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# Lipid Anchor Oligomer Synthesis

## Resin Loading

The 2-chlorotrityl chloride resin was loaded as described before (Schaffert, Badgujar, & Wagner, 2011). In brief, 0.5 g resin (1.56 mmol/g) was swollen in dry dichloromethane (DCM) for 30 min. Meanwhile, 0.45 mmol Fmoc-L-azidolysine was dissolved in 3.5 ml (1:2.33) dimethylformamide (DMF) and DCM with the addition of 1.35 mmol diisopropylethylamine (DIPEA). After removing the dry DCM from the now swollen resin, the solution containing the amino acid was added and everything was agitated for 1 h. Since the free attachment points on the resin are in threefold excess over the amino acid, the unreacted 2-chlorotrityl units needed to be capped with methanol. To this end, the amino acid solution was removed and replaced by a 1:1.75 mixture DCM and MeOH with 2.74 mmol DIPEA for at least 30 min. Afterwards, the resin was washed with 3x1 ml DMF and 3x1 ml DCM before an aliquot of 70-100 mg was taken and dried inside an exsiccator for loading determination. Roughly 7 mg of dried resin was weighted into each of three 1.5 ml tubes, agitated for 75 min with 1 ml 20 % piperidine in DMF at room temperature (RT) and diluted 1:40 with DMF. The absorption at 301 nm was measured against a DMF blank and an extinction coefficient of 7800 was used to determine the concentration of free fmoc in solution and thereby the amount of bound amino acid per g resin in mmol/g. The fmoc protected amino acid on the main resin batch was deprotected by agitating it 4x10 min with 20 % piperidine in DMF. Complete deprotection was validated by performing a Kaiser test after the resin had been washed with 3x1 ml DMF and 3x1 ml DCM. For the Kaiser test, two drops of each solution (5% ninhydrin in ethanol (w/v), 80% phenol in ethanol (w/v), 2 ml 0.001 M KCN in 98 ml pyridine) were added to a few resin beads and heated to 100° C for 1 – 3 min. If free amines are present on the resin, the solution will turn blue. Afterwards, the remaining resin was dried in an exsiccator and stored at 7 °C. Alternatively, it was directly used for the intended oligomer synthesis.
Usually, the first amino acid is loaded up to a concentration of 0.25 mmol/g resin to enable fast and near quantitative conversion of reactants by allowing the usage of a fourfold excess of target amino acid. For the synthesis of the lipid anchor oligomers here, the ratio between concentration of resin and Fmoc-amino-PEG12-COOH was almost reversed in order to save expensive PEG reagents. Specifically, a higher resin loading was chosen (~ 0.5 mmol/g) and reacted with 120 µmol amino-PEG12-COOH for 12 h to achieve a final loading of resin-azidolysin-PEG12-amino-Fmoc of 0.25 mmol/g resin. This approach leaves some unreacted amines on the resin which were inactivated with acetic anhydride. In detail, the resin with an initial loading of 0.5 mmol/g was agitated for 1 h with 2.5 mmol acetic anhydride and 5.0 mmol DIPEA in DCM. After a washing step with 3x DMF and 3x DCM the successful coupling and capping was confirmed with a Kaiser test. The oligomer was deprotected as described above and synthesis was continued as described in the next paragraph.

## Lipid Anchor Oligomer Synthesis

Oligomer synthesis is carried out with a pre-loaded resin (cf. a) Resin Loading) and repeated cycles of coupling and deprotection steps. The resin loaded with the first amino acid (L-azidolysine) and amino-dPEG12 is swollen in DCM for 30 min. The oligomer chain elongation consists of two important steps for each additional amino acid. In the first step, 4 equivalents (eq, relative to mol of free amines on the resin) of the desired amino acid is dissolved in 1 ml DCM with 8 eq. DIPEA while the activation agents PyBOP (4 eq) and HOBt (4 eq) are dissolved in 1 ml DMF. Both solutions are introduced into a syringe containing the resin and the mixture is agitated for 4 h. Afterwards, the reaction mixture is discarded, and the resin is washed three times with DMF and three times with DCM. A Kaiser-test is performed (cf. a) Resin Loading) to validate the success of the coupling step. If the test is positive, i.e. free amines are still present on the resin, the previous coupling step will be repeated. If the test is negative, deprotection of the current terminal amino acid will be done. To this end, 1 ml 20 % piperidine in DMF was added to the resin, incubated for 10 min and was discarded. This step is repeated four times. Afterwards, the resin was washed with DMF and DCM, three times each. A consecutive Kaiser test needed to be positive to proceed with coupling the next amino acid.

