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A simple visual assay based on small molecule hydrogels for detecting inhibitors of enzymes

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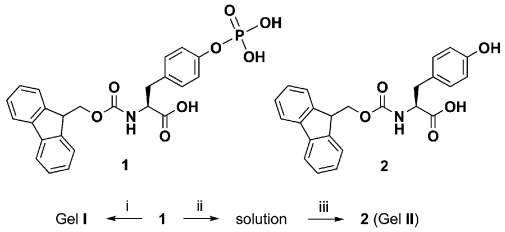
Here we report a simple approach to develop assays based on the hydrogelation of small molecules for quick detecting inhibitors of enzymes.

Enzymes, as a class of highly efficient and specific catalysts, dictate a myriad reactions that constitute various cascades in biological systems.[1] Identifying effective inhibitors of an enzyme is the essential step for the control and applications of enzymatic reactions. Although many useful methods have been used successfully to screen the inhibitors of enzymes, few of them allow the direct visual screening of inhibitors without the assistance of appropriate instruments. Hamachi *et al.* recently reported a semi-wet peptide/protein microarray to screen inhibitors using a small molecule hydrogel, based on the enhanced fluorescence when the fluorophore deposits on the self-assembled fibers that make up the matrices of the hydrogel.[2] Encouraged by this result and other works on the hydrogels[3] and organogels[4] that stem from the self-assembly of small molecules,[5] including our work on enzymatic formation of supramolecular hydrogels,[6] we decided to explore the possibility of using enzymatic hydrogelation directly as a visual assay to screen inhibitors for an enzyme.

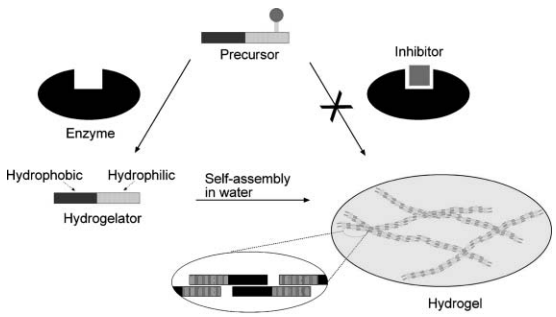
Scheme 1 illustrates the design of the visual assay. The precursor, which acts as the substrate of an enzyme, transforms into a hydrogelator when the enzyme catalyzes its conversion. Then, the self-assembly of the hydrogelators in water induces the formation of hydrogel. When inhibitors competitively bind with the active site of the enzyme and block the conversion of the precursor catalyzed by the enzyme, no hydrogel forms. Therefore, the macroscopic sol–gel transition (which can be observed visually) of the solution of the precursor reports the inactivation of the enzyme by the inhibitors. This approach has a unique feature—it enlists water molecules as part of the report system. In addition, no spectrometer is required for observing the sol–gel phase transition. This simple and inexpensive method should be useful not only for screening the inhibitors, but also for detecting the presence of enzymes when appropriate precursors are used. To verify the feasibility of the design shown in Scheme 1, we use a simple amino acid derivative (**1**), which can be converted into a hydrogelator (**2**) by dephosphorylation, to screen the inhibitors for an acid phosphatase.

As summarized in Scheme 2, we first examined the properties of hydrogelation of **1** and **2**. Heating the suspension of **1** (40 mM) to about 60 °C resulted in a clear solution, and hydrogel I formed after cooling the solution back to room temperature. Adjusting the pH value of hydrogel I to >3.5 or raising the temperature to 47 °C caused the gel–sol phase transition, which was reversible to the changes of pH and temperature. At pH = 6.0, adding 10 μL of an acid phosphatase (50 U of acid phosphatase suspended in 1.0 mL of buffer, buffer: 3.2 M $(NH_4)_2SO_4$, pH = 6.0, stabilized with BSA) to the solution of **1** and keeping it at 37 °C for about 10 minutes resulted in the formation of hydrogel II, which was also thermoreversible (the gel–sol phase transition occurs at ~52 °C). Fig. 1(A) shows the linear viscoelastic frequency sweep response of two hydrogels. Both samples exhibit weak frequency dependence from 0.2 to 100 rad s^{-1} (hydrogel I: $G' \propto (\text{frequency})^{0.11}$; hydrogel II: $G' \propto (\text{frequency})^{0.05}$), with G' dominating G'', indicating that the hydrogels are solid-like and highly elastic. The dynamic storage modulus of hydrogel II is an order of magnitude larger than that of hydrogel I, which indicates that the networks in hydrogel II exist in higher density than that of hydrogel I. This result is also consistent with the morphologies of the hydrogels, as proved by an electron micrograph (Fig. 2). Rheological experiments also determine the proper incubation time for the enzymatic hydrogelation. As shown in Fig. 1(B), hydrogel II starts to form in less than 10 minutes, as indicated by the storage modulus (G') dominating the loss modulus (G''). This enzyme-catalyzed hydrogelation completes in 30 minutes, as indicated by the storage modulus (G') reaching the plateau. ^{1}H NMR indicates that 66% of **1** is converted into **2** at this stage.

