

Supplementary Materials for

Long-term spatiotemporal biological and mechanobiological dynamics of *Staphylococcus aureus* biofilms

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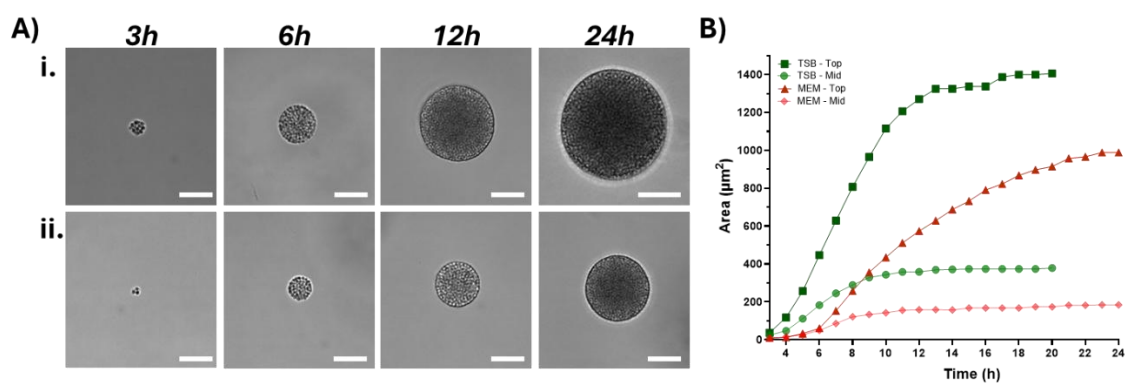
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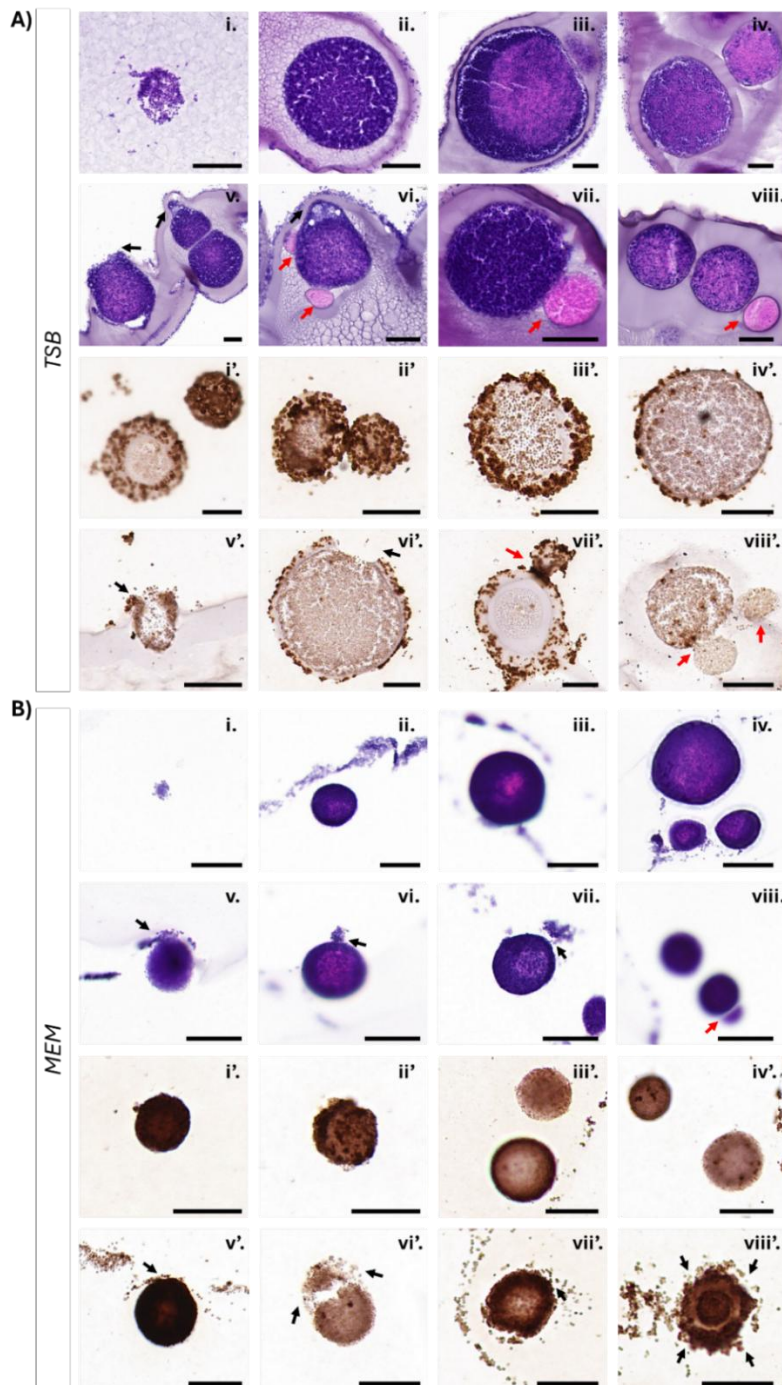
Supplementary Figures 1 to 5

