Electronic supplementary information

Enhanced antifungal activity of siRNA-loaded anionic liposomes against the human pathogenic fungus *Aspergillus fumigatus*

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Materials

Acetonitrile (ACN; HPLC grade), calcofluor white M2R (fluorescent brightener 28, for microbiology), cholesterol (Chol, Sigma grade, ≥ 99%), diethyl pyrocarbonate (DEPC, 96% [NT]), ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2, for molecular biology), hydrogenated soy phosphatidylcholine (HSPC, >99% [TLC]), methanol (MeOH, HPLC grade, 99.8%), protamine sulfate (from salmon, grade X), sodium chloride (NaCl, ACS grade, ≥ 99.5%), and TWEEN 80 (from tallow) were purchased from Sigma Aldrich (St. Louis, MO, USA). Hydrochloric acid (HCl, ACS grade, 37%) was purchased from Bernd Kraft (Duisburg, Germany). 1,2-distearoyl-sn-glycero-3-phosphoglycerol, sodium salt (DSPG, ⩾98%) was purchased from was purchased from Avanti Lipids via Merck KGaA (Darmstadt, Germany). Chloroform ($CHCl_3$, ACS grade) was purchased from Fisher Scientific UK Ltd. (Loughborough, UK). Amphotericin B (*Streptomyces nodosus*; AmB), nuclease-free water (not DEPC-treated), and rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamin triethylammonium salt (rhodamine-DHPE, ⩾ 95 %) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

For all experiments, Milli-Q water with a resistivity of 18.2 mΩ·cm at 25°C was used. DEPC-treated water (DEPC-H_2O) was prepared as follows: 1 mL DEPC was added to 1 L deionized water; the mixture was stirred overnight, and then autoclaved.

5X siRNA Buffer was purchased from Horizon Discovery Biosciences Limited (Cambridge, UK) and diluted with nuclease-free water to obtain a 1X siRNA buffer (filtered sterilized) before use.

1X phosphate-buffered saline (PBS) was prepared by dissolving PBS tablets (Thermo Fisher Scientific) in Milli-Q water according to manufacturer's protocol, and autoclaved.

Synthesis of liposomes

Liposomes (LIP) were prepared using a thin film hydration technique. The LIP were composed of 1,2-distearoyl-sn-glycero-3-phosphoglycerol, sodium salt (DSPG), hydrogenated soy phosphatidylcholine (HSPC), and cholesterol (Chol). The total lipid content was 15 µmol, with the ratio of Chol:DSPG:HSPC being 1:0.8:2.

In the standard procedure, lipids (1.530 mg Chol, 2.530 mg DSPG, and 6.190 mg HSPC) are dissolved in 1 mL of a $CHCl_3$:MeOH mixture (2:1; v:v) each, using a thermo-shaker (1200 U/min; until the temperature reaches 65°C). Subsequently, the dissolved lipids are transferred into a 50 mL flask, and the total volume is increased to 10 mL by adding 7 mL of the $CHCl_3$:MeOH mixture. The solvent is removed using a rotary evaporator (60°C; 30 min at 686 mbar; 30 min at 1 mbar) to create a thin lipid film at the bottom of the flask. The lipid film is then dried under vacuum using a Schlenk line for 2 h. To create liposomes, the lipid film is rehydrated with 1.5 mL of Milli-Q water, incubated in an ultrasonic bath (Sonorex RK 255;

Bandelin electronic GmbH, Berlin, Germany) for 30 min, followed by extrusion of the suspension[1] using the Avanti Mini Extruder at 80°C (to ensure the sample temperature exceeds the phase transition temperatures of HSPC and DSPG[2,3] for easier extrusion), with 21 passes through a 100 nm polycarbonate membrane.

Fluorescent labeling of liposomes

To label the liposomes with dye, the synthesis was performed as described above. Additionally, 2% of the total lipid content (equivalent to 0.3 µmol) of rhodamine-DHPE (rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamin, triethylammonium salt) dissolved in 500 µL of $CHCl_3$ (concentration: 0.8 mg/mL) was added.

