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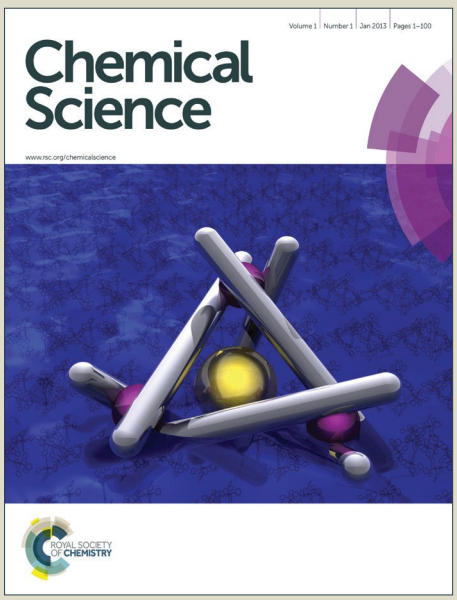

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ARTICLE

Simple and Ultrastable All-Inclusive Pullulan Tablets for Challenging Bioassays

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Many biodetection systems employ labile enzymes and substrates that need special care, making it hard to routinely use them for point-of-care or field applications. In this work we provide a simple solution to this challenging problem through the creation of all-inclusive pullulan assay tablets. The proposed tablet system not only enhances the long-term stability of both enzymes and organic substrates, but also simplifies the assay procedure. The enhanced stability is attributed to two factors: the restriction of the molecular motion of proteins and impermeability to molecular oxygen afforded by the tables. These tablets dissolve rapidly upon addition to testing samples, making the test very easy to perform. Using the ATP-detecting luciferase-luciferin system as an example, we show that the tablet-based assay can achieve highly sensitive detection of ATP in biological samples and that the activity of the assay tablets remains unchanged for over a month at room temperature.

Introduction

Chemical biology, drug development, medical diagnosis and environmental monitoring have employed a large number of biological assays.[1-5] Many of these assays use labile enzymes and bioreactive organic molecules to generate easily-tracked signals such as color changes,[6] fluorescence, chemiluminescence[7] and electrochemical readouts.[8, 9] For such assays, maintaining the long-term stability of enzymes and signal-generating small molecules remains a significant challenge, which is particularly important in the context of point-of care-diagnostics, where reagents need to be used in the field and far away from the well-controlled conditions of a laboratory. A widely used assay that remains particularly challenging is the luminescence assay for ATP, [10-17] which utilizes firefly luciferase,[11, 18] and its luminescent substrate luciferin.[12] A major limitation associated with this assay is the instability of both luciferase and luciferin.[19-21] The activity of luciferase decreases significantly over time even at low temperatures.[22] Methods to stabilize this enzyme have included the use of osmolytes/stabilizers,[23] immobilization of luciferase on solid supports such as pre-coated polymer films,[24] dendrimers,[25] and glass rods,[26] and entrapment into sol-gel derived silica.[27-30] Although these methods are useful in slowing the loss of luciferase activity, they are not effective in maintaining the long-term storage stability of luciferase. In addition, most of these methods involve very complex procedures, making them of limited practical use. Furthermore, none of these methods address the instability issue of luciferin, which is prone to degradation as a result of oxidation.[31]

Recently, our groups reported on a new method for stabilizing labile enzymes and substrates by entrapment into pullulan, a polysaccharide produced by the fungus *Aureobasidium pullulans*.[32, 33] Herein, we extend the study of pullulan-entrapped reagents to the very labile luciferase/luciferin assay system, and investigate whether an "all-inclusive" tablet containing all required reagents and co-factors (luciferase, luciferin, $MgSO_4$, $MgCO_3$, dithiothreitol (DTT), ethylenediaminetetraaceticacid (EDTA), co-enzyme A (CoA) and tricine) could be used for facile detection of ATP using a mix-and-read assay. Pullulan tablets were prepared by casting and drying a 10% (final concentration) pullulan solution containing all seven components at defined concentrations (Figure 1). Optimal intensity and stability of luminescence was achieved by using a pH of 7.8, as reported previously in the literature.[34, 35] In addition, the buffer was added directly to the tablet formulation to ensure that the pH was maintained at a constant value during drying and vitrification. These formed optically clear, glass-like materials that could be easily handled.