Other Supplementary Materials for this manuscript include the following:

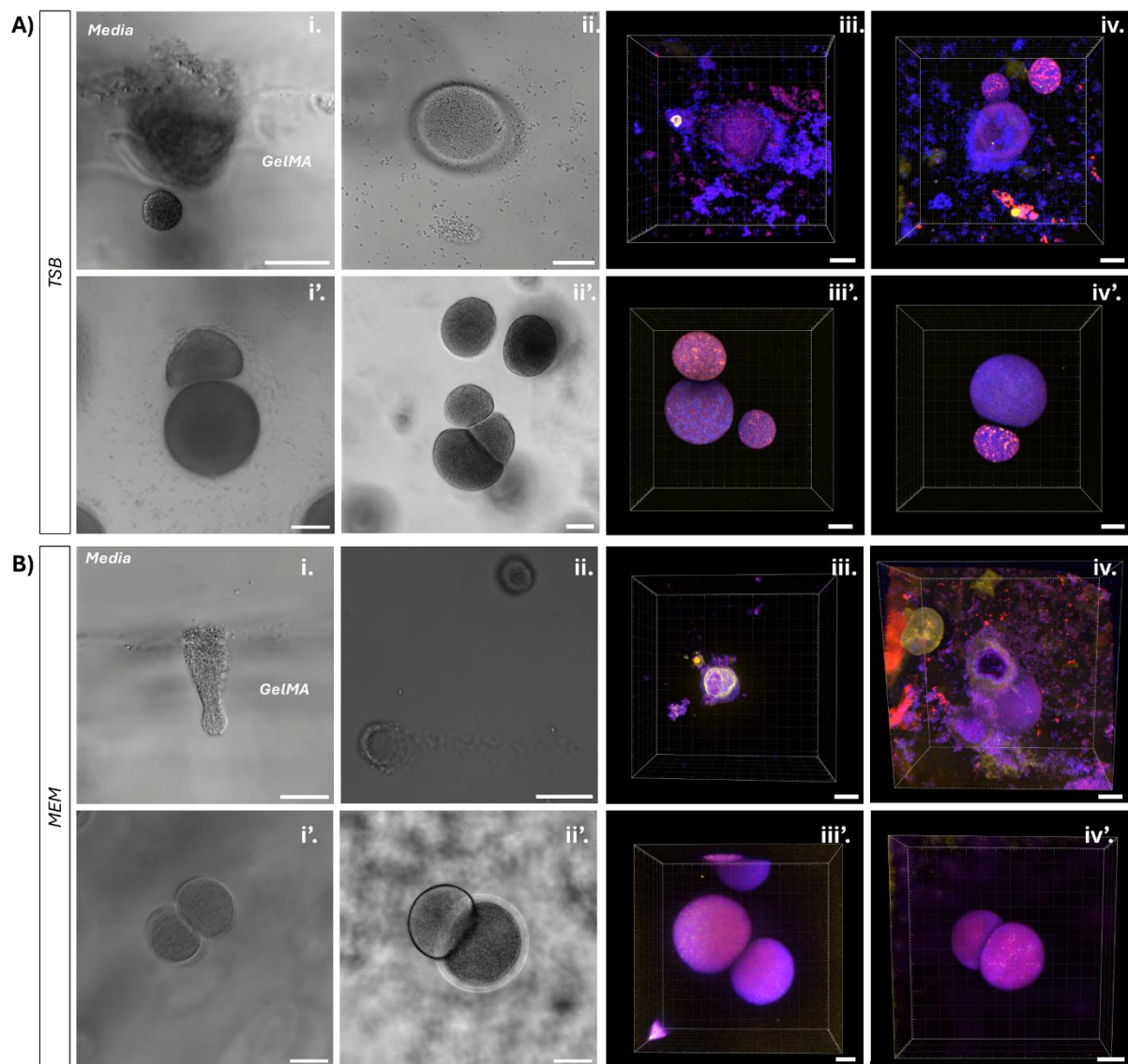
Supplementary Movies 1 to 4



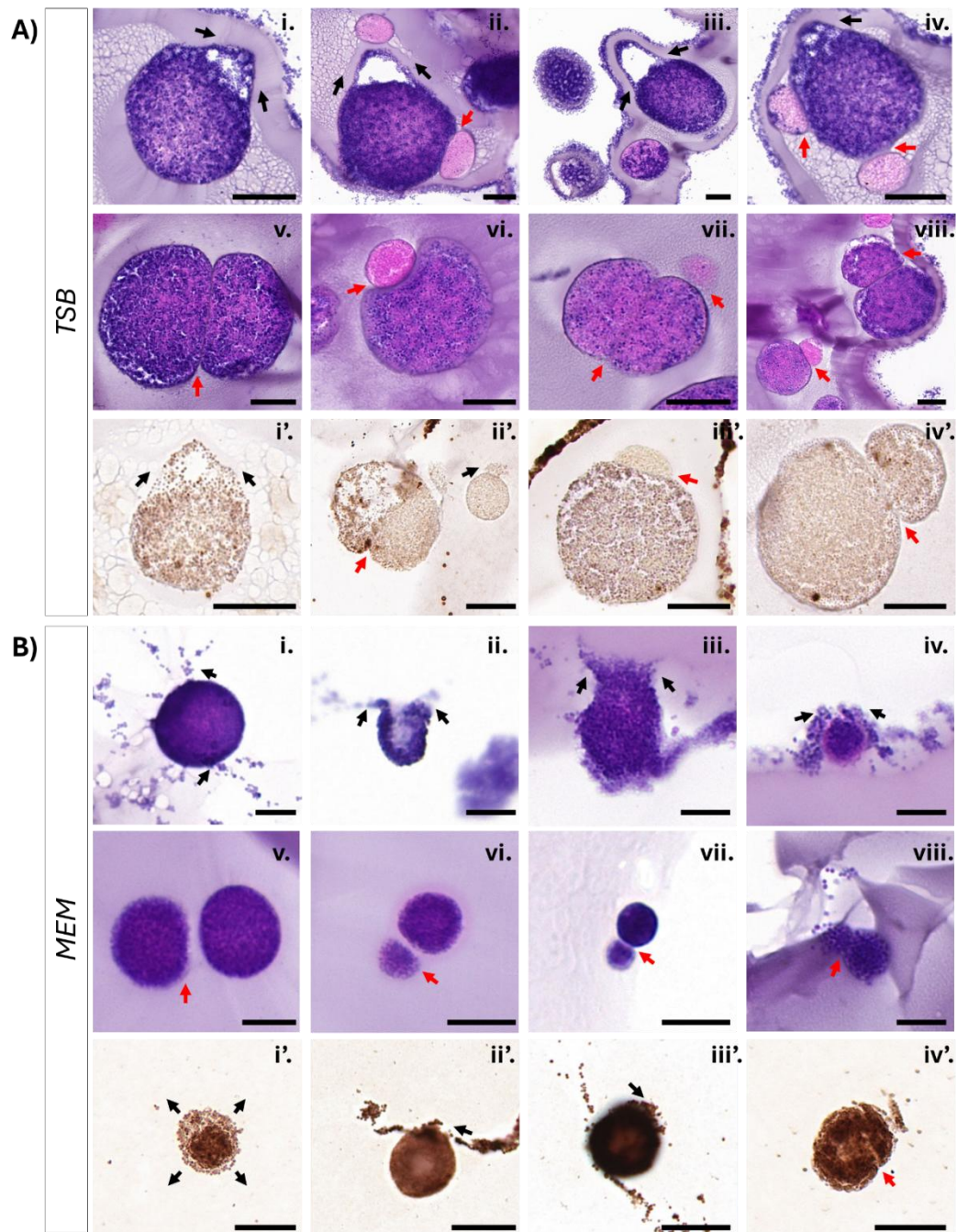
Supplementary Figure 1. Live imaging of *S. aureus* aggregates over 24 h. (A) Representative time-lapse brightfield microscopy images showing the development of individual *S. aureus* aggregates embedded in GelMA hydrogels and cultured in (i) TSB or (ii) MEM over a 24 h period. Scale bars: 5 μm. (B) Quantification of aggregate area for clusters located near the top of the hydrogel (Top; 0–300 μm from the nutrient interface) and for clusters located deeper within the hydrogel (Mid; 300–600 μm).



