Supporting information for:

Active droplets in a hydrogel release drugs with a constant and tunable rate.

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Materials and methods

Materials. We purchased (*E*/*Z*)-2-Buten-1-ylsuccinic anhydride (C_4 anhydride), (*E*/*Z*)-2-hexen-1-ylsuccinic anhydride (C_6 anhydride), (*E*/*Z*)-2-decen-1-ylsuccinic anhydride (C_{10} anhydride) from TCI Chemicals. (*E*/*Z*)-2-Octen-1-ylsuccinic anhydride (C_8 anhydride), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, trifluoroacetic acid (TFA) and Nile Red were purchased from Sigma-Aldrich and used without any further purification unless otherwise indicated. HPLC grade acetonitrile (ACN) was purchased from VWR. We purchased Agar-agar from Carl-Roth and synthesized the succinic acid derivatives (C_4-C_{10}) as described below.

Synthesis of the succinic acid derivatives. We synthesized the precursor acids by treating the corresponding anhydrides with two (mass)-equivalents of MQ water and stirred overnight at room temperature. We found no starting material by HPLC. We lyophilized the mixture, and it stored at -20 °C until further use.

Sample preparation. We prepared stock solutions of the precursors by dissolving the acid in 0.2 M MES buffer, after which we adjusted the pH to pH 6.0, 7.4 or 8.0. Stock solutions of EDC were prepared by dissolving the EDC powder in MQ water. We prepared the stock solutions of 1.0 M EDC freshly. Reaction cycles were started by addition of the high concentration EDC to the acid solution. We carried out all experiments at 25 (±0.5), 37 (±0.5) or 45 (±0.5) ºC

Kinetic model. We used a kinetic model for predicting the evolution of the anhydride concentration over time. Supporting Notes 1 describes the model briefly. More details can be found in our previous work.[1] The rate constants we used in this work are given in Supporting Table S3.

HPLC. We monitored the kinetics of the chemical reaction cycles over time by analytical HPLC (HPLC, Thermofisher Dionex Ultimate 3000, Hypersil Gold 250 x 4.8 mm). A 1.0 mL sample was prepared as described above and placed into a screw cap HPLC vial. Samples of the solutions were directly injected without further dilution (injection volume: 25 µL) and tracked with a UV/Vis detector at 220 and 240 nm. All compounds involved were separated using a linear gradient of H_2O: ACN each with 0.1% TFA.
Method 1: H_2O: ACN from 40:60 to 2:98 in 8 min for C_4 and C_6; Method 2: H_2O: ACN from 98:2 to 2:98 in 12 min for C_8 and C_{10};
Both methods were followed by 2 min 98% ACN. Calibration curves for the EDC (in MQ water), acids (in MES buffer), anhydrides (in ACN) and drugs (in ACN or MQ-water) were performed with the corresponding method in triplicate. Retention times and calibration values are given in Supporting Table S1, S2 and S4.

UV/Vis Spectroscopy. The UV/Vis measurements were carried out using a Multiskan FC (ThermoFisher) microplate reader. Samples (200 µL) were directly prepared into a 96 well-plate (tissue culture plate non-treated). The Temperature (25 (±0.5), 37 (±0.5) or 45 (±0.5) ºC) was set 30 min before starting the measurement. Each experiment was performed at 500 nm and in triplicate.

Confocal Fluorescence Microscopy. We used a Leica SP5 confocal microscope using a 63x oil immersion objective to image the droplets. We prepared samples as described above but with 25 μM Nile Red as s dye. 20 μL of the sample was deposited on a PEG-coated glass slide and covered with a 12 mm diameter coverslip. Samples were excited with 543 nm laser and imaged at 580-700 nm.

Fluorescence Microscopy. We used a Leica DMi8 inverted wide-field microscope (63x Oil objective) with TXR filter (Exc. 540-580; Em. 592-668; DC 585). The samples were prepared as described above for the confocal fluorescence microscopy.

ESI. We used a Varian 500 MS LC ion trap to perform ESI-MS measurements. The samples were diluted in acetonitrile before injection into an acetonitrile carrier flow (20 µL min-1).