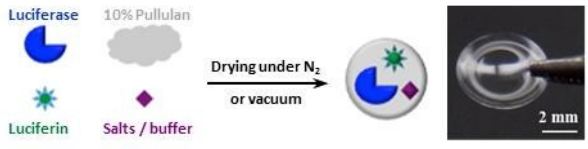

Fig 1. Method to produce all-inclusive pullulan assay tablets for ATP detection. A photograph of an all-inclusive pullulan tablet is shown on the right, where the tablet is a disc-shaped film with a diameter of 6 mm.

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Results and Discussion

Initial studies focused on the thermal stability of the luciferase within the pullulan tablets relative to solution (Figure 2A). It was found that the entrapped enzyme maintained similar activity in the tested temperature range (22-65 °C), suggesting that the pullulan entrapment method is useful for conducting assays for field applications that often have varying ambient temperatures. In contrast, the solution-based assay mixture began to lose activity even at 30 °C and became completely inactive at 50 °C.

To probe into the mechanisms of stabilization, we examined the steady-state emission spectra of luciferase and the model protein human serum albumin (HSA) and the steady-state fluorescence anisotropy of HAS to probe protein conformation and dynamics. HSA was chosen as a surrogate for luciferase for dynamics studies because it contains a single tryptophan, which is required for analysis of anisotropy data, and because the relationship between HSA dynamics and conformation is well understood.[36] Steady-state emission spectra for luciferase and HSA are shown in Figure S1. Both proteins show identical spectra in aqueous solution and in solutions with 10% (w/v) pullulan, showing that pullulan has no effect on protein conformation. In the pullulan tablet, both proteins show small blue-shifts in the emission maximum, but retain the same overall spectral contour. These data show that the entrapped proteins do not aggregate, and that the Trp residue is in an environment where molecular motion is restricted, and thus dipolar solvent relaxation cannot occur.[37] As shown in Figure 2B, the steady-state anisotropy value of pullulan-entrapped HSA (~0.29) remains close to the limiting anisotropy value (0.31) even up to 60 °C, whereas the anisotropy of HSA in solution starts at a lower value (0.18) and drops by a factor of ~2 at 60 °C. This further demonstrates that the rotational motion of the entrapped protein and the surrounding pullulan molecules is highly restricted, consistent with the inability of protein to unfold within pullulan sugar-glass.[38] The restricted molecular motion is thus likely to be a major contributor to the enhanced stability of luciferase in pullulan tablets. In addition, the high glass transition temperature of pullulan glass (T_g > 100 °C) is likely to be a contributing factor as well, as it aids in maintaining the restricted mobility of the entrapped enzyme even at high temperatures.[39]

Further analysis of the effect of pullulan on other assay components (Figure S2) demonstrates that the presence of the sugar does not affect the signal generated by the assay, demonstrating that pullulan is an inert polymer that does not interact with any of the components used in the assay. An unexpected feature of pullulan tablet is the ability to better protect labile reagents compared with other sugar-glasses, such as those formed from dextran, a structurally similar polysaccharide. Production of pullulan and dextran tablets was done in ambient air and at room temperature (simplest possible method for casting) and these were dried for 24 hr. As shown in Figure 2C, the pullulan tablets both preserved the activity of luciferase and prevented the oxidation of luciferin, as evidenced by the increase in light emission associated with the dissolution of the tablets in the presence of ATP. In contrast, the dextran tablets produced <10% of the emission intensity, indicative of a much lower activity of luciferase and/or significant oxidation of luciferin. Direct inhibition of luciferase by dextran was ruled out as the luminescent assay showed full activity in fresh dextran solutions (Figure S3). These results indicate that pullulan has a highly unique ability to stabilize luciferase and protect luciferin during the tablet forming (i.e. drying) process. We also created polyethylene glycol (PEG, MW ~6000) tablets (see ESI) containing luciferase and luciferin, but no signal was obtained when performing the assay with these films, further indicating that pullulan has a unique ability to stabilized (bio)molecules.