## Cleavage Conditions

To separate the lipid anchor oligomers from the resin, a cleavage mixture of 95:2.5:2.5 TFA:TIS:H2O (TFA: trifluoroacetic acid, TIS: triisopropylsilane) was used. The dried resin was incubated and agitated with 1.5 ml cleavage mixture for 90 min. Afterwards, the solution was added dropwise to 50 ml of a -80 °C cold solution of 75:25 n-hexane:tBME (tert-butylmethylether) to precipitate the crude oligomer while the scavengers and protecting groups remain dissolved. The mixture was centrifuged, solvent was decanted, and the precipitate was dried under nitrogen flow.

## Oligomer Purification

The crude product was dissolved in 2 ml 50 % acetone in purified water and purified by size exclusion chromatography with an ÄKTA system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and a sephadex G10 column using a mixture of 7:3 acetonitrile:H2O with 10 mM HCl as mobile phase. The fractions of the first peak exhibiting 214 nm absorbance were collected, combined and lyophilized. The identity of the oligomers was confirmed by mass spectrometry.

## Oligomer Analysis: MALDI Mass Spectrometry

Lyophilized oligomers were dissolved in purified water with 50 % acetone (5 mg/ml). Sample preparation was done the following way: First, 1 µl matrix solution (Super-DHB: 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid in purified water with 50 % acetonitrile and 0.1 % (v/v) TFA) was spotted on a MTP AnchorChip (Bruker Daltonics, Bremen, Germany) and allowed to crystallize. Second, 1 µl sample solution was added to the spot with the crystallized matrix solution. Samples were analyzed using an Autoflex II mass spectrometer (Bruker Daltonics, Bremen, Germany). All spectra were recorded in positive mode.

# Chemical Structures

Both core oligomers (CO and CON) and two PEG-ligand oligomers (DP24F and DP48F) have been published by Klein et al. (Klein et al., 2018) and accordant MALDI mass spectra can be found there.

## Core Oligomers

### CO (id: 991)

**Supplemental Figure S1: CO’s chemical structure.**
Calculated molecular weight: 3081.07 Da

### CON (id: 1106)

**Supplemental Figure S2: CON’s chemical structure.**Calculated molecular weight: 3235.24 Da

## Lipid Anchor Oligomers

### LA (id: 1203)

**Supplemental Figure S3: LA’s chemical structure and MALDI mass spectrum.**
Measured in positive mode. Calculated molecular weight: 2929.16 Da. Mass found: 2930.73 Da.

### LAE (id: 1223)

**Supplemental Figure S4: LAE’s chemical structure and MALDI mass spectrum.**
Measured in positive mode. Calculated molecular weight: 3187.39 Da. Mass found: 3181.00 Da.

## PEG-Ligands

### DF (id: 1323)

**Supplemental Figure S5: DF’s chemical structure and MALDI mass spectrum.**
Measured in positive mode. Calculated molecular weight: 884.95 Da. Mass found: 881.89 Da.

### DP3F (id: 1324)

**Supplemental Figure S6: DP3F’s chemical structure and MALDI mass spectrum.**
Measured in positive mode. Calculated molecular weight: 1187.32 Da. Mass found: 1183.42 Da.

### DP12F (id: 1325)

**Supplemental Figure S7: DP12F’s chemical structure and MALDI mass spectrum.**
Measured in positive mode. Calculated molecular weight: 1484.67 Da. Mass found: 1479.96 Da.