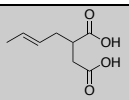
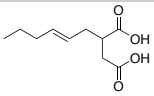
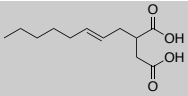
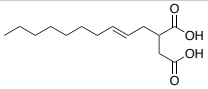
Nile Red Assay. The Nile Red assay was performed on a Jasco (FP-8300) spectrofluorometer with external temperature control (MCB-100). Samples were directly prepared in the 10 mm quartz cuvette (Precision Cells Inc.) by mixing different concentrations of precursor (from 0.01 to 100 mM in 0.2 M MES buffer) with Nile Red (5 μM). The fluorescence intensities were measured at 635 nm with an excitation at 550 nm. The corresponding blue shift was calculated by subtracting the intensity of the blank (0.2 M MES buffer with 5 μM Nile Red) from the corresponding sample.

Drug Release Experiments. A 2% agar-agar stock in 0.2 M MES buffer pH 6.0 was heated to 90 °C. For the first-order release experiments, X μL of a 5 mM stock solution of the drug (in acetonitrile for Nimesulide/Nitrendipine, in dimethylsulfoxide for Acyclovir) was added to 500 μL 7.5 mM C_{10} precursor in 0.2 M MES buffer pH 6.0. 500 μL of the hot agar was added, and 60 μL of this mixture was immediately put on the bottom of a 96 well plate. After 1 min 120 μL of 0.2 M MES buffer was added as a supernatant. The release of precursor and drug to the supernatant was measured via HPLC. For every HPLC injection, a separate well was prepared. For the zero-order release experiments, the precursor solution was activated as described above. The drug was added after 30 min and agar after 45 min. We carried out the rest of the experiment as described above.

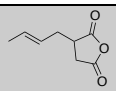
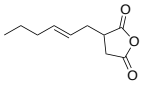
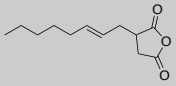
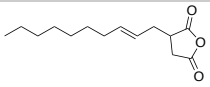
Spin Down Experiments. 500 μL of the precursor solution was activated as described above. After 30 min, X μL of a 5 mM stock solution of the drug (in acetonitrile for Nimesulide/Nitrendipine, in dimethylsulfoxide for Acyclovir) was added. One hour in the experiment, the solution was put in a centrifuge for 5 min (rcf = 5.5). The aqueous phase (200 μL) was measured by HPLC to determine the amount of drug left.

Supporting Tables

Supporting Table S1. Characterization of precursors.

name	structure	mass calculated [g/mol]	mass observed [g/mol]	retention time [min] @220nm	calibration value (mAU/mM)
C_4 acid		Mw = 172.1 $C_8H_{12}NO_4$	171.1 $[Mw-H]^-$	4.16 (method 1)	2.83
C_6 acid		Mw = 200.1 $C_{10}H_{16}N_2O_4$	199.1 $[Mw-H]^-$	5.60 (method 1)	2.78
C_8 acid		Mw = 228.1 $C_{12}H_{24}NO_4$	227.1 $[Mw-H]^-$	10.96 (method 2)	3.22
C_{10} acid		Mw = 256.2 $C_{14}H_{24}NO_4$	255.1 $[Mw-H]^-$	11.83 (method 2)	3.25

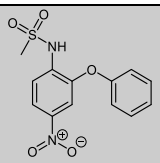
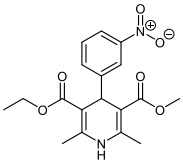
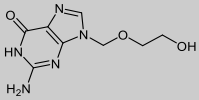
Supporting Table S2. Characterization of products.

name	structure	mass calculated [g/mol]	mass observed [g/mol]	retention time [min] @220nm	calibration value (mAU/mM)
C_4 anhydride		Mw = 154.1 $C_8H_{10}NO_3$	155.2 $[Mw+H]^+$	6.68 (method 1)	4.78
C_6 anhydride		Mw = 182.1 $C_{10}H_{14}N_2O_3$	183.1 $[Mw+H]^+$	7.78 (method 1)	5.19
C_8 anhydride		Mw = 210.1 $C_{12}H_{18}NO_3$	211.2 $[Mw+H]^+$	12.84 (method 2)	5.04
C_{10} anhydride		Mw = 238.2 $C_{12}H_{11}NO_5$	239.2 $[Mw+H]^+$	13.39 (method 2)	5.48

