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Simple and Ultrastable All-Inclusive Pullulan Tablets for Challenging Bioassays

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Supporting Information

Experimental Section

Materials. Luciferase, luciferin, co-enzyme A (CoA), adenosine triphosphate (ATP), tricine, magnesium carbonate ($MgCO_3$), magnesium sulfate ($MgSO_4$), DL-dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), human serum albumin (HSA; fatty acid and globulin free, ≥99%) and dextran (MW ~148000) were purchased from Sigma-Aldrich. Luciferase Cell Culture Lysis Reagent (CCLR), 5X was purchased from Promega. Polyethylene glycol (PEG, MW ~6000) was purchased from Fluka. Pullulan (MW ~200000 Da) was purchased from Polysciences.

Preparation of 'all-inclusive' pullulan tablets. All reagents for the luciferase assay, except for ATP, were casted in a single pullulan tablet. For preparation of 100 the luminescent tablets, aqueous solutions of 20 mM luciferin, 0.4 mg/mL luciferase, 54 mM CoA, 340 mM DTT, 20 mM EDTA, 214 mM $MgCO_3$, and 534 mM $MgSO_4$ were prepared. 50 µL of each solution was added to 2 mL of 12 w/v% pullulan solution (prepared by dissolving 120 mg pullulan 200kDa in 1 mL of Tricine buffer, 200 mM, pH= 7.8). Lastly, for each tablet, 23.5 µL of the final solution was pipetted onto a PET film and dried for a minimum of 2 hours in a glove box under nitrogen. The dry tablets were then stored at room temperature.

Buffer preparation. A buffer solution containing ATP was prepared to test the activity of the luciferase tablets. For the buffer solution, 16 mL of water was added to 2 mL of 2.5 mM ATP and 2 mL of 200 mM Tricine and the pH was adjusted to pH 7.8. Further dilution of this solution was done as needed for assays.

Cell culturing. *E. coli* DH5α cell cultures were initiated from a glycerol stock and grown in Müller Hinton (MH) media at 37 °C, 250 rpm, for 18 hours. A 1:50 dilution of the overnight culture in MH broth was created and grown until log phase (OD_{600} = 0.3). At this point, a 10 µL aliquot of the culture was serially diluted by 10^5 in sterile PBS and plated, in triplicate, on LB agar plates for enumeration. Cell lysis was performed right before the luminescence testing by adding CCLR 1X in a volume ratio of 1:4 (cell culture:CCLR 1X) and was incubated for 10 minutes at room temperature. 100 µL of the lysed cells was added into the well containing the luminescence assay tablet. Stock CCLR 1X lysis reagent was prepared by adding 4 volumes of Milli-Q water to 1 volume of luciferase Cell Culture Lysis Reagent (CCLR 5X).

Assessing the activity of tablets. The activity of the tablets was measured by placing a single tablet into a well (Falcon 96 Flat Bottom Transparent/Black Polyethylene), followed by the addition of 100 µL of 250 µM ATP, and then measuring the luminescence using a TECAN M1000 (with the settings of: Mode: Luminescence; Interval Time: Minimal; Attenuation: NONE; Integration Time: 5000 ms; Settle Time: 1000 ms; Shaking – Orbital - Duration: 1 s; Shaking – Orbital - Amplitude: 6 mm). The integrated luminescent signal (RLU) collected over time.

Thermal stability test. Reagents in solution and pullulan tablets were incubated at each temperature set point for 30 minutes using a hot plate to adjust temperature. Samples were allowed to cool to room temperature, after which the luminescence reading was taken using 100 µL of 250 µM ATP.

ATP assay. A series of standard ATP solutions with concentration of 5, 10, 20, 50, 100, 500 and 1000 pM were prepared from a 100 mM ATP stock (tris buffered) using Milli-Q water. Each solution (100 µL) was tested in triplicate in a 96 well plate (clear bottom, Greiner sensoplate) containing luminescence assay

tablets and the signal was measured as a function of time. The luminescence signal (RLU) was integrated over the time course of the signal evolution and plotted against ATP concentration.

Detecting ATP in cell assays. Cell assays were done in a similar manner as described above by adding 100 μL of cell lysate solutions with increasing cell density to wells containing a luminescence assay tablet. Cell lysis was done by mixing the cell suspension with CCLR (1X) in a volume ratio of 1:4 and allowing it to incubate at room temperature for 10 minutes, followed by centrifugation at 4000 rpm for one minute before adding lysates into the wells. The integrated luminescent signal (RLU) was plotted against cell density (CFU/μL).