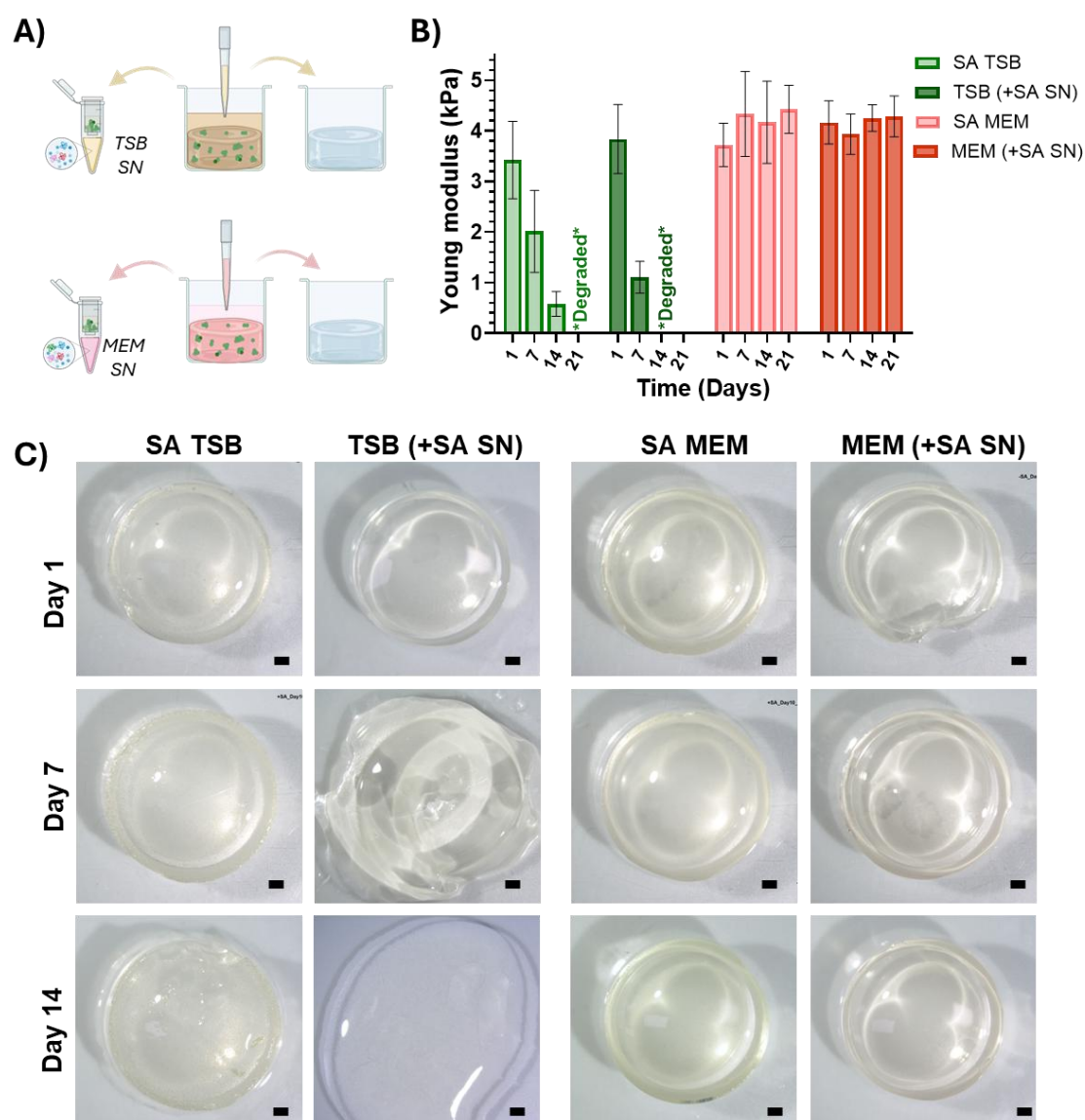
Supplementary Figure 2. Histological analysis of *S. aureus* morphological adaptation within GelMA hydrogels. Cross-sectional H&E (purple = bacteria/nucleic acids; pink = GelMA/ biofilm matrix) and IHC (brown :*S. aureus*) staining of hydrogels cultured in TSB or MEM at Day 1 and Day 14. A) TSB-grown clusters, Day 1 clusters are small and compact (i, i'). By Day 14, aggregates form large macro-colonies with a dense peripheral rim and hollow or sparsely populated cores (ii–iv; ii'–iv'). Dispersal, including cluster fracturing and satellite budding, is evident (v–viii; v'–viii'). B) MEM-grown Clusters remain smaller and highly compact. By Day 14, some aggregates exhibit core hollowing, and dispersal through fracturing or budding is also observed (iii–viii; iii'–viii'). Scale bars: 20 μ m



