

Pullulan Encapsulation of Labile Biomolecules to Give Stable Bioassay Tablets**

Sana Jahanshahi-Anbuhi, Kevin Pennings, Vincent Leung, Meng Liu, Carmen Carrasquilla, Balamurali Kannan, Yingfu Li, Robert Pelton, John D. Brennan,* and Carlos D. M. Filipe*

Abstract: A simple and inexpensive method is reported for the long-term stabilization of enzymes and other unstable reagents in premeasured quantities in water-soluble tablets (cast, not compressed) made with pullulan, a nonionic polysaccharide that forms an oxygen impermeable solid upon drying. The pullulan tablets dissolve in aqueous solutions in seconds, thereby facilitating the easy execution of bioassays at remote sites with no need for special reagent handling and liquid pipetting. This approach is modular in nature, thus allowing the creation of individual tablets for enzymes and their substrates. Proof-of-principle demonstrations include a Taq polymerase tablet for DNA amplification through PCR and a pesticide assay kit consisting of separate tablets for acetylcholinesterase and its chromogenic substrate, indoxyl acetate, both of which are highly unstable. The encapsulated reagents remain stable at room temperature for months, thus enabling the room-temperature shipping and storage of bioassay components.

Almost all bioassays make use of bioreagents (such as enzymes and small-molecule substrates) that are labile to various degrees and require special shipping and storage. The instability of these molecules can arise from either thermal denaturation or chemical modification,^[1] such as oxidation or hydrolysis.^[2] Because of these issues, they often have to be shipped on dry ice with special packaging, which is costly. These reagents also have to be stored in bulk in refrigerators or freezers to minimize loss of activity, but they must be retrieved, thawed, and aliquoted for intended tests that are often performed at room temperature. Repeated freezing and

thawing can result in significant loss of activity, which often leads to less reliable test results. These problems make running such assays in resource-limited settings a significant challenge.

One approach to address this problem is to place the assay reagents into a tablet or capsule, which both provides premeasured quantities of reagents and allows the addition of preservatives that can prolong the shelf life of the reagents. Such an approach is used in several commercial assay kits that use nonbiological reagents but has thus far not been extended to biological agents like enzymes, which are often the key components of bioassays.^[3] This is in part due to a lack of a suitable material that can meet the following three conditions: 1) it allows the encapsulation of biomolecules in a form suitable for shipping; 2) it provides outstanding protection for entrapped biomolecules against thermal denaturation and chemical modification during shipping and storage;^[4] and 3) it is readily soluble in aqueous solution, allows the release of the encapsulated molecules, and does not interfere with the assay itself.^[5] In this work we report on the use of pullulan to create bioassay tablets (cast, not compressed) that meet these three conditions.

Pullulan is a natural polysaccharide produced by the fungus *Aureobasidium pullulans*.^[6] It readily dissolves in water but resolidifies into films upon drying.^[6a,b,7] The film-forming property of pullulan has been utilized in some unique applications in the pharmaceutical and food industries, such as breath fresheners and food additives.^[6b,8] Recent studies have found that pullulan coatings applied to food packaging can act as oxygen barriers to prolong the shelf life of various foods.^[7,9] In addition, pullulan has been shown to preserve the viability of bacteria under various storage conditions.^[8]

Given the above findings, we hypothesized that pullulan might be suitable for producing assay tablets with encapsulated enzymes or other labile molecules, and more importantly, that these tablets may not only allow the long-term storage of unstable molecules at room temperature but may also provide a simplified platform for carrying out bioassays in resource-limited settings. Herein, we demonstrate the use of pullulan to create tablets to facilitate two different enzymatic reactions: a single-tablet system for DNA amplification through the polymerase chain reaction (PCR) with Taq DNA polymerase (TaqDP); and a two-tablet system for pesticide detection, where one tablet contains acetylcholinesterase (AChE) and the other contains indoxyl acetate (IDA, a chromogenic substrate for AChE).