As shown in Fig. 2A, the scanning electron micrograph (SEM) of the cryo-dried hydrogel I exhibits entangled irregular fibers (widths from 150 nm to more than 1 μm), which provide the matrices for the hydrogel. Hydrogel II has a different morphology, and its fibers, compared to those in hydrogel I, are more uniform,



Scheme 2 The chemical structures of the molecules for hydrogelation and the schematic gelation process. Conditions of hydrogelation: (i) 40 mM, pH = 2.5; (ii) Na_2CO_3, pH = 6.0; (iii) enzyme in buffer, 37 °C.



Scheme 1 The illustration of the design for identifying inhibitors of an enzyme by hydrogelation.

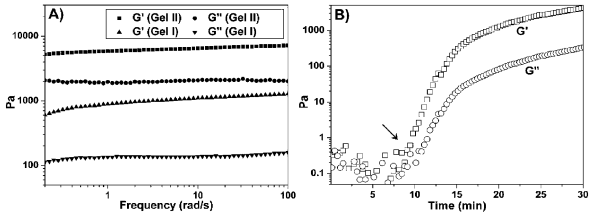


Fig. 1 (A) Frequency dependence of the dynamic storage moduli (G') and the loss moduli (G'') of hydrogels I and II; (B) oscillatory rheology of a solution containing 40 mM of **1** and 10 μL of enzyme solution, pH = 6.0, 37 °C. The arrow indicates the gelation point.

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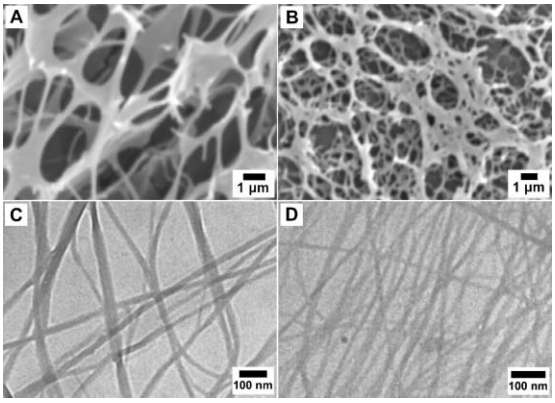

Fig. 2 (A), (B) SEM images and (C), (D) TEM images of hydrogels I and II.

with widths of 200–600 nm (Fig. 2B). Transmission electron micrographs (TEM) reveal that the nanofibrils in both hydrogels I and II have widths of 20–25 nm. As shown in Fig. 2C, these fibrils tend to form big bundles (50–100 nm in width) in hydrogel I, but remain as fine fibrils with a width of 20 nm in hydrogel II. The higher density of the self-assembled nanofibers in hydrogel II than that of hydrogel I also agrees the higher elasticity of hydrogel II.

The circular dichroism (CD) and fluorescence spectra provide some useful information on the molecular arrangements of **1** or **2** in the hydrogels. As shown in Fig. 3A, the Cotton effect at about 200 nm and 240 nm in hydrogels I and II indicates the superhelical arrangements of the amino acid residues, which induce the helical orientation of the fluorenyl groups (the Cotton effects at 250–320 nm) in the hydrogels. By comparing the emission spectra of both hydrogels and their solutions, we infer that the fluorenyl groups provide π–π interactions in several modes. For example, the emission peak of the fluorenyl group at 346.8 nm in solution phase shifts to ~351 nm in hydrogel I, suggesting that the fluorenyl groups of **1** overlap with the phenyl groups. The shoulder at ~380 nm likely originates from the antiparallel dimerization of the fluorenyl group, with the small peak at ~400 nm due to the small amount of the fluorenyl groups overlapping in parallel fashion.[7] In hydrogel II, the fluorenyl group also has an emission peak at 348 nm, which indicates that some of **2** exists in the monomeric form in the gel phase. Hydrogel II has the more pronounced broad peaks centered at 420 and 450 nm, implying that multiple fluorenyl groups aggregate *via* π-stacking. The two sharp peaks at 380 nm and 400 nm are likely caused by the antiparallel and parallel dimerization of fluorenyl moieties in the gel phase.

