ADVANCED MATERIALS

Supporting Information

for *Adv. Mater.*, DOI: 10.1002/adma.201603270

Layer-by-Layer Encapsulation of Probiotics for Delivery to the Microbiome

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Supplementary Information

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Experimental Methods

Bacteria Modifications and Growth

Bacillus coagulans (BC) (ATCC #7050, Manassas, Virginia) were grown at 37°C in Difco nutrient broth or on Difco nutrient agar (VWR, Radnor, Pennsylvania) and stored in 25% glycerol, 25% water, and 50% nutrient agar at -80°C while in the exponential phase for all experiments. Counting of BC was performed using the standard plate counting method streaked with glass beads, where each plate had between 30-100 colonies. Prior to plating, all formulations were first resuspended and washed twice in PBS. BC, either plain or encapsulated, were grown under shaking conditions (225 RPM) at 37°C using an innova44 incubating shaker (New Brunswick, Hamburg, Germany). OD600 readings were obtained using a SpectraMAX Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA) with media-only backgrounds subtracted. For in vivo imaging experiments, bacteria were fluorescently labeled with vivotag-S 750 (PerkinElmer, Waltham, MA) by mixing vivotag-S 750 (20 µl, 10 mg/ml) in DMSO (1 ml) with BC (5 mg) in bicarbonate buffer (980 µl, pH 8.5, 210 mM) under rotation at room temperature for 1 hour. Transformation of BC with pGEN-luxCDABE (a gift from Harry Mobley; Addgene plasmid # 44918)[1] was performed following a standard heat-shock protocol. Briefly, BC (150 µl, 2 mg) were incubated on ice with pure plasmid DNA (5 ng) for 30 minutes, heat-shocked for 45 seconds at 42°C, and placed back on ice for 2 minutes. Next, SOC medium (800 µl) was added to the tubes and incubated for 1 hour at 37°C. The final suspension (50 µl) was plated on LB agar plates with ampicillin and incubated over night at 37°C. Individual colonies were selected, and luminescence was verified using a PerkinElmer Spectrum-bioluminescent and fluorescent In Vivo Imaging System (IVIS).

Layer-by-layer Synthesis and Characterization

Glycol chitosan (2 mg/ml), alginate (2 mg/ml) (Sigma, St. Louis, Missouri), and L100 (2 mg/ml) (Evonik, Piscataway, New Jersey) were prepared in NaCl (0.5 M). The pH of chitosan and alginate solutions were 6.0 and HCl and/or NaOH were used to adjust pH. L100 was prepared in NaCl (0.5 M) by first dissolving L100 in pH 9 NaCl and titrating to a final pH of 6.0 NaCl. BC (5 mg/ml) were first suspended in NaCl (0.5 M) and washed twice. Cationic polymers (e.g. EPO or chitosan) were layered on plain-BC, for 30 minutes at room temperature under constant rotation. BC were then washed 2x in NaCl (0.5 M) and then coated identically with anionic polymers (e.g. L100 or alginate) for 30 minutes at room temperature under constant rotation. This process was repeated as necessary to synthesize LbL-probiotics of up to 3 bilayers (6 total layers). A Malvern zeta sizer (Worcestershire, UK) was used to measure zeta potentials in either pH 1.5 or pH 7 pure water. Fluorescent chitosan and alginate polymers (PEGWorks, Chapel Hill, North Carolina), were coated identically to methods described above. A Tecan SAFIRE (Medford, Massachusetts) plate reader was used for all fluorescent measurements. A Carl Zeiss Axiovert 200M was used for taking brightfield images of probiotics and LbL-probiotics.

Probiotic Stability and Response to Insults

Plain or LbL-coated BC were subjected to either bile salt solution (4% w/v) (Sigma, St. Louis, Missouri) in PBS or SGF (Ricca Chemical, Arlington, Texas) in a water bath at 37°C for up to 2

hours. After 2 hours, bacteria were pelleted via centrifugation (3000g), resuspended, and washed twice in PBS. BC were plated in sequential dilutions of ten and allowed to grow for at least 48 hours at 37°C and counted as described above. For stability studies, plain or LbL-coated BC were stored in water and in the dark at either room temperature or 4°C.