Preparation of HSA-pullulan and lucifersase-pullulan films and solutions for fluorescence studies. HSA was dissolved into either Tris buffer (100 mM, pH 7.5) or Tris buffer containing 10% pullulan to give a final concentration of 60 μM HSA. 500 μL of the HSA-pullulan solution was then carefully pipetted onto a quartz slide (32 x 8 mm) followed by air drying overnight at ambient conditions (21 °C and 48% RH) to produce the pullulan film samples. For luciferase-pullulan films, 62.5 μL of 16 μM luciferase was mixed with 937.5 μL pullulan (12% w/v in buffer) to give a final concentration of 1 μM luciferase. 300 μL of this luciferase solution was then carefully pipetted onto a quartz slide followed by air drying overnight at ambient conditions (21 °C and 48% RH) to produce the pullulan film samples. Blank films were prepared exactly the same way but without luciferase, and were used for background correction. For the fluorescence measurements in solution phase, HSA or luciferase solutions (1 μM) was prepared in buffer or 10% w/v pullulan solutions and tested in quartz cuvetted (1 cm^2).

Fluorescence Emission Spectra. Fluorescence data for HSA were acquired using a Cary Eclipse fluorescence spectrophotometer. Solution samples were measured in quartz cuvettes and continuously stirred throughout the experiments. Film samples were suspended in quartz cuvettes at a 45° angle to the excitation light using specialized holders which reflected excitation light away from the detector and collected emission through the slide and into the monochromator/PMT.[1] For fluorescence emission spectra, samples were excited at 295 nm (to

ensure that the light was absorbed almost entirely by the lone tryptophan residue) and emission was collected from 310 – 450 nm in 1 nm increments, using a 5-nm bandpass for both excitation and emission paths and an integration time of 0.1 s. To assess HSA thermal stability, spectra were collected from 20 – 90 °C and the integrated intensity was plotted against temperature to derived a thermal unfolding curve. In all caes the spectra from both solution and film-based samples were corrected for light scattering by blank subtraction of signals originating from buffer or pullulan/quartz materials, respectively, without HSA present. All the spectra were also corrected for deviations in emission monochromator throughput and PMT response and smoothed by the Savitzky-Golay method, using a factor of 5 and an interpolated factor of 5. Steady-state fluorescence anisotropy measurements were performed in the L-format with excitation at 295 nm and emission at 342 nm, using a 5-nm bandpass and a 3.0 s integration time. All anisotropy values represent the average of three separate samples, which were corrected for a G factor that was generated beforehand.

Fluorescence Emission Measurements for Luciferase. Fluorescence emission spectra for luciferase were collected in buffer, pullulan solution and pullulan films collected using a SPEX Fluorolog-3 Spectrofluorometer with double-grating monochromators on both the excitation and emission paths. Samples were excited at 295 nm, and emission was collected from 320 – 450 nm in 1 nm increments with an integration time of 1 s, using 1-nm bandpasses in both the excitation and emission paths. Spectra were corrected for deviations in both excitation and emission double monochromator throughput. The results are shown in Figure S1.

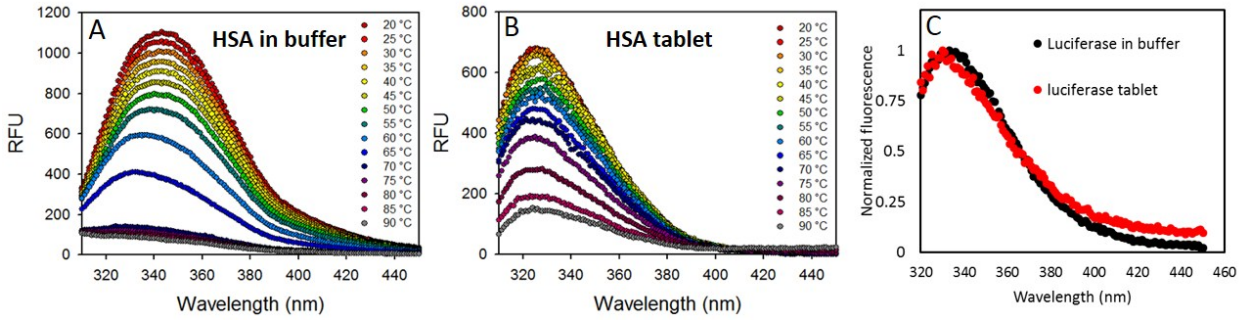

Figure S1. Fluorescence emission spectra of HSA (Trp residue) in A) buffer and B) tablets exposed to

different temperatures, ranging from 20° to 90° C. C) Fluorescence emission spectra of luciferase (Trp residues) in buffer and entrapped in a pullulan tablet – experiments done at room temperature.