Further evaluation of assay performance over time revealed that the production of pullulan tablets under ambient conditions resulted in poor long-term stability, likely owing to oxidation of luciferin by oxygen that was entrapped in the tablets. This hypothesis was verified through mass spectrometry, which showed that oxyluciferin was indeed formed in such tablets (data not shown). To avoid this problem, assay tablets were produced under nitrogen and dried under vacuum. As shown in Figure 2D, these pullulan tablets retained high activity (>97%) when stored at room temperature for up to 30 days, whereas the assay components in solution lost all activity within a few hours. These results further confirm the observation that pullulan films are oxygen-impermeable.[32] Interestingly, entrapment of luciferase alone (Figure S4) resulted in a loss of 20% activity in 30 days, demonstrating that the presence of the additional assay components helped to stabilize the entrapped enzyme (Figure 2D).

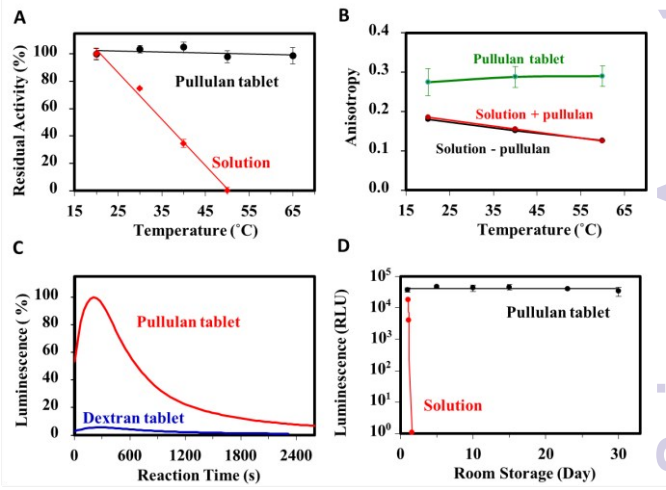

Fig 2. A) Thermal stability of all-inclusive tablet vs. solution. B) Steady-state anisotropy of Trp within HSA in pullulan tablet, pullulan solution and buffer solution. C) Comparison of luminescence of all-inclusive pullulan vs. dextran tablets cast under ambient conditions (refer to Figure S5 for the data obtained when luciferase and luciferin were included in separate pullulan tablets). D) Long-term stability of pullulan tablet produced under nitrogen vs. solution.

Important issues when using reagent tablets are the degree to which the formulation can be manipulated to optimize the response of the assay, and how the rate of dissolution might affect the overall output from the assay. To address the first issue, we examined tablets prepared with varying levels of $MgSO_4$, $MgCO_3$ and CoA. Variations in the levels of the salts had a minor effect on the assay performance (data not shown), while alterations in CoA concentration had a significant effect. The role of CoA in the luciferase-luciferin assay is to react with dehydroluciferyladenylate

(L-AMP), a byproduct formed in the presence of oxygen and a potent inhibitor of luciferase,[40] generating luciferyl-coenzyme A (L-CoA) through thiolysis with CoA. L-CoA is a much weaker inhibitor of luciferase than L-AMP, hence adding CoA to the assay substantially increases signal generation. As shown in Figure 3A, increasing CoA concentration significantly improved assay sensitivity, in agreement with results obtained in solution-based assays.[34] As shown in Figure 3B, when using optimal amounts of CoA, the tablet based assay has sensitivity that is similar to solution, though the maximum intensity decreases somewhat (ca. 22%). On the other hand, the maximum intensity occurs later owing to the dissolution time of the tablet, making it easier to detect the maximum signal reproducibly. It is worth noting that following the attainment of the maximum signal; the rate at which the signal decreases is virtually identical to that from the solution-based assay. In addition, the integrated intensity of the tablet-based assay is within 10% of the solution assay.

