Molecular Syringe for Cargo Photorelease: Red-Light-Triggered Supramolecular Hydrogel

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Photochomatic supramolecular hydrogels are versatile materials that show macroscopic effects upon irradiation, like liquefaction or shape changes. Here, we demonstrate a simple photochromic cyclic dipeptide (2,5-diketopiperazine-based) supergelator, composed of (S)-lysine and an azobenzene analogue of phenylalanine, that forms supramolecular hydrogels even at 0.1 wt% loading. The gels can physically encapsulate cargo molecules and release them to the environment in a controllable manner upon irradiation with red light, thus working as a “molecular syringe”. As the material is biocompatible and operational in the “therapeutic window” of light (>650 nm) that deeply penetrates soft human tissues, it is applicable to smart drug-delivery systems.

Phototriggered smart materials[1] hold increased promise for the delivery of therapeutic compounds[2] or solar-thermal energy storage.[3] For applications in a biological context, it is optimal to work with visible-light-responsive systems.[4] The most suitable range (650-900 nm) is called the “therapeutic window”, as negligible absorption of these frequencies by hemoglobin and tissues enables deep penetration of soft human tissues.[5]

An increasing collection of molecular photoswitches operating in that range of excitation frequencies comprises tetra-ortho-substituted azobenzenes bearing alkoxy groups or halogen atoms,[6] as well as heterodiazocines,[7] dihydroxybenzenes,[8] indigoids,[9] arydehydrazones,[10] or imidazole-based systems.[11]

Here, we decided to combine the tetra-ortho-chloroazobenzene photoswitch with a simple hydrogelator motif[12] based on cyclic dipeptides (CDPs).[13] Previously,[14] we demonstrated efficient and reversible photodissipation of similar azobenzene-decorated CDPs with UV or green light, and their applicability for encapsulation and light-controlled release of drugs and biopolymers. Now, we developed a red-light-triggered “molecular syringe” based on the CDP 1, which ejects previously encapsulated cargo in response to red light (Figure 1).

To synthesize the tetra-ortho-chloroazobenzene chromophore in 1 we applied the late-stage chlorination strategy,[15] which – along the addition of organolithium reagents to diazonium salts[16] – is among the most efficient synthetic strategies for these sterically hindered azobenzenes. Briefly, (S)-phenylalanine (Phe-OH) was nitrated in the para position, N-acetylated, and the nitro group subsequently reduced to amine with H₂ on Pd/C. The product 4 was subjected to Mills reaction with nitrosobenzene, yielding the azobenzene amino acid 5 in multigram scale (Scheme S1 in the Supporting Information). The subsequent key step – tetra-ortho chlorination of 5 (Scheme 1) – was performed in a sealed tube, 20°C above the boiling point of acetic acid, with excess of NCS resulting in 6 (54% yield) on a gram scale. Our previous attempts to use the unprotected version of 5 or its N-Boc analogue as substrates were unsuccessful. Next, the acetyl group was removed from 6 under acidic conditions (reflux in 6 M aq. HCl for 24 h). The precipitated product 7 was unfortunately fully racemized, as confirmed by chiral RP HPLC (Figure S22). The racemic 7 was

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Figure 1. The hydrogel (orange) formed from 1 (here 0.3 wt%) in aqueous buffer can physically encapsulate cargo substances (yellow) and act as a molecular syringe. Upon irradiation with biocompatible red light (660 nm), the buffer with cargo is ejected from the gel, while in darkness the composition remains stable.
Boc-protected to 8, then coupled with a commercial Boc-protected (S)-lysine derivative H-Lys(Boc)—OMe·HCl, yielding a mixture of diastereomeric dipeptides 9a and 9b. Then, the Boc protecting groups were removed quantitatively, resulting in crude 10, which was directly subjected to the final cyclization that ultimately yielded the diastereomeric mixture (denoted “1”) of CDPs (Scheme 1). The final diastereomeric ratio of 7 : 3 in precipitate isolated after the reaction (Figure S23) indicates different cyclization rate or solubility of the diastereomers in the reaction medium. Part of the mixture 1 has been separated by preparative RP-HPLC onto the more polar major product 1a, and the less polar diastereomer 1b (minor component), which were characterized separately. Most meaningful differences were the chemical shift of the hydrogen atom located at the stereocenter in the 1H NMR spectroscopy – 1a (3.81 ppm) and 1b (3.65 ppm; Figure S30), as well as the C=O stretching vibration of the conjugated ketones in the IR spectroscopy (Figure S31).

