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A Self-Assembled Delivery Platform with Post-production Tunable Release Rate

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Supporting Information

ABSTRACT: Self-assembly of three molecular components results in a delivery platform, the release rate of which can be tuned after its production. A fluorophore-conjugated gelator can be hydrolyzed by an enzyme, resulting in the release of a fluorescent small molecule. To allow the release to be tunable, the enzyme is entrapped in liposomes and can be liberated by heating the system for a short period. Crucially, the heating time determines the amount of enzyme liberated; with that, the release rate can be tuned by the time of heating.

External control over catalyst activity could play a major role in the development of responsive materials and controlled release applications. Recent developments in soft matter self-assembly show several opportunities for application in medicine.[1] For instance, self-assembling peptide amphiphiles have been successfully applied in regenerative medicine,[2] and thermosensitive liposomes can be used for localized[3] or targeted[4] drug delivery as well as other applications.[5] Furthermore, nanotechnology has been exploited to tune drug release rates from polyelectrolyte films,[6] gels,[7] block-copolymer micelles,[8] and vesicles.[9] In the latter examples, controlled release comes from the intrinsic properties of the material or the method of preparation. As a result, the release rate cannot be tuned after the preparation of the delivery platform. It remains a challenge to develop a delivery system, the release rate of which can be tuned after its production. Such a material would be useful for applications in personalized medicine and could potentially lead to the development of customizable medications where the end user, either a health-care professional or the patient, can tune the drug release rate as needed. Likewise, this concept has potential for the development of implants where the drug release rate can be set externally, or in materials science for controlling the reactivity of multicomponent adhesives.

In this work we show that multicomponent self-assembly[10] can be exploited to develop a delivery platform where the release rate can be tuned, after production and shortly before use, to the user's needs. In the platform described in this paper, controlled heating of the platform liberates an enzyme into the system, which hydrolyzes a gelator and thereby releases detectable small molecules from the scaffold. The amount of liberated enzyme determines the small-molecule release rate and, crucially, can be controlled by the length of the heating period.

To develop a model delivery platform with a tunable release rate, we designed a system comprised of three major components: a hydrogelator, a proteolytic enzyme, and a phospholipid. The low-molecular-weight hydrogelator **1**,[11] based on a cyclohexane-tris-amide core,[12] is covalently connected to the fluorogenic molecule 6-aminoquinoline (6-AQ) via an enzymatically hydrolyzable amide linker (Figure 1a). The excitation wavelength of 6-AQ shifts drastically when the amide is hydrolyzed to an amine, which makes it highly suitable for spectroscopic release studies.[13] Above its critical gelation concentration (cgc) of 1.9 mM, gelator **1** assembles into fibrous aggregates, forming a hydrogel and thereby immobilizing the gelator-conjugated 6-AQ in the material. Gelator **1** can be hydrolyzed by the protease α-chymotrypsin (α-chy), resulting in the release of the small molecule 6-AQ.[14]

In order to tune the release rate after production of the gel, the α-chy should initially be isolated from **1** in a separate compartment. Gelator **1** and liposome-forming lipids can self-assemble orthogonally within a single system, with each preserving its own characteristics.[10a] Thus, loading a gel of **1** with liposomes that contain α-chy in their inner aqueous compartment physically isolates the enzyme and its substrate, gelator **1** (Figure 1b). In such a system the triggered liberation of α-chy from its compartment will initiate the hydrolysis of **1** and thus the release of the fluorescent 6-AQ. The liberation of liposomal content can be brought about by a series of triggers, such as heating the liposomes to their phase transition temperature (T_m),[15] changing the pH,[16] using an external magnetic field,[17] adding co-surfactants such as Triton-X,[18] or stimulating with ultrasound.[19] In the latter examples, controlled release comes from the intrinsic properties of the material or the method of preparation. As a result, the release rate cannot be tuned after the preparation of the delivery platform. Examples of platforms, the drug release rate of which can be tuned post-productionally do exist, but they often require permanent changes in the environment of the platform, such as the concentration of plasticizer[20] or the pH.[21] Here we used thermosensitive liposomes to encapsulate α-chy (Figure 1b). We postulated that controlled heating of the samples would liberate α-chy into the gel matrix, after which hydrolysis of **1** by the liberated enzyme can take place, also at lower temperatures ($T < T_m$). The amount of liberated enzyme, and thus the small-molecule release rate, can be controlled by the heating time. Interestingly, the presence of the fibrillar network of **1** may

