Electronic supplementary information (ESI)

Polyhexamethylene Biguanide Assembly-assisted Strategy of Dentin Bonding Greatly Promotes Bonding Effect and Caries Treatment

Chang Shu [a,†], Yadong Chen [a,†], Zhenzhen Zhang [a,†], Yuqing Lei [a], Jiajia Xu [a], Weiwei Lao [a], Qingping Xi [a], Qiang Sun [a], Xiaojun Li [a], Hongbing Liao [b], Qiaojie Luo [a,], Xiaodong Li [a]*

[a] Stomatology Hospital, School of Stomatology, Zhejiang University School of Medicine, Zhejiang Provincial Clinical Research Center for Oral Diseases, Key Laboratory of Oral Biomedical Research of Zhejiang Province, Cancer Center of Zhejiang University, Engineering Research Center of Oral Biomaterials and Devices of Zhejiang Province, Hangzhou 310000, P. R. China.

[b] Guangxi Key Laboratory of Oral and Maxillofacial Rehabilitation and Reconstruction, College of Stomatology, Guangxi Medical University, Nanning 530021, P. R. China

† The authors contribute equally to this article.

* Correspondence:luoqiaojie@zju.edu.cn (Q, L)

Tel: +8657188208151

Fax: +8657187217433

Postal address: 166# Qiutao North Road, Hangzhou 310000, P. R. China

Experimental section

Bacterial culture

A loop of glycerol stock of Streptococcus mutans (S. mutans, UA159) was streaked onto a brain heart infusion (BHI, QDRS Biotec, CHN) medium agar plate and statically incubated at 37 °C in an atmosphere of 5% for 24-48 h. A single colony was picked and grown in the BHI liquid medium at 37 °C in an atmosphere of 5% until it reached the exponential phase for bacterial growth. Then, a loop of bacteria solution was streaked onto a BHI

medium agar plate and incubated statically. After 24-48 h, the plate with single colony bacteria was sealed with parafilm and stored at 4 °C.

To prepare bacterial suspension, a single colony was picked and grown in the BHI liquid medium and statically incubated for 18-24 h until it reached the exponential phase for bacterial growth. 1 ml of bacteria liquid was centrifuged at 4 °C, 12000 rpm for 5 min. The centrifugated deposit was resuspended in 0.1 M PBS. Bacterial concentration was measured by bioMerieux (DensiCHEK Plus, bioMerieux Inc., Germany).

Screening of PHMB solution concentration

Antibacterial activity

5 mm × 5 mm dentin slices were treated with 0, 0.01 mg/ml, 0.1 mg/ml, 1 mg/ml, 2 mg/ml, 5 mg/ml, 10 mg/ml PHMB solution for 30 seconds, then placed into 48-well plate after UV disinfection. Bacterial was adjusted to 1.0×10^6 CFU/ml with BHIS. 1 ml of bacteria suspension was seeded into each slice. After statically incubated for 24 h, the slices were washed with PBS for 3 times. Samples were successively dehydrated for 15 min each in solutions with an increasing concentration of ethanol (50, 70, 90, 100%), followed by immersion in hexamethyldisilazane (HMDS, Sigma-Aldrich, St. Louis, MO, USA) for 30 min. The morphological observation was performed using the scanning electron microscopy (SEM, Nova Nano 450, Thermo FEI, USA) at 5 kV.

Micro-tensile bond strength test

The Spectrum Bond (Dentsply, Konstanz, Germany) was applied according to the manufacturer's instructions. Resin composite (Filtek Z350 XT, 3M ESPE, St. Paul, MN, USA) was buildup on the bonded surfaces in 4 increments of 1.0 mm thick each and each one was individually light activated for 20s. The restored crown segments were sectioned longitudinally in mesiodistal and buccal-lingual directions perpendicular to the bonded interface with a slow-speed diamond saw. The cross-sectional area of each resin-dentin bonded stick was measured using a digital caliper to the nearest 0.01 mm and recorded for subsequent calculation of the μTBS (Bisco, Schaumburg, IL, USA). The resin–dentin bonded sticks were tested under tension at 1.0 mm/min until failure. The μTBS values (MPa) were calculated by dividing the load at failure by the cross-sectional bonding area.

Screening of PHMB solution action time

Antibiofilm activity

8 mm × 8 mm coverslips were placed into 24-well plate after UV disinfection. 300 μl Bacteria-Free Saliva (BFS) was added into each well and incubated at 37 °C for 1 h to prepare acquired salivary pellicle. Bacterial was adjusted to 1.0×10^6 CFU/ml with BHIS. 1 ml of bacteria suspension was seeded into each coverslip. After statically incubated for 24 h, BHI medium was pipetted. The coverslips were washed with PBS for 3 times, each 5 min on

horizontal rotator. Then 500 μL of BHI contain 5 mg/ml PHMB was seeded onto each coverslip for 30 s, 2 min, or 5 min. BHI without any antibiotic reagent was used as negative control. Then samples were stained with LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Invitrogen, USA) and observed by confocal laser scanning microscope (CLSM, Leica, TCS SP8, Germany).

Antibacterial activity

5 mm × 5 mm dentin slices were treated with 5 mg/ml PHMB solution for 0, 30 s, 2 min, or 5min, then placed into 48-well plate after UV disinfection. Bacterial was adjusted to 1.0×10^6 CFU/ml with BHIS. 1 ml of bacteria suspension was seeded into each slice. After statically incubated for 24 h, the slices were washed with PBS for 3 times, each 5 min on horizontal rotator. Samples were stained with LIVE/DEAD™ BacLight™ Bacterial Viability Kit and observed by CLSM (Leica, TCS SP8, Germany).

Scanning electron microscopy (SEM)

Dentin samples were fixed directly or after drying. Then, they were successively dehydrated with an increasing concentration of ethanol (30, 50, 70, 90, 100%), followed by immersion in hexamethyldisilazane (HMDS, Sigma-Aldrich, St. Louis, MO, USA). Each specimen was examined the occlusal section (the observed surface was perpendicular to the tubule axis). The morphological observation of dentin was performed using the SEM (Nova Nano 450, Thermo FEI, USA) at 5 kV.

X-ray Photoelectron Spectroscopy (XPS)

XPS (Kratos AXIS Ultra DLD) was used to determine the surface elemental composition of each group. The excitation source is Al Kα X-rays (energy, hυ ~ 1486.6 eV), the emission current is 3 mA, and the working voltage is 15kV. Use C 1s (284.8 eV) for correction.

The nanomechanical properties of the hybrid layer

The nanomechanical properties of the hybrid layer were tested using QNM-AFM (MultiMode 8, Bruker, USA), operated under the contact mode with a 1.0 Hz scan rate, using RTESPA-525 (Bruker, USA), which was calibrated with silicon before measuring mechanical properties. Then the software NanoScope Analysis V3.0 was used to calculate the Young's modulus.

Failure mode (FM) analysis

The failure mode of the resin-dentin bonded sticks was evaluated under a stereomicroscope (SZ61, Olympus, Japan). Two representative fractured sticks from each group with a μTBS close to the mean bond strength of that

group were platinum sputter-coated for micromorphological evaluation under a SEM (Nova Nano 450, Thermo FEI, USA). Differences in fracture modes between the groups were tested with the Chi-Square test ($\alpha = 0.05$).

The ultrastructure of the hybrid layer

The ultrastructure of the hybrid layer was examined by a transmission electron microscope (TEM, JEM-1230, JEOL, Tokyo, Japan) at 90 kV. Samples were prepared according to established specimen preparation procedures, including fixation, dehydration, and embedding in epoxy resin. Ultrathin sections (70-90 nm) were cut utilizing a diamond knife (Diatome, Bienne, Switzerland) in an ultramicrotome (Boeckeler Instruments, Tucson, USA). Stained (5% uranyl acetate for 5 min and saturated lead citrate for 5 min) sections were observed.