Our first case study involves using AChE and IDA for the detection of malathion, a widely used organophosphate pesticide that has been implicated in causing reduced neuro-

[*] S. Jahanshahi-Anbuhi, K. Pennings, V. Leung, Dr. M. Liu, C. Carrasquilla, Dr. B. Kannan, Prof. Dr. Y. Li, Prof. Dr. R. Pelton, Prof. Dr. J. D. Brennan, Prof. Dr. C. D. M. Filipe
Departments of Chemical Engineering, Chemistry & Chemical Biology, and Biochemistry and Biomedical Sciences
McMaster University
1280 Main Street West, Hamilton, ON, L8S 4M1 (Canada)
E-mail: brennanj@mcmaster.ca
filipe@mcmaster.ca

[**] We thank the Natural Sciences and Engineering Research Council of Canada and Sentinel Bioactive Paper Network for funding. The authors also thank the Canada Foundation for Innovation and the Ontario Innovation Trust for support of this work. R.P. holds the Canada Research Chair in Interfacial Technologies. J.D.B. holds the Canada Research Chair in Bioanalytical Chemistry and Biointerfaces. We would also like to thank Dr. Clemence Sicard for her useful consultation. We also acknowledge the McMaster Biointerfaces Institute (BI) where part of the experiments was performed.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201403222>.

logical function, eventually leading to Alzheimer's, ADHD, reduced IQ, or even death.^[10] IDA can be hydrolyzed by AChE into hydroxyindole and acetic acid: in the presence of oxygen, hydroxyindole spontaneously changes into blue-colored indigo (Figure 1a).^[11] Malathion is known to be an inhibitor of AChE^[12] and the AChE–IDA combination has been shown to be an excellent system for the colorimetric detection of malathion.^[13] However, the main challenge for

followed by the addition of an IDA tablet (Figure 1c). If malathion is present, the sample remains colorless or turns faint blue (dependent on the concentration of malathion, as discussed below). In the absence of malathion, IDA is fully hydrolyzed by AChE and the test sample turns deep blue (Figure 1c). Experimental details are provided in the Supporting Information.

The tablet system can not only be used to achieve qualitative colorimetric detection of malathion by eye, it can also provide quantitative analysis of the pesticide concentration in a test sample when using a smartphone and image-processing software (such as ImageJ).^[13b,c,17] Figure 2 shows a plot of the dose-dependent inhibition of AChE by malathion, with data obtained using a smartphone. This simple method can be used to detect malathion at levels as low as 64 nM (S/N = 3).

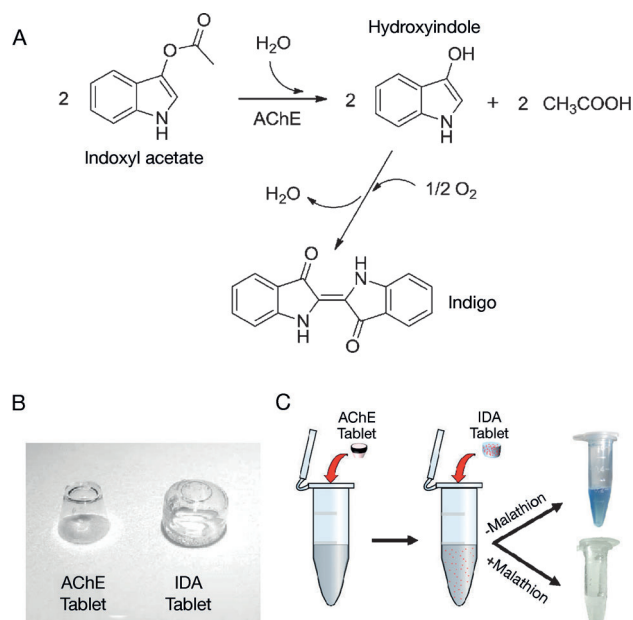


Figure 1. Tablet assay kit for malathion. a) Hydrolysis of IDA by AChE. b) A photograph of the AChE and IDA pullulan tablets. c) Operational principle of the assay kit.

this assay is the instability of both AChE and IDA.^[14] When stored at room temperature, AChE loses its enzymatic activity and IDA becomes oxidized within a few hours.^[14b,15] After being left in air, unprotected IDA turns slightly pink as a result of indirubin formation. There are two competing oxidative reactions involved in the hydrolysis of IDA. One results in the formation of the indigo dimer (the desired product of the assay), while the other produces isatin, which then dimerizes with indoxyl to give indirubin (pinkish).^[16] Therefore, fresh solutions must be prepared prior to each test, thus making this test unsuitable for on-site applications. Given these issues, the AChE–IDA system offers an excellent case study for examining the utility of pullulan tablets.