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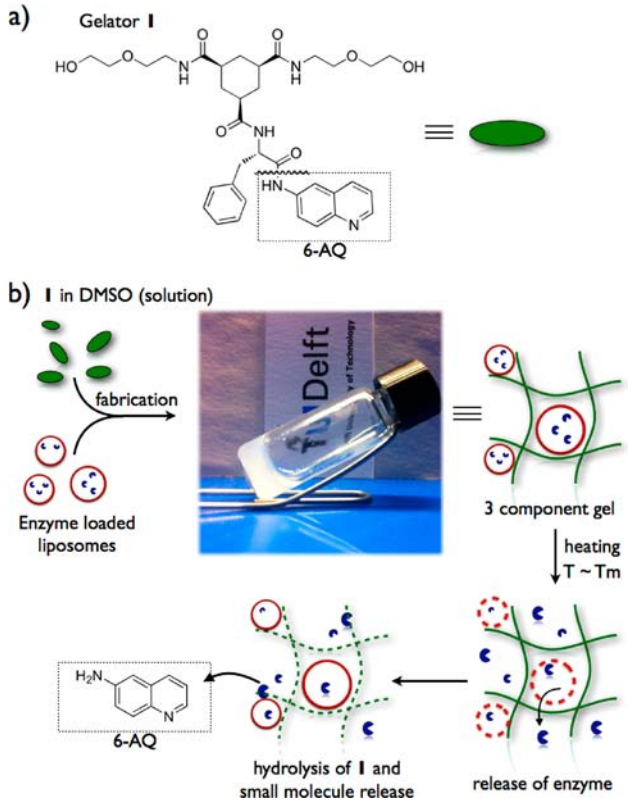

Figure 1. (a) Structure of gelator **1** bearing fluorogenic 6-AQ. α-Chymotrypsin can selectively hydrolyze the amide bond between L-phenylalanine and 6-AQ, resulting in release of the fluorescent molecule. (b) Self-assembled multicomponent controlled-release system: a solution of **1** (green) in DMSO is added to an aqueous solution of the enzyme α-chy (blue) loaded in liposomes (red), resulting in the formation of a gel network with embedded liposomes. Briefly heating the gel at the T_m of the liposomes results in partial liberation of the enzyme into the gel matrix, allowing for release of 6-AQ through enzymatic hydrolysis of the amide bond. The heating time dictates the amount of enzyme liberated and thus the small-molecule release rate.