Cytotoxicity measurements

Mouse fibroblast (L929 cells) were used for the biocompatibility assessment. Cells were cultured in DMEM high Glc (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) at 37 °C in 5 % CO_2 atmosphere. When the cells reached a high confluence (> 80 %), the cells were subcultured or collected with trypsin enzyme-digesting technique. Cell density was adjusted to 1×10^4 cells/ml. 100 µL of cell suspension was seeded into each well of the 96-well plate containing dentin slices. Prior to cell seeding, the dentin slices from all groups were sterilized by UV radiation. After culturing for 24 h or 48 h, the cell viability was determined by a Cell Counting Kit-8 (CCK-8, APExBIO, USA). The cells were washed twice with PBS. Subsequently, 200 µl of the CCK-8 solution (10% in medium) was added to the culture wells. After incubation for 4 h at 37 °C in a 5% CO_2 atmosphere, the optical density readings of the CCK-8 reaction medium were subsequently performed at 450 nm by using a microplate reader (SpectraMax i3, Molecular Devices, USA).

The morphology of the L929 cells after the 24-h or 48-h incubation period was observed using SEM (Nova Nano 450, FEI, USA). The samples with cultured cells were rinsed with PBS and fixed with 2.5% glutaraldehyde. Subsequently, the samples were dehydrated for 15 min using an ethanol dilution series (30%, 50%, 70%, and 90%) before the final dehydration with 100% ethanol. After dehydration, the samples were immersed in HMDS for 30 min and dried at room temperature. Gold sputtering was performed for 60 s before SEM observation.

Supplementary figures

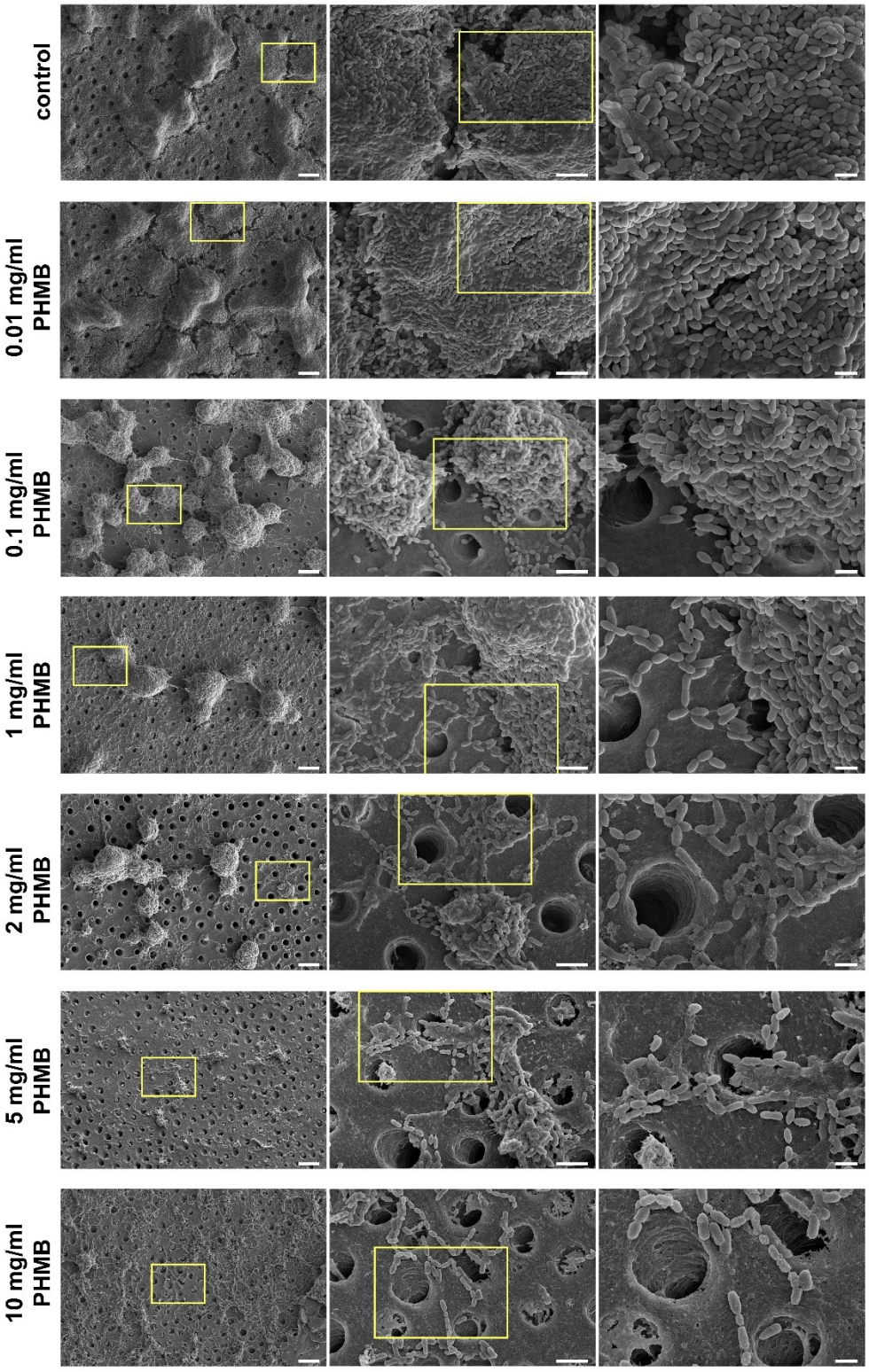

Figure S1. Inhibition of PHMB solution with different concentrations on Streptococcus mutans, observed by scanning electron microscope. Scale bar, 10 μm (left), 3 μm (middle), and 1 μm (right).

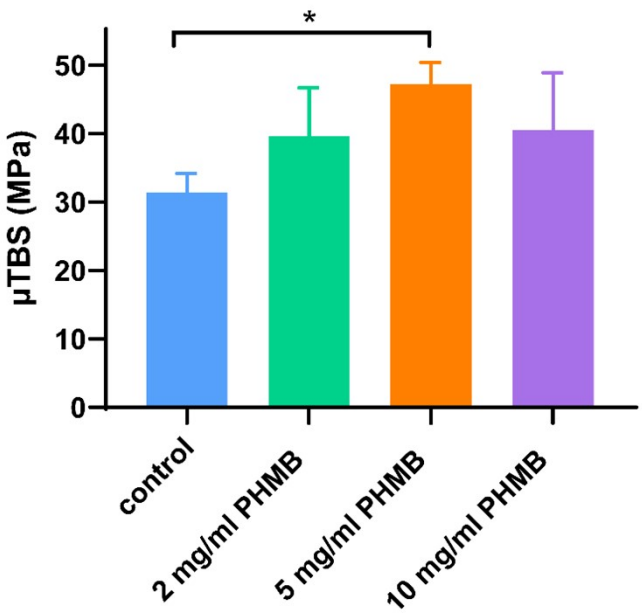

Figure S2. Micro-tensile strength (μTBS) of demineralized dentin matrix pretreated with PHMB solution of different concentrations for 30 seconds. * $p < 0.05$.

