Supporting Information for:

Title: Hydrolyzable emulsions as dual release platform for hydrophobic drugs and DNA

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Supporting Figures

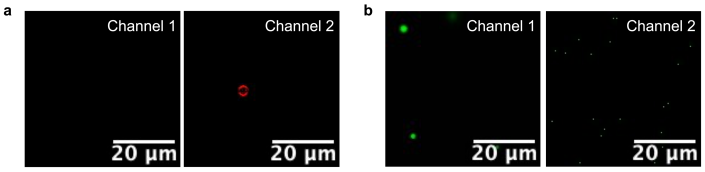

Figure S1: a) Confocal image of 5.00 mM C_6C_6 oil droplets loaded with 30.00 nM chol-T_{15}-cy5 embedded in a 0.6% agarose gel. The sample is excited at 488 nm and imaged at 500 -520 nm (Channel 1) as well as 638 nm and 650 - 750 nm (Channel 2), respectively. Chol-T_{15}-cy5 is only visible in channel 2, confirming that the DNA only accumulates on the droplet surface. b) Confocal image of 5.00 mM C_6C_6 oil droplets loaded with 10.00 µM BODIPY 493/503 embedded in a 0.6% agarose gel. The sample is excited at 488 nm and imaged at 500 - 520 nm (Channel 1) as well as 638 nm and 650 - 750 nm (Channel 2), respectively. BODIPY 493/503 is only visible in channel 1, confirming that the dye partitions into the oil droplets.

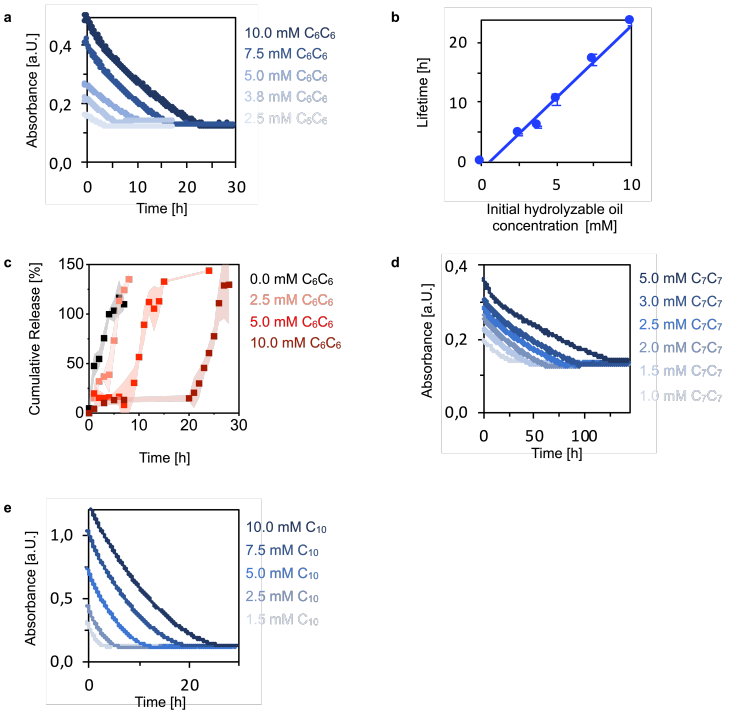

Figure S2: a) UV/Vis measurement showing the hydrolysis of 10.00, 7.50, 5.00, 3.80 and 2.50 mM C_6C_6 oil droplets embedded in a 0.6% agarose gel over time. b) The lifetime of the hydrolyzable oil is directly proportional to the initial oil concentration

that is embedded in the hydrogel. To determine the lifetimes, the absorbance was measured at 500 nm over time for 2.50, 3.75, 5.00, 7.50 and 10.00 mM C_6C_6. c) Release profile of 30.00 nM chol-T_{15}-cy5 released from an agarose gel containing 2.50, 5.00, 10.00 mM C_6C_6 droplets (red), as well as no oil droplets (black), respectively. d) UV/Vis measurement showing the hydrolysis of 5.00, 3.00, 2.50, 2.00, 1.50 and 1.00 mM C_7C_7 oil droplets embedded in a 0.6% agarose gel over time. e) UV/Vis measurement showing the hydrolysis of 10.00, 7.50, 5.00, 2.50 and 1.50 mM C_{10} oil droplets embedded in a 0.6% agarose gel over time. All experiments were performed at 25°C in PBS in triplicate.