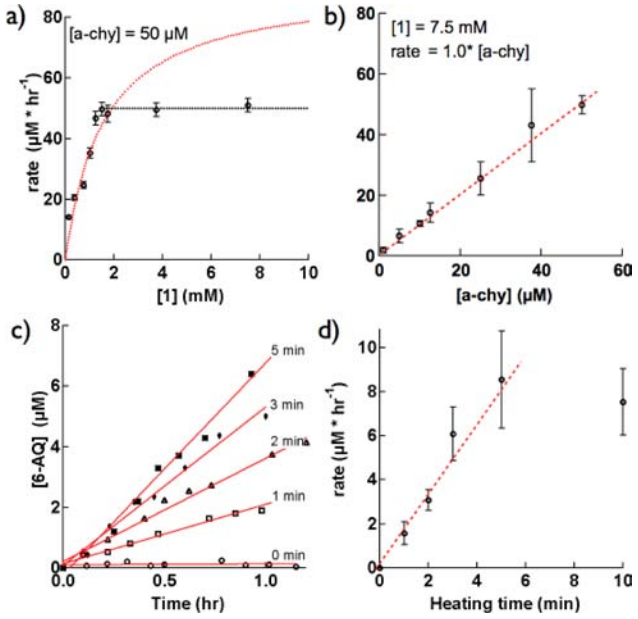

Figure 2. (a) Rate of hydrolysis of **1** as a function of substrate concentration. The rate of hydrolysis remains constant above 1.5 mM **1** at a rate of 50 μM h^{-1}. Black line, added to guide the eye; red line: theoretical Michaelis–Menten curve calculated from K_m and V_{max}. (b) Rate of hydrolysis of **1** as a function of enzyme concentration. The rate of hydrolysis is first-order in α-chy, with rate constant k = 1.0 h^{-1}. (c) Concentration of 6-AQ in gels against time for different heating times (indicated in minutes at each data set) (black markers) and their linear fit (red lines). (d) 6-AQ release rate, derived from (c) and its duplicate experiment, against heating time (black markers). Red line added to guide the eye. Error bars: standard deviation over two individual experiments. All amide hydrolysis experiments were performed at 25 °C.

stabilize the enzyme-loaded liposomes, thereby increasing the stability (and thus the shelf life) of the platform.

We first investigated the kinetics of the enzymatic cleavage of **1** by α-chy using fluorescence spectroscopy. In line with previous results,[14] addition of 50 μM α-chy to a gel of **1** resulted in the release of 6-AQ. This reaction follows Michaelis–Menten kinetics as long as the substrate concentration ([**1**]) remains below 1.5 mM (Figure 2a). Above 1.5 mM, a value that nicely agrees with the cgc of **1**, the hydrolysis remains zeroth-order in substrate **1**, and the rate (V_h) remains constant at 50 ± 5 μM h^{-1} with increasing concentration of **1**. All further experiments were done in the concentration regime [**1**] = 7.5 mM. We also confirmed that, at substrate concentrations above the cgc, the rate of hydrolysis is linearly proportional to the concentration of α-chy, with a first-order rate constant of 1.0 h^{-1} (Figure 2b). This linear relationship between the hydrolysis of **1** and the concentration of α-chy allows us to tune the small-molecule release rate by controlling the concentration of substrate-accessible enzyme.

Next, we synthesized the controlled-release platform as depicted in Figure 1. Thermosensitive liposomes, designed to have a phase transition temperature of 42 °C,[3a,22] were prepared and loaded with 1.6 mM α-chy in their interior (see Supporting Information for details). Addition of these enzyme-loaded liposomes to a concentrated solution of gelator **1** in DMSO resulted in the rapid formation of turbid gels (Figure 3a), typically within seconds.

To liberate the enzyme from the liposomes, and thus initiate the release of 6-AQ, the gels containing the enzyme-loaded liposomes were heated for 5 min at 42 °C,[23] which matches the phase transition temperature of the liposomes (vide infra).[3a] After heating, the gels were cooled to room temperature, stabilized for 30 min, and studied by fluorescence spectroscopy. The release rate of 6-AQ was found to be 8.6 μM h^{-1} (Figure 2c,d). From the previously found k value (1.0 h^{-1}), it can be calculated that the concentration of released α-chy is 8.6 μM. Heating the gels for longer times did not result in higher release rates, but instead decreased the release rate. From this observation we conclude that, after 5 min, all the available enzyme has been released, and heating for longer than 10 min most likely results in autodigestion of the enzyme.[23] Crucially, if the gels were heated for less than 5 min, the observed 6-AQ release rates were lower (Figure 2c). For instance, heating a gel for 1 min resulted in a release rate of 1.6 μM h^{-1}. Moreover, between 0 and 5 min of heating, the release rate increased linearly with heating time (Figure 2d). These results clearly show that it is possible to set the small-molecule release rate to any required value between 0 and 8.6 μM h^{-1} by heating the system for a predetermined amount of time. Thus, the orthogonal self-assembly of a molecular hydrogelator with liposomes containing enzymes has been successfully exploited