Since the acid phosphatase catalyzes the conversion of **1** to **2** and leads to hydrogelation at pH = 6.0 and 37 °C, the event of hydrogelation can indicate the activity of inhibitors for the acid phosphatase itself. We chose pamidronate disodium (**3**), Zn^{2+} (**4**), and sodium orthovanadate (Na_3VO_4, **5**) to estimate their minimum inhibition concentrations for the acid phosphatase. We mixed the three compounds first with the enzyme at a series of concentrations, respectively, and then added **1** to the solutions 10 minutes after the mixing. After an additional 30 minutes' incubation, the sol–gel phase transition indicates the minimum inhibition concentration of the compounds. From the changes of rows 2, 3, and 4 in Fig. 4, we observed that the minimum inhibition concentrations of **3**, **4**, and **5** for the acid phosphatase are 33 mM, 0.33 mM, and 3.3 mM,

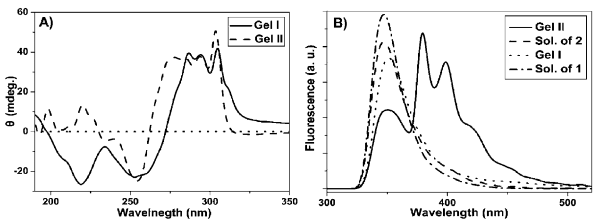

Fig. 3 (A) The CD spectra of hydrogels and (B) the emission spectra (λ_{ex} = 265 nm, slit width = 2.5 nm) of the hydrogels and the solutions of **1** and **2**.

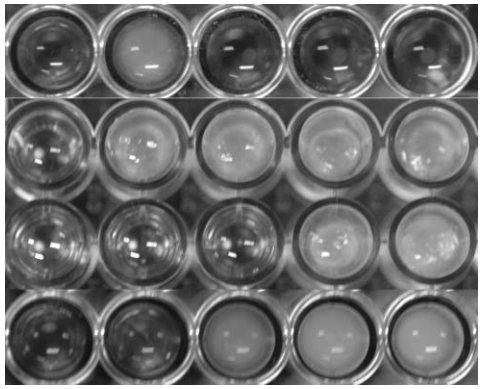

Fig. 4 Results of activities of three inhibitors: row 1) Left to right: sol. of **1**; sol. of **1** and enzyme; sol. of **1** + **3**; sol. of **1** + **4**; and sol. of **1** + **5** ([**3**] = [**4**] = [**5**] = 33 mM); row 2) pamidronate; row 3) Zn^{2+}; and row 4) Na_3VO_4. (Left to right, conc. = 33; 3.3; 0.33; 0.033; 0.0033 mM).

respectively. This result is very close to the literature values for this enzyme,[8] which validates our design.

Compared to the existing colorimetric or fluorescent assays, this system remains to be improved for determining an accurate IC_{50} value of an enzyme. This semi-quantitative assay, however, should be useful for the initial screening of inhibitors. Similar to other assays, this assay also requires that the reporting event (*i.e.* hydrogelation) should not be obstructed by the inhibitors themselves. Although the system investigated in this work is a specific assay for an acid phosphatase, the principle should be able to be extended to other enzymes (*e.g.* peptidases, aldolases, reductases, *etc.*) by designing proper precursors.

In summary, we have developed a simple visual assay for screening the activities of inhibitors for an enzyme (acid phosphatase) based on the hydrogelation of small molecules. This approach can be adapted easily into a parallel assay to allow many inhibitors to be tested efficiently. This is the first time that inhibitors of enzymes have been screened by directly coupling hydrogelation with enzymatic reactions. We believe that this approach will be useful for reporting enzymatic processes for bioanalytical applications.

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