Mucoadhesive Assay

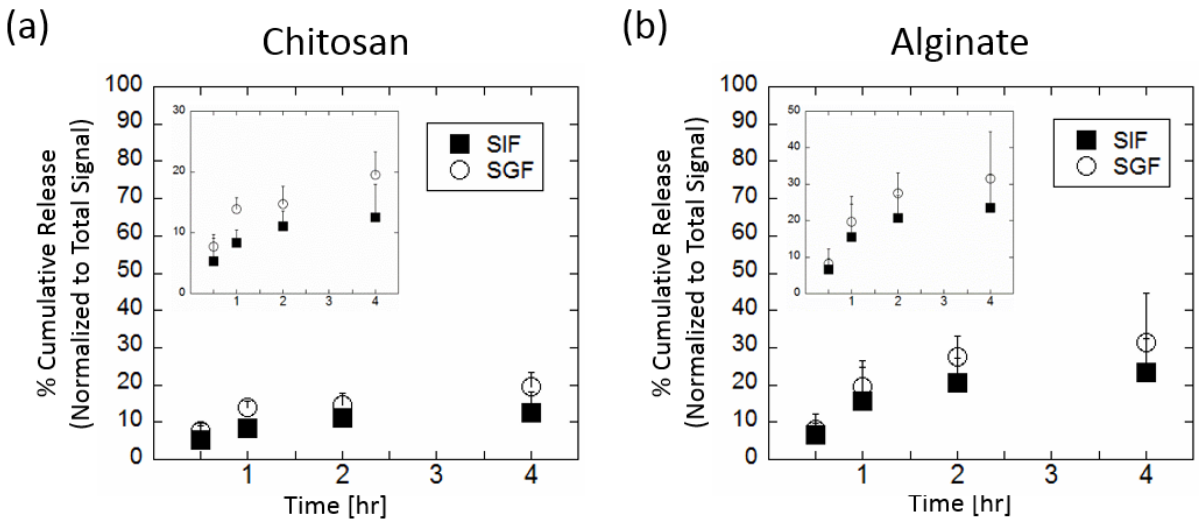
Freshly isolated porcine small intestine was cleaned and sectioned. Vivotag-S 750 stained probiotics (100 µl, 5 mg/ml) was pipetted directly onto the inner wall of the small intestines. Samples were then incubated for 1 hour at 37°C before being imaged using an IVIS. Analysis and spectral unmixing were performed using Living Image Software (PerkinElmer).

MatTek Tissue Experiments

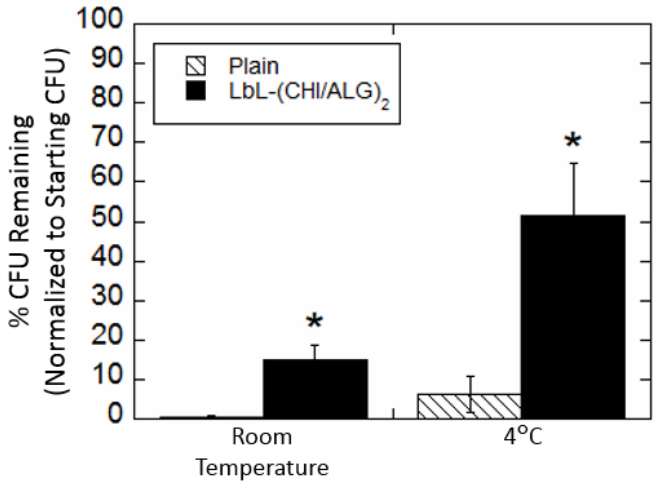
EpiIntestinal tissues were purchased from MatTek (Ashland, Massachusetts) and used as recommended under antibiotic-free conditions. For growth experiments, BC ($1x10^7$ CFU) were applied to the apical side of the EpiIntestinal tissue (n = 3 per group) in 50% nutrient broth and 50% maintenance medium (MatTek, Ashland, Massachusetts). After 1 hour of incubation at 37°C and 5% CO_2, non-adherent BC were washed 3x using sterile PBS. IVIS images of MatTek tissues with BC were taken at specific time points.