Resuspension of siRNA

Lyophilized siRNA was resuspended in a 1X siRNA buffer, as previously described,[4] to yield a 100 µM stock solution. This stock solution was either directly used or further diluted in 1X siRNA buffer to yield a 10 µM intermediate solution. This intermediate solution was then diluted in nuclease-free water to yield a 1 µM working solution, ensuring a consistent dilution factor of 10 at each step.

Cargo loading into liposomes

Amphotericin B (AmB) was loaded into liposomes by incorporating it directly into the lipid film, while siRNA was introduced via the rehydration solution.

50 µg of AmB (54 µmol) were suspended in 1 mL of the $CHCl_3$:MeOH mixture in parallel with the lipid dissolution, using a thermo-shaker (1200 U/min; until temperature reached 65°C). Subsequently, the AmB suspension was added to acidified DSPG (1.7 µL of 2.5 M HCl) to form a complex via electrostatic attraction between AmB (negatively charged) and acidified DSPG (positively charged), as described in the preparation of the AmBisome® formulation.[5] The formation of a DSPG/AmB complex was achieved by shaking the mixture using a thermo-shaker (1200 U/min; 50°C; 20 min). After complexation, the dissolved lipids, the DSPG/AmB complex, and 6 mL of the $CHCl_3$:MeOH mixture were added to a 50 mL flask. The following steps were performed as previously described.

To load siRNA into liposomes (LIP), the lipid dry film (with or without AmB) was rehydrated with an aqueous siRNA/protamine complex solution. This complex solution was created by mixing 5 µL of siRNA (100 µM) with 20 µL of DEPC-H_2O, followed by the addition of 13.3 µL of protamine solution (300 µg/mL in DEPC-H_2O). After allowing complexation for 30 min at room temperature (RT), the volume of the rehydration solution was brought up to 1 mL by adding 987 µL of DEPC-H_2O, resulting in a final input siRNA concentration of approximately 0.5 µM (0.488 µM). The resulting lipid suspension was centrifuged using a Vivaspin 2 centrifugal

concentrator (Sigma Aldrich) to separate the LIP from unencapsulated siRNA. To avoid degradation of the loaded siRNA, the ultrasound treatment was omitted, and the final extrusion was performed as described earlier, with the modification that the entire procedure was conducted at RT. For fluorescently labeld siRNA (DY547-siRNA), which required a higher concentration for visualization experiments, the procedure was slightly modified: 50 µL of DY547-siRNA (100 µM) was mixed with 200 µl of DEPC-H_2O and 133 µL of protamine solution. The rehydration volume was then adjusted to 1 mL by adding 617 µL of DEPC-H_2O, resulting in a final input siRNA concentration of 5 µM.

Determination of loaded AmB concentration

The concentration of loaded AmB in LIP was determined using a Shimadzu HPLC system. Prior to HPLC analysis, 10 µL of 2.5 M HCl were added to 200 µL the LIP suspension. After a 10-min incubation at RT, the solution was added to 800 µL of HPLC solvent (ACN:0.02 M EDTA-Na_2;45:55, v:v), adjusted to pH 5.0). The measurement conditions were as follows: injection volume of 50 µL, flow rate of 1 mL/min, using a Luna 5 µm C18(2) column (150 x 4.6 mm), with the temperature set at 40°C. An SPD-M20A photodiode array detector was used at a wavelength of 407 nm. The measurement was performed in a solvent mixture of ACN:0.02 M EDTA-Na_2 (45:55, v:v), adjusted to pH 5.0 based on a previously established protocol.[6] Using the calibration curves for free AmB (Fig. S6), we calculated the limits of detection (LOD) and quantification (LOQ) as LD = 9 ± 7 µg/mL and LQ = 27 ± 21 µg/mL, respectively. Although the standard deviations are relatively high, the measured AmB concentrations in the samples fall within the LOD and LOQ ranges, ensuring reliable quantification.

siRNA encapsulation efficiency

The concentration of encapsulated DY547-siRNA was determined using a microplate reader (Tecan, Männedorf, Switzerland) based on a calibration curve created with free DY547-siRNA (Fig. S5), with the input siRNA concentration set at 100%. The concentration of encapsulated functional siRNA was measured using the Quant-it™ RiboGreen RNA Assay (Thermo Fisher Scientific) according to the manufacturer's instructions, with the input siRNA concentration also set at 100%.