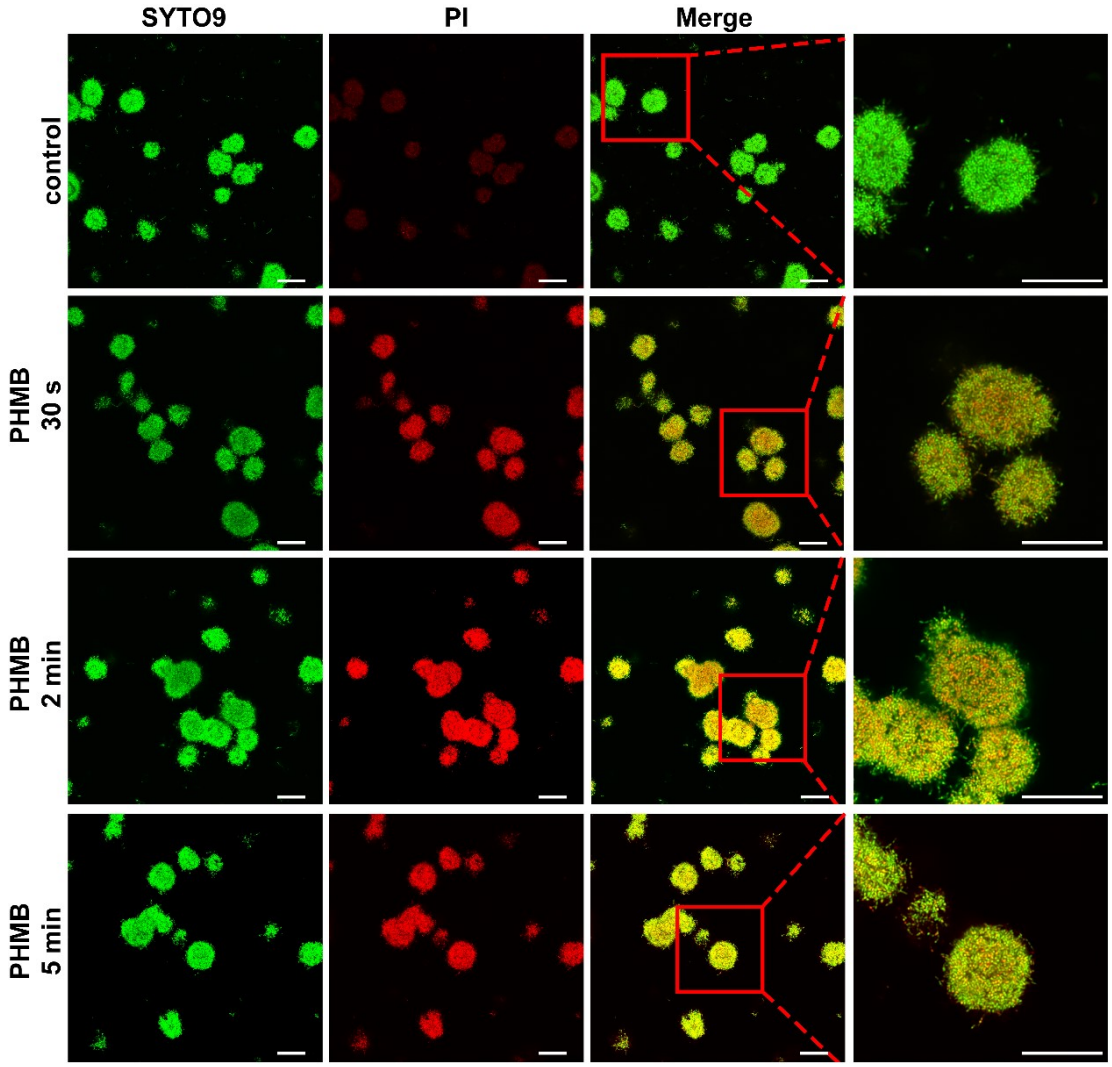

Figure S3. Effect of PHMB action time on live/dead bacteria in the biofilm of *S. mutans*, measured with Live/Dead Baclight Bacterial Viability kits by confocal laser scanning microscopy. Scale bar, 20 μm.

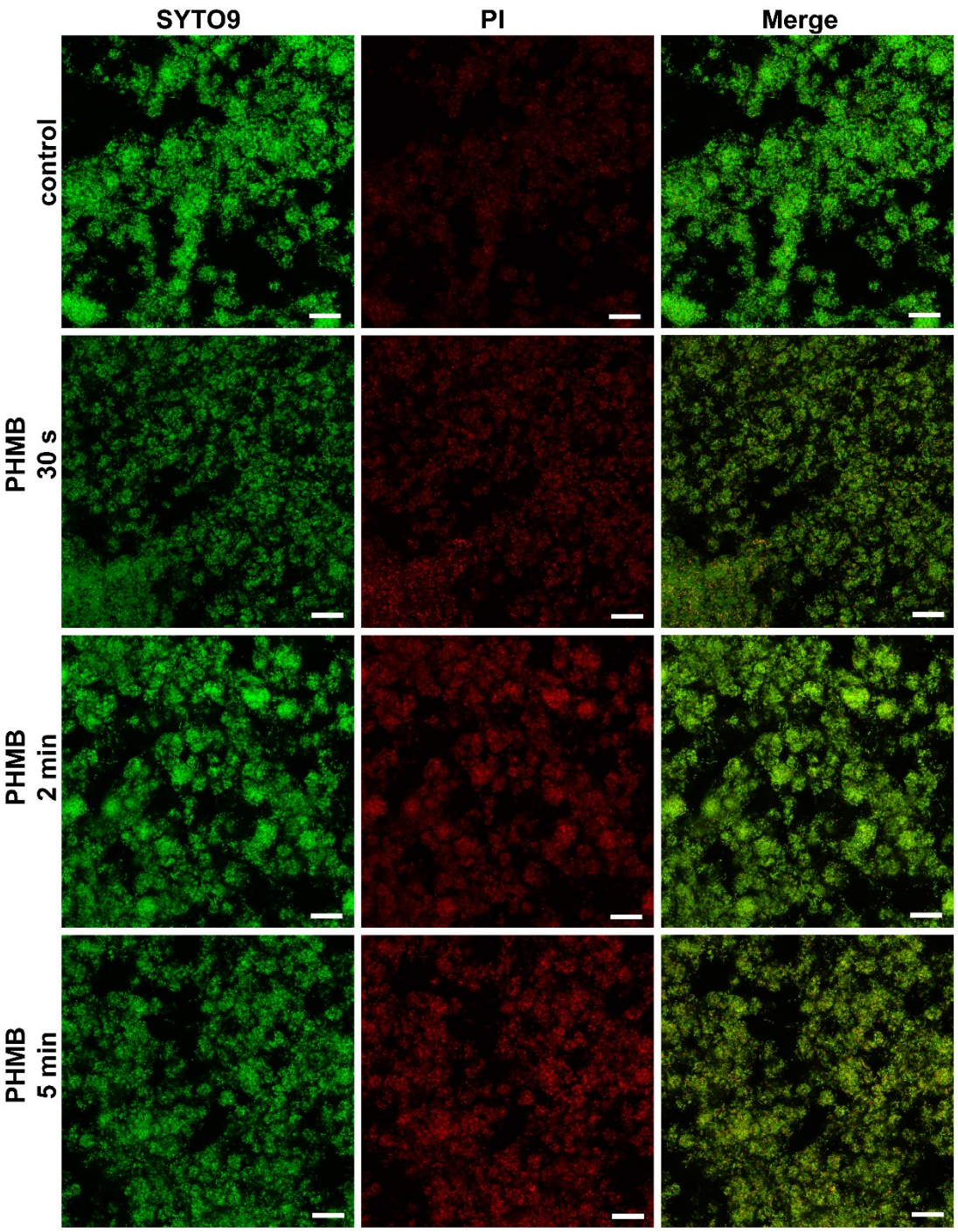

Figure S4. Effect of PHMB action time on biofilm formation of *S. mutans*. Scale bar, 20 µm.