Supplementary Figure 3. Dynamic and structural visualization of *S. aureus* dispersal in 3D GelMA hydrogels. Representative brightfield images and confocal visualizations of dispersal events in GelMA hydrogels cultured in TSB (A) or MEM (B). Active dispersal at the hydrogel–media interface (i) and within the hydrogel matrix (ii). Brightfield images of budding events (i'–ii'). Three-dimensional confocal reconstructions (iii–iv) depict dispersal events characterized by matrix fracture and release of bacterial cells, while (iii'–iv') show budding events involving detachment of small satellite aggregates from parent microcolonies. Scale bars: 20µm



Supplementary Figure 4. Dispersal phenotypes in 3D hydrogels. Additional histological sections (i-viii, H&E and i'-iv', IHC) displaying two primary dispersal mechanisms: fracture-mediated release (black arrows) and budding/satellite formation (red arrows). These features were consistently observed across multiple biological replicates and tissue depths ($n \geq 10$), distinguishing them from processing artifacts. Notably, bacteria at dispersal sites remain intact and spherical, with no evidence of mechanical smearing or cellular distortion typically associated with microtome sectioning; Scale bars: 20um.



Supplementary Figure 5. Indirect degradation assay. (A) Schematic representation of the assay workflow. Created with BioRender.com. Supernatants collected from hydrogels cultured with *S. aureus* in TSB or MEM were filtered to remove bacteria and subsequently used to culture fresh, bacteria-free hydrogels to assess the presence of degradative factors (e.g., proteases) in the conditioned media. (B) Young's modulus determined by uniaxial compression testing, showing a pronounced decrease in stiffness for hydrogels cultured in *S. aureus*-conditioned TSB supernatants. Data are presented as mean $\pm$ SD. (C) Representative images of hydrogels over time, illustrating progressive sample degradation in the TSB-conditioned groups, with visible degradation evident by day 14; Scale bar: 1 mm

Supplementary Movie 1. (separate file) Dynamic visualization of *S. aureus* dispersal in 3D GelMA hydrogels cultured in TSB. Video showing active dispersal at the hydrogel–media interface. Scale bar: 20um

Supplementary Movie 2. (separate file) Dynamic visualization of *S. aureus* dispersal in 3D GelMA hydrogels cultured in TSB. Video showing active dispersal within the hydrogel matrix Scale bar: 20um

Supplementary Movie 3. (separate file) Dynamic visualization of *S. aureus* dispersal in 3D GelMA hydrogels cultured in MEM. Video showing active dispersal at the hydrogel–media interface. Scale bar: 20um

Supplementary Movie 4. (separate file) Dynamic visualization of *S. aureus* dispersal in 3D GelMA hydrogels cultured in MEM. Video showing active dispersal within the hydrogel matrix Scale bar: 20um