Characterization of liposomes

The particle size and zeta-potential of liposomes were measured using a Zetasizer Nano-ZSP (Malvern Panalytical). For these measurements, samples were diluted to a ratio of 1:81 in Milli-Q water and analyzed at a laser wavelength of 633 nm at 25°C, with a measurement angle of 173°.

Particle concentrations were determined using a the Nanosight NS300 (Malvern Panalytical). For these measurements, samples were diluted to either 1:1000 or 1:10000 in Milli-Q water and analyzed at a laser wavelength of 642 nm at 25°C.

In vitro interaction of liposomes with *Aspergillus fumigatus*

To investigate the interaction of liposomes with the human fungal pathogen *Aspergillus fumigatus in vitro*, rhodamine B-labeled liposomes (RhoB-LIP) were used. Co-incubation was conducted using the *A. fumigatus strain* A1160p+ (MFIG001)[7] in *Aspergillus* minimal medium[8] (AMM; prepared in nuclease-free water). Unlabeled LIP served as the negative control.

Fresh spores of the A1160p+ strain were prepared as previously described.[8] Co-incubation was conducted in a 48-well plate, with each well containing 200 µL of medium, $2x10^4$ fungal spores, and $3x10^{11}$ particles of LIP. After 16 h of co-incubation at 37°C, the samples were washed three times with 200 µL of PBS at each step (centrifugation for 5 min at 2850 xg, 4°C; the same below). After the final washing step, 200 µL of fresh PBS was added to each well. Hyphae were manually detached from the wells using a sterile 200 µL pipette tip. Finally, 90 µL of each sample was mixed with 3 µL of calcofluor white M2R (0.35% w/v stock in water) in a 1.5 mL microtube and transferred into a µ-Slide VI 0.5 with a glass bottom (ibidi) for microscopic examination.

In vitro interaction of siRNA-loaded liposomes with *A. fumigatus*

To observe the interaction of siRNA-loaded liposomes with *A. fumigatus in vitro*, DY547-labeled siRNA (DY547-siRNA) was encapsulated into LIP or LIP containing AmB (LIP x AmB), resulting in LIP x DY547-siRNA and LIP x AmB x DY547-siRNA formulations, respectively. Empty LIP (without siRNA and AmB) were used as the negative control.

The co-incubation was conducted in a 48-well plate, with each well containing 200 µL of AMM, $2x10^4$ fungal spores (A1160p+), and encapsulated siRNA at a final concentration of approximately 0.4 µM or $1x10^{11}$ particles of the empty LIP. After 16 h of incubation at 37°C, samples were washed three times as described earlier. After the third washing, each well received 180 µL PBS and 6 µL of calcofluor white M2R (0.35% w/v stock in water), followed by additional centrifugation. The washing step was repeated two more times, and samples were subsequently maintained in 200 µL of fresh PBS. Hyphae were manually detached from the wells as described earlier. Finally, 90 µL of each sample was transferred into a µ-Slide VI 0.5 with a glass bottom (ibidi) for microscopic examination.

Quantification of RNA knockdown effects by naked siRNAs on *A. fumigatus*

Spores of the A1160p+ strain were pre-incubated at 37°C in AMM ($2x10^4$ spores/mL, 1.5 mL/well in a 24-well plate) for 6 h, followed by the addition of naked siRNA at desired

concentration (20-30 nM). After 24 h of further incubation, the plates were centrifuged at 2850 xg and 4°C for 10 min. The supernatant was discarded, and the hyphae were manually detached from each well as described earlier. The detached hyphae were transferred into 1.5 mL microtubes, followed and briefly centrifuged to remove any residual AMM. RNA was extracted from the hyphae using the RiboPure-Yeast Kit (Thermo Fisher Scientific) following the manufacturer's protocol. RNA quality and quantity were assessed by UV-Vis spectrophotometry using a Nanodrop 2000 (Thermo Fisher Scientific). For cDNA synthesis, 200 ng RNA per sample was used with the iScript cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR) was conducted using a CFX Connect Real-Time PCR system and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), as per the manufacturer's guidelines. Primers listed in Table S4 were employed to quantify target mRNA levels. All reactions were performed in technical triplicates. Quantitative analyses were carried out using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$ method),[9] with β-tubulin gene (*tubA*, AFUA_1G10910) serving as the reference transcript.