Supporting Table S3: Rate constants used in the kinetic model.

precursor	k_1 $[M^{-1}*s^{-1}]$	k_2	k_3	k_4 $[s^{-1}]$	CAC [mM]
C_4 acid	0.2	$1xk_1$	$0.25xk_1$	$3.5*10^{-3}$	40
C_6 acid	0.2	$1xk_1$	$0.25xk_1$	$3.5*10^{-3}$	3.5
C_8 acid	0.2	$1xk_1$	$0.25xk_1$	$3.5*10^{-3}$	0.3
C_{10} acid	0.2	$1xk_1$	$0.25xk_1$	$3.5*10^{-3}$	0.03

Supporting Table S4: Characterization of drugs.

name	structure	mass calculated [g/mol]	mass observed before the cycle [g/mol]	mass observed after the cycle [g/mol]	retention time [min]	calibration value (mAU/mM)
Nimesulide		Mw = 308.31 $C_{13}H_{12}N_2O_5S$	307.0 $[Mw-H]^-$	307.0 $[Mw-H]^-$	11.10 (220 nm) (method 2)	0.340
Nitrendipine		Mw = 360.36 $C_{18}H_{20}N_2O_6$	359.1 $[Mw-H]^-$	359.2 $[Mw-H]^-$	11.48 (240 nm) (method 2)	0.530
Acyclovir		Mw = 225.21 $C_8H_{11}N_5O_3$	248.1 $[Mw+Na]^+$	248.1 $[Mw+Na]^+$	5.38 (240 nm) (method 2)	0.192

Supporting Table S5: Percentage of Nimesulide measurable in the aqueous phase after 1h for 7.5 mM C_{10} with different amounts of EDC and different initial concentrations of Nimesulide. The error depicts the standard deviation (n=3).

EDC [mM]	**c (Nimesulide)** [µM]		
	50	**100**	**150**
7.5	19.5 ± 1.2 %	24.0 ± 3.2 %	19.0 ± 8.8 %
10	14.5 ± 0.7 %	19.2 ± 1.1 %	17.1 ± 0.3 %
15		17.7 ± 1.5 %	15.2 ± 1.2 %

Supporting Notes

Supporting Notes 1. Description of kinetic model

A kinetic model was written in Matlab that described each reaction involved in the chemical reaction network. The concentrations of each reactant were calculated for every 1 second in the cycle. The model was used to the obtained fit HPLC data that described the evolution of the concentration of anhydride, EDC and acid over time. The concentration of the C_4-, C_6-, C_8-precursor was 10 mM and of the C_{10}-precursor was 7.5 mM.
The model described five chemical reactions:
Direct hydrolysis of carbodiimide with a first order rate constant of 1.3×10^{-5} sec^{-1} as determined in previous work.[1]
The formation of O-acylisourea by reaction with EDC (k_1). This second order rate constant was determined for each precursor by HPLC, by monitoring the EDC consumption.
The formation of the anhydride with a first order rate constant. This rate constant could not be determined because the O-acylisourea was never observed. It was therefore set to be twice the rate of k_1. As a result, the O-acylisourea did never reach concentrations over 1 μM in the model.
Direct hydrolysis of O-acylisourea (k_3). This reaction rate could not be obtained because the O-acylisourea was not observed. The ratio of k_2 and k_3 (anhydride formation and competing direct hydrolysis of O-acylisourea) was varied to fit the HPLC data for several concentrations of $[fuel]_0$ and $[di\text{-}acid]_0$.
Hydrolysis of anhydride in solution proceeded with a first order rate (k_4) The rate constant was determined by HPLC for kinetic experiments where no assemblies were reached.

The rate of the hydrolysis reaction was calculated by multiplying the first order rate constant k_4 with the concentration of anhydride. When the concentration reached values above the CAC, the hydrolysis rate was calculated by multiplying the rate constant by the CAC.

Supporting Notes 2. Preparation of the activated gels.