### DP24F (id: 1139)



**Supplemental Figure S8: DP24F’s chemical structure.**
Calculated molecular weight: 2013.30 Da.

### DP48F (id: 1140)



**Supplemental Figure S9: DP24F’s chemical structure.**
Calculated molecular weight: 3141.65 Da

# Channel Layout

The channels leading to the first Y - junction of the single meander channel were 50 µm, 100 µm, and 50 µm wide (left, middle, and right). They lead into the main channel which was 100 µm wide and ~ 166 cm long. Inner and outer turn radius of the meander’s curves were 200 µm and 300 µm, respectively. The inlets leading to the first Y - junction of the double meander channel were 100 µm, 200µm, and 100 µm wide (left, middle, and right inlet), the inlets leading to the second Y - junction had a width of 100 µm as well. The main channel was 200 µm wide and 2x ~ 166 cm long. Inner and outer turn radius of the meander’s curves were 150 µm and 350 µm, respectively. With maximal flow rates of 1500 µl/h, Reynolds numbers were Re ≈ 3 for the single meander channel and Re ≈ 2.5 for the double meander channel. These numbers indicate a laminar flow profile inside both channels. Dean numbers at curvatures and with flow rates of 1500 µl/h were De ≈ 1.23 and De ≈ 1.25 in single and double meander channels, respectively, indicating negligible influence of lateral flows.

## Single Meander Channel

**Supplemental Figure S10: Channel design of the single meander channel.**Circles on the left represent inlets, a circle on the right an outlet. Liquids are pumped from left to right. The inserts a, b, and c present the details of the regions marked with squares in the channel sketch.

## Double Meander Channel

**Supplemental Figure S11: Channel design of the double meander channel.**
Circles represent inlets, except the circle on the bottom of the left side, which is an outlet. Liquids are pumped from top left to bottom left. The inserts a, b, and c present the details of the regions marked with squares in the channel sketch.

# Manual Formulation of Core – Lipid Anchor Polyplexes

## Addition of Lipid Anchor Oligomers to Pre-Formed Core Polyplexes and Addition of siRNA to Pre-Mixed Core and Lipid Anchor Oligomers

**Supplemental Figure S12: DLS data of core (CO + siRNA) – lipid anchor polyplexes produced by bulk mixing.**
The mixing order is denoted on the x-axis. At first, the components written in the first line were mixed. Second, the third component was added to the mixture by rapid pipetting. Whenever CO and siRNA were mixed together, was it in the first or second step, the mixture was incubated for 45 in before the next step. Shape and color indicate which lipid anchor was used: Blue circle: LA, orange cube: LAE. A: Mean hydrodynamic diameter (z-average). B: Mean polydispersity index (pdi). C: Mean zeta potential, measured in HBG pH 7.4. Statistics: A, B: Error bars correspond to 95 % confidence intervals. C: Error bars correspond to mean zeta deviations. N = 3.

# Polyplex Production at a T-junction

**Supplemental Figure S13: DLS data of core (CO + siRNA) polyplexes produced at a T-junction.**
siRNA is dissolved in HBG pH 7.4, CO is dissolved either in HBG pH 7.4 (panels 1 and 2) or HBG pH 7.4 with 50 % [v/v] acetone (panels 3 and 4). Subfigures are divided into four panels. Panel “hand”: Bulk mixed polyplexes for comparison. Remaining panels: Depict the remaining amount of acetone in the final formulation. For panels 2 and 4, solutions were pumped at equal flow rates, while solutions depicted in panel 3 were pumped with a flow rate (FR) ratio of 1:10 (CO:siRNA). A: Mean hydrodynamic diameter (z-average). B: Mean polydispersity index (pdi). Statistics: Error bars correspond to 95 % confidence intervals. N = 3.

# Controlled Production of Core – Lipid Anchor – PEG-Ligand Polyplexes from their Single Components

**Supplemental Figure S14: Automated production of core (CO + siRNA) – lipid anchor – PEG-ligand polyplexes.**Solvent with or without acetone, double meander channel. First junction: CO:siRNA = 1:10, siRNA in HBG pH 7.4, CO in HBG pH 7.4 ± 50 % acetone. 1.0 ml/h total flow rate. Second junction: Core polylex:lipid anchor oligomer (±PEG-ligand) = 1:11, lipid anchor or lipid anchor – PEG-ligand oligomer in HBG pH 7.4 ± 50 % acetone, 1.1 ml/h total flow rate. Formulation key: core: core polyplex, LA/LAE: lipid anchors, Px: total number of ethylene oxide repetitions (in LA/LAE + PEG-ligand), F: folic acid. See Fig. 1A for detailed structures A: Mean hydrodynamic diameter (z-average) in nm. B: Mean polydispersity index (pdi). Grey spheres: CO was dissolved in HBG pH 7.4. Blue cubes: CO was dissolved in HBG pH 7.4 with 50 % acetone. Error bars correspond to 95 % confidence intervals; n = 3.

