Supplementary Information for:

A self-assembled delivery platform with post-production tunable release rate

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Materials

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine and (DSPC) 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] ammonium salt (DSPE-PEG-2000) were purchased from Avanti-Polar Lipids. α-chy and all other chemicals were purchased from Sigma-Aldrich and used without further purification. Gelator **1** was synthesized via previously described routes.[1,2]

Synthesis of fluorescent probe for 1: 1-fluorescein-isothiocyanate. Free hydroxyl labeling of **1** with fluorescein-isothiocyanate was done by dissolving 75 mM **1** (24.9 mg) in 500 μL DMSO and adding 75 mM fluorescein-isothiocyanate (14.6 mg). The mixture was stirred for 24 hrs and was used as such.

Synthesis of fluorescent probe for α-chy: α-chy-Rhodamine-isothiocyanate. Nonspecific labeling of α-chy with rhodamine-isothiocyanate was performed by adding the rhodamine-isothiocyanate to an enzyme solution in a 10:1 molar ratio in 0.2 M phosphate buffer at pH 8.0. The reaction was carried out for 30 min at room temperature and the unreacted dye was removed by column chromatography over a Supelco PD-10 Sephadex column eluted with MQ-water, after which the collected fractions were lyophilized.

Methods

Liposome preparation. Thermosensitive liposomes were prepared from DPPC: DSPC: Cholesterol: DSPE-PEG in the following molar ratio: 77: 5: 15: 3 with a final total concentration of 60 mM. The correct amounts were weighed in a 25 mL flask and dissolved in 1 mL chloroform. The chloroform was evaporated under reduced pressure to form a thin film, which was subsequently dried under high vacuum for at least 6 hours. Liposomes were created by rehydrating the thin film with the enzyme solution (1.5 mM) in buffer (100 mM Tris HCl at pH 7.4 in presence of 2 mM $CaCl_2$ to increase the activity of α-chy[3]). The resulting turbid solution was subjected to 5 freeze thaw cycles to increase encapsulating efficiencies. Thereafter, the solution was extruded through 1000 nm, 400 nm and finally 100 nm polycarbonate filters at 45 °C ± 2°C. Finally, the non-entrapped enzyme was separated from the liposomes by gel permeation on a Sepharose 4B column eluted with 100 mM Tris HCl at pH 7.4 with 2 mM $CaCl_2$.

Gel preparation. All gels were prepared by placing 5 μL DMSO with varying concentration **1** on the bottom of a vial or cuvette. 195 μL of buffer solution (100 mM Tris HCl at pH 7.4 with 2 mM $CaCl_2$) was pipetted on top, after which the mixture was directly vortexed for 3 seconds. As a consequence all gels contained 2.5 v/v% DMSO. Gelation typically took places within seconds.

For the hydrolysis experiments the same procedure was used, however, in these experiments the buffer solution contained the appropriate concentration of α-chy. For the liposome bearing gels, again the same procedure was used, however, in these experiments the DMSO solution was diluted with 195 μL of the liposome solution described above.

Critical gelation concentration determination. The critical gelation concentration of **1** in 2.5 v/v% DMSO solutions of buffer was determined by an inverted tube test. Gels were prepared as described above and allowed to stand overnight. The tube was inverted to see if a gel had formed. The cgc (1.9 mM ± 0.3) is the point in between the last point where a gel was formed (2.2 mM) and the concentration where the solvent could not longer be supported (1.6 mM).

Fluorescence spectroscopy. Fluorescence spectroscopy was performed on a Jasco 815 spectrophotometer. Gel samples were prepared as described above in a 3 mm fluorescence cuvette. All samples were excited at 341 nm and with a slit width of 5 nm. The response time time was set at 32 milliseconds and the photomultiplier tube voltage was 700 V. All samples were measured at 25°C ± 0.1°C and the [6-AQ] was determined at 550 nm, using a calibration curve (Figure S1a and b).

Hydrolysis of 1 by α-chy. All hydrolysis rates of **1** by α-chy were determined by measuring the concentration of 6-AQ against time using fluorescence spectroscopy for at least 30 minutes. Each experiment was performed in duplo.

Tunable liberation of α-chy and hydrolysis of 1. For the controlled liberation of α-chy, the gels were prepared as described above. Subsequently the cuvettes were heated in the spectrophotometer using a Jasco PTC-423L heating element to 42 °C ± 0.1°C for the predetermined time after which the cuvettes were cooled down to 25°C ± 0.1°C inside the spectrophotometer. To ensure a uniform temperature throughout the entire cuvette and to allow the gel to settle after the thermal shock the samples were stabilized for 30 minutes at 25°C ± 0.1°C after which the [6-AQ] was determined against time. Because the concentration of **1** in solution is equal to the cgc of **1**, this concentration remains constant even if **1** in solution is hydrolyzed. Therefore, also the rate of hydrolysis remains constant for the first hours and allowing the sample to stabilize does not have consequences for the measured hydrolysis rate. The hydrolysis rate was determined as described above. The entire experiment was performed in duplo with a two individual batches of enzyme loaded liposomes. Because the liposome preparation required gel permeation chromatography to remove the un-encapsulated enzyme, the exact concentration of liposomes varies from batch to batch and as a result of the different liposome concentration the error bars in the tunable drug release study are relatively large. However, the trend (increasing drug release rate with increasing heating time) is valid and reproducible.

Stability test. For the stability test, two enzyme bearing liposome gels were prepared as described above in a cuvette. These cuvettes were stored at 5°C ± 2°C to minimize autodegradation of the proteolytic enzyme. For two weeks the hydrolysis rate was checked according to the previously described method. These hydrolysis rates were compared to freshly prepared gels, made of the same batch of enzyme loaded liposomes also stored at 5°C ± 2°C (Figure S3).