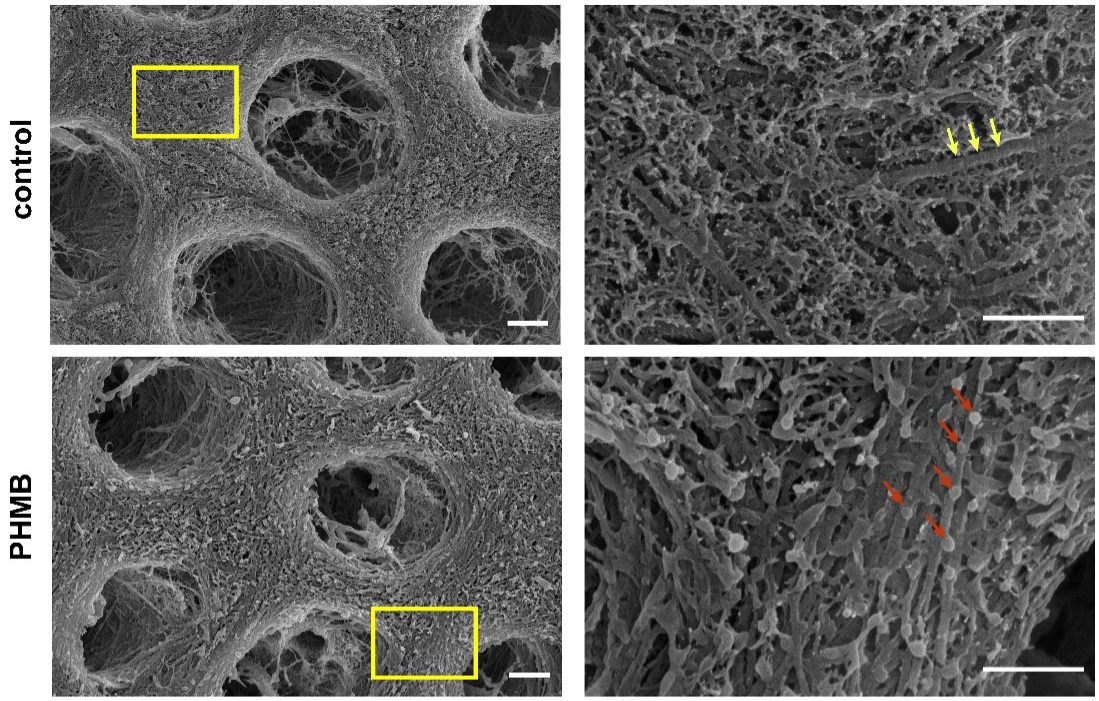

Figure S5. Representative SEM images of dentin surface treated with PHMB solution then kept moist and magnified images of the dentin morphology (right) within the region indicated by yellow rectangles. Yellow arrows indicate the periodic band structure of dentin type I collagen. Red arrows indicate the spherical protrusions which are arranged on the surface along the long axis of collagen. Scale bar, 1 μm (left) and 500 nm (right).

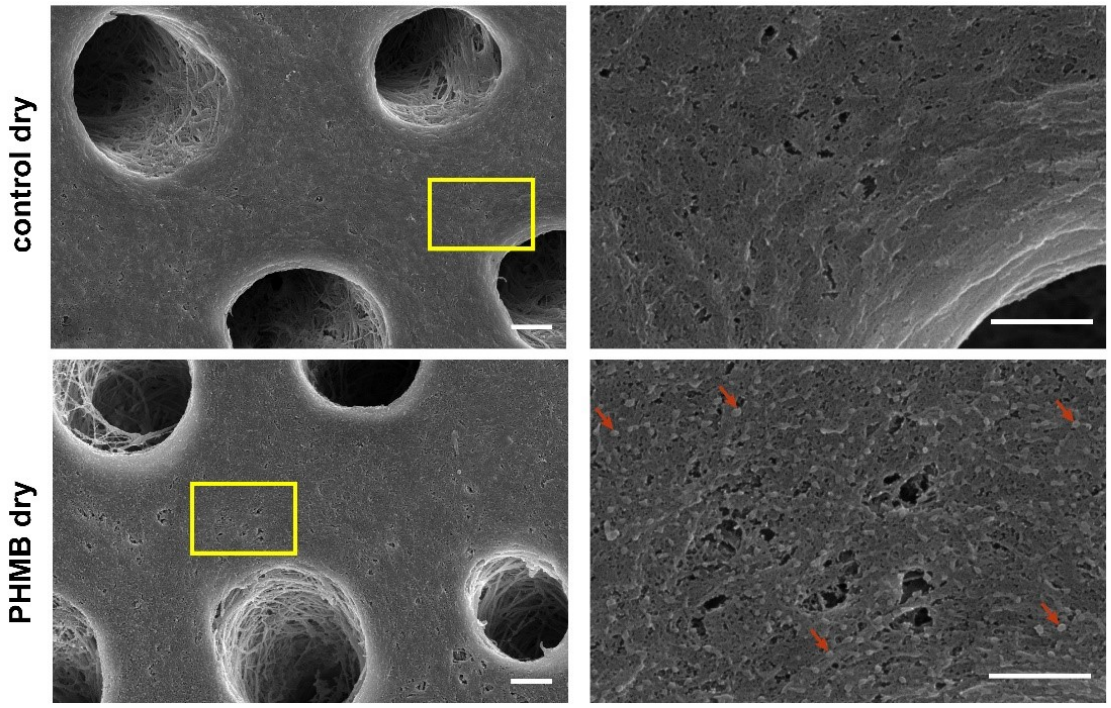

Figure S6. Representative SEM images of dentin surface treated with PHMB solution then air-dried and magnified images of the dentin morphology (right) within the region indicated by yellow rectangles. Red arrows indicate the spherical protrusions. Scale bar, 1 µm (left) and 500 nm (right).

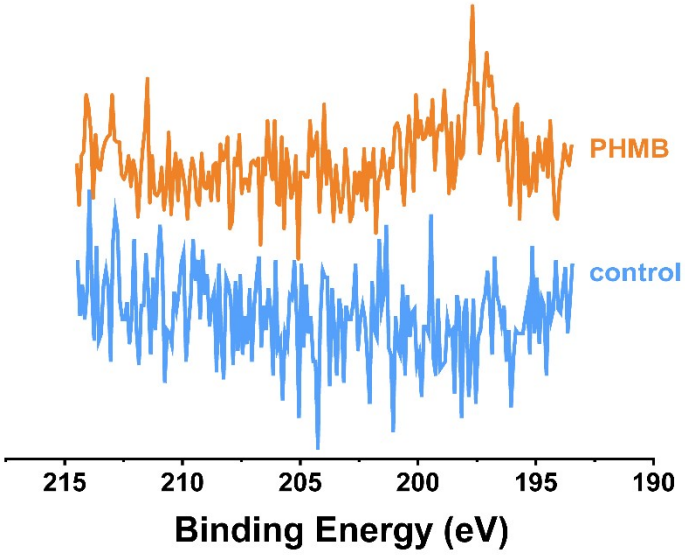

Figure S7. Representative XPS spectra of chlorine.

