LSCM)

The BMSCs were seeded on tissue culture plastic (noted as Blank), glass, PDA, PD1 and PD10 on 6-well plates (5×10^3 cells/well) and cultured in the basic medium (high-DMEM supplemented with 5% FBS) for 24 h. The cells were fixed in 4% paraformaldehyde for 30 min and incubated with Phalloidin-TRITC (10 mg mL^{-1}) for 2 h. The sections were counterstained with Hoechst 33342 (5 mg mL^{-1}) to identify the nuclei. Image collection and superimposition were processed by A1R MP (Nikon Corporation, Japan). The assays were repeated three times.

1.5.3 Cytotoxicity assay and cell proliferation

To evaluate the toxicity of the samples, MTT assay was conducted on the L929 cell line. The L929 cells were seeded on tissue culture plastic, glass, PDA, PD1, and PD10 samples on 6-well plates (5×10^4 cells/well) for 24 h and then 50 μL of the 0.5 mg mL^{-1} MTT solution was added to each well and incubated for another 4 h. After removing the culture media, the cells were mixed with DMSO. The absorbance value of the formazan-DMSO solution was monitored at 490 nm on the iMark™ Microplate Absorbance Reader (Bio-rad, USA). To assess cell proliferation on the samples, the

BMSCs (10^3 cells/well) were incubated on the samples on 24-well plates and harvested on the 2nd, 4th, 6th, and 8th day. The MTT assay was done as described above and the absorbance values were summarized and analyzed. The assays were repeated three times.

1.5.4 Flow cytometry (FCM) analysis of apoptosis

The BMSCs were cultured on tissue culture plastic (Blank), glass, PDA, PD1, and PD10 samples on 6-well plates (2×10^4 cells/well). The 5 groups of BMSCs were harvested after incubation for 24 h and the cells were collected. Apoptosis was measured by Annexin V-FITC/PI kit according to manufacturer's instructions. After incubation for 24 h, the cells were spun for 4 min and the supernatant was decanted. The cells were re-suspended in 400 μL of the binding buffer and 5 μL of Annexin V-FITC and was left in darkness at room temperature for 15 min. Following incubation, 10 μL of prodium iodide were added to each sample and left in darkness at 0 °C for 5 min. Ten thousand cells were acquired by the flow cell sorter (FACSAria-III, BD, USA) and the assays were repeated three times.

1.5.5 Quantitative real time-polymerase chain reaction (RT-PCR)

The BMSCs were cultured on tissue culture plastic (Blank), PD1, and PD10 samples on 6-well plates (2×10^4 cells/well) for 3 days. The total RNA was isolated from the BMSCs using the Trizol reagent (Invitrogen). Approximately 2-5 μg of the total RNA were converted to cDNA by using the Super Script First Strand Synthesis kit (Invitrogen). The RT-PCR was performed using the QuantiTect SYBR Green PCR kit (Toyobo, Osaka, Japan) and Applied Biosystems 7500 RT-PCR detection system. Three independent experiments were performed for each reaction in triplicate. The primer sequences for RT-PCR are as follows.

ALP: (F)5'-ACAGTGACAGCTGCCCGCAT-3',

(R)5'-TTGCATCGCGTGCGCTCAGT-3';

RUNX-2: (F)5’-AGGGCGCATTCCTCATCCCAGT-3’,

(R)5’-AAGACAGCGGCGTGGTGGAA-3’;

OPN: (F)5’-TGGCACCACCGTTTAGGGCA-3’,

(R)5’-TTTGGAGCAGCTGTGCCGTC-3’;

BSP: (F)5’-AGACCATGCAGAGAGCGAG-3’,

(R)5’-ACGTCTGCTTGTGTGCTGG-3’;

OCN: (F)5’-TGGCACCACCGTTTAGGGCA-3’,

(R)5’-TTTGGAGCAGCTGTGCCGTC-3;

GAPDH: (F)5’-GGCACAGTCAAGGCTGAGAATG-3’,

(R)5’- ATGGTGGTGAAGACGCCAGTA-3.

1.5.6 Immunofluorescence staining

The BMSCs were seeded on a 24-well plate (5×10^3 cells/well) and cultured in the basic medium (high-DMEM supplemented with 5% FBS) for 5 day. The cells were fixed in 4% paraformaldehyde for 0.5 h, incubated with alkaline phosphatase (ab54778, Abcam, CA, USA), Runx-2 (ab76956, Abcam, CA, USA), bone sialoprotein (ab52128, Abcam, CA, USA) and osteocalcin (ab93876, Abcam, CA, USA) for 2 h, and then incubated with FITC- or rhodamine-conjugated anti-rabbit or anti-mouse secondary antibodies. The sections were counterstained with Hoechst 33342 (5 mg mL^{-1}; Sigma) to identify the nuclei. Image collection and superimposition were processed by A1R MP (Nikon Corporation, Japan) and DP Manager. For the isotype-matched control, antibodies were used under the same conditions. The experiments were repeated three times.