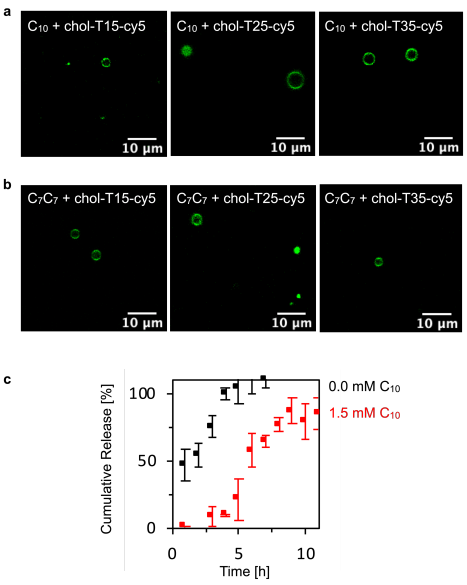

Figure S3: a) Confocal images of 30.00 nM chol-T_{15}-cy5, chol-T_{25}-cy5 and chol-T_{35}-cy5, respectively, in 10.00 mM C_{10} oil droplets embedded in a 0.6% agarose gel. b) Confocal images of 30.00 nM chol-T_{15}-cy5, chol-T_{25}-cy5 and chol-T_{35}-cy5, respectively, in 10.00 mM C_7C_7 oil droplets embedded in a 0.6% agarose gel. c) release of 10.00 nM chol-T_{15}-cy5 from an emulgel containing 1.50 mM C_{10} oil droplets (red) and from a pure agarose gel (black), respectively.

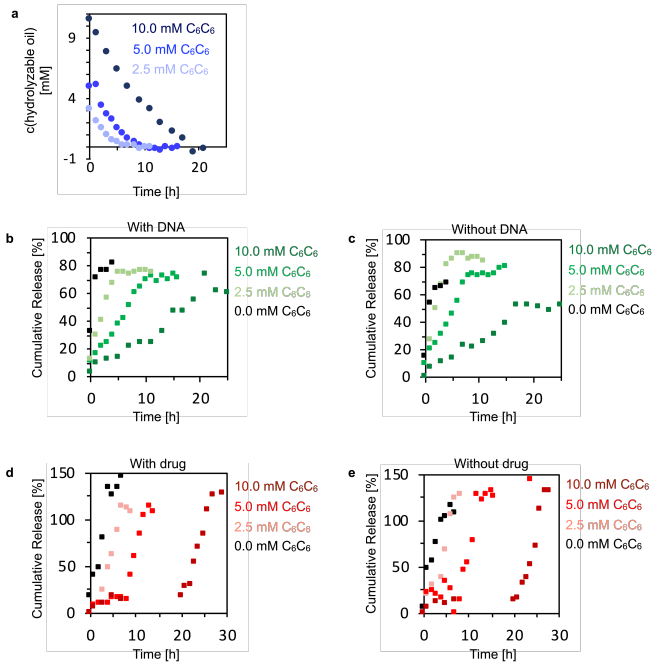

Figure S4: a) Evolution of the hydrolyzable oil concentration over time for emulgels containing initial concentrations of 10.00, 5.00 and 2.50 mM C_6C_6. The oil concentration was calculated by subtracting the measured hydrolysis product concentration of the initially used hydrolyzable oil concentration. The value is then divided by 2 and multiplied by 3, to take into account

that one anhydride molecule hydrolyses to two corresponding acid molecules and the dilution through the supernatant addition (emulgel/supernatant = 1/2). b) Release profile of 25.00 µM Nitrendipine from an emulgel containing 10.00, 5.00, 2.50 and 0.00 mM C_6C_6 oil droplets, respectively, as well as 30.00 mM chol-T_{15}-cy5. c) Release profile of 25.00 µM Nitrendipine from an emulgel containing 10.00, 5.00, 2.50 and 0.00 mM C_6C_6 oil droplets, respectively, without any DNA strands. d) Release profile of 30.00 nM chol-T_{15}-cy5 from an emulgel containing 10.00, 5.00, 2.50 and 0.00 mM C_6C_6 oil droplets, respectively, as well as 25.00 µM Nitrendipine. e) Release profile of 30.00 nM chol-T_{15}-cy5 from an emulgel containing 10.00, 5.00, 2.50 and 0.00 mM C_6C_6 oil droplets, respectively, without any hydrophobic drug.