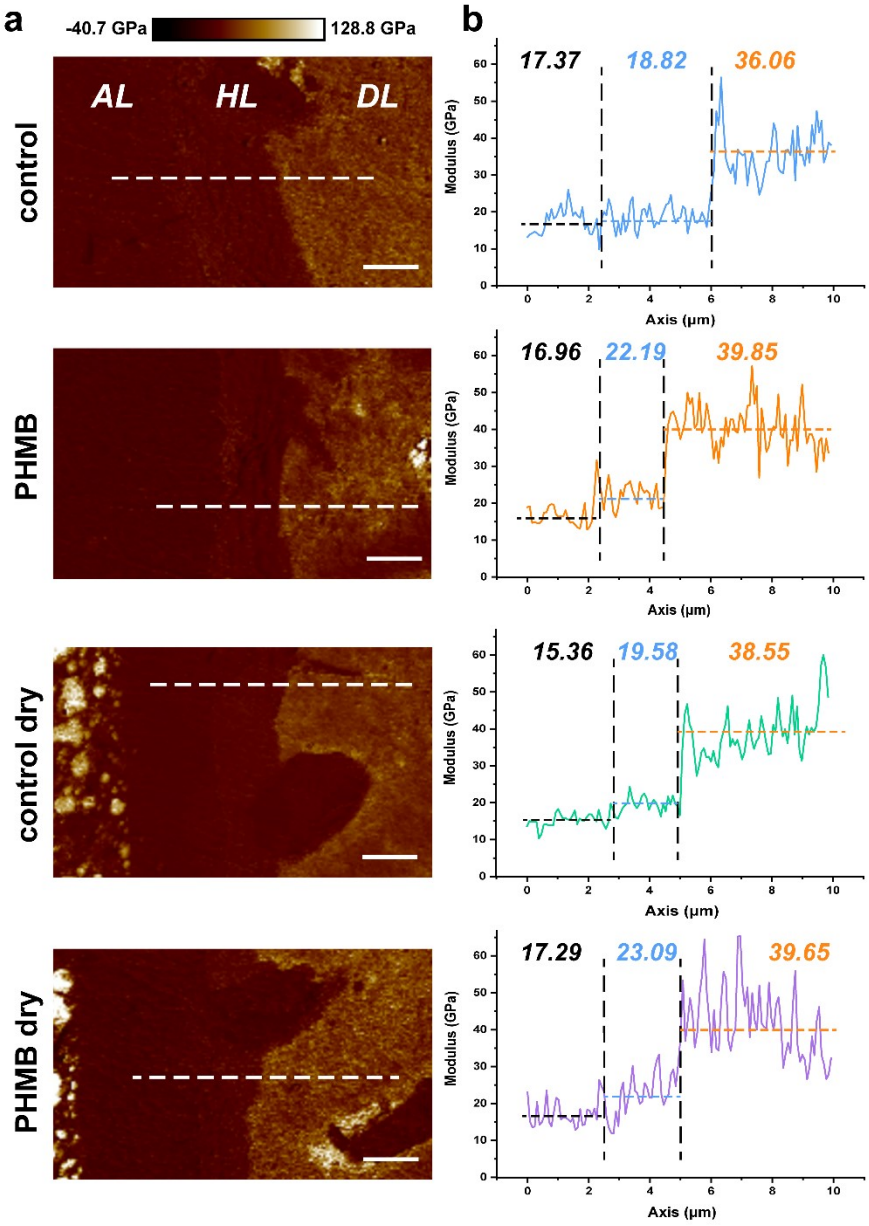

Figure S8. PHMB-assembled DDM greatly improves the modulus of hybrid layer using Spectrum Bond. (a) Representative AFM modulus mapping graphs. AL, adhesive layer; HL, hybrid layer; DL, dentin layer. Scale bar, 2 μm. (b) Section analyses of Young's modulus along the white line of the corresponding AFM property map.

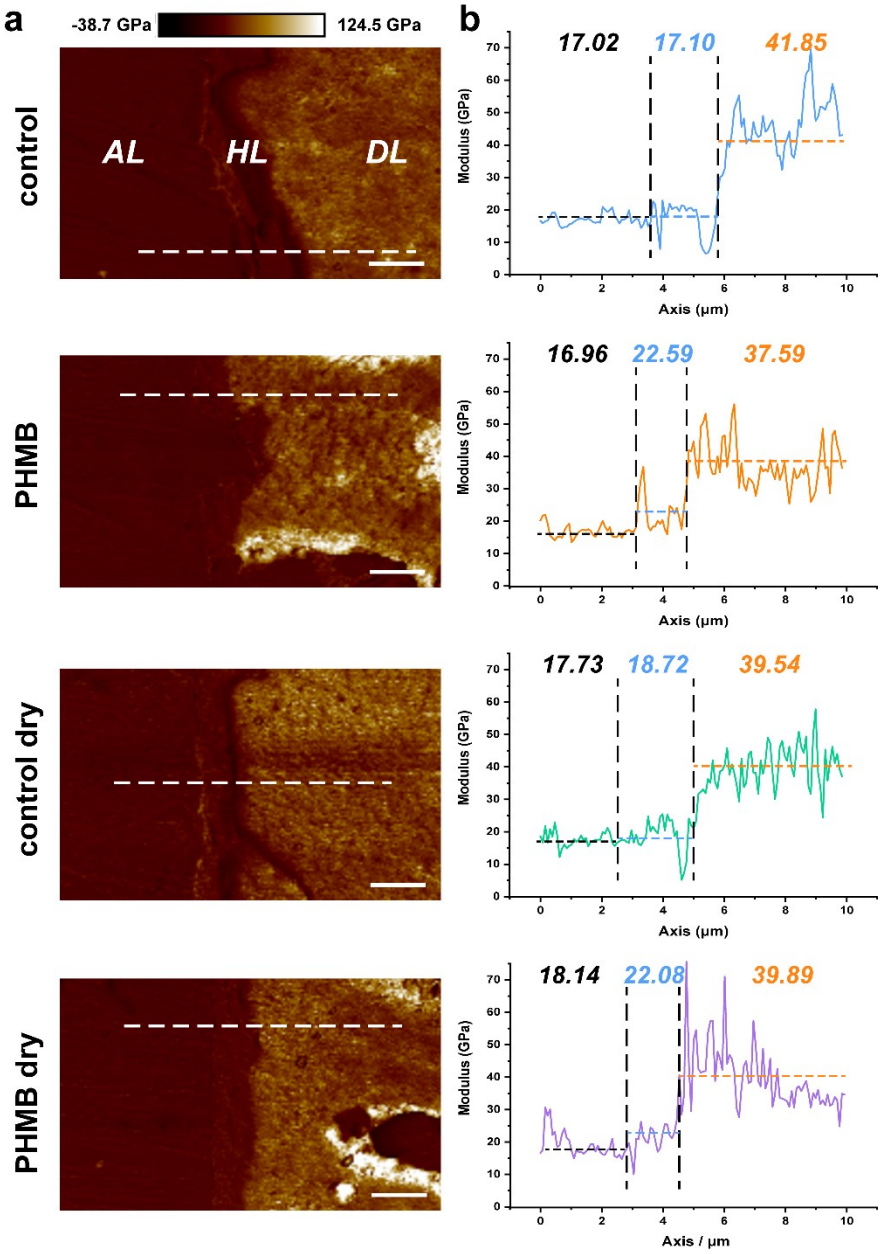

Figure S9. PHMB-assembled DDM greatly improves the modulus of hybrid layer using Dentex Bond V. (a) Representative AFM modulus mapping graphs. AL, adhesive layer; HL, hybrid layer; DL, dentin layer. Scale bar, 2 μm. (b) Section analyses of Young's modulus along the white line of the corresponding AFM property map.

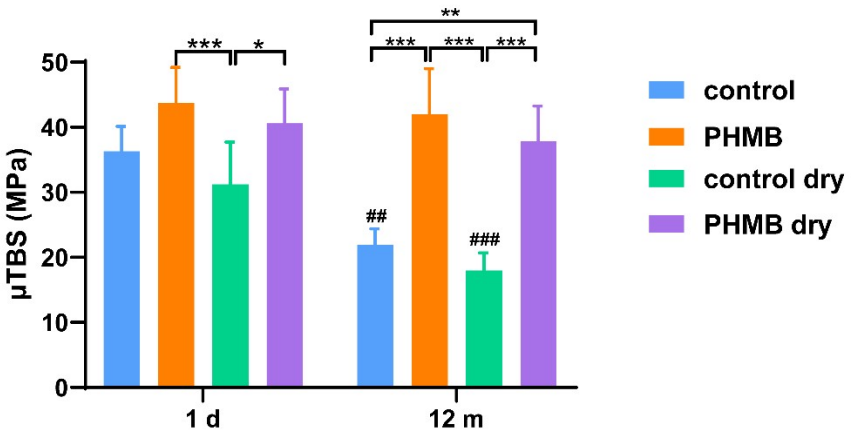

Figure S10. Micro-tensile strength (μTBS) of dentin bonding at different aging times using Spectrum Bond adhesive. Data is mean ± SD. # indicate significant differences according to the Bonferroni test comparing different aging periods for each group; * indicate significant differences according to the Bonferroni test by comparison of different groups for each aging period. ## $p < 0.01$, ### $p < 0.001$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