Assessing the inhibitory effects on fungal growth by naked and encapsulated siRNAs

Spores from specified *A. fumigatus* strains (A1160p+, Af293, D141, or ATCC46645) were pre-incubated at 37°C in AMM ($2x10^4$ spores/mL, 150 µL/well in a 96-well plate) for 6 h. Naked or encapsulated siRNA(s) were then added at the desired concentrations (20-30 nM for individual naked siRNAs, approximately 1.5 nM for individual encapsulated siRNAs). After 18 h of further incubation, the plates were centrifuged at 3750 xg, RT for 10 min. The supernatant was discarded, and each well was replenished with 150 µL of iron-depleted AMM (AMM-Fe), followed by further incubation. If incubation conditions involved iron-repletion, the centrifugation and medium change steps could be omitted. If a second siRNA treatment is required, the desired siRNA(s) should be mixed into the AMM-Fe before being added to the respective wells.

The optical density (OD) of the fungal culture was measured using a microplate reader at a wavelength of 600 nm (OD_{600}). Following the siRNA treatment, measurements were taken at 37°C at 10-min intervals.

Statistical analysis

Size and zeta-potential were compared using the normality test (Shapiro-Wilk) and all pairwise multiple comparison procedures (Tukey Test) with the SigmaPlot software (v.12.5), *p*-values smaller than 0.05 were deemed statistically significant.

Quantification of DY547-siRNA uptake by fungal cells, based on pixel brightness distribution within hyphae, was analysed using the Kruskal-Wallis test (multiple comparisons), with

statistical significance set at $p < 0.05$. All analyses were performed using GraphPad Prism (v.8.4.3).

Growth curves derived from OD_{600} measurements were analyzed using a two-way ANOVA with multiple comparisons (Dunnett's test) at each time point, utilizing GraphPad Prism software (v.8.4.3), *p*-values less than 0.05 were considered statistically significant.

Supplementary Tables & Figures

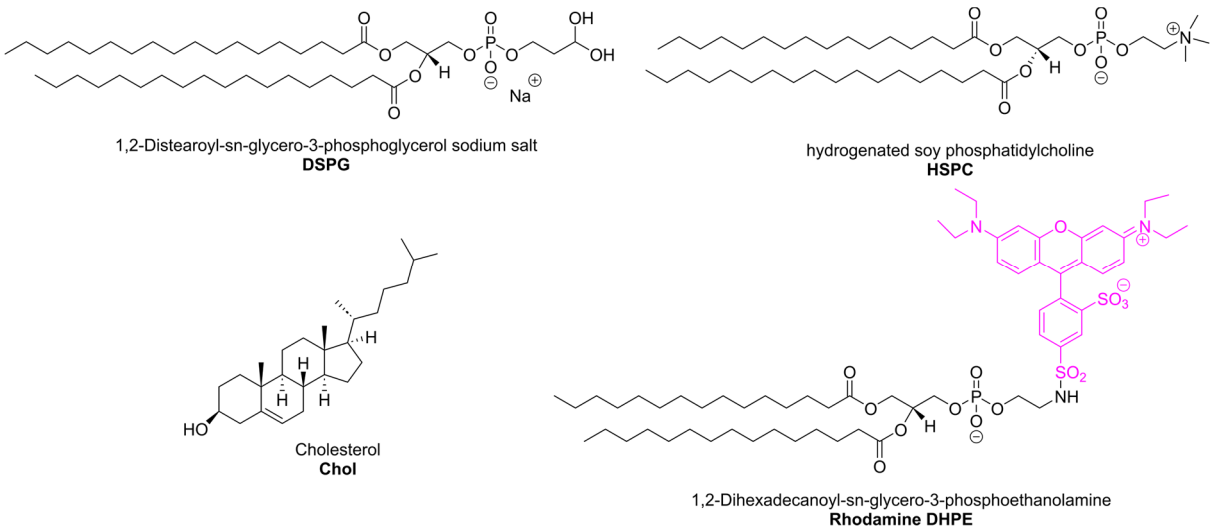

Figure S1 Chemical structures of phospholipids utilized in the formulation of anionic liposomes (LIP) in this study. Rhodamine DHPE was used exclusively for the synthesis of rhodamine B-labeled LIP (RhoB-LIP).