The ability of luminescent tablets to detect free and cell-based ATP is demonstrated in Figure 4. Luminescence (RLU) was measured as a function of ATP concentration in tricine buffer. The limit of detection was calculated to be 5.6 pM at 3σ above the background, which corresponds to an ATP concentration of 5 attomoles per µL. This value is comparable to the ultrasensitive ATP detection limit reported using sol-gel entrapped luciferase and ~500 times better than a recently reported voltammetric ATP detection method.[41]

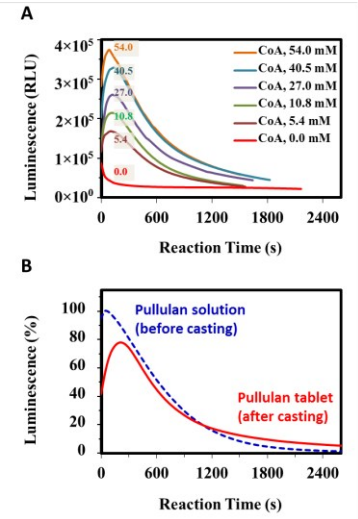

Fig 3.. A) Effect of CoA concentration on assay performance (values are final concentrations after tablet dissolution). 54 mM represents the optimal concentration of CoA in our all-inclusive assay system. B) Comparison of signal evolution from a tablet-based assay and a solution assay.

The ability of the all-inclusive pullulan tablets to detect the ATP from lysed *E. coli* cells was also tested (Figure 4B). The detection limit was found to be 3 x 10^3 cells/mL for the lysed cells, which was close to the LOD for a highly sensitive commercially available assay kit (2 x 10^3 cells/ mL).[42] However, only the all-inclusive tablet method provided a simple, one-step assay format that was both reproducible and user-friendly. By contrast, the Promega BacTiter-Glo™ technology involved preparing a substrate solution from a freeze-dried substrate and then mixing with a buffer solution where both the buffer and substrate required storage at -20 °C.

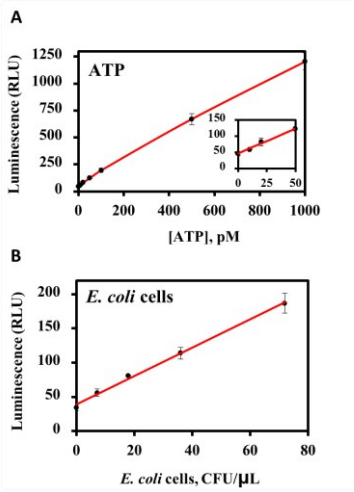

Fig 4. A) ATP detection using all-inclusive pullulan tablets. B) Detection of lysed *E. coli* cells using the all-inclusive luminescent tablets.

In addition to the ATP assay using the luciferase/luciferin system, we expect that pullulan tablets should also be useful for the fabrication of highly stable bioassay kits containing other fragile biomolecules and enzymes. This has already been demonstrated for pesticide analysis using acetylchholieserase and the indoxyl acetate substrate,[32] but should be capable of providing a generic platform for point-of-care (POC) diagnostics. Such tablet-based assays could significantly improve health-care support in resource-limited settings, and allow multiple applications across various fields such as environmental monitoring, food safety, and clinical diagnostics that require the use of labile (bio)reagents. This strategy should also decrease the number of steps needed to complete an analysis and the degree of user intervention in current multi-step assays.

Conclusions

In conclusion, we have succeeded in entrapping the highly unstable luciferase-luciferin system into pullulan tablets to produce a highly stable, all-inclusive ATP assay system with excellent reproducibility and user-friendliness. The basis of the enhanced stability is shown to be a combination of restricted molecular motion of the entrapped protein, and oxygen impermeability, which maintains the molecular structure of oxygen-sensitive reagents. The dissolution rate of pullulan delays the onset of maximum signal evolution, which makes ATP detection more reproducible. It is noteworthy that the method for producing these tablets is particularly cost-effective (~1 USD per 100 tablets). Given the importance of ATP detection, it is conceivable that the all-inclusive tablets can be adopted for a broad range of applications ranging from inexpensive on-site ATP detection to high-throughput kinase activity analysis where simplicity, sensitivity and stable light emission are desired.

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RP holds the Canada Research Chair in Interfacial Technologies. Y.L. holds the Canada Research Chair in Chemical Biology of Nucleic Acids.