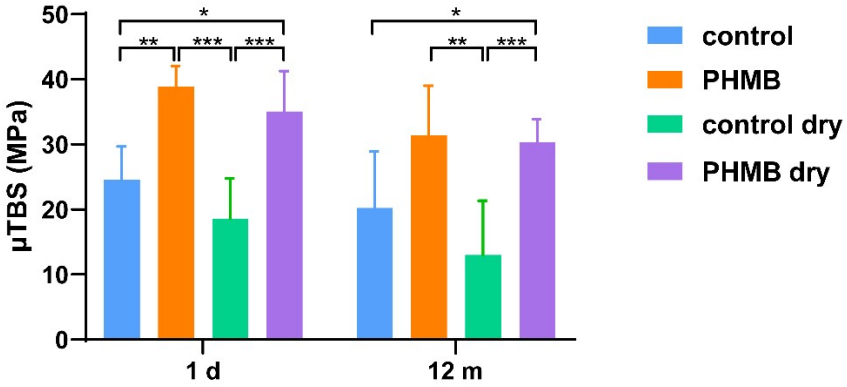

Figure S11. Micro-tensile strength (μTBS) of dentin bonding at different aging times using Dentex Bond V adhesive. Data is mean ± SD. * indicate significant differences according to the Bonferroni test by comparison of different groups for each aging period. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

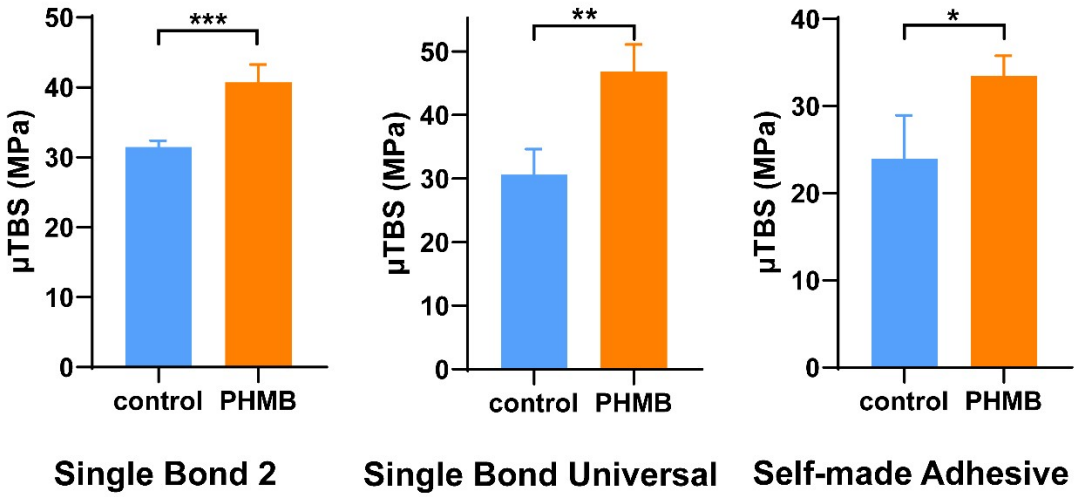

Figure S12. Micro-tensile strength (μTBS) of dentin bonding after 1 day using Single Bond 2, Single Bond Universal or Self-made adhesive. Data is mean ± SD. * indicate significant differences according to the t test by comparison of two groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

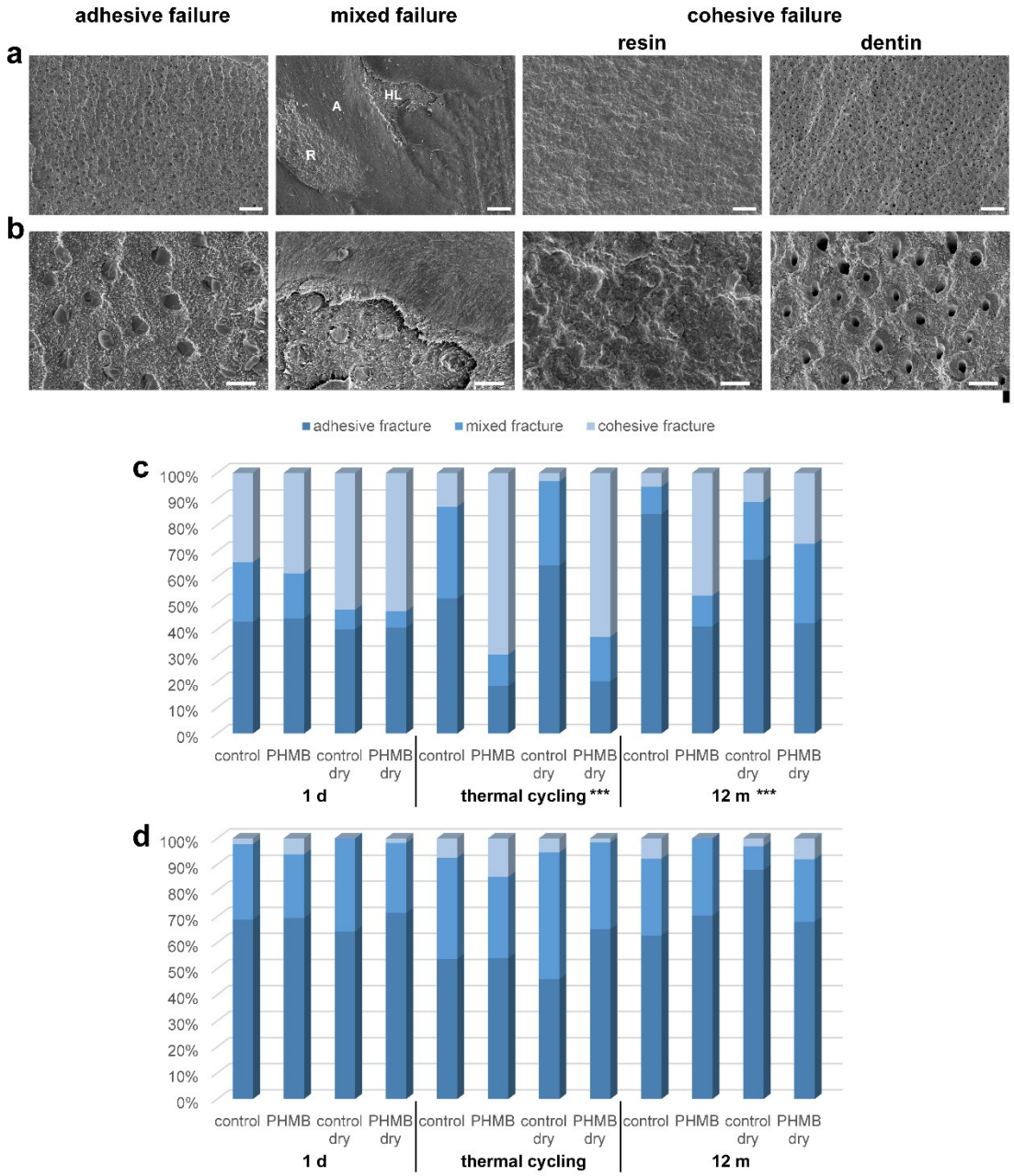

Figure S13. Fracture mode of the resin-dentin sticks. (a) Three different fracture modes: adhesive failure (failure at the resin/dentin interface), cohesive failure (failure exclusively within dentin or failure exclusive within resin composite) and mixed failure (failure at the resin/dentin interface with partial cohesive failure of the neighboring substrates). Scale bar, 20 µm. (b) A higher magnification view of the same specimen in (a). R, resin; A, adhesive; HL, hybrid layer. Scale bar, 5 µm. (c, d) Analysis of the proportion of the three fracture modes with different aging mode after using Spectrum Bond adhesive (c) and Dentex Bond V adhesive (d). Statistical analysis was performed using the Chi-Square test in each aging time, $\alpha = 0.05$, *** $p < 0.001$.