# FRET Control Experiments

**Supplemental Figure S15: FRET control measurements of core (CO + siRNA) – lipid anchor polyplexes and their components.**
Title of each panel indicates dye measured; Color indicates dyes used in this formulation. FRET: excites Atto488 (485 nm), measures Cy5 (680 nm). ‘formulation’ specifies formulation composition (e.g. ‘core + LA’: conventionally prepared core polyplex with 20 mol % LA lipid anchor oligomer added with microfluidics). Cy5 is coupled to siRNA’s sense strand. Atto488 is coupled via azide – alkyne click chemistry to the azide of LA or LAE oligomers. Measured fluorescence is divided by gain’s value to exclude amplifier effects.

# Gel Shift Assay

**Supplemental Figure S16: Agarose gel shift assay.**
Core (CO + siRNA) – lipid anchor (LA/LAE) – PEG-ligand (FolA) polyplexes. Key:Px: total amount of ethylene oxide repetitions (LA/LAE + PEG-ligand). Solvent: 100 % HBG or HBG + 90 % fetal bovine serum (FBS), up to 24 h incubation at 37 °C. Gel: 1 % agarose in 1x TBE buffer with 0.1 % GelRed®. 17 % loading buffer. Runtime: 1 h, 80 V. t: Time from formulation until measurement; t > 0: incubated at 37 °C. First row: 100 % HBG pH 7.4, other rows: 10 % HBG pH 7.4, 90 % FBS.

# Ethidium Bromide Displacement Assay

**Supplemental Figure S17: Ethidium bromide displacement assay ± heparin stress.**
Core (CO + siRNA) – lipid anchor (LA/LAE) polyplexes were incubated with ethidium bromide and increase of fluorescence relative to siRNA with dye alone was measured. Resistance to anionic stress was investigated with the addition of heparin. Key: LA: core (CO + siRNA) + 20 mol % LA; LAE: core (CO + siRNA) + 20 mol % LAE; none: core (CO + siRNA) alone. Sample’s mean fluorescence minus the mean fluorescence of the negative control (HBG alone) is reported. Error bars correspond to 95 % confidence intervals; n = 3.

# MTT Assay of Core – Lipid Anchor – PEG-Ligand Polyplexes

**Supplemental Figure S18: MTT assay of core (CO + siRNA) – lipid anchor (LA/LAE) – PEG-ligand polyplexes.**
All polyplexes were prepared using microfluidics. Values are calculated relative to values of buffer treated cells. Colors indicate type of siRNA used: Light color: control siRNA, saturated color: siGFPLuc siRNA. ‘core polyplex’ (green bars): core polyplex formulation used for all subsequent modifications with 20 mol % lipid anchors and lipid anchor-PEG-ligands. Formulation key: P12: core polyplex with unmodified lipid anchor. Px: PEGx, F: Folate. Detailed PEG-ligand description in Fig. 1A. A: Polyplexes with LA lipid anchor (blue bars). B: Polyplexes with LAE lipid anchor (orange bars). Statistics: Error bars correspond to 95 % confidence intervals. N = 5.

# Luciferase Activity Assay: Comparison between LA and LAE containing samples

**Supplemental Figure S19: Luciferase activity assay of core (CO + siRNA) – lipid anchor – PEG-ligand polyplexes.**All polyplexes were prepared using microfluidics. Luciferase enzyme activity is measured in relative light units (RLU) and is shown relative to values of buffer treated cells. siGFPLuc siRNA was used in all samples. Core polyplexes with 20 mol % lipid anchors and lipid anchor - PEG-ligands were used. PEG-ligand Formulation key: P12: core polyplex with unmodified lipid anchor. Px: PEGx, F: Folate. Detailed PEG-ligand description in Fig. 1A. Color key: Blue bars: polyplexes with LA, orange bars: polyplexes with LAE. Statistics: Tips of horizontal lines indicate compared samples. Samples were compared with a two-sided student’s t – test with HOLM correction. N = 5. Key: NS: not significant at α = .05; \*\*\*: α < .001. Error bars correspond to 95 % confidence intervals.