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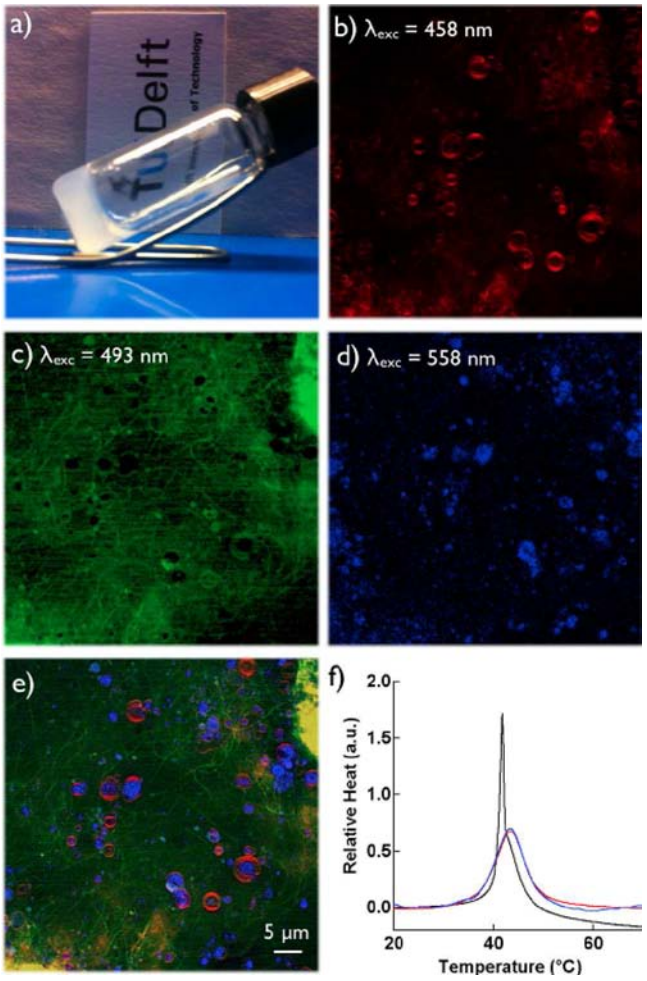

Figure 3. (a) Photograph of a gel of **1** embedded with enzyme-loaded liposomes. (b) Confocal laser scanning micrograph of α-chy entrapped in giant multilamellar liposomes embedded in a gel matrix. Micrographs are taken with an excitation wavelength (λ_{exc}) of (b) 458, (c) 493, and (d) 558 nm for the lipid probe, probe for **1**, and probe for α-chy, respectively. (e) An overlay of all three micrographs. (f) DSC traces of liposomes (black), enzyme-loaded liposomes (red), and enzyme-loaded liposomes in a gel of **1** (blue).

to control catalyst activity through addressable compartmentalization. Coupling the catalytic activity to the release of a detectable small molecule shows that such a platform could be applied to tunable drug delivery systems in the future.

The previous results showed that no significant amount of 6-AQ was released after preparation of the gels, which is a good indication that the enzyme remains entrapped in the liposomes during preparation of the gel. We observed, however, that freshly prepared gels made using a solution of 4-day-old liposomes showed a small but significant release of 6-AQ (see Supporting Information). Further experiments showed that the 6-AQ release rate of the gels increased with the age of the liposome solution, suggesting that α-chy-loaded liposomes in solution tend to be unstable and will partially leak their contents over time. In contrast, freshly prepared liposomes entrapped in a gel network of **1** did not show any significant release of 6-AQ, even after standing for 2 weeks, indicating that the fibrillar network of **1** serves as a stabilizing matrix for the liposomes, potentially increasing the shelf life of these and other liposome-based materials and drug formulations.