Unequivocal assignment of 1a and 1b to the respective configuration was not possible so far. The overall low solubility in volatile solvents hampered the approaches to grow crystals. And slow evaporation of solutions in mixtures of water and acetone resulted in amorphous material, which overall prevented X-ray structure determination.

Photochromism of 1a and 1b (Figures S1 and S2) was similar to other known tetra-ortho-chloroazobenzene derivatives.[8a–d,16–17] Even though the molar extinction coefficient \( \epsilon_{365 \text{ nm}} \) is below 10 M\(^{-1}\) cm\(^{-1}\), red light (660 nm) irradiation resulted in the strongest shift of the molar absorptivity (Figures S3 and S4). The ratio of photoisomers at the respective position of the conjugated ketones in the DKP ring in the IR spectroscopy (Figure S31).

Fluoro derivatives, but higher than the fluorinated azobenzenes with extended \( \pi \)-electron system triggered with red light.[18]

We also investigated biological stability of our gelator 1 (0.1 mM) using standard conditions that mimic intracellular reducing potential with 10 mM reduced glutathione and 5 mM TCEP in PBS buffer pH 7.4 with \( 5\% \) (v/v) DMSO at 25 or 37°C. We did not observe any significant degradation over 24 h (Figure S8).

Next, the crude “1” (diastereomeric mixture of 1a/1b 7:3, produced in the cyclization reaction) was suspended in PBS buffer (phosphate-buffered saline, pH 7.4; Table S3) and boiled for a short period of time, which resulted in homogenous hydrogels at the decreasing concentrations from 2.0 wt% until 0.1 wt%, stable upon via inversion (Figure 2a). 0.1 wt% is more than ten times lower than the critical gelation concentration of the fluorinated analogue.[19] Further decrease of concentration to 0.05 wt% resulted in mechanically unstable viscous material. Hydrogels prepared in Ringer’s solution (0.1–0.3 wt%) were generally less stable (Table S4) and therefore disregarded in further investigation. Hydrogels prepared in PBS buffer at the concentration of 0.3 wt% of 1 or higher were stable in a boiling water bath, while at lower concentration upon increasing temperature the gel shrank and released slightly colored liquid (Figure 2b). This process was reversible by heating the samples over the boiling point in a closed vessel.

Recombination of the HPLC-purified diastereomers to the originally isolated ratio of 7:3 (1a:1b) resulted in stable gel formation at the concentration of 0.1 wt% and higher, with similar shrinking behavior observed upon heating. Equimolar mixture of 1a and 1b (1:1) formed stable gels at 0.2 wt%.

Pure 1a formed gels at 0.2 wt% and shrinking was observed starting from 90°C. To obtain a material with comparable behavior, 0.3 wt% of pure 1b were necessary (Table S5, all samples in PBS buffer). Thus, we conclude that both individual diastereomers 1a and 1b are efficient super-hydrogels, but their combination at the 7:3 ratio (“1”) shows at least similar, if not better, properties. The thermal half-life at 60°C in AcOH is 4.39 h for (Z)-1a and 5.07 h for (Z)-1b (Figures S5–S7) – lower than for simple tetra-ortho-fluoro derivatives,[18] but higher than the fluorinated azobenzenes with extended \( \pi \)-electron system triggered with red light.[18]

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not slightly superior gelating behavior, and therefore isomer separation is not critical for further experiments.