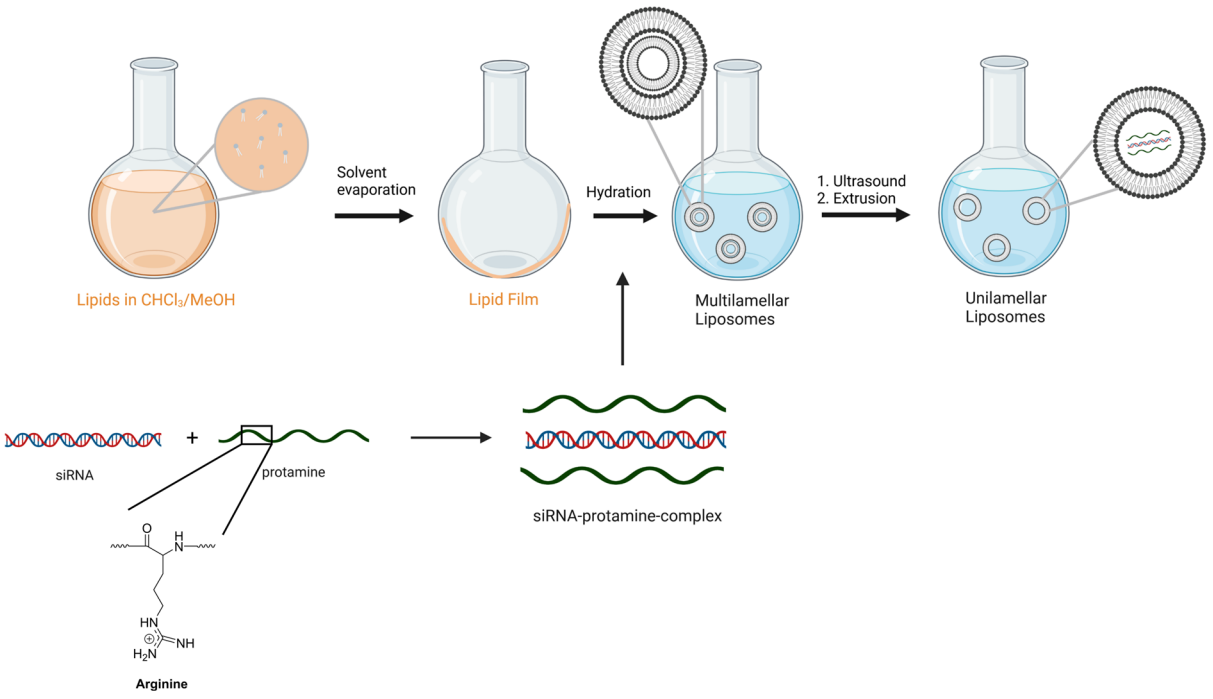

Figure S2 Schematic illustration of liposome synthesis via the "thin film hydration" technique, including the loading step of hydrophilic cargo with the aqueous rehydration solution. Scheme was created using BioRender.

Table S1 Characteristics of identical liposome samples before and after extrusion

Number of extrusion cycles	Size [nm]	PDI	Count Rate [kpcs]
0	832 ± 62	0.576 ± 0.22	183 ± 2
5	143 ± 2	0.057 ± 0.02	437 ± 1
11	137 ± 3	0.052 ± 0.02	360 ± 1
21	130 ± 2	0.040 ± 0.01	352 ± 1