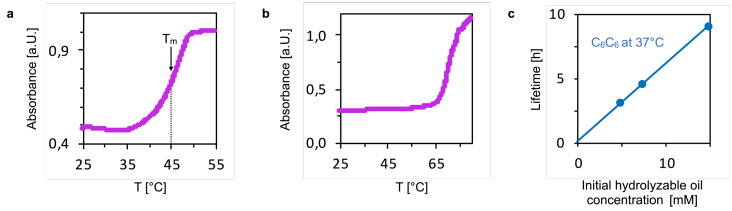

Figure S5: a) Melting curve of DNA-AuNP aggregates formed with 1.50 pmol A*-AuNPs, 1.50 pmol B*-AuNPs and 60.00 pmol linker in PBS containing 3.00 mM $MgCl_2$, 0.01%SDS and 0.05% NaN_3. b) Melting curve of the linker-target duplex. c) Lifetime of 5.00, 7.50 and 15.00 mM C_6C_6 droplets embedded in 0.6% agarose gel with a PBS supernatant containing 3.00 mM $MgCl_2$ at 37°C.

Table S1: Sequence of the used DNA strands.

DNA	DNA sequence
Chol-T_{15}-cy5	5'-(CholTEG) TTT TTT TTT TTT TTT cy5-3'
Chol-T_{25}-cy5	5'-(CholTEG) TTT TTT TTT TTT TTT TTT TTT TTT T cy5-3'
Chol-T_{35}-cy5	5'-(CholTEG) TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TT cy5-3'
A* for AuNP-A*	5'-(HS) AAAAAAAAAA ATG GAA TCA-3'
B* for AuNP-B*	5'-ATC GAA CAA AAA AAA AAA A(SH)-3'
Linker DNA	5'-AAAAA TTG TTC GAT TGA TTC CAT AAAAA-3'
Target DNA	5'-(CholTEG) TTTTT ATG AAG TCA ATC AAG CAA TTTTT-3'

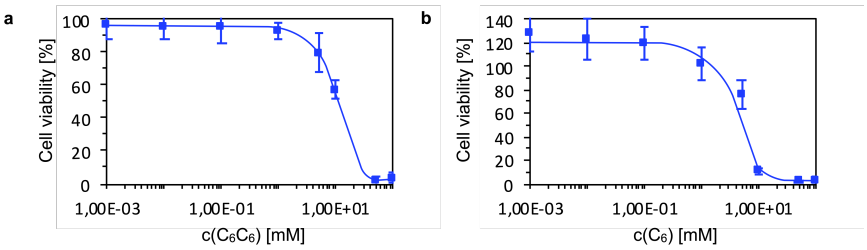

Figure S6: Cell viability of a) the hydrolyzable oil C_6C_6 with an IC_{50} value of 14.00 mM and b) the corresponding acid C_6 with an IC_{50} value of 6.25 mM with Human epithelial cells (HeLa). For both compounds, the tested concentrations were 0.001 mM, 0.01 mM, 0.10 mM, 1.00 mM, 5.00 mM, 10.00 mM, 50.00 mM and 100.00 mM.

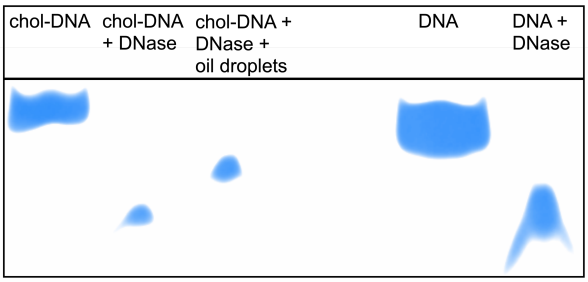

Figure S7: Polyacrylamide gel image of 1.00 nmol cholesterol-conjugated DNA and 1.00 nmol cholesterol-conjugated DNA incubated with 20 U DNase I with and without 2.50 mM C_6C_6 oil droplets as well as 1.00 nmol DNA of the same sequence without cholesterol modification with and without DNase I incubation. The gel image indicates the complete degradation of all DNA strands in the presence of DNase I. All samples were incubated for 30.00 min at 37°C and analyzed by gel electrophoresis with a 25% polyacrylamide gel and stained with methylene blue.

