Bacterial Inhibition

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Intracellular Hydrogelation of Small Molecules Inhibits Bacterial Growth**

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Self-assembly, a fundamental process on all scales,[1] not only plays a vital role in nature but also offers a powerful strategy for constructing biomaterials. For example, rationally designed oligopeptide amphiphiles spontaneously form nanofibers which gel biological fluids, potentially leading to a new class of biomaterials with applications in regenerative medicine.[2] Similarly, hydrogels of small organic molecules,[3] the result of hierarchical molecular self-assembly in an aqueous environment, are finding applications in tissue engineering,[4] drug delivery,[5] wound healing,[6] and semiwet protein arrays.[7] In fact, a large variety of small organic molecules are capable of self-assembling in solvents to yield three-dimensional fibrillar nanostructures and form molecular gels.[3,8] A recent mechanistic study[9] has revealed that the formation of such nanofibers in gels, like the occurrence of cellular nanostructures (for example, actin filaments, microtubules, and viral capsids), follows a nucleation-growth mechanism. Such a common feature leads to a fundamental, intriguing, and yet unexplored question: How does a cell respond to the intracellular hydrogelation that results from the self-assembly of small organic molecules?

Forming a hydrogel of small molecules (also called "supramolecular hydrogel") usually requires a physical or chemical perturbation (for example, a change of temperature, pH, or ionic strength) that unavoidably disrupts cellular processes and prevents precise evaluation of intracellular hydrogelation. This difficulty can easily be overcome by enzymatic supramolecular hydrogelation,[10] in which an enzyme catalytically converts a precursor into a hydrogelator and triggers molecular self-assembly. As a new method of making biomaterials, the design and application of enzyme-catalyzed[10,11] or -regulated[12] hydrogelation has already shown promise in screening inhibitors of enzymes,[13] assisting biomineralization,[14] typing bacteria,[15] and developing smart drug-delivery systems.[16] Since it integrates molecular self-assembly in water (hydrogelation) with the wide range of biological processes involving enzymes, enzymatic hydrogelation promises to be a unique method of exploring the hydrogelation of small molecules inside a cell and addressing the intriguing question raised above.

In this study, we used an enzymatic hydrogelation[10] inside *E. coli*, which served to demonstrate the general concept and allowed examination of the cellular response to the intracellular hydrogelation of small molecules. Overexpressed phosphatase in *E. coli* was found to catalyze the formation of the hydrogelator inside the bacterium, and the subsequent intracellular hydrogelation inhibited its growth. This new methodology—enzyme-regulated intracellular self-assembly of small molecules for creating artificial nanostructures and thus controlling the fate of cells—could lead to a new technique for managing cellular processes, understanding cellular functions, and developing new therapeutics at the supramolecular level.

As shown in Figure 1a, the precursor of a hydrogelator enters a cell by a diffusion process. An enzyme then converts the precursor into a hydrogelator that self-assembles into nanofibers (a simple nanostructure). Later, the formation of the nanofibers induces hydrogelation, which changes the viscosity of the cytoplasm and thus stresses the cell.

Implementing the design illustrated in Figure 1a, the hydrogelation of a series of small molecules bearing the general formula $C_{10}H_7CH_2C(O)$-ZZ (where Z is the residue of an amino acid) was evaluated. $C_{10}H_7CH_2C(O)$-L-Phe-L-Phe exhibited the lowest minimum gelation concentration (mgc; ca. 0.4%).[12] This result is consistent with the observation of Gazit and co-workers that the Phe-Phe motif is prone to self-assembly in water.[17] To be accessible to an enzyme, the molecule is phosphorylated at its C terminal with tyrosine phosphate to afford $C_{10}H_7CH_2C(O)$-L-Phe-L-Phe-Tyr-$(PO(OH)_2)$ (**1a**; Figure 1b). A tyrosine phosphatase cleaves the phosphate residue from **1a** and generates the more hydrophobic $C_{10}H_7CH_2C(O)$-L-Phe-L-Phe-Tyr (**2a**), which then self-assembles into nanofibers to form a hydrogel.

It was necessary to first verify that an alkaline phosphatase could convert **1a** into **2a**, lead to the formation of nanofibers, and induce hydrogelation. Adding alkaline phosphatase (700 U mL^{-1}) to a phosphate saline buffer (PBS) solution of **1a** (0.5 wt%, 6.91 mM) resulted in a hydrogel. Rheological tests (Figure 2a) revealed that the hydrogel started to form almost instantly after the phosphatase was added at room temperature, as indicated by the storage modulus (G') dominating the loss modulus (G''). According to HPLC analysis, about 48% of **1a** transformed into **2a** at the gelling point. The transparency of hydrogel **2a** (Figure 2b) suggests that no microcrystalline aggregates formed

