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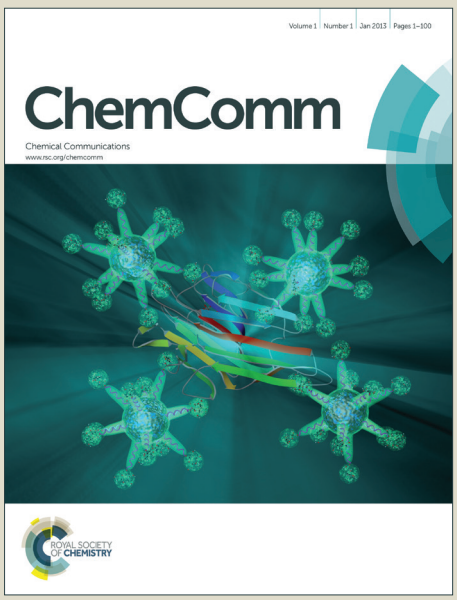

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A bistable switch in pH in urease-loaded alginate beads

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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A bistable switch from a low pH (unreacted "off") state to a high pH (reacted "on") state was obtained in enzyme-loaded gel beads in response to supra-threshold substrate concentrations.

Feedback is prevalent in biological systems and is exploited in cellular switches when a sharp transition is required from one chemical state to another in response to an external signal. One of the first examples of feedback was demonstrated in frog oocyte maturation[1], where above a critical stimulus (the concentration of a hormone) a transition to an activated "on" state was observed. In this case, feedback resulted in an irreversible bistable switch so that following removal of the stimulus the cell remained in the activated state.

There has been much interest in the reproduction of feedback-driven behaviour in synthetic biology, for example genetic circuits were designed to create a toggle switch and oscillations in E. coli cells[2]. Autocatalysis has also been obtained using biological components, DNA and enzymes, in artificial reaction networks[3]. In synthetic chemical systems, oscillations have been produced in iron-catalyst loaded particles or gels in a solution of inorganic substrates[4] and inorganic reactions that display bistability and oscillations in pH have been coupled with chemo-responsive gels in flow reactors[5]. Such systems were proposed for use in, for example, periodic drug delivery[6]. Here, we designed a bistable switch between a low pH "off" and high pH "on" state driven by chemical feedback in an enzyme-loaded bead.

Particles with immobilised enzymes have widespread applications, such as sensors, drug delivery devices and bio-reactors, and enzyme reactions have also been proposed for logic gates in microfluidic reactors or bio-fuel cells[7]. Feedback in enzyme-loaded particles offers potential advantages such as amplification of a chemical signal above a threshold (transistor) or an irreversible response to a signal (one-way switch)[8]. Some time ago, it was suggested that feedback might be obtained in enzyme catalysed reactions in non-buffered environments as a result of the bell-shaped rate-pH curve[9]. A small number of enzyme-catalysed reactions have been shown to display feedback via this route[10], including the urea-urease reaction[11]:

$$CO(NH_2)_2 + H_2O \xrightarrow{urease} 2NH_3 + CO_2$$

This well-studied reaction[12] has a maximum rate at pH 7 so if the initial pH is lower than 7 (by addition of acid) then the reaction accelerates as it proceeds. In experiments in a closed reactor, the solution remained at low pH (~4) for a period of time, the clock time, before rapidly changing to high pH (~10). This particular reaction is of interest as it is used by bacteria such as H. pylori to elevate the pH and protect them from the acidic environment of the stomach and has also been exploited in applications such as sensors or bio-reactors for the production of calcium carbonate crystals[13].

Parameter values for bistability were obtained in simulations of a simplified model of the urea-urease reaction. The urease was contained in a reaction cell and urea and acid diffused in from the enzyme-free surrounding solution (see supplementary information for more details on the simulations). The phase diagram in Figure 1a shows the behaviour of the cell as a function of the total enzyme activity E and substrate concentration at the boundary, $[S]_0$. The final state of the cell was low pH (unreacted) for low E and $[S]_0$ or high pH (reacted) for high E and $[S]_0$. In the region of bistability the two states co-exist for the same parameters.