# Gel Shift Assay: Uncropped pictures

**Supplemental Figure S20: Gel shift of core (CO + siRNA) – lipid anchor – PEG-ligand (FolA) polyplexes at t = 0 h**
Solvent: with (top) and without (bottom) 90 % FBS in HBG. Order: (left to right) siRNA, Core polyplex (CO + siRNA), core polyplex + lipid anchor (LA), core polyplex + LA + PEG-ligand (DF), core polyplex + LA + PEG-ligand (DP3F), core polyplex + LA + PEG-ligand (DP12F) , core polyplex + LA + PEG-ligand (DP24F), core polyplex + LA + PEG-ligand (DP48F) , core polyplex + LAE, core polyplex + LAE + PEG-ligand (DF) , core polyplex + LAE + PEG-ligand (DP3F) , core polyplex + LAE + PEG-ligand (DP12F) , core polyplex + LAE + PEG-ligand (DP24F) , core polyplex + LAE + PEG-ligand (DP48F).

**Supplemental Figure S21: Gel shift of core (CO + siRNA) – lipid anchor – PEG-ligand (FolA) polyplexes at t = 1 h**
Solvent: 90 % FBS in HBG. Incubation temperature: 37 ° C, Order: (left to right) siRNA, Core polyplex (CO + siRNA), core polyplex + lipid anchor (LA), core polyplex + LA + PEG-ligand (DF), core polyplex + LA + PEG-ligand (DP3F), core polyplex + LA + PEG-ligand (DP12F) , core polyplex + LA + PEG-ligand (DP24F), core polyplex + LA + PEG-ligand (DP48F) , core polyplex + LAE, core polyplex + LAE + PEG-ligand (DF) , core polyplex + LAE + PEG-ligand (DP3F) , core polyplex + LAE + PEG-ligand (DP12F) , core polyplex + LAE + PEG-ligand (DP24F) , core polyplex + LAE + PEG-ligand (DP48F).

**Supplemental Figure S22: Gel shift of core (CO + siRNA) – lipid anchor – PEG-ligand (FolA) polyplexes at t = 4 h**
Solvent: 90 % FBS in HBG. Incubation temperature: 37 ° C, Order: (left to right) siRNA, Core polyplex (CO + siRNA), core polyplex + lipid anchor (LA), core polyplex + LA + PEG-ligand (DF), core polyplex + LA + PEG-ligand (DP3F), core polyplex + LA + PEG-ligand (DP12F) , core polyplex + LA + PEG-ligand (DP24F), core polyplex + LA + PEG-ligand (DP48F) , core polyplex + LAE, core polyplex + LAE + PEG-ligand (DF) , core polyplex + LAE + PEG-ligand (DP3F) , core polyplex + LAE + PEG-ligand (DP12F) , core polyplex + LAE + PEG-ligand (DP24F) , core polyplex + LAE + PEG-ligand (DP48F).

**Supplemental Figure S23: Gel shift of core (CO + siRNA) – lipid anchor – PEG-ligand (FolA) polyplexes at t = 24 h**
Solvent: 90 % FBS in HBG. Incubation temperature: 37 ° C, Order: (left to right) siRNA, Core polyplex (CO + siRNA), core polyplex + lipid anchor (LA), core polyplex + LA + PEG-ligand (DF), core polyplex + LA + PEG-ligand (DP3F), core polyplex + LA + PEG-ligand (DP12F) , core polyplex + LA + PEG-ligand (DP24F), core polyplex + LA + PEG-ligand (DP48F) , core polyplex + LAE, core polyplex + LAE + PEG-ligand (DF) , core polyplex + LAE + PEG-ligand (DP3F) , core polyplex + LAE + PEG-ligand (DP12F) , core polyplex + LAE + PEG-ligand (DP24F) , core polyplex + LAE + PEG-ligand (DP48F).

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