PDI: polydispersity index; kpcs: kilo counts per second

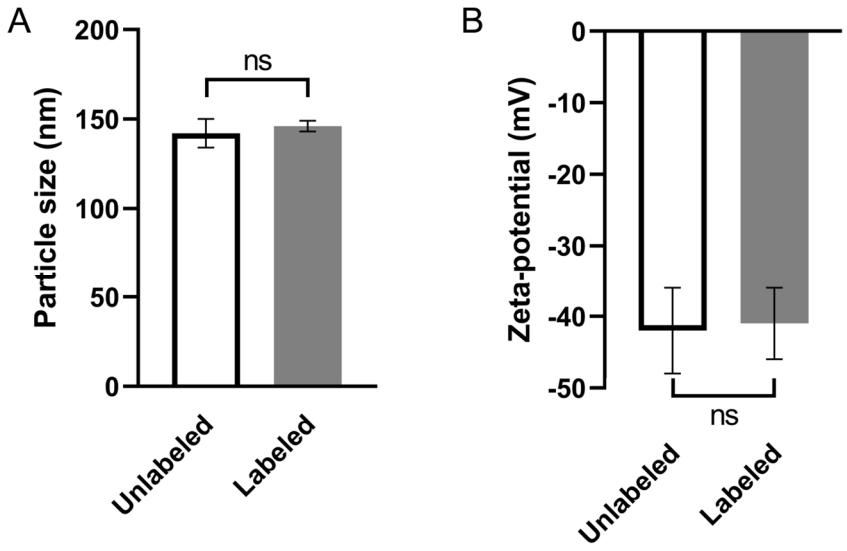

Figure S3 Comparison of (A) particle size and (B) zeta-potential between unlabeled and rhodamine B-labeled liposomes across three different batches; ns: not significant ($p > 0.05$).

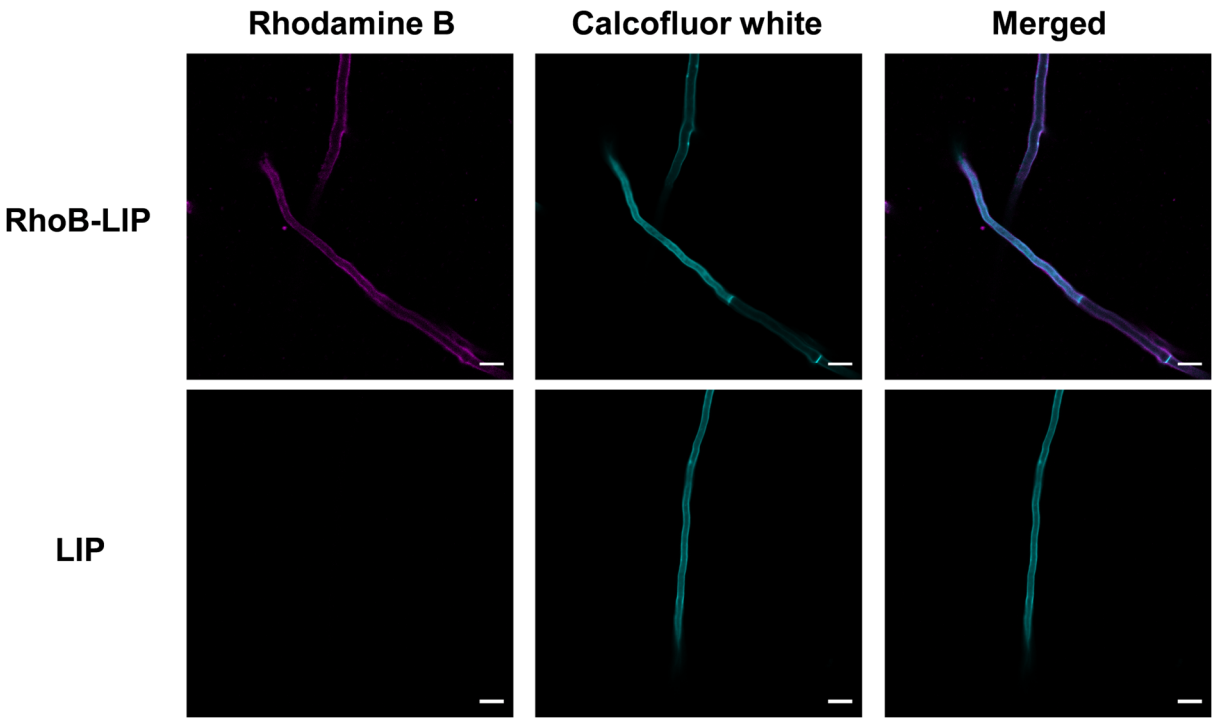

Figure S4 Interaction of rhodamine B-labeled liposomes (RhoB-LIP) with *A. fumigatus* strain A1160p+, using unlabeled LIP as a negative control. $2x10^4$ fungal spores were co-incubated with $3x10^{11}$ particles of the specified LIP type for 16 h at 37°C in *Aspergillus* minimal medium (AMM). Following the co-incubation, samples were washed and stained with calcofluor white to visualize the fungal cell wall. Scale bars = 10 µm.