In Vivo

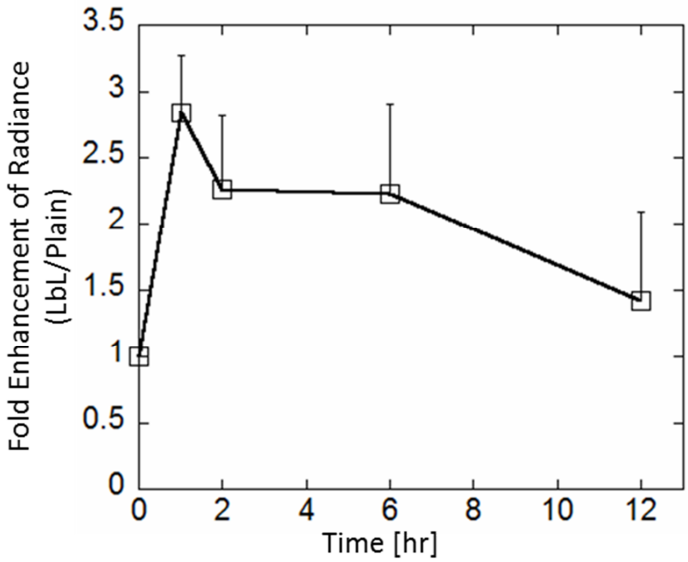
Plain or LbL-BC ($5x10^8$ CFU) were administered to randomly grouped 8-10 week old female BALB/c mice via oral gavage (n = 4 mice per group) in a non-blind fashion. After 1 hour, mice were sacrificed via CO_2 asphyxiation and the GI tract (stomach to colon) was harvested. The GI tract was then opened with a razor blade to expose the BC residing in the lumen to oxygen for bioluminescent imaging.[1-2] All animal procedures were performed according to MIT Animal Care and Use Committee-approved protocols.



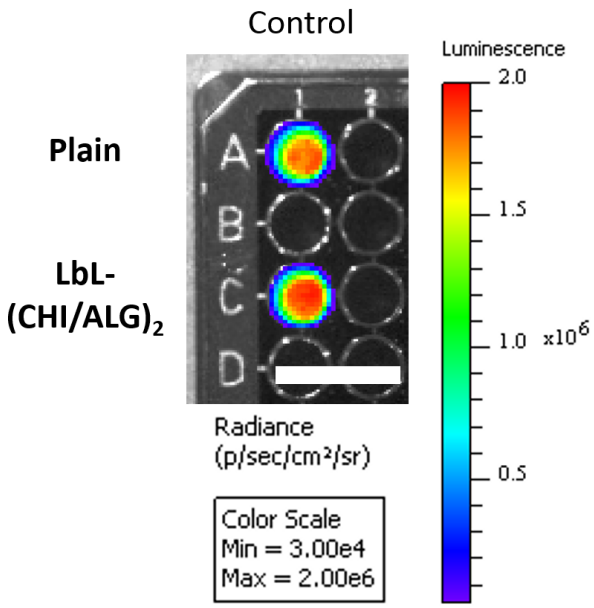
SI Figure 1: Release of (a) chitosan and (b) alginate from the surface of *Bacillus coagulans* following coating of 2 bilayers of chitosan and alginate $(CHI/ALG)_2$ in simulated intestinal fluid (closed squares) and simulated gastric fluid (open circles).