Effect of pullulan on assay performance. To confirm that pullulan was chemically inert and did not interact with any of the assay components, two assays for ATP detection where performed, one using the same conditions as those used in Figure 3B (with pullulan in the background solution) and the other test containing all the reagents but with no pullulan present. The results shown in Figure S2 show that virtually no change in signal intensity was observed between the two reactions, except for a slight decrease in reaction rate owing to the increased solution viscosity. These results confirm that the pullulan did not interfere in the assay.

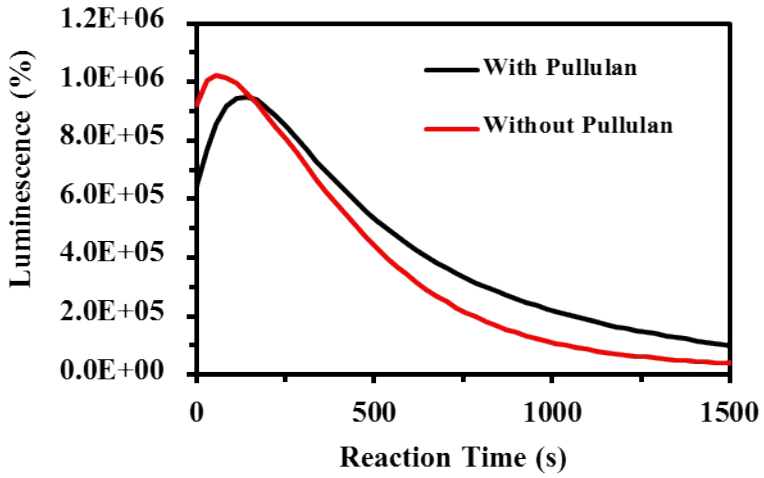

Figure S2. Effect of the presence/absence of pullulan in the liquid phase on the assay performance. Data shown the luminescence intensity with time for the all-inclusive assay using 250 μM ATP with (black) and without (red) pullulan in the reaction mixture.

Assessing the stability in dextran and PEG tablets. The effect of dextran and PEG on luciferase activity was investigated. Dextran and PEG tablets were prepared using the same procedure as was used for the pullulan tablets (see section *Preparation of 'all-inclusive' pullulan tablets*). In place of the pullulan solution, 12 w/v% Dextran and 12 w/v% PEG solutions were used to create dextran and PEG tablets respectively (10% w/v final concentration). We found that dextran solution cast under bench top conditions lost substantial activity after drying (see Figure S3 – data for PEG not shown as the activity was essentially zero). When formed under nitrogen and vacuum dried, the dextran was not able to

produce tablets in contrast to pullulan, which formed highly stable tablets. We also created tablets with luciferase only and with all the cofactors added to the solution being assayed, to be sure that it was the luciferase and none of the other reagents that was degraded - the same lack of activity after making the tablets was observed (data not shown).

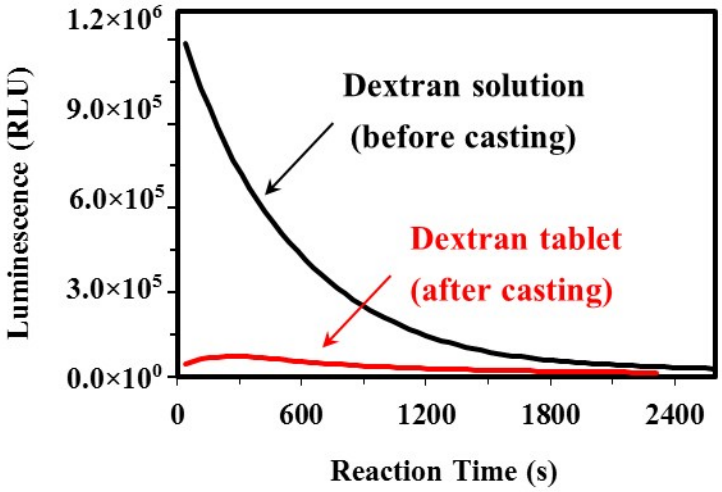

Figure S3. Assessing the effectiveness of using dextran tablets to preserve the activity of luciferase. Activity of fresh luciferase in dextran solution before being cast are shown in red, and luciferase activity in dextran-tablet format are shown black color.