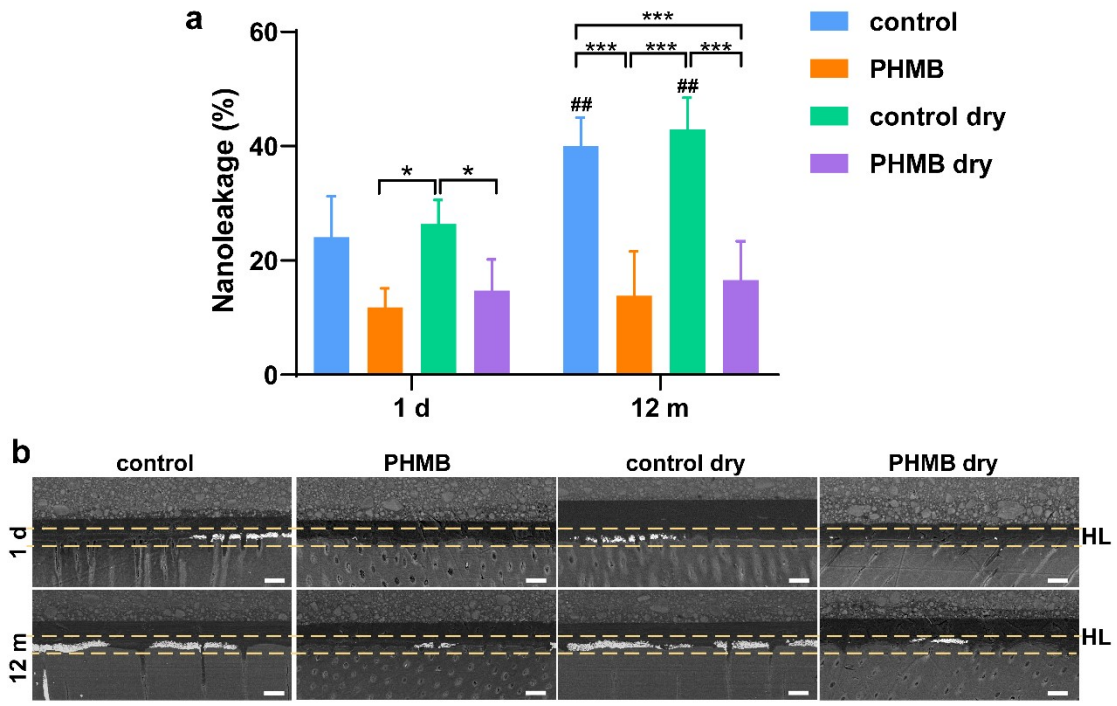

Figure S14. PHMB-assembled DDM reduces the nanoleakage of dentin bonding using Spectrum Bond adhesive. (a) Semiquantitative nanoleakage results of dentin bonding at different aging times. Data are mean ± SD. # indicate significant differences according to the Bonferroni test comparing different aging periods for each group; * indicate significant differences according to the Bonferroni test by comparison of different groups for each aging period. ## $p < 0.01$; * $p < 0.05$, *** $p < 0.001$. (b) Representative backscattered SEM micrographs illustrating the most common nanoleakage features traced by silver. HL: hybrid layer. Scale bar, 10 µm.

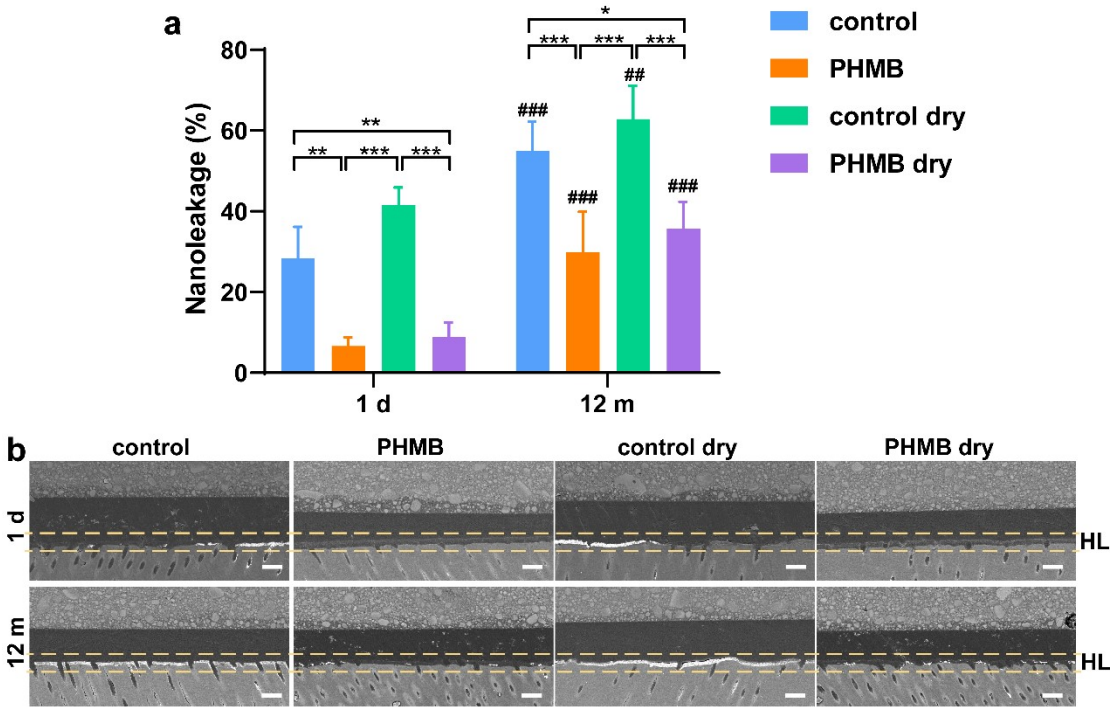

Figure S15. PHMB-assembled DDM reduces the nanoleakage of dentin bonding using Dentex Bond V adhesive. (a) Semiquantitative nanoleakage results of dentin bonding at different aging times. Data are mean ± SD. # indicate significant differences according to the Bonferroni test comparing different aging periods for each group; * indicate significant differences according to the Bonferroni test by comparison of different groups for each aging period. ## $p < 0.01$, ### $p < 0.001$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (b) Representative backscattered SEM micrographs illustrating the most common nanoleakage features traced by silver. HL: hybrid layer. Scale bar, 10 μm.