The behavior of our droplets is sensitive to temperature (Fig. 3e). We thus explain here the preparation of the gels with active droplets for the drug release experiments in more detail.
We ensure that the solution of droplets was kept at room temperature until the agar gel was added. We add 500μL of the liquified agar gel at 90°C to 500 μL of emulsion at 25°C in an Eppendorf tube. The total solution of 1000 μL can, theoretically, never exceed a temperature between 55 and 60°C when mixing is done rapidly. This estimation is a high estimate because it does not take into account the thermal capacity of the walls of the tube, or the pipet tips. Then, immediately after mixing, we pipette 60 μL of the solution on the bottom of a well in a 96 well-plate which is at room temperature. There it directly solidifies because of the further drop in temperature. Taken together, the upper temperature of the droplets is significantly below 60°C, and it experiences this temperature for less than a minute. In that minute, we assume that the droplet do not hydrolyze significantly faster than the rest of the experiment.

Supporting Figures

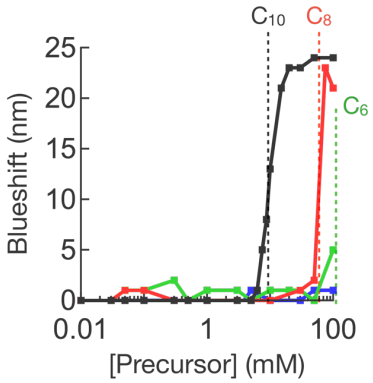


Fig. S1. Nile Red Assay at fluorescence spectroscopy for precursor C_6, C_8 and C_{10}.