Methods

1. Materials

We purchased (E/Z)-2-Decen-1-ylsuccinic (C_{10} anhydride) and heptanoic anhydride (C_7C_7 anhydride) from TCI Chemicals. Hexanoic (C_6C_6 anhydride), Difluoro(2-[1-(3,5-dimethyl-2H-pyrrol-2-ylidene-N)ethyl]-3,5-dimethyl-1H-pyrrolato-N)boron (BODIPY 493/503), Poly(vinyl alcohol) (PVA, Mw = 89.000-98.000), trifluoroacetic acid (TFA), Nitrendipine, PBS tablets and Gold(III)-chloride solution (HAuCl4) were purchased from Sigma-Aldrich. The agarose powder (TopVision low melting point) was purchased by Thermo Scientific. Chol-T_{15}-cy5, chol-T_{25}-cy5, chol-T_{35}-cy5, chol- TTTTT AAC AAG CTA ACT AAG GTA TTTTT-cy5 and chol-target were synthesized by Merck KgAa. All chemicals were used without any further purification. HPLC grade acetonitrile (ACN), ethanol and PES filter units (pore size: 0.45 µm) were purchased from VWR. MilliQ-water was received from a Milli-Q®Direct 8 water purification system. DNase I (RNase free) was purchased from Thermo Fischer.

The DNA strands for the gold nanoparticles, the linker DNA strands, and the gold nanoparticles were synthesized. All chemicals used for the DNA and the DNA-AuNP synthesis as well as the Glen-Pak Cartridges were purchased by Glen Research. PD-10 columns were purchased from GE Healthcare.

2. Emulgel sample preparation and release experiments

The DNA release experiments were performed in PVA-passivated microtubes. The passivation was performed with a 5% w/v PVA solution in water following a protocol by L. Reese and coworkers.[1]

The 100.00 µM and 5.00 µM stock solutions of the cholesterol-conjugated DNA (chol-T_{15}-cy5, chol-T_{25}-cy5, chol-T_{35}-cy5, chol-TTTTT ATG AAG TCA ATC AAG CAA TTTTT-cy5) were prepared by dissolving the DNA in MilliQ-water and stored at -20°C. The 5.00 mM hydrophobic drug stock solution was prepared by dissolving the Nitrendipine in acetonitrile and stored at 8°C until further use.

The precursor stock solutions were prepared by emulsifying the anhydride (C_6C_6, C_7C_7, C_{10}) in PBS (pH 7.4) through sonication with a Branson UltrasonicsTM SonifierTM SFX250 at 25% for two minutes in an ice bath. These precursor emulsions were prepared freshly for each experiment.

The emulgels were prepared by adding 6.00 µL of the 5.00 µM DNA stock solution and 2.50 µL of the 5.00 mM drug stock solution, respectively, or both to 250.00 µL of the precursor stock solution (Scheme 1a). 250.00 µL of a 1.2% agarose stock, that was heated to 60°C, was added to the mixture and 100.00 µL of this emulgel was put on the bottom of a PVA-passivated microtubes. Subsequently, the emulgel was covered with 200.00 µL PBS and the cumulative DNA and drug release were measured in the supernatant by fluorescence spectroscopy and HPLC, respectively. All experiments were performed at 25°C in triplicate.

3. Synthesis of the DNA strands and the DNA-conjugated gold nanoparticles

The DNA strands A* and B* used for the modification of the gold nanoparticles were synthesized by solid-phase synthesis with an Applied Biosystems Model 392 DNA/RNA Synthesizer. All bases used to synthesize DNA were standard nucleotide phosphoramidites. Additionally, Thiol-Modifier C6 and 3' Thiol-Modifier C3 S–S CPG were used to synthesize thiolated strands. Once the synthesis was done, overnight deprotection of the strands was performed with 1.00 mL of ammonium hydroxide (NH_4OH) followed by purification using the DMT-On procedure with Glen-Pak Cartridge. The purified DNA was lyophilized before placing in the freezer (−20°C) for future use.

Concentration calculations and kinetic and thermal denaturation experiments were conducted with an µDrop™ on a Multiskan FC microplate reader (ThermoFisher) or a HP 8453 diode- array spectrophotometer equipped with a HP 89090A Peltier temperature controller. All DNA concentrations were determined from their absorbance at a wavelength of 260 nm with an extinction coefficient determined by Oligocalc.[2] All samples, buffers, and solutions were dissolved in Milli-Q water.

Gold nanoparticles with 13.00 nm diameters were synthesized following the established Turkevich Synthesis.[3-5] Briefly, all glassware used in the synthesis was soaked in aqua regia, rinsed multiple times with Milli-Q water and then oven-dried at 100°C. Gold(III)-chloride solution ($HAuCl_4$, 0.085 g, 0.25 mmol) was dissolved in Milli-Q water (250.00 mL) and heated to reflux. After 20 min, an aqueous solution of trisodium citrate (25.00 mL, 38.80 mM) was added. This mixture was allowed to reflux for another 10 min followed by cooling and filtering through a PES filter unit with a pore size of 0.45 µm. The nanoparticles were stored in a plastic container in the dark at room temperature. The nanoparticles were characterized by UV/Vis absorbance spectroscopy revealing the characteristic peak at λ = 519 nm of citrate capped 13.00 nm AuNPs.