We produced individual AChE–pullulan tablets and IDA–pullulan tablets (Figure 1b) by using a simple process that involves 1) the mixing of a pullulan solution with either an AChE or IDA solution, 2) the casting of each mixture into a polypropylene mold with small wells (3 mm in diameter $\times$ 3 mm in depth), and 3) air-drying. Note that defined concentrations of AChE and IDA were chosen to achieve a maximum rate of color formation (see Figure S1 in the Supporting Information). To conduct the assay, an AChE tablet is added to the sample to allow preincubation with the pesticide,

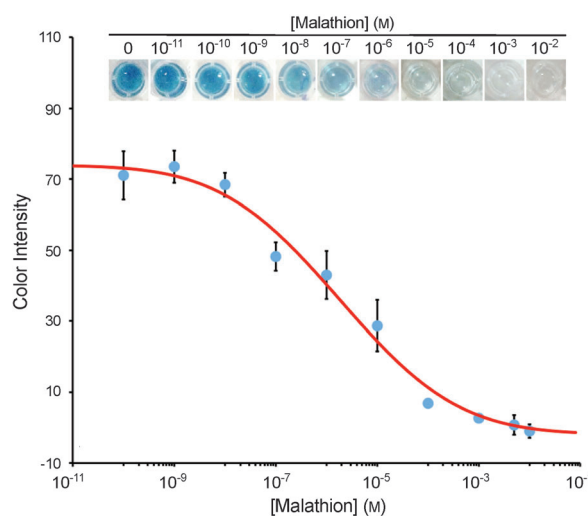


Figure 2. Dose-dependent inhibition of AChE at varying concentrations of malathion.

We next evaluated the long-term stability of both the AChE and IDA tablets. As shown in Figure 3, AChE stored in solution at room temperature became completely inactive within 3 days. Similarly, IDA in solution at room temperature lost 70 % of its activity within one day and become completely inactive within a week. In sharp contrast, both AChE and IDA in tablet form remained fully active for at least 2 months when stored at room temperature. In the case of IDA, the loss in performance was related to oxidation.^[16] Our data suggests that pullulan acts as a strong barrier to oxygen, an effect that is consistent with previous findings.^[7,9] In theory, an antioxidant could be used to prevent the oxidation of IDA during storage at room temperature. However, the antioxidant would also inhibit the formation of indigo during the assay.

The loss of AChE activity, however, is attributed to thermal denaturation. To further examine the role of pullulan in stabilizing AChE, we monitored the activity of AChE as a function of temperature. For this experiment, native (unencapsulated) AChE and the corresponding pullulan tablet were treated at a given temperature for 30 min, followed by activity assessment at room temperature. Figure 4 shows that the free AChE became completely

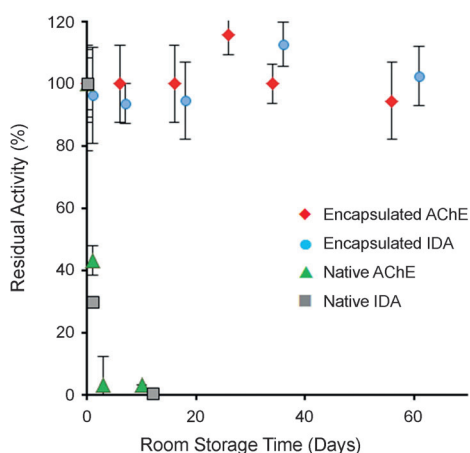


Figure 3. Evaluation of the long-term stability of AChE and IDA tablets stored at room temperature. Normalized activity of encapsulated AChE in reaction with fresh IDA, encapsulated IDA in reaction with fresh AChE, unencapsulated (native) AChE in reaction with fresh substrate, and unencapsulated IDA in reaction with fresh AChE.

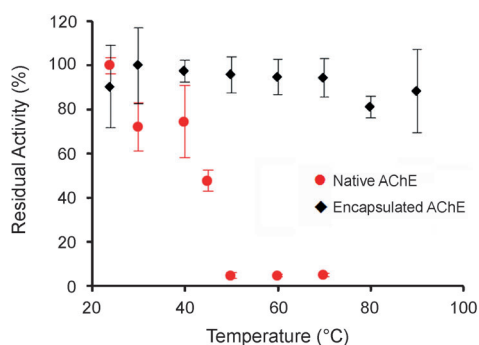


Figure 4. Loss of AChE activity as a function of temperature. Both encapsulated AChE and native enzyme were assessed.

inactive following a 30-minute heat treatment at 50°C or above. In stark contrast, AChE tablets retained ca. 90% of their initial activity even after a 30-minute incubation at 90°C.