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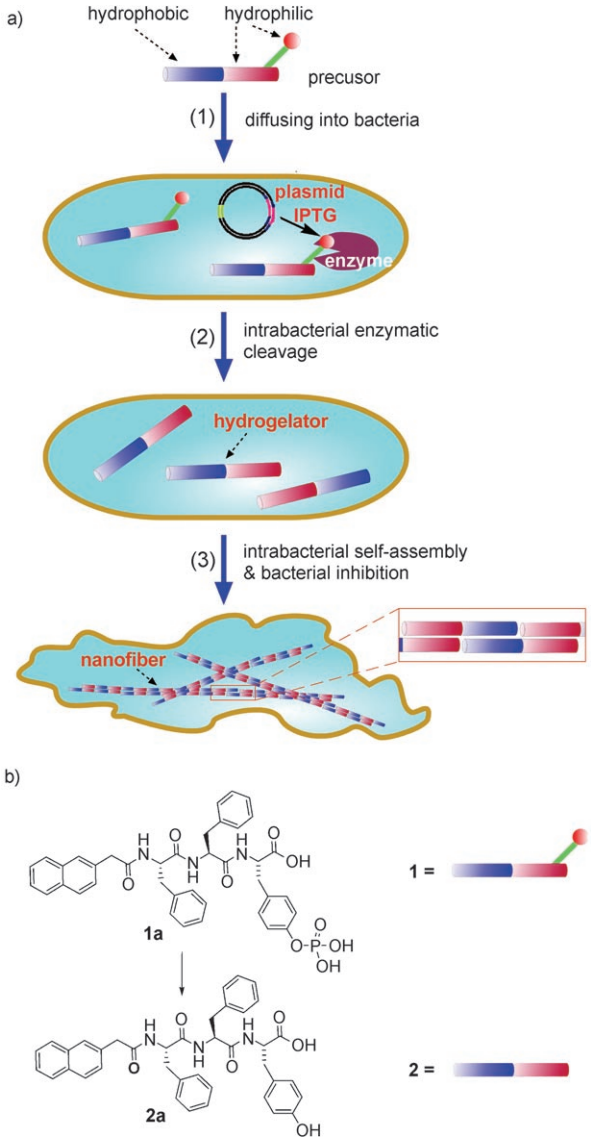

Figure 1. a) Schematic representation of intracellular nanofiber formation leading to hydrogelation and the inhibition of bacterial growth. b) The chemical structures and graphic representations of a typical precursor (**1a**) and the corresponding hydrogelator (**2a**). The structures of isomers of **1a**, $C_{10}H_7CH_2C(O)$-D-Phe-D-Phe-Tyr$(PO(OH)_2)$ (**1b**), and $C_{10}H_7CH_2C(O)$-β^3-HPhg-β^3-HPhg-Tyr$(PO(OH)_2)$ (**1c**; β^3-HPhg: a beta amino acid named β^3-homophenylglycine[21]) are shown in the Supporting Information.

in the hydrogel to scatter visible light. A transmission electron micrograph (TEM) of the hydrogel (Figure 2c) showed that the diameter of the nanofibers formed by the self-assembly of **2a** was about 26 nm. The addition of the phosphatase to solutions of **1a** (Figure 2d) indicated that the mgc of **2a** was between 0.025 and 0.05 wt% in the PBS solution.

To demonstrate intracellular enzymatic hydrogelation, isopropyl-β-D-thiogalactopyranoside (IPTG) and plasmids were used to induce the overexpression of phosphatase according to a literature protocol.[18] After confirming the successful overexpression of phosphatase within the *E. coli* (BL21) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Figure 3a), **1a** (75 $\mu g\,mL^{-1}$, 10.4 μM) was added to the culture medium to generate **2a** intracellularly, leading to the self-assembly and hydrogelation. After incubation for 24 h at 18 °C, the bacteria were harvested, washed three times with PBS buffer, and lysed in dimethyl sulfoxide to dissolve the organic compounds from the cells for HPLC analysis.

The results indicated significant conversion of **1a** into **2a** within the bacteria: The total concentration of **1a** plus **2a** was 230 $\mu g\,mL^{-1}$ within the bacteria (BL21, plasmid +, IPTG +) and 75 $\mu g\,mL^{-1}$ in the culture medium (Figure 3b). The intracellular concentration of **2a** ($[\mathbf{2a}] = 148\ \mu g\,mL^{-1}$) was significantly higher than that in the culture medium ($[\mathbf{2a}] = 15\ \mu g\,mL^{-1}$), confirming not only the successful enzymatic conversion of **1a** into **2a**, but also its accumulation inside the bacteria. The concentration of **2a** inside the cells also agreed with the fact that **2a** is more hydrophobic than **1a**. Once formed inside a cell by the enzymatic reaction, **2a** tended to stay and self-assemble inside the cell. Though lower than the mgc of **2a** (500 $\mu g\,mL^{-1}$) required to gel the PBS buffer solution, the concentration of **2a** inside the *E. coli* (148 $\mu g\,mL^{-1}$) is higher than the mgc (125 $\mu g\,mL^{-1}$) needed for gelling the bacterium's cytoplasm, as determined in a control experiment (see Figure S2 in the Supporting Information). This result indicates that hydrogelation was able to proceed intracellularly.