The loading of thiolated DNA A* and B*, respectively, onto the AuNPs followed a modified procedure of Hurst et al.[6] Briefly, by reacting the disulfide terminated DNA strand A* or B* at room temperature with a dithiothreitol (DTT) solution (0.10 M DTT, 0.18 M phosphate buffer (PB), pH 8) for 2 h, then purifying through a PD-10 column with buffer (50.00 mM PB, 0.05% SDS, 2.5% NaN_3, pH 7), the thiolated DNA was generated. All fractions with thiolated DNA were combined and the concentrations were determined based on the absorbance at 260 nm using OligoCalc.[2] Then 15.00 nmol of the thiolated DNA was added to 1.00 mL of AuNPs in 3.00 mL water. Elution buffer was added to reach a total volume of 5.00 mL. After 20 min the thiolated DNA and gold nanoparticle mixture was salted up to 0.05 M NaCl with a salt solution containing 10.00 mM PB, 0.01% SDS and 0.5% NaN_3 (pH 7) and leaving it overnight. The next day, we further salted up to 0.70 M NaCl by adding 0.01 M NaCl solution in increments while maintaining the buffer concentration until the desired salt concentration was reached.

The purification of the DNA-AuNP A* and B* were performed as outlined by Hurst et al. with few modifications.[6] Briefly, the AuNP probes were centrifuged at 20000 RCF for 20 min and the supernatant was removed. The AuNPs were resuspended in 0.10 M NaCl buffer (0.10 M NaCl, 10.00 mM PB, 0.01% SDS, 0.5% NaN_3, pH 7). These steps were repeated 3 times. Finally, the DNA-AuNPs were resuspended in 0.10 M NaCl buffer (0.10 M NaCl, 10.00 mM PB, 0.01% SDS, 0.5% NaN_3, pH 7). The concentration of gold nanoparticles was determined via UV−visible absorbance spectroscopy at a wavelength of 525 nm with a molar coefficient ε of $2.4 \times 108\ M^{-1}cm^{-1}$.[7]

4. Preparation of the gold nanoparticle aggregates

The DNA-AuNP A*-B*-aggregates were freshly prepared for every experiment by adding 1.50 pmol AuNP-A*, 1.50 pmol AuNP-B* and 60.00 pmol linker DNA to a 10.00 mM PBS containing 0.01% SDS, 0.5% NaN_3 and 3.00 mM magnesium chloride to get a total volume of 1.00 mL. After 1 day incubation at room temperature, the gold nanoparticle aggregates are formed and were used in experiments.

5. Fluorescence spectroscopy

The concentration of the released cholesterol-conjugated and cy5-tagged DNA in the supernatant was determined by fluorescence spectroscopy with Jasco Spectrofluorometer FP-8300. The samples were excited at 600 nm and the emission was measured at 665 nm at 25°C in triplicate. Calibration curves for all DNA strands were performed with the same method in PBS in triplicate.

6. Microscopy

Confocal fluorescence microscopy was performed on a Leica TCS SP8 confocal microscope using a 63× water immersion objective. 1.00 µL BODIPY 493/503 or 1.50 µL cholesterol-conjugated DNA or both was added to the freshly prepared hydrolyzable oil emulsion. The emulgel samples were prepared as previously described. The samples were excited at 638 nm and imaged at 650 – 750 nm with a HyD detector (pinhole: 1 a.u., laser power: 0.03, gain: 150.0%) for chol-T_n-cy5. For the BODIPY 493/503 dye the samples were excited at 488 nm and imaged at 500 – 520 nm with a PMT detector (pinhole: 1 a.u., laser power: 1.00, gain: 400.0%). The measurements were performed at 25°C.

7. Analytical HPLC

The released drug and acid concentrations were determined by analytical high-performance liquid chromatography (HPLC, Thermo- fisher Dionex Ultimate 3000, Hypersil Gold 250 × 4.8 mm) using a linear gradient of MilliQ-water and acetonitrile, each with 0.1% TFA. All compounds were detected with a UV/Vis detector at 220 and 240 nm. The method we used was programmed to run a H2O:ACN gradient from 98:2 to 2:98 in 13 min (Table 1). Calibration curves for the drug (in ACN) and the carboxylate (in PBS) were conducted with the corresponding method in triplicate. All experiments were performed at 25°C in triplicate.