Significant thermal stabilization was also observed for human serum albumin (HSA; Figure S2), where the unfolding temperature of the protein, as determined by tryptophan emission intensity, increased by 20°C, thus demonstrating that the stabilizing effects of pullulan are generic.

To show that pullulan encapsulation is a general strategy for increasing the long-term stability of enzymes, we carried out a second case study with TaqDP, which has been widely used in molecular biology laboratories around the world to achieve DNA amplification and has been increasingly explored for disease diagnosis and pathogen detection.^[18] Even though TaqDP is a much more stable enzyme than AChE, it still can lose significant activity when stored at room temperature. As shown in Figure 5 (see also Figure S3), the encapsulation of TaqDP in pullulan tablets resulted in the retention of 90% of initial activity after storage at room temperature for 50 days versus only 40% for native TaqDP. Once again, the data indicate that the pullulan tablet strategy offers a general method for enzyme stabilization. It is

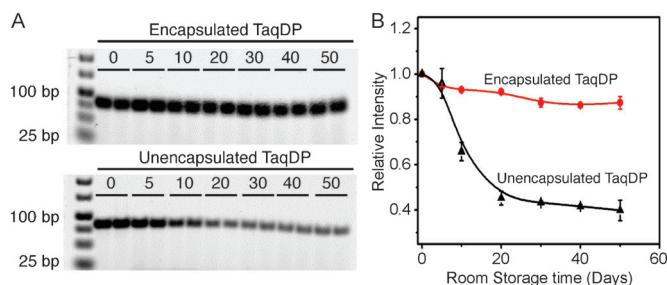


Figure 5. TaqDP activity loss during room-temperature storage with and without pullulan encapsulation.

noteworthy that when combined with portable PCR systems, TaqDP–pullulan tablets should facilitate the on-site detection of DNA.

In summary, we demonstrate that pullulan is an excellent material for producing water-soluble bioassay tablets containing labile enzymes and small molecules. Most significantly, the pullulan tablets provide exceptional protection for the entrapped reagents against thermal denaturation or chemical modification, thereby allowing room-temperature storage for extended periods of time. Equally important, the tablet strategy provides a general platform for carrying out bioassays with minimal steps and user intervention, which is ideal for resource-limited regions, particularly in the developing world.

Received: March 11, 2014

Published online: April 24, 2014

Keywords: carbohydrates · encapsulation · enzymes · pullulan · stabilization

- [1] T. Arakawaa, S. J. Prestrelskia, W. C. Kenneya, J. F. Carpenter, *Adv. Drug Delivery Rev.* **2001**, *46*, 307–326.
- [2] K. D. Miller, R. Barnette, R. W. Light, *Chest J.* **2004**, *126*, 1933–1937.
- [3] a) F. Caruso, M. Lehmann, R. Renneberg, D. Trau, Vol. US20040014073A1, **2004**; b) N. Milani, *Apidologie* **1995**, *26*, 415–429; c) R. A. Setterquist, G. K. Smith, *Nucleic Acids Res.* **1996**, *24*, 1580–1581; d) V. Siegmund, O. Adjei, P. Racz, C. Berberich, E. Klutse, F. Vloten, T. Kruppa, B. Fleischer, G. Bretzel, *J. Clin. Microbiol.* **2005**, *43*, 271–276.
- [4] a) C. Ó'Fágáin, *Enzyme Microb. Technol.* **2003**, *33*, 137–149; b) A. Y. Kolosova, W.-B. Shim, Z.-Y. Yang, S. A. Eremin, D.-H. Chung, *Anal. Bioanal. Chem.* **2006**, *384*, 286–294; c) M. K. Goyal, I. Roy, A. Amin, U. C. Banerjee, A. K. Bansal, *J. Pharm. Sci.* **2010**, *99*, 4149.
- [5] P. V. Iyer, L. Ananthanarayan, *Process Biochem.* **2008**, *43*, 1019–1032.
- [6] a) M. Shibata, M. Asahina, N. Teramoto, R. Yosomiya, *Polymer* **2001**, *42*, 59–64; b) R. S. Singh, G. K. Saini, J. F. Kennedy, *Carbohydr. Polym.* **2008**, *73*, 515–531; c) A. Imeson, *Food Stabilisers, Thickeners and Gelling Agents*, Wiley, Hoboken, **2011**, pp. 267–274.
- [7] S. Farris, L. Introzzi, J. M. Fuentes-Alventosa, N. Santo, R. Rocca, L. Piergiovanni, *J. Agric. Food Chem.* **2012**, *60*, 782–790.
- [8] A. A. Krumnow, I. B. Sorokulova, E. Olsen, L. Globa, J. M. Barbaree, V. J. Vodyanov, *J. Microbiol. Methods* **2009**, *78*, 189–194.
- [9] S. Wu, J. Chen, *Int. J. Biol. Macromol.* **2013**, *55*, 254–257.