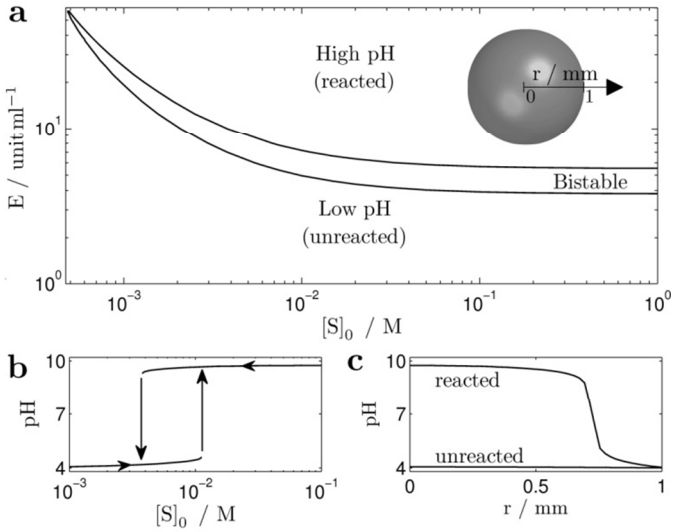

Fig. 1. Reaction-diffusion simulations of the urea-urease reaction in a spherical cell of total enzyme activity E and radius 1 mm in contact with a solution of fixed substrate, $[S]_0$, and acid, $[H^+]_0 = 1 \times 10^{-4}$ M. (a) Phase diagram of behaviour in the cell (pH at r = 0, centre of the cell) showing parameter values for the high pH state, low pH state and bistability between the two states. (b) pH of the cell at r = 0 as a function of $[S]_0$ with E = 7 units/ml showing the high pH state (upper branch, pH > 9) and low pH state (lower branch, pH < 5). (c) Steady state pH profile along a radial coordinate for a reacted and unreacted cell.

Figure 1b shows what happens as the concentration of substrate was increased. With low substrate, the cell was in the unreacted state (pH ~ 4 at r = 0). When the concentration of the substrate was increased above the threshold value of $[S]_0 = 0.01$ M, the cell switched to the high pH reacted state (pH ~ 10 at r = 0) after several minutes. The steady state pH profile across the cell is shown in Figure 1c. In the reacted state the cell was pH ~ 10 from the centre to r = 0.7 mm then it dropped to pH 4 because of the fixed high acid concentration at the boundary of the cell. When the substrate concentration was decreased from high values, the cell remained in the reacted state until $[S]_0 = 0.006$ M then it dropped back to the low pH state. Thus the cell was bistable for the values of $[S]_0 = 0.006 – 0.01$ M and its pH profile for a particular substrate concentration depended on its history.

In order to obtain bistability in experiments, beads were prepared from urease solution, sodium alginate and calcium chloride (see supplementary information). With this preparation method, the enzyme was physically entrapped in the resulting calcium alginate gel matrix[14]. In a typical experiment, ten urease-loaded beads were added to compartments containing a solution of sulphuric acid and urea (Fig. 2a). With initially the same pH and number of beads in each compartment, the beads were of similar size (~3 mm) and the size did not change during an experiment. The beads and the substrate solution contained indicator cresol red (pKa = 8.3) in order to visualise the low (yellow) and high (red) pH states. The intensity profile along a cross section of a bead depended on the substrate concentration (Figure 2b). In the compartments with lower substrate concentration, the outer edge of the beads remained yellow. This was also the case in simulations (Fig 1c).

Immobilisation of an enzyme results in a change of activity compared to solution phase experiments[15]. The activity of the urease-loaded beads is possibly best characterised by the clock time, defined as the time for the average intensity in the central area of the bead to fall to half its initial value. There was some variation in clock time between beads in the same compartment which may arise as result of differences in enzyme loading, microstructure or size of the beads[16]. A histogram of the clock times for different substrate concentrations is shown in Figure 2c. The average clock time increased with decreasing substrate from 7.0 minutes in A to 17.3 minutes in B and 25.7 minutes in C. Beads in compartment D did not change colour.

In the experiment shown in Figure 2, all the beads reached steady intensity profiles within 40 minutes. The beads in compartments B and C were removed and the remainder were distributed between all compartments. The average intensity in time for beads 1 - 12 is shown in Figure 3a. Beads that were moved between compartments A and D changed state: beads 1 and 2 were moved from D to A and reacted (high pH, red); the clock time was 15 minutes. The time taken for some of the beads to reach new steady intensity profiles took longer than initially as a result of the different initial concentrations. Beads 7 and 8 went to the unreacted, low pH state (yellow).

In compartments B and C, beads 3 - 6 which were initially yellow remained in an unreacted state (high intensity). Beads 9 - 12 which were initially red remained in a reacted state (low intensity) but the intensity profiles across the beads altered and the outer edges of the reacted beads became yellow. Thus, for the concentrations in B and C, the final state of the bead (reacted or unreacted) depended on the initial state and hysteresis was observed.