To further prove that hydrogelation occurred within the cells, bacteria were incubated with **1a**, collected, and lysed by using ultrasound. The resulting suspension formed a supramolecular hydrogel (Figure 3c) whose TEM image also confirmed the presence of nanofibers (Figure 3d). There was no phosphatase overexpression observed when BL21 with plasmids was treated with compound **1a** without IPTG. Further, the absence of **1a** resulted in neither a hydrogel nor nanofiber formation (see Figures S3 and S4 in the Supporting Information). After confirming that Congo red selectively stained the hydrogel of **2a**, Congo red was used to stain the *E. coli* after they had been incubated with **1a** for 24 h. The Congo red could only stain *E. coli* with overexpressed phosphatase and incubation with **1a** (see Figure S5 in the Supporting Information), indicating the formation of a hydrogel of **2a**. These results clearly suggest that the overexpression of phosphatase dictates the formation of nanofibers and of a hydrogel of **2a** within the bacteria.

After confirming that hydrogelation was taking place inside the bacteria, **1a** was tested at various concentrations to examine the effective concentration required for bacteria inhibition. As shown in Table 1, **1a** exhibited an IC_{50} value of 2.77 μM (20 $\mu g\,mL^{-1}$) against the bacteria that overexpressed phosphatase. This value was more than 100-fold lower than the IC_{50} value of **1a** (greater than 2000 $\mu g\,mL^{-1}$, 277 μM) against the bacteria without the overexpression of phosphatase. In a control experiment, **1a** showed no inhibition effects ($IC_{50} > 2000\ \mu g\,mL^{-1}$, 277 μM) on the bacteria (BL21 with plasmid or without plasmid) in the absence of IPTG, thus confirming that the up-regulation of the enzymes is critical for the formation of hydrogelators and the inhibition of bacterial growth. In addition, HPLC analysis showed that the concentrations of **1a** (75 $\mu g\,mL^{-1}$) and **2a** (3 $\mu g\,mL^{-1}$) inside the

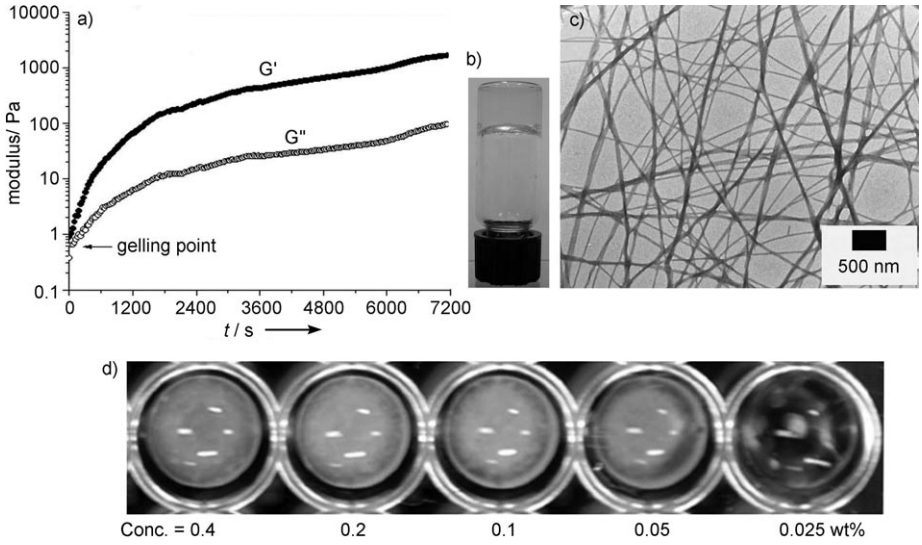

Figure 2. a) Oscillatory rheology of a PBS buffer solution containing 6.91 mM (0.5 wt%, 5000 $\mu g\,mL^{-1}$) of **1a** and 10 μL of enzyme solution, pH 7.4, 25 °C; b) optical and c) TEM images of the hydrogel formed by **2a** through enzymatic gelation in PBS buffer solution (0.5 wt%); d) enzymatic conversion of **1a** into **2a** with the addition of alkaline phosphatase (700 $U\,mL^{-1}$) to PBS buffer solutions containing different concentrations of **1a**.