- [10] a) S. Ciesielski, D. P. Loomis, S. Rupp Mims, A. Auer, *Am. J. Public Health* **1994**, *84*, 792; b) DailyNewsBlog in *Beyond Pesticides* **2013**; c) B. Eskenazi, A. R. Marks, A. Bradman, K. Harley, D. B. Barr, C. Johnson, N. Morga, N. P. Jewell, *Environ. Health Perspect.* **2007**, *115*, 792–798; d) S. Giri, S. B. Prasad, A. Giri, G. D. Sharma, *Mutat. Res.* **2002**, *514*, 223–231; e) S. M. Ross, I. C. McManus, V. Harrison, O. Mason, *Crit. Rev. Toxicol.* **2013**, *43*, 21–44.
- [11] M. Pohanka, M. Hrabanova, K. Kuca, J. P. Simonato, *Int. J. Mol. Sci.* **2011**, *12*, 2631–2640.
- [12] a) J. E. Casida, *Science* **1964**, *146*, 1011–1017; b) H. Soreq, S. Seidman, *Nat. Rev. Neurosci.* **2001**, *2*, 294–302.
- [13] a) N. Hwa-Young, K. Young Ah, L. Yong Tae, L. Hye-Sung, *Anal. Chim. Acta* **2007**, *594*, 37–43; b) S. Jahanshahi-Anbuhi, P. Chavan, C. Sicard, V. Leung, S. M. Z. Hossain, R. Pelton, J. D. Brennan, C. D. M. Filipe, *Lab Chip* **2012**, *12*, 5079–5085; c) S. Jahanshahi-Anbuhi, A. Henry, V. Leung, C. Sicard, K. Pennings, R. Pelton, J. D. Brennan, C. D. M. Filipe, *Lab Chip* **2014**, *14*, 229–236; d) M. Yuqing, H. Nongyue, Z. Jun-Jie, *Chem. Rev.* **2010**, *110*, 5216–5234.
- [14] a) K. M. Somashekar, McMaster University (Hamilton), **2013**; b) G. Pico, *Biochem. Mol. Biol. Int.* **1995**, *36*, 1017–1023.
- [15] S. Cotson, S. J. Holt, *Proc. R. Soc. London Ser. B* **1958**, *148*, 506–519.
- [16] U. H. Brinker, J.-L. Mieusset, *Molecular Encapsulation Organic Reactions in Constrained Systems*, Wiley, Vienna, **2010**, chap. 3.
- [17] C. Liu, Q. Jia, C. Yang, R. Qiao, L. Jing, L. Wang, C. Xu, M. Ga, *Anal. Chem.* **2011**, *83*, 6778–6784.
- [18] a) C. Ding, C. R. Cantor, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 7449–7453; b) F. Feng, L. Liu, S. Wang, *Nat. Protoc.* **2010**, *5*, 1255–1264; c) T. Ishige, S. Sawai, S. Itoga, K. Sato, E. Utsuno, M. Beppu, K. Kanai, M. Nishimura, K. Matsushita, S. Kuwabara, F. Nomura, *J. Hum. Genet.* **2012**, *57*, 807–808; d) R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, H. A. Erlich, *Science* **1988**, *239*, 487–491; e) R. K. Saiki, S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, N. Arnheim, *Science* **1985**, *230*, 1350–1354.