Confocal laser scanning microscopy (CLSM) was used to further investigate the structure of the self-assembled multicomponent platform and to corroborate the release mechanism proposed above. For this study, fluorescent probe-labeled versions of the three components (gelator, fluorescein; lipid, nitrobenzoxadiazole (NBD); enzyme, rhodamine) were used (see Supporting Information for details). To ensure the visibility of the liposomes, giant multilamellar liposomes were prepared, rather than the 100 nm liposomes used in all other studies. Excitation of the lipids at 458 nm clearly shows the presence of giant multilamellar liposomes (Figure 3b). Excitation of the probe for **1** reveals the presence of fibers, and excitation of the probe for α-chy at 558 nm shows localized domains of higher enzyme concentration (Figure 3c,d). Overlaying these images clearly shows the successful encapsulation of the α-chy by the liposomes (Figure 3e). Also, CLSM confirms that the presence of neither **1** nor the enzyme visually interferes with the liposomes. Vice versa, the presence of the enzyme-loaded liposomes does not prevent gelator **1** from forming fibers, confirming that self-assembly of **1** and the liposomes takes place orthogonally. Finally, CLSM shows that all observed liposomes are filled with α-chy, which confirms that spontaneous leaking of liposomal content does not take place to any significant extent.

Differential scanning calorimetry (DSC) experiments were carried out to study the extent of orthogonal self-assembly of the different components. DSC on thermosensitive liposomes without enzyme showed a sharp phase transition temperature of 41.9 °C, in agreement with literature.[3a] In contrast, the enzyme-loaded liposomes displayed a broadened phase transition temperature with a maximum at 43.4 °C, indicating that the enzyme somehow interacts with the liposome bilayer (Figure 3f). Because the T_m shifted only slightly and became broader in the presence of α-chy, heating at 42 °C, as used for the controlled enzyme release experiments, is suitable to partially melt the bilayer and induce leakage of the liposomes as described above. Comparing DSC traces of enzyme-loaded liposomes embedded in a gel network to DSC traces of the same liposomes in solution showed no significant differences, from which we can conclude that the self-assembly of the enzyme-loaded liposomes and gelator **1** is fully orthogonal, in agreement with the CLSM results. It should be noted that the increased stability of the enzyme-loaded liposomes in a gel network is most likely the result of mechanical interactions between the liposomes and the fibrous network at the submicrometer length scale, rather than interactions at the molecular level.[10a]

In conclusion, we have shown that orthogonal multicomponent self-assembly can be used to create a functional system in which the catalytic activity can be tuned after its production. With the catalytic activity coupled to the release of a small molecule, this prototype system can be envisioned as a future delivery platform with post-production tunable release rate. The small-molecule release rate can be tuned using a simple trigger: the heating time at a constant temperature. This system is created by compartmentalizing an enzyme in liposomes which in turn are entrapped in a fibrillar network of a self-assembled gelator. A fluorescent small molecule is connected to the hydrogelator by an amide bond, which can be hydrolyzed selectively by the enzyme. Controlled heating of the sample around the T_m of the liposomes results in liberation of the enzyme into the gel, leading to release of the fluorophore. Crucially, the amount of liberated enzyme, and thus the

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fluorophore release rate, can be tuned by the heating time of the platform. In future work, other enzymes, catalysts, and associated addressable chemical bonds might be applied to enhance biocompatibility and selectivity. In combination with drug-carrying gelators,[7,24] such a concept could find use in future applications in personalized medicine or medical implants, where the drug release rate can be tuned before intake of the medicine or by locally induced hyperthermia.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and fluorescence spectroscopy data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(22) The temperature to induce release from thermosensitive liposomes can be set over a broad range by the bilayer gel−fluid phase transition temperature T_m (see also ref 15). Here, a T_m of 42 °C was chosen deliberately to ensure that the liposomes do not leak their contents at body temperature; leakage of content can be induced by controlled heating of the sample.

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