Irradiation of all the aforementioned gel samples with red light resulted in shrinking (Figure 3 sample b) similar to the reaction upon exposure to heat (Figure 2b). Further irradiation with blue light (455 nm) restored the majority of (E)-1, but it did not reconstitute the original gel (Figure 3 sample c). The ejected liquid remained unabsorbed. The closed vial was then warmed up until boiling for short time and cooled down again. This yielded a stable gel that absorbed all previously expelled liquid, with all parameters comparable to the original non-irradiated sample.

In comparison with relatively flat fluorinated or unsubstituted (E)-azobenzene chromophores,[14a,b,19] the sterically hindered ortho-chlorinated (E)-azobenzene shows a twisted structure that can assume three conformations in crystals.[6a]

To exclude the photothermal effect, another gel sample (0.1 wt%) was wrapped in aluminium foil and irradiated for 90 min with 660 nm under the same conditions. There, we observed no ejection of the liquid. We postulate that the photoinduced shrinking effect and ejection of the aqueous buffer is caused by conformational changes resulting from E→Z photoisomerization with red light. Yet, the E isomer restored with blue light might assume another sterically less favorable conformation within the hydrogel network — in comparison with the E isomer obtained upon thermal equilibration. Therefore, only boiling in a closed vessel restores the original gel constitution.

Mechanical stability of the hydrogels formed from 1 (1a and 1b at the 7:3 ratio) was assessed by rheological measurements at the concentrations of 1.0 and 0.3 wt% of the gelator in PBS buffer pH 7.4. The values of $G'$ and $G''$ were, respectively, $6 \times 10^3$ and $7 \times 10^3$ Pa for 1.0 wt% (tan $\delta \approx G''/G' = 0.10$ at 1.14 Hz; Figure S12) or $1.3 \times 10^3$ and $2.5 \times 10^3$ Pa for 0.3 wt% (tan $\delta \approx G''/G' = 0.14$ at 1.14 Hz; Figure S11). Thermal stability was also conferred (Figure S13). The results indicate that the compound 1 forms proper hydrogels with mechanical stability comparable (yet, at the significantly lower critical gelation concentration) to other supramolecular hydrogels developed by our group.[14,19]

Scanning electron microscopy (SEM) revealed porous networks (Figure 4a) with density decreasing proportionally to the initial gelator concentration (Figures S14–S16). At the lowest concentration achieved for stable hydrogel, the observed porous structures were only rudimentary (Figure S14). We observed similar structures previously.[20]

Transmission electron microscopy (TEM) images show fibrous networks comparable to other hydrogelators investigated in our group (Figure 4b, Figure S19).[14a,b,19] Significant morphology changes have been perceived upon exposure of the samples on heat or on 660 nm irradiation, however the fiber network is conserved (Figures S17 and S18). It corroborates with lack of the complete light- or heat-induced gel dissolution to fluid — the process that was previously observed for non-halogenated and ortho-fluorinated LMWGs of similar design.[14,19]

Drug delivery is the desired application for stimuli-responsive soft materials. So far, upon irradiation of photochromic hydrogels produced in our group,[14,19] the complete liquefaction of the material occurred concomitant cargo release. Here we demonstrate release of cargo previously encapsulated in a hydrogel made of 1 upon its light-induced shrinking. The cargo — a fluorescent dye 5(6)-carboxyfluorescein (2) – has been added (50 μg of 2 per vial) to a hydrogel formed from 0.2 wt% of 1 to induce the release of 2. Vials were mounted upside down in an irradiation chamber and irradiated at 660 nm until the gel visibly reduced to 25–50% of its former size and fluorescent liquid was released (Figure S10a). The liquid accumulated at the bottom of the vial, while the shrunk gel remained attached to its top surface (Figure 5). We quantified (Figure S9 and Table S6) the amount of 2 released to the non-viscous liquid at the bottom of vials — after 2.5 h, we obtained 17 μg (30 %) of 2, and after 4 h: between 29 (59 %) and 35 μg (66 %) of 2 in two independent experiments. To exclude the photothermal effect, another gel sample was wrapped in aluminium foil and irradiated for 4 h under the same conditions without expelling the liquid (Figure S10d).