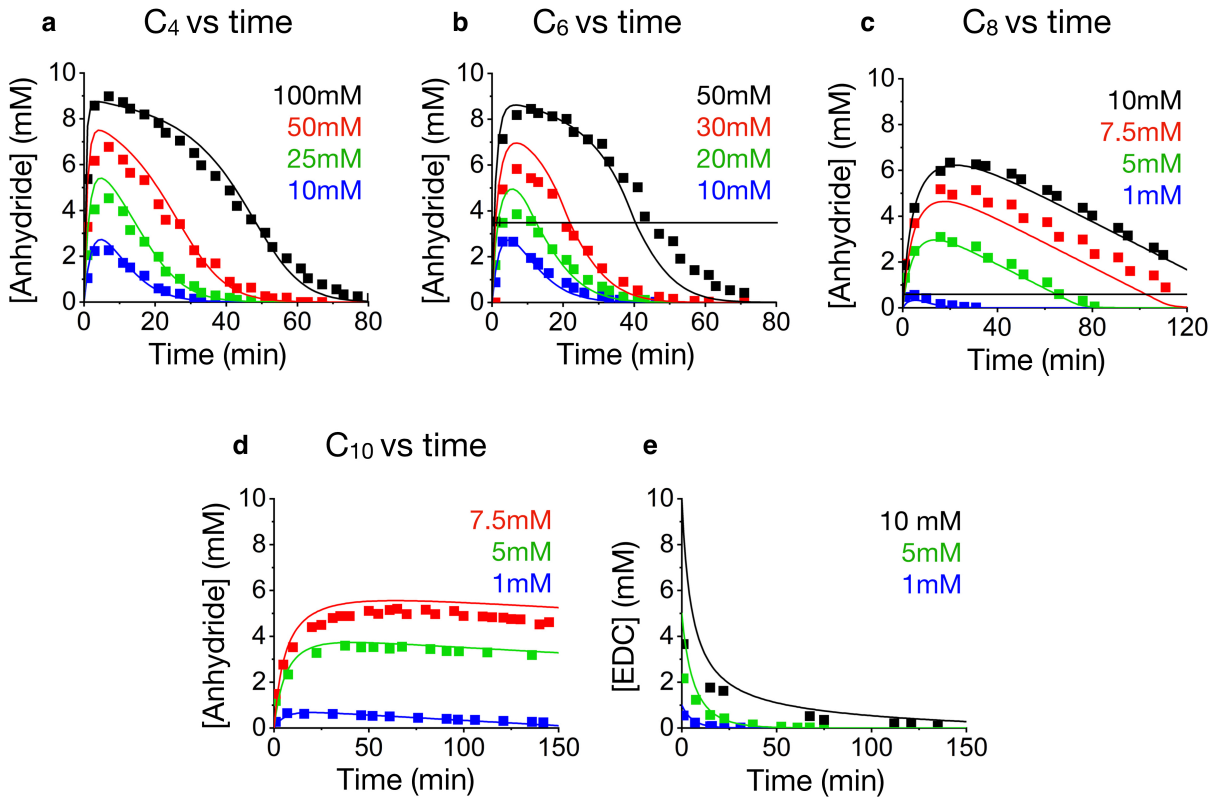


Fig. S2. HPLC kinetics for different precursor with different concentrations EDC, markers represent HPLC data, while lines represent the kinetic model **a)** 10 mM C_4 acid **b)** 10 mM C_6 acid **c)** 10 mM C_8 acid and **d)** 7.5 mM C_{10} acid; EDC consumption of **e)** 7.5 mM C_{10} acid with different concentrations of EDC.

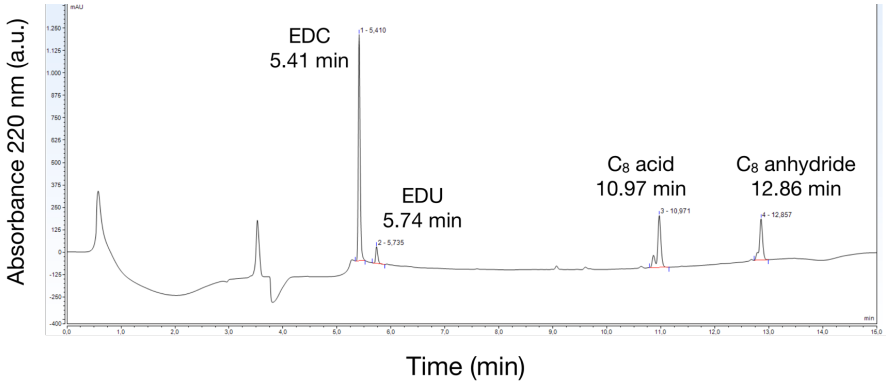


Fig. S3. Representative HPLC trace of 10 mM C_8 acid after 15 min of EDC addition (5 mM).

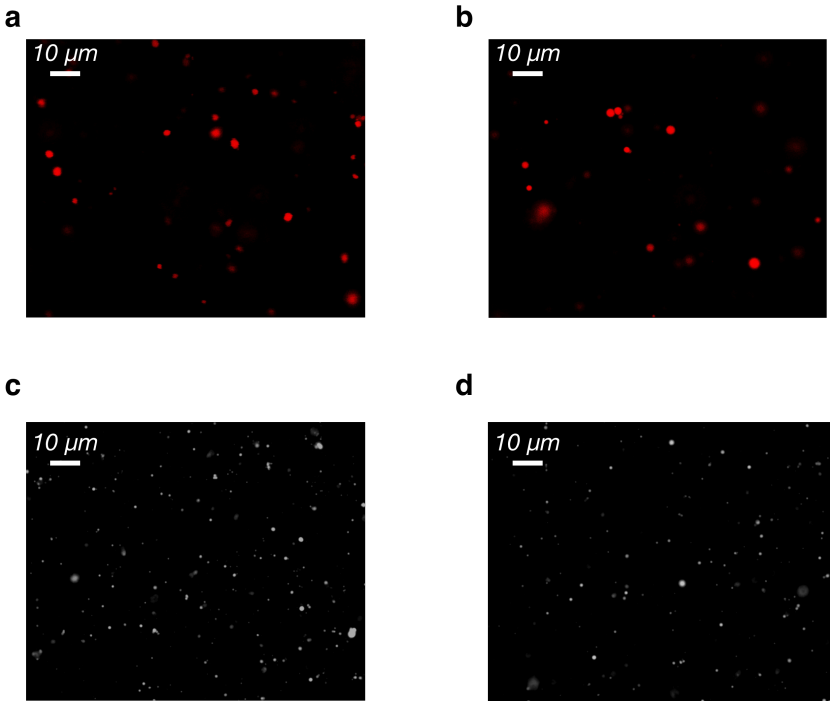


Fig. S4. Confocal micrographs of 7.5mM C_{10} acid with 10mM EDC **a)** directly before gel addition (44min) and **b)** directly after gel addition (46min). Fluorescence micrographs of 7.5mM C_{10} acid with 10mM EDC **c)** with 50 µM Nimesulide and **d)** with 200 µM Nimesulide both directly after gel addition.