Testing stability at room temperature. Once the tablets were dried (2 hours drying under nitrogen,) they were collected in dark bottles. The tablets were then stored at room temperature and the activity of the tablets was tested over time. Each test was performed with three replicates. Tablets were also produced with luciferase only (no cofactors, luciferin, etc), to determine the effect of storage time on the activity of the enzyme using reagents present in the Promega kit. Figure S4 shows that the enzyme retained over 50% activity when stored for 4 months at room temperature.

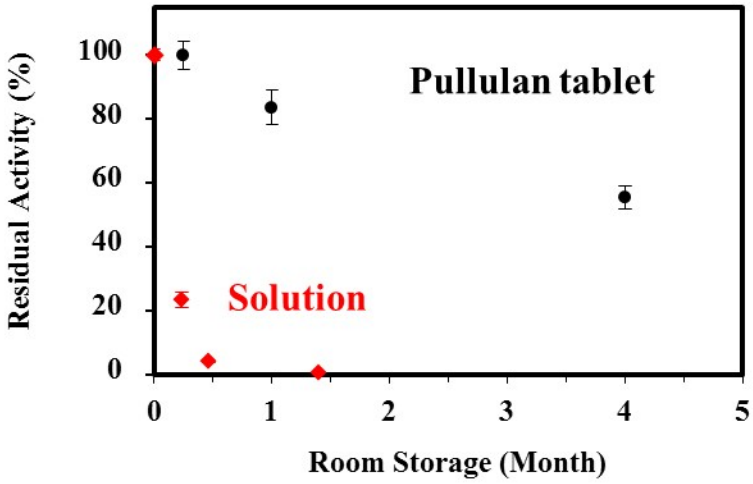

Figure S4. Evaluation of the long-term stability of luciferase-only either in pullulan tablets or buffer solution when stored at room-temperature. The error bars representing the standard deviations based on triplicate repeats.

Luciferase and luciferin performance in separate tablets. To confirm that pullulan both preserved the activity of luciferase and prevented the oxidation of luciferin, the luminescence generated by pullulan tablets either containing only luciferase or only luciferin was evaluated. As shown in Figure S5, for both cases including the luciferase-alone and luciferin-alone tablets, full light intensity was retained, confirming the preservation of both analytes when entrapped in pullulan tablets, indicating that pullulan protected the enzyme and prevented the oxidation of the substrate.

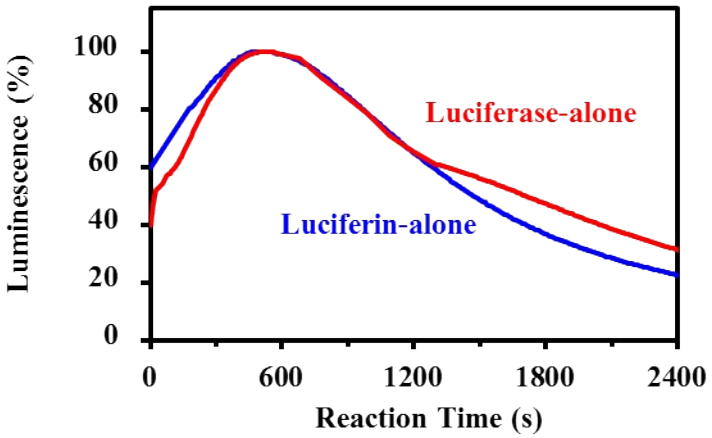

Figure S5. The luminescence generated with pullulan tablets that contained either luciferase only (red), or luciferin only (blue), while all other required reagents ($MgCO_3$, $MgSO_4$, EDTA, CoA, DTT, and luciferin

or luciferase) were in freshly prepared solution that also included 250 μM ATP .

References

1. L. Zheng, W. R. Reid and J. D. Brennan, *Anal. Chem.*, 1997, **69** 3940–3949.