This process is depicted schematically in Figure 6. We hypothesize, that the hydrogel fibers depicted on Figures S17–S19 are stabilized by the hydrogen bonding network. In the dark state (Figure 6a), larger distance between the (E)-azobenzene fragments might enable accommodation of water and cargo molecules. The irradiation turns most of the fragments into more sterically demanding Z isomer (Figure 6b), which

Figure 3. Hydrogel “a” formed from an equivalent mixture of diastereomers 1a and 1b (total conc. 0.1 wt%) was irradiated with red light (660 nm, 90 min) (sample “b”) causing gel shrinking with liquid ejection that was, however, not reversible upon irradiation with blue light (455 nm; sample “c”), even though the majority of the E isomer of 1 was restored under these conditions.

Figure 4. Electron microscopy imaging of the hydrogel samples prepared from 1 in PBS buffer at pH 7.4. a) SEM image of a xerogel composed of 1.0 wt% of 1; scale bar: 250 μm. b) TEM image of a hydrogel composed of 0.1 wt% of 1; scale bar: 3 μm.
results in ejection of the cargo and water from the fiber network.

Additional reasons for the observed macroscopic effect can be, say, light-induced polarity changes, or photomodulation of the helical pitch inside the supramolecular fibers.

Finally, we determined the cytotoxicity of 1 using cell viability assays (MTT assays). Human cancer cell line (HeLa) was treated with increasing concentrations of the gelator 1 (the 7:3 mixture), as well as each diastereomer 1a and 1b separately, to determine the respective IC\(_{50}\) values. In each case, we have investigated the pure \(\epsilon\) isomers that form hydrogels in aqueous solutions, as well as the mixtures obtained upon irradiation (60 min) of the respective stock solutions with red light (660 nm). In all cases (Tables S7–S9), the IC\(_{50}\) values were around or above 100 \(\mu\)M, indicating negligible toxicity (Figure S20) – apart from 1b, where they oscillated around 10 \(\mu\)M (Figure S21).

To conclude, we have demonstrated synthesis and characterization of a new red-light-responsive supramolecular low-MW hydrogelator 1 based on a cyclic dipeptide bearing ortho-chlorinated azobenzene as the light-sensitive component. The hydrogelator undergoes efficient photoisomerization upon irradiation with 660 nm light, and thus is compatible with in vivo biological applications.\(^{[5]}\) 1 formed mechanically stable hydrogels under physiologically relevant conditions (PBS buffer, pH 7.4) at exceptionally low concentrations (significantly below 1 wt%) – both as separated diastereomers, and as their mixture resulting directly from the synthetic process. Irradiation of the hydrogels with red light (660 nm) caused shrinking of the material with concomitant release of the aqueous buffer.

When the hydrogel has been quantitatively pre-loaded with fluorescent cargo (2), majority (up to 66\%) of the guest was released to the aqueous solution upon red light irradiation, whereas in darkness the cargo remained firmly encapsulated inside the gel. The cytotoxicity of our material is generally low, and it demonstrated stability in reducing environment. Thus, the demonstrated “molecular syringe” is a promising system for light-induced drug release.

In the future, we will investigate techniques of injectable micro/nanogel formation from 1 and use bioactive cargo (anticancer or antimicrobial agents) in order to explore the therapeutic applications. Furthermore, we will explore the possibility of enantioselective synthesis for the photochromic amino acid 6, as then it may find broader application for synthesis of photoresponsive peptides using solid-phase techniques.

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**Conflict of Interests**

The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available in the supplementary material of this article.

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Red-y, steady, drop: Molecular syringe is a peptide-derived photochromic hydrogel that can be loaded with cargo and then release the guest when biocompatible red light illuminates it under physiological conditions.