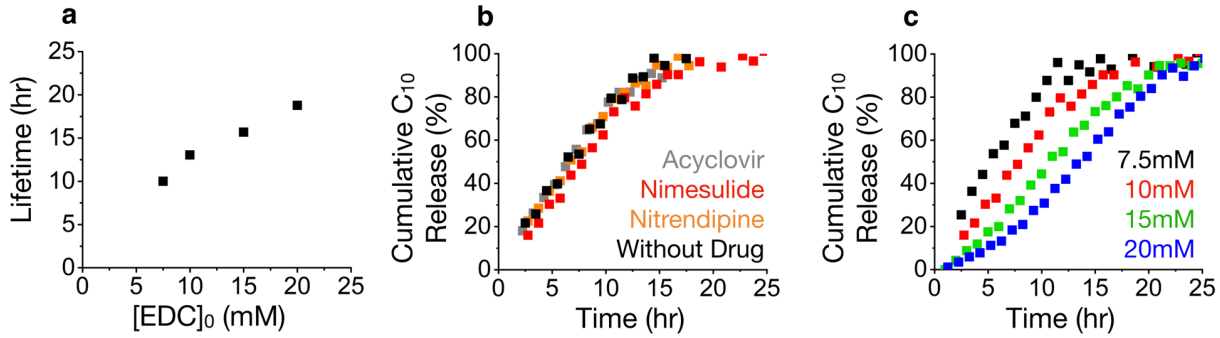


Fig. S5. a) Lifetimes of the active emulsion in gel vs. starting concentrations EDC determined by UV/Vis. Cumulative C_{10} release measured by HPLC from the active emulsion following zero-order release for **b)** 7.5mM C_{10} acid with 50 µM of different drugs or without drug with 10mM starting concentration EDC **c)** 7.5 mM C_{10} acid with different starting concentrations EDC. All release profiles are measured in a 1% agar gel at pH 6.0 and 25 °C.

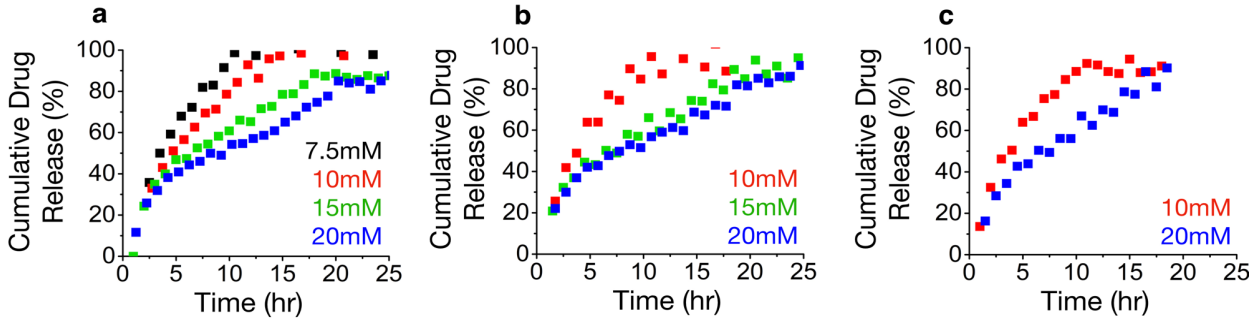


Fig. S6. Cumulative drug release measured by HPLC from the active emulsion following zero-order release for **a)** 50 µM Nimesulide with different starting concentrations EDC, **b)** 100 µM Nimesulide with different starting concentrations EDC, **c)** 200 µM Nimesulide with different starting concentrations EDC. All release profiles are measured in a 1% agar gel at pH 6.0 and 25 °C.

References

[1] Tena-Solsona, M.; Rieß, B.; Grötsch, R. K.; Löhrer, F. C.; Wanzke, C.; Käsdorf, B.; Bausch, A. R.; Müller-Buschbaum, P.; Lieleg, O.; Boekhoven, J., Non-equilibrium dissipative supramolecular materials with a tunable lifetime. *Nature Communications* **2017,** *8*, 15895.