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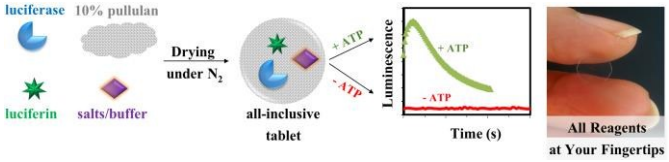

A glowing example: simple to use, highly stable and cost-effective luciferase/luciferin assays using rapidly solubilizing pullulan tablets.

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 8 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, 47 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, HAS 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.

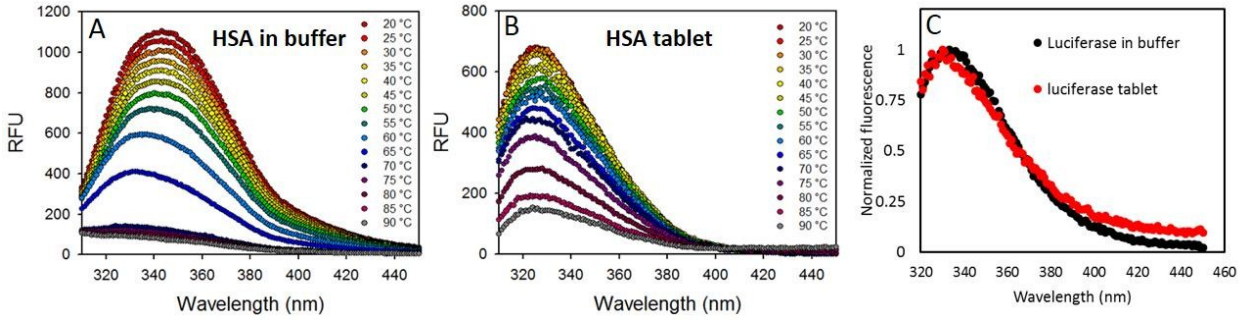

Figure S1. Fluorescence emission spectra of HSA (Trp residue) in A) buffer and B) tablets exposed to different temperatures, ranging from 20° to 90° C. C) Fluorescence emission spectra of luciferase (Trp residues) in buffer and entrapped in a pullulan tablet – experiments done at room temperature.

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.

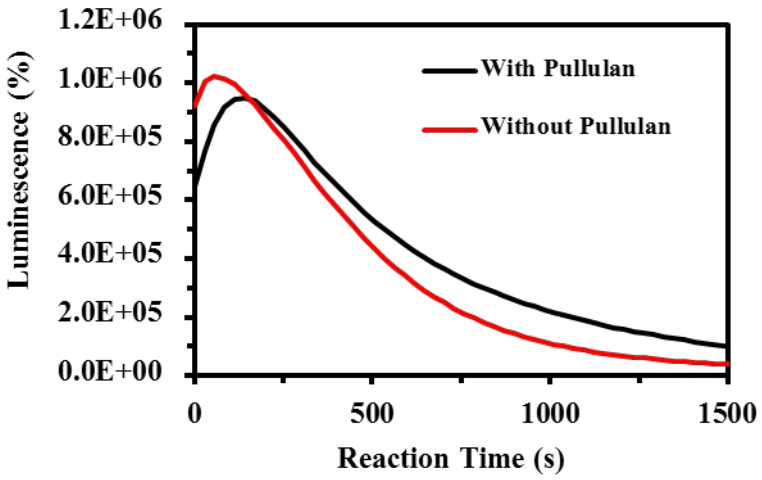

Figure S2. Effect of the presence/absence of pullulan in the liquid phase on the assay performance. Data shown the luminescence intensity with time for the all-inclusive assay using 250 μM ATP with (black) and without (red) pullulan in the reaction mixture.

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).