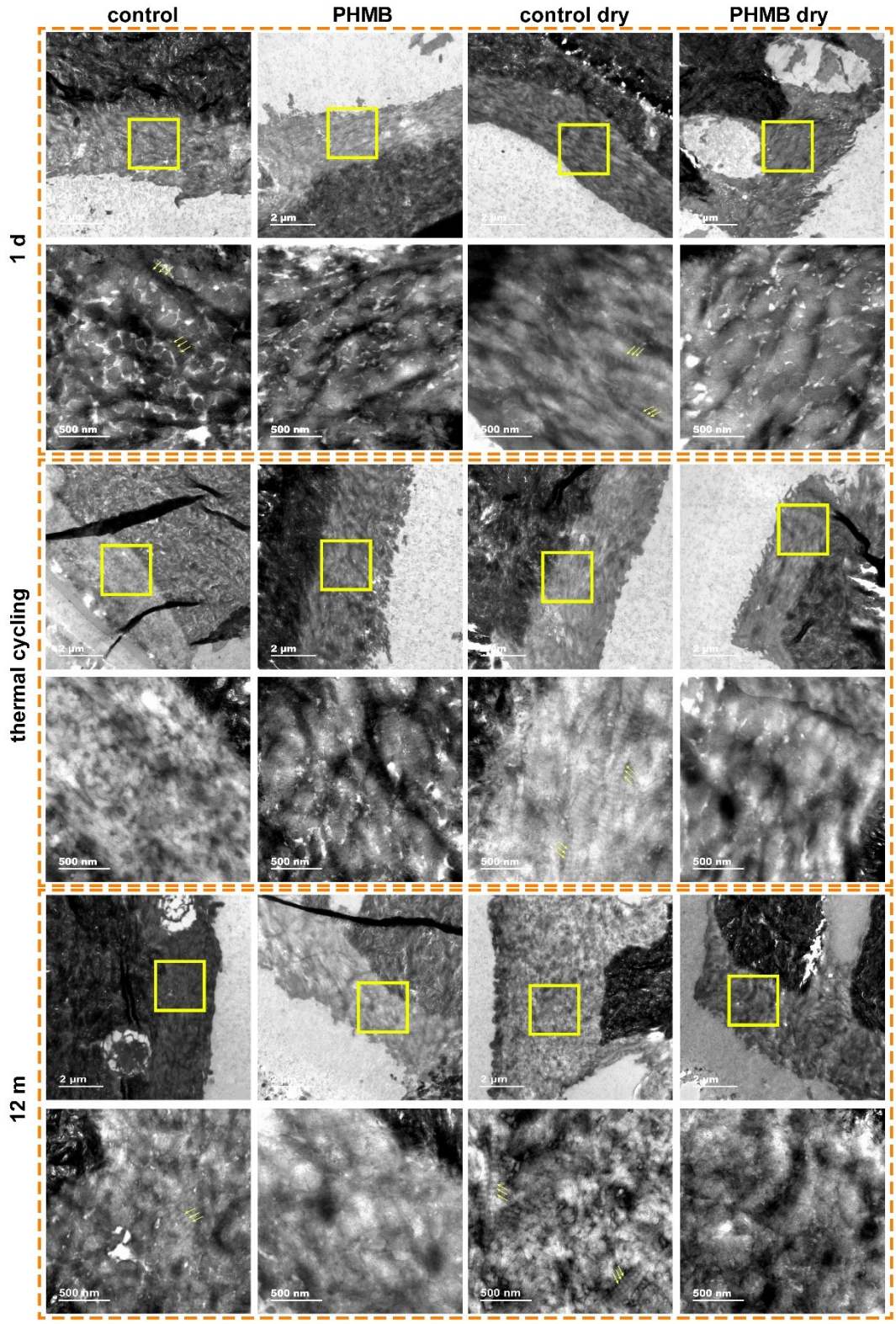

Figure S16. Representative TEM images of resin-dentin interface in 1 d, thermal cycling, and 12 m using Spectrum Bond adhesive. The morphology of the hybrid layer in the yellow rectangle is amplified. Yellow arrows indicate the periodic band structure of dentin collagen.

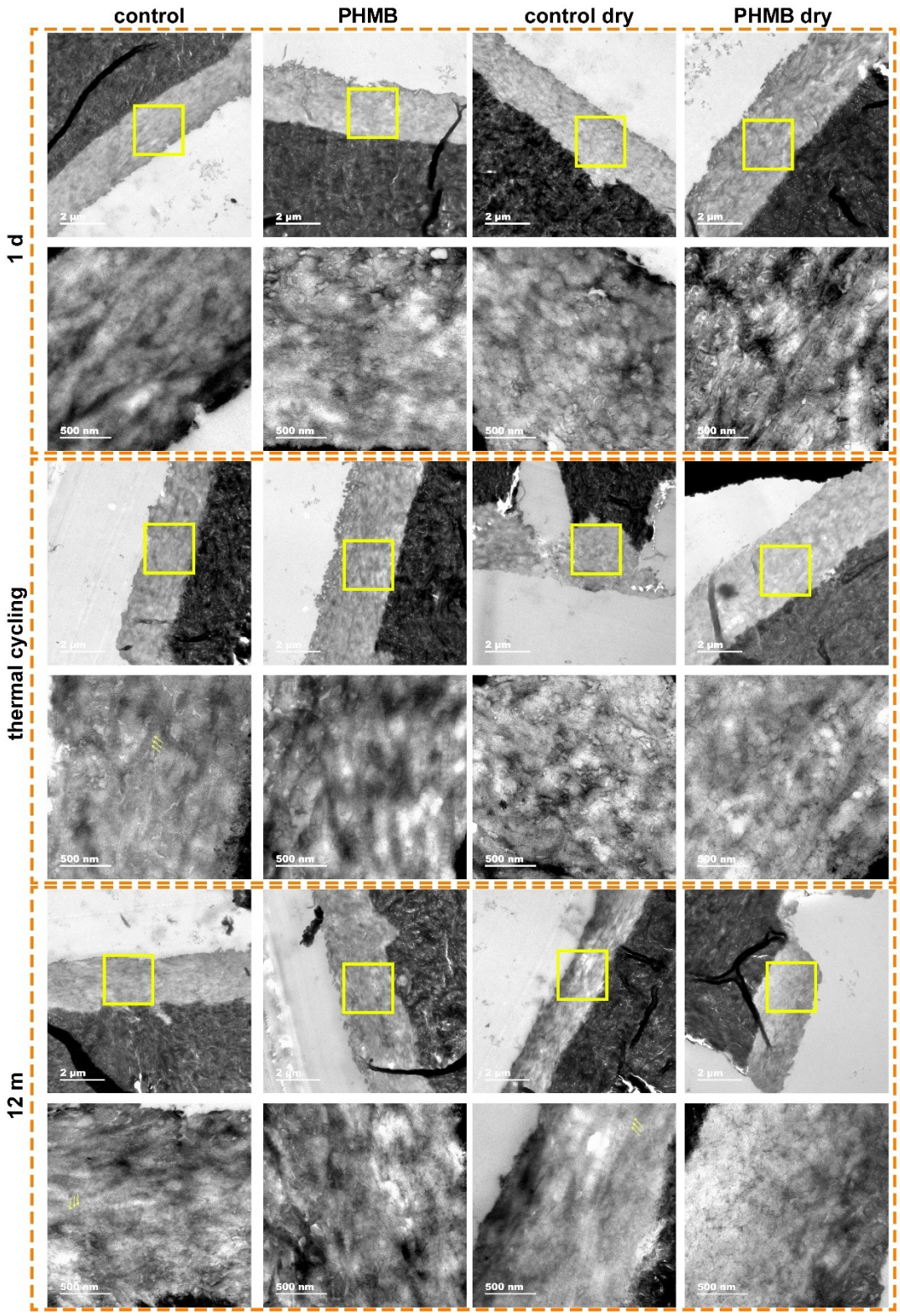

Figure S17. Representative TEM images of resin-dentin interface in 1 d, thermal cycling, and 12 m using Dentex Bond V adhesive. The morphology of the hybrid layer in the yellow rectangle is amplified. Yellow arrows indicate the periodic band structure of dentin collagen.

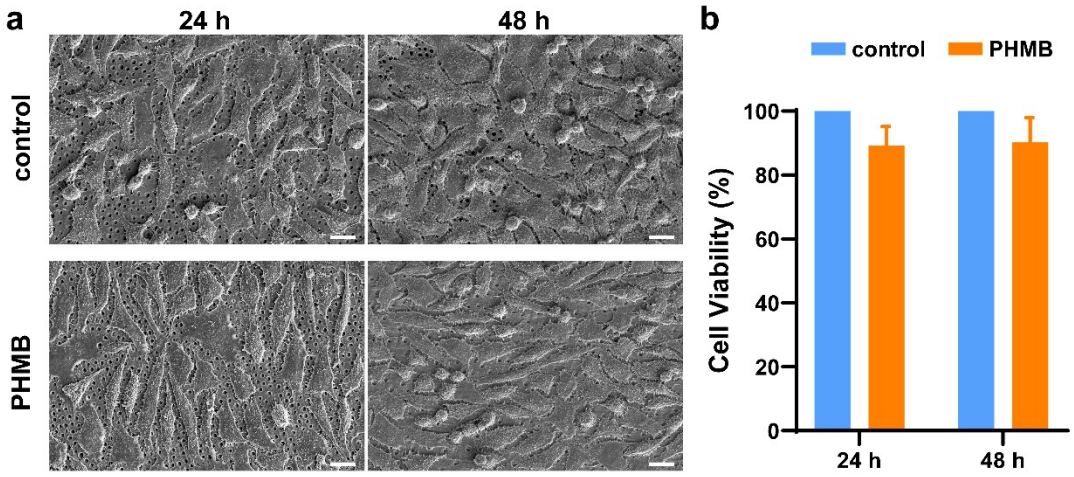

Figure S18. PHMB-assembled DDM exhibits good cyto-compatibility. (a) Representative SEM images of cells on dentin slices. L929 cells are spindle-shaped, and there is no significant difference in cell morphology between the two groups. Scale bar, 20 μm. (b) CCK-8 test results show that the PHMB group has comparable L929 biocompatibility with the control group.