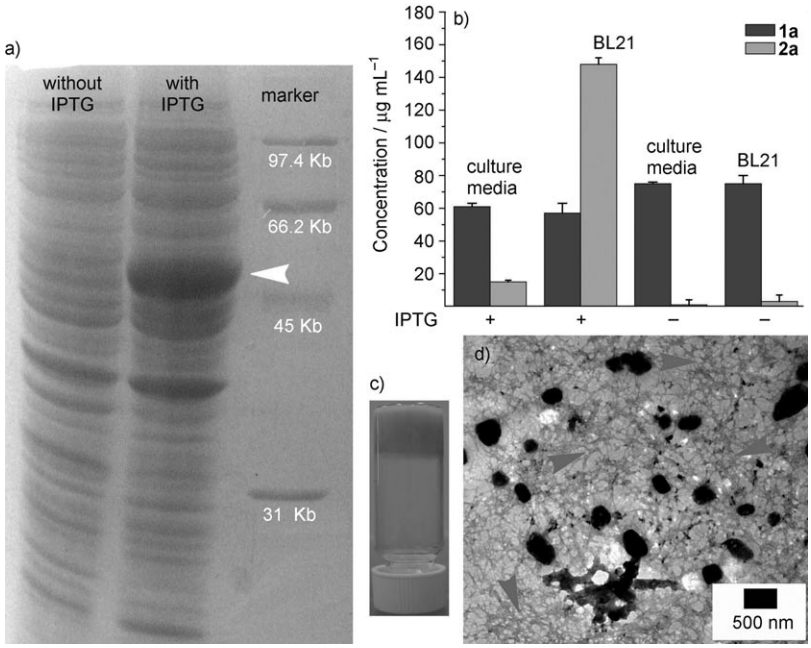

Figure 3. a) SDS-PAGE distribution of cell lysates of *E. coli* BL21 to prove the expression of phosphatase (indicated by the arrow); b) concentrations of **1a** and **2a** in the culture medium and within the cells (BL21, plasmid+, IPTG+ or IPTG-); c) optical and d) TEM images of the hydrogel formed inside the bacteria after culturing with **1a** for 24 h (arrows indicate the nanofibers formed by **2a**).

Table 1: IC_{50} values of different precursors of hydrogelators against *E. coli* with/without overexpression of phosphatase (expression of phosphatase controlled by the addition of plasmids and IPTG, Y(p) = tyrosine phosphate).

E. coli			IC_{50} ($\mu g\,mL^{-1}$)		
Plasmid	IPTG	Overexpression of phosphatase	**1a** Nap-FFY(p)	**1b** Nap-DFDFY(p)	**1c** Nap-β^3-HPhg-β^3-HPhgY(p)
+	+	yes	20	20	20
+	–	no	> 2000	> 2000	> 2000
–	+	no	> 2000	> 2000	> 2000

E. coli (BL21, plasmid+, IPTG–) without the overexpression of phosphatase were almost same as those in the culture medium ([**1a**] = 75 $\mu g\,mL^{-1}$ and [**2a**] = 1 $\mu g\,mL^{-1}$), suggesting that the phosphatase-catalyzed accumulation of **2a** within the bacteria was critical for the inhibition. Moreover, the structural variants of **1a**, precursors **1b** and **1c**, which formed hydrogels upon the addition of the phosphatase,[16] exhibited inhibitory effects (Table 1, $IC_{50} < 2.77$ μM) similar to that of **1a** against the *E. coli* that overexpressed phosphatase. Because of their different stereo- and regiochemical structures, the inhibitory properties of **1a**, **1b**, and **1c** could not be ascribed to a specific ligand–receptor interaction. The results confirm that the formation of supramolecular nanofibers and hydrogelation within the bacteria inhibit bacterial growth.

These experiments have demonstrated for the first time that intracellular enzymatic formation of supramolecular nanostructures can control the fate of cells (for example, inhibiting bacterial growth or regulating the cell death of mammalian cells[19]). Unlike hydrogelation triggered by enzymatic cross-linking polymers,[20] enzymatic supramolecular hydrogelation should be suitable for probing intracellular processes, since small molecules enter cells more easily than polymers. In these experiments, inhibition was observed only in bacetria overexpressing phosphatase, suggesting that the up-regulated expression or activity of an enzyme may confer the specificity needed for further applications. In addition, it may be possible to design a substrate that is susceptible to multiple enzymes to achieve more sophisticated control (for example, both spatial and temporal control) over the formation of supramolecular hydrogels inside cells. In essence, the target of intracellular hydrogelation is water in the cytoplasm, thus allowing the inhibition of multiple cellular processes (or pathways) at the same

time. Therefore, the principle and strategy demonstrated in this work may ultimately lead to a new type of therapeutic agent based on kinetics of enzymatic reactions rather than specific and tight ligand–receptor binding.

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