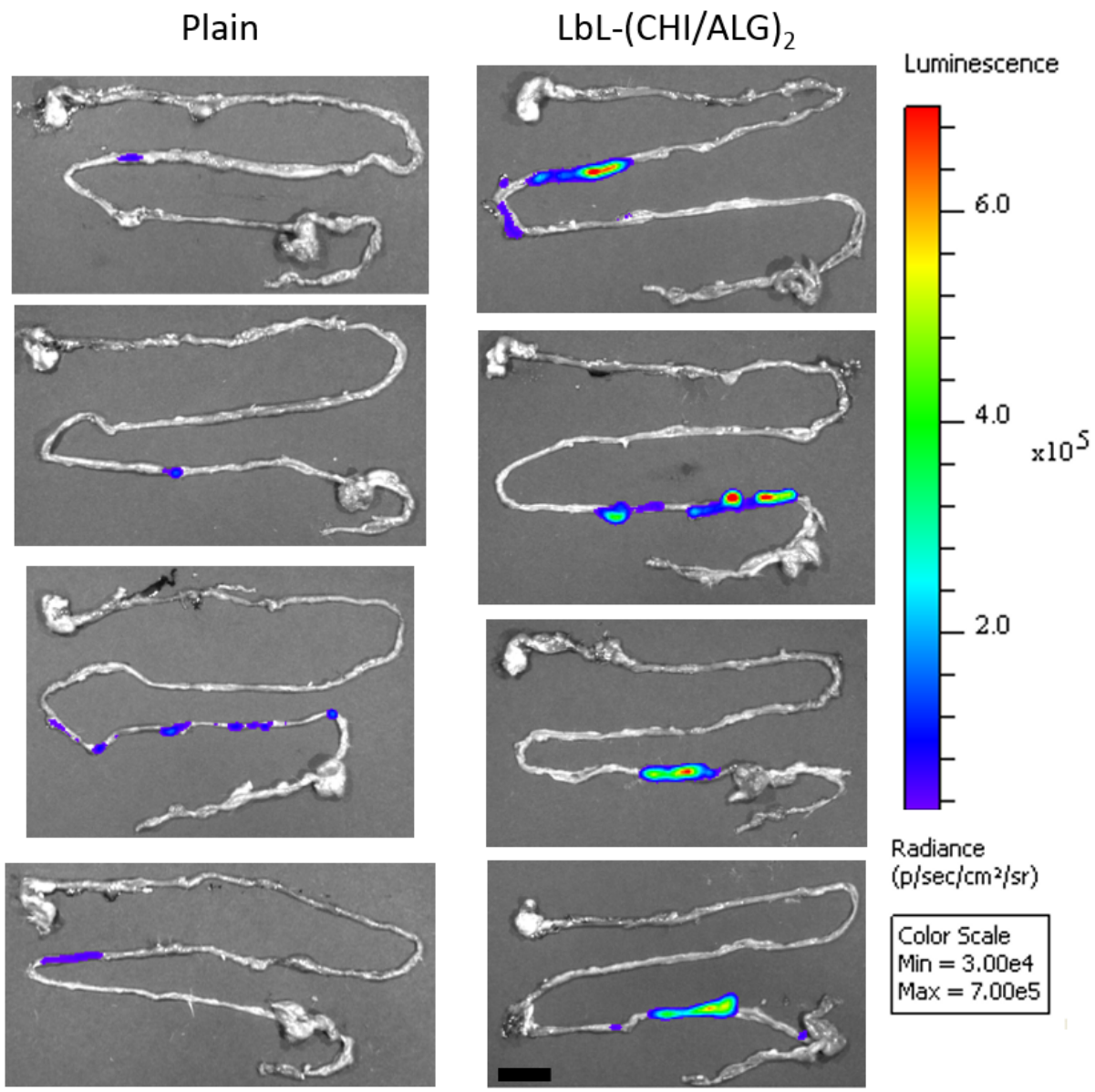
SI Figure 2: Viability of plain (hatched bars) and LbL-BC (black bars) in water after 1 week storage at room temperature and 4°C. * denotes statistical difference ($P < 0.05$) using Student's t-test between plain and LbL groups.



SI Figure 3: Fold-enhancement (LbL-BC/Plain-BC) of radiance at each timepoint.



SI Figure 4: IVIS images of the LbL- and plain-BC controls prior to oral gavage. Scale bar = 1.5 cm.



SI Figure 5: IVIS images of the GI tract of mice following oral gavage of plain-BC and LbL-BC. n = 4 for each group. Scale bar = 1.5 cm.

References

[1] M. C. Lane, C. J. Alteri, S. N. Smith, H. L. Mobley, *Proceedings of the National Academy of Sciences* **2007**, *104*, 16669.
[2] S. Wiles, K. M. Pickard, K. Peng, T. T. MacDonald, G. Frankel, *Infection and immunity* **2006**, *74*, 5391.