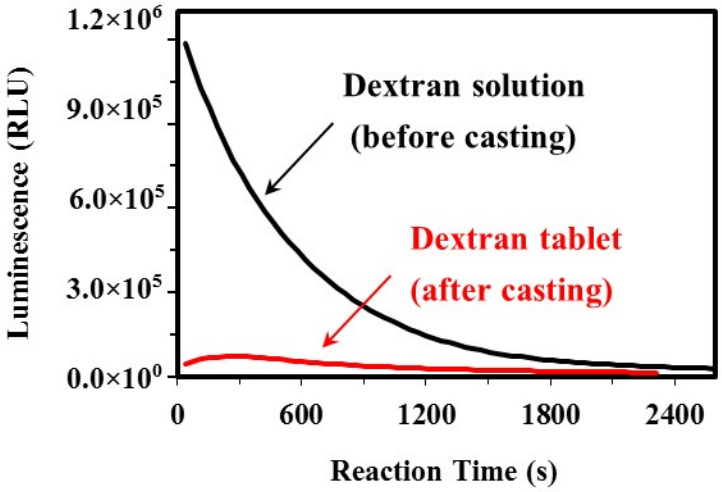

Figure S3. Assessing the effectiveness of using dextran tablets to preserve the activity of luciferase. Activity of fresh luciferase in dextran solution before being cast are shown in red, and luciferase activity in dextran-tablet format are shown black color.

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.

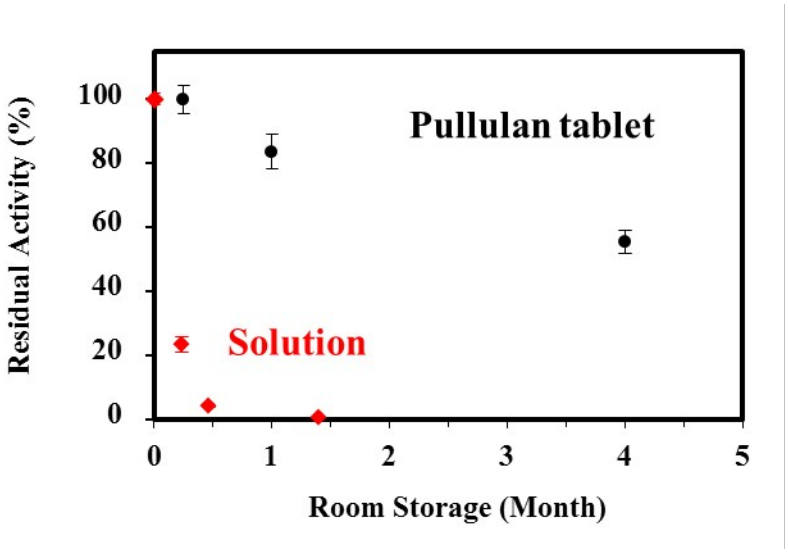

Figure S4. Evaluation of the long-term stability of luciferase-only either in pullulan tablets or buffer solution when stored at room-temperature. The error bars representing the standard deviations based on triplicate repeats.

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.

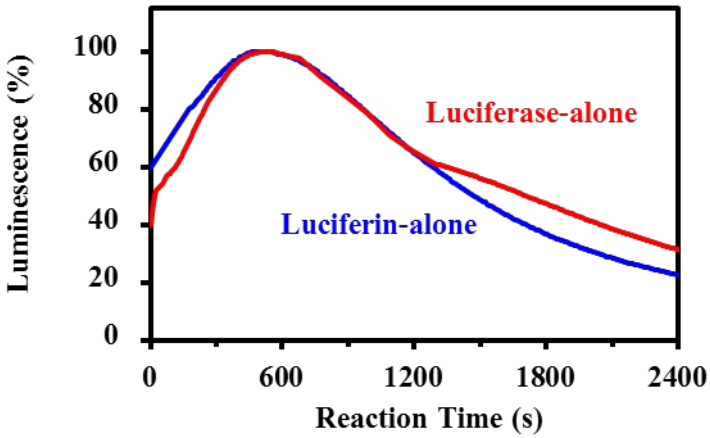

Figure S5. The luminescence generated with pullulan tablets that contained either luciferase only (red), or luciferin only (blue), while all other required reagents ($MgCO_3$, $MgSO_4$, EDTA, CoA, DTT, and luciferin or luciferase) were in freshly prepared solution that also included 250 μM ATP .

References

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