Table S2: Chemical compounds and the in the HPLC measured retention time, the wavelength at which the compound was detected, the calibration value and molar mass.

Compound	Retention time [min]	Wavelength [nm]	Calibration value	M [g/mol]
Nitrendipine	11.29	240	0.52	360.36
C_{10} acid	11.79	220	3.25	238.32

8. UV/Vis-spectroscopy

A Multiskan FC microplate reader (ThermoFisher) was used for UV/vis measurements. The emulgels were pipetted on the bottom of a 96-well-plate (tissue culture plate non-treated) and subsequently covered with PBS to measure the turbidity over time. The measurements were performed at a wavelength of 500 nm at 25°C in triplicate.

To measure the absorbance of the gold nanoparticle aggregates over time, 1.00 mL gold nanoparticle aggregate suspension was pipetted into quartz glass cuvette. The absorbance at a wavelength of 525 nm was measured every 10 min at 37°C. Before and after measurements the sample was mixed carefully to prevent precipitation of the aggregates. When the target DNA was released from the emulgel, the samples were prepared as previously described and 100.00 µL of the emulgel was pipetted on the bottom of the quartz glass cuvette. Subsequently, the emulgel was covered with the gold nanoparticle aggregate suspension. The absorbance was measured at 525 nm at 37°C and the supernatant was mixed carefully to prevent precipitation of the aggregates before and after measurements. When the target DNA strand was added manually, the DNA was added after 50 min to make sure that the nanoparticle aggregate solution was equilibrated and the disassembly only starts after the target addition. All experiments were performed in triplicate.

9. Cell viability studies

Human epithelial cells (HeLa) were cultured in minimum essential medium (MEM; Sigma-Aldrich) containing 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), and 1% (v/v) of a non-essential amino acid solution (Sigma-Aldrich), and 100 U/mL penicillin-streptomycin (Sigma-Aldrich) at 37 °C in a humidified atmosphere with 5% CO2.

The viability of HeLa cells after incubation with the hydrolyzable oil C6C6 and the corresponding acid C_6 was assessed by a WST-1 assay (5015944001, Sigma-Aldrich). First, 5,000 cells were seeded into each well of a 96-well plate and incubated for 24 h. Then, the medium was replaced by a medium containing different concentrations of C_6C_6 or C_6, respectively: 0.001 mM, 0.01 mM, 0.10 mM, 1.00 mM, 5.00 mM, 10.00 mM, 50.00 mM, and 100.00 mM. Cells incubated with a cell culture medium were used as a negative control group. Cells incubated with 50% (v/v) methanol were used as a positive control group. After a further incubation time of 24 h, the cells were washed twice with Dulbecco's phosphate-buffered saline (D-PBS, Sigma-Aldrich). To determine the cell viability of the treated cells, a cell culture medium enriched with 2% (v/v) water-

soluble tetrazolium (WST-1) solution was incubated with the cells for 1 h. Afterward, the metabolized medium was pipetted bubble-free into a fresh well plate and the absorbance of the solutions was measured at 450 nm with a plate reader (SpectraMax ABS Plus, Molecular Devices, San José, USA). Finally, the cellular viability was calculated by normalizing the absorbance value of a test group to those of the negative control group. Each group has 6 replicates of independent samples. All values obtained were used for the determination of the half-maximal inhibitory concentration (IC_{50}) of C_6C_6 or C_6.

10. DNA degradation by DNase I

1.00 nmol of cholesterol-conjugated DNA or 1.00 nmol DNA of the same sequence but without cholesterol modification were incubated with 20 U DNase I (RNase free) in a reaction buffer containing $MgCl_2$. Additionally, 2.5 mM C_6C_6 oil droplets were added to some samples. The reaction mixtures were incubated for 30 min at 37°C and subsequently frozen at -20°C and lyophilized. To analyze the samples via gel electrophoresis, the samples were diluted with 2.00 µL MQ water and 0.6 µL 6x running dye (0.25% (w/v) bromophenol blue and 40% (w/v) sucrose). The samples were loaded into a 25% polyacrylamide gel. After 50 min gel electrophoresis at 250V, the gels are stained with 0.02% methylene blue staining solution for 30 min and imaged with a GelDoc EZ Imager (BioRad).

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