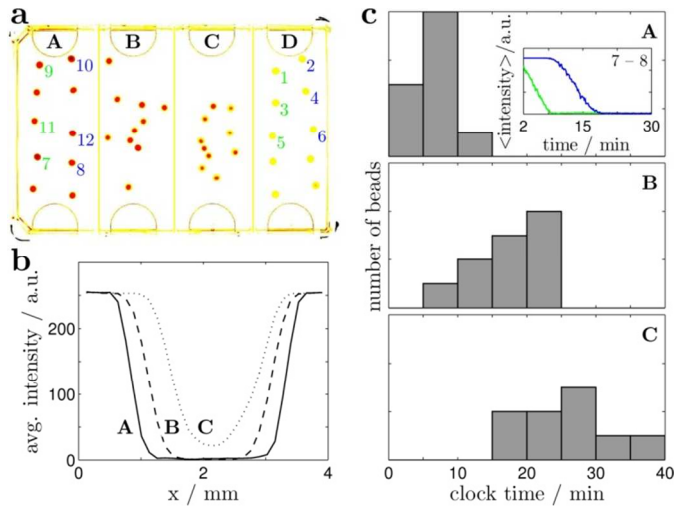

Fig. 2. Urease-loaded alginate beads with cresol red indicator in compartments of size 8 × 3 cm containing urea: A = 0.01 M; B = 0.004 M; C = 0.003 M; D = 1×10^{-4} M and sulphuric acid, pH = 4.7. T = 24 °C. (a) Image (enhanced) of beads at t = 42 mins where red = high pH (reacted) and yellow = low pH (unreacted). (b) Intensity profile averaged across all beads in compartments A, B or C (low intensity = high pH). (c) Histogram of clock times in compartments A, B and C. Inset shows the average intensity in the centre of a bead (circular area of 1.2 mm^2) in time for beads 7 and 8.

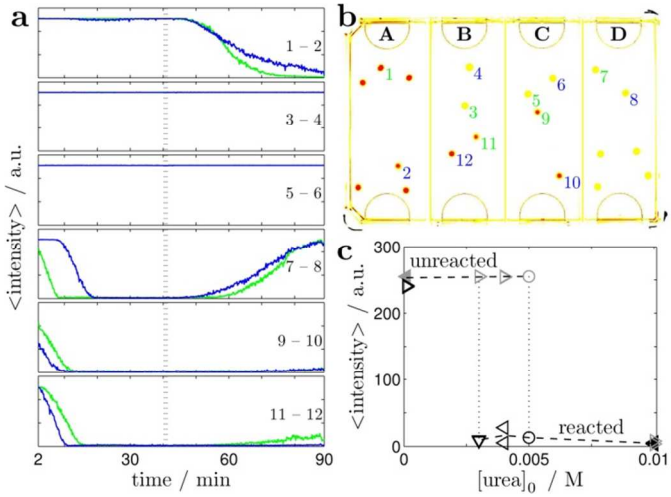

Fig. 3. (a) Average intensity in the centre of a bead (circular area of 1.2 mm^2) in time for alginate beads labelled 1 – 12 in Fig. 2 (a). The dashed line at t = 40 minutes indicates when beads were moved between compartments (b). Image at t = 90 minutes. (c) Bistability plot constructed from average intensity of beads before (t = 42 minutes) and after they were moved (t = 92 minutes) in the experiment shown (triangles) and other experiments (circles) with different concentrations of urea.

The average intensities of the particles at t = 40 and t = 90 minutes were used to construct a bistability plot (Fig. 3c) with two possible states of the beads, low pH or high pH, between [urea] = 0.003 and 0.005 M. The switch in pH was not only sensitive to the concentrations but also the temperature and the preparation method of the beads (see supplementary information). The region of bistability separates the region of no reaction to that of reacted state (Fig. 1a), so small decreases in substrate or enzyme lead to complete collapse of activity. Also, the range of bistability is relatively small in enzyme concentration (up to 3 enzyme units in Fig. 1a) making experimental observation challenging. However, simulations showed that the range increased as the bead size was decreased (up to 10 enzyme units, see supplementary information) thus suggesting that the bistable switch will be more robust in smaller beads.

Conclusions

A bistable switch in pH was obtained in mm-sized urease-loaded alginate beads immersed in urea and acid solution. In the bistable regime, the final pH of the bead was low (unreacted) or high (reacted), depending on the initial state of the bead. A supra-threshold amount of substrate was required for beads to switch to the high pH state. The beads changed colour after a period of time, the clock time, and there was a variation in clock times that may arise from differences in enzyme loading or structure of the gel matrix. These results illustrate that feedback-driven behaviour is possible in enzyme reactions in beads and may have important consequences in the use of enzyme-loaded particles for biotechnological applications and drug delivery.

Acknowledgements

AFT and TB acknowledge grant EPSRC EP/K030574/1 and Marie Curie International Incoming Fellowship (PIIF-GA-2010-274677), FM acknowledges funding from Czech Ministry of Education and Youth and Sports.

Notes and References

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Electronic Supplementary Information (ESI) available: experimental and numerical simulation details. See DOI: 10.1039/c000000x/

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