Laser Scanning Confocal Microscopy. Confocal Laser Scanning Microscopy (CLSM) micrographs were obtained on a Zeiss LSM 700 confocal laser scanning microscope. Excitation was done with 458 nm (NBD-PE), 493 nm (1-FITC) and 558 nm (α-chy-RhITC) laser lines. The laser beam was focused on a 40X oil immersion objective and the sensitivity of detectors and filters were adjusted in order to obtain maximum signal to noise ratio.

Samples were prepared by mixing 4.9 μL 300 mM 1 in DMSO with 0.1 μL 75 mM **1**-FITC in DMSO (0.5%), this solution was diluted with 195 μL enzyme loaded liposomes. These liposomes were prepared by the previously described method, however in this case 0.7% NBD-PE was added before preparing the thin film. After that the thin film was hydrated with 1.6 mM α-chy solution with 5% α-chy-FITC. No extrusions were performed to keep the liposomes at a size visible by microscopy.

Differential Scanning Calorimetry. Phase transition temperatures of the thermosensitive liposomes were determined using ultrasensitive Differential Scanning Calorimetry (Nano II-DSC, Calorimetry Sciences corp., Lindon, UT). Per samples, four scans (2x heating and 2x cooling) were performed, with a rate of 1°C/min, from 20°C to 70°C, against a reference capillary cell filled with water. The first and second heating and cooling scans did not show significant differences. Three different samples were scanned: thermosensitive liposomes (5 mM), enzyme loaded liposomes (exact concentration unknown) and enzyme loaded liposomes (exact concentration unknown) embedded in a gel of **1** (7.5 mM).

Supporting Graphs

Up to a concentration of at least 250 μM the emission of 6-AQ at 550 nm is linear with concentration.

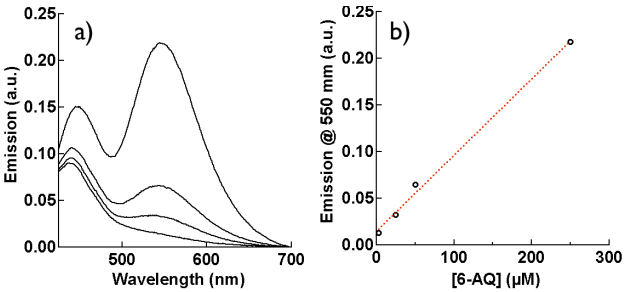

Figure S1. (a) Fluorescence spectra of 6-AQ at different concentrations. b) Emission of 6-AQ at 550 nm against concentration 6-AQ.

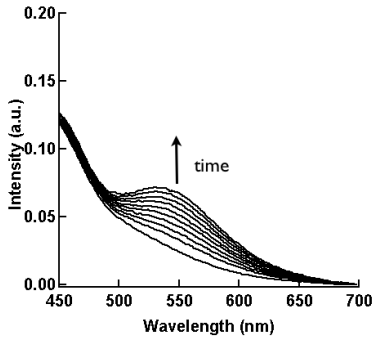

Figure S2. (a) Fluorescence spectra of two component gel at different time intervals ranging from t=0 to t=70 minutes. Conditions: [**1**] = 7.5 mM, [α-chy] = 50 uM.

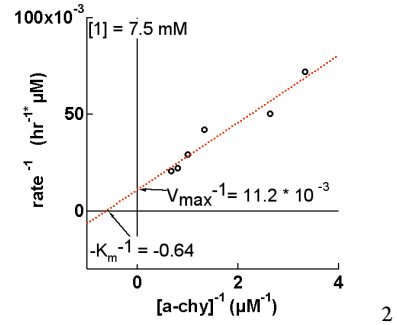

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Figure S3: Lineweaver-Burk plot giving a V_{max} 89 μM * hr^{-1} and a K_m of 1.6 mM.

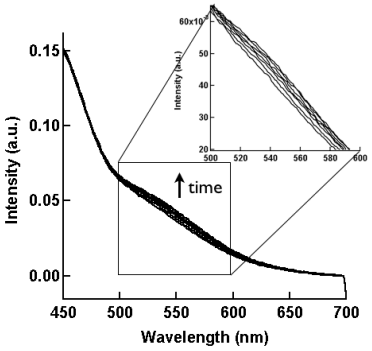

Figure S4. Fluorescence spectra of a three component gel heated for 5 minutes at 42°C, cooled down to 25°C and stabilized for 30 minutes. Different time intervals are plotted ranging from t=0 to t=60 minutes.

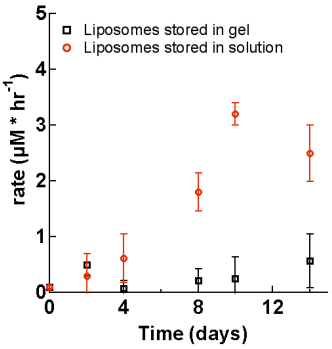

Figure S5. Hydrolysis rates of gels formed from liposomes stored in a gel matrix (squares) and of gels prepared from liposomes stored in solution (circles). Storing liposomes in solution for 4 days resulted in a significant release rate. Liposomes stored in a gel matrix of **1**, on the other hand, show only a marginal release rate, implying that no significant release of α-chy has taken place.

References

[1] Bommel, K. J.; Stuart, M. C.; Feringa, B. L.; van Esch, J.; *Org Biomol. Chem.* **2005**, 3, 2917

[2] Brynes, P. J.; Bevilacqua, P.; Green, A.; *Anal. Biochem.* **1981**, *116*, 408

[3] Jennings, R. R.; Kerr, R. J.; Niemann, C.; *Biochim.Biophys. Acta* **1958**, *28*, 144