1.6 Statistical analyses

SPSS version 19.0.0 (Chicago, IL, USA) software was used for statistical analysis. Data were expressed as means ± SD, and $p < 0.05$ was considered statistically significant. The least significant difference (LSD) test was used to determine differences between groups.

2. EXPERIMENTAL RESULTS

Zone of Bacterial Inhibition

To fully remove the free DA and PHMB molecules from the polydopamine mediated coatings, the samples were ultrasonically cleaned in distilled water and ethanol thoroughly before use. Afterwards, the bacterial suspension with 10^7 cfu mL^{-1} (0.2 mL) was dispersed uniformly on the NA plate in the culture dish. The blank group (glass without bio-interface) and samples (16.4 mm diameter) were laid on the NA plate with the bacteria and cultured at 37 °C. After 24 h, the diameters of inhibition against the bacteria were measured and compared. The images in Figure S1 confirm that free PHMB has been removed from the cationic bio-interface and no zone of bacterial inhibition exists on PD10 as well as glass.

As mussel adhesive protein, PDA with multiple reactive sites can adhere to a variety of materials. Therefore, this simple approach is applicable to different types of biomaterials (Figure S2a) such as PET, nylon, ceramics, Ti alloys in antibacterial (Figure S2b) and cytocompatible (Figure S2c) applications. Moreover, some functional proteins such as bone morphogenetic protein-2, vascular endothelial growth factor, bovine serum albumin, and heparin can be embedded in the cationic bio-interface to perform other functions.

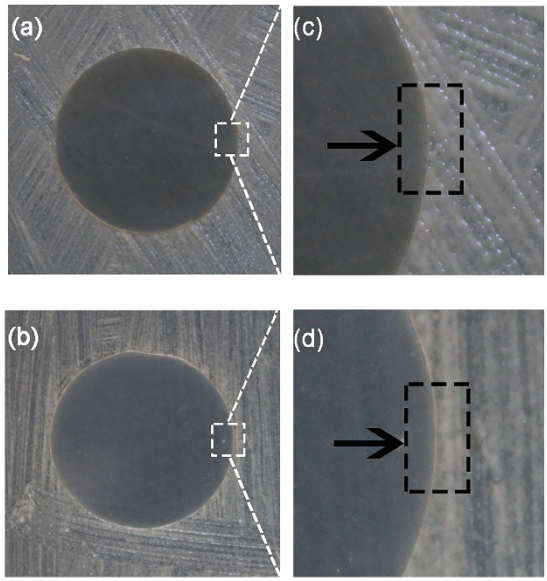

Figure S1. No zone of bacterial inhibition around (a) PD10 and (b) glass after 24 h contacting with S.aureus, (c) and (d) are their magnified images.

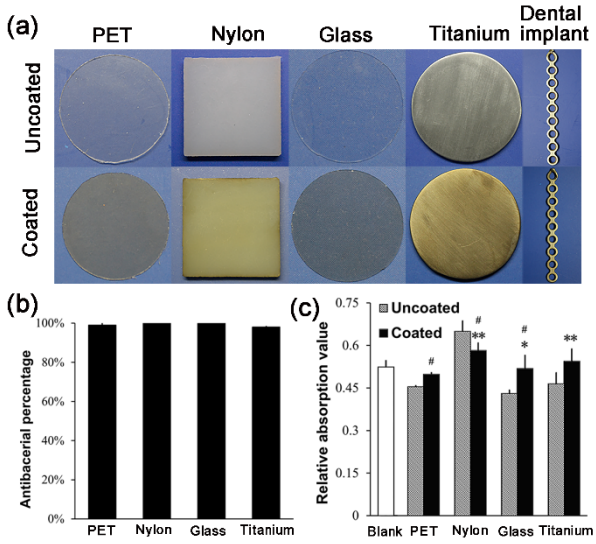

Figure S2. Cationic-π interaction mediated cationic surfaces on different types of medical implants for antibacterial activity and osteocompatibility. (a) Digital images of the uncoated (up) and coated (down) medical implants. (b) Inhibition of S. aureus growth with the initial amount of 1.83×10^4 cfu cm^{-2} after 24 h incubation. (c) MTT analysis of BMSCs cultured after 24 h incubation. (*) and (**) denote statistical significance compared to blank ($p < 0.05$ and $p < 0.01$), and (#) denotes statistical significance compared to the uncoated materials ($p < 0.05$).