Table S2 Small interfering RNAs (siRNAs) used in this study

Name	Sequences/Notes	Manufacturer
DY547-siRNA	Accell red non-targeting control siRNA (commercially available)	Horizon Discovery Biosciences Limited
hapB-1	Sense: 5´-GaAcUuUcCuCaGuCaUaUdTdT-3´ Antisense: 5´-aUaUgAcUgAgGaAaGuUcdTdT-3´	BioSpring GmbH
hapB-S	Sense: 5´-GuAaAgAuGcGgGaUaUaAdTdT-3´ Antisense: 5´-uUaUaUcCcGcAuCuUuAcdTdT-3´	BioSpring GmbH
hapX-3	Sense: 5´-GgUcUgGuUcCaAuAaCaAdTdT-3´ Antisense: 5´-uUgUuAuUgGaAcCaGaCcdTdT-3´	BioSpring GmbH
hapX-S	Sense: 5´-GgGaCuGaCuUuAaCcUaAdTdT-3´ Antisense: 5´-uUaGgUuAaAgUcAgUcCcdTdT-3´	BioSpring GmbH
sreA-1	Sense: 5´-GgAcAuUcGuGcAgUaAuUdTdT-3´ Antisense: 5´-aAuUaCuGcAcGaAuGuCcdTdT-3	BioSpring GmbH
sreA-S	Sense: 5´-GgAaUcCuCgAaCcAcUaUdTdT-3´ Antisense: 5´-aUaGuGgUuCgAgGaUuCcdTdT-3´	BioSpring GmbH

N: RNA; n: 2'OMethyl-RNA; dT: deoxythymidine

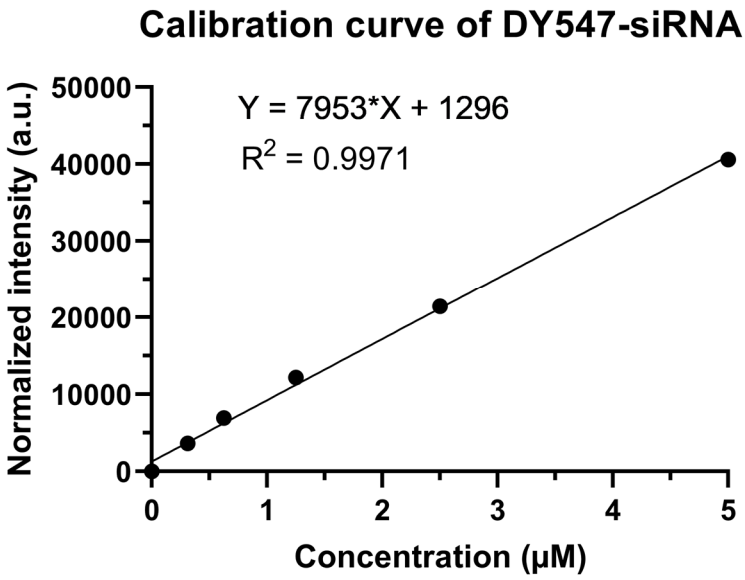

Figure S5 Calibration curve of free DY547-siRNA, generated using simple linear regression analysis (GraphPad Prism, v.8.4.3). The equation obtained from the regression is displayed, indicating the relationship between concentration and fluorescence intensity.

Table S3 Characteristics of empty liposomes and liposomes loaded with DY547-labeled siRNA, with or without protamine complexation prior to loading

Cargo	Batch	Size [nm]	PDI	Zeta-potential [mV]	Encapsulation efficiency
-	-	149 ± 1	0.094 ± 0.02	-41 ± 4	-
siRNA	1	150 ± 1	0.060 ± 0.01	-47 ± 4	86%
	2	176 ± 1	0.146 ± 0.02	-43 ± 1	41%
	3	150 ± 1	0.077 ± 0.01	-43 ± 3	12%
siRNA x protamine	1	165 ± 5	0.178 ± 0.08	-45 ± 2	60%
	2	153 ± 1	0.102 ± 0.03	-36 ± 1	84%
	3	143 ± 1	0.130 ± 0.01	-38 ± 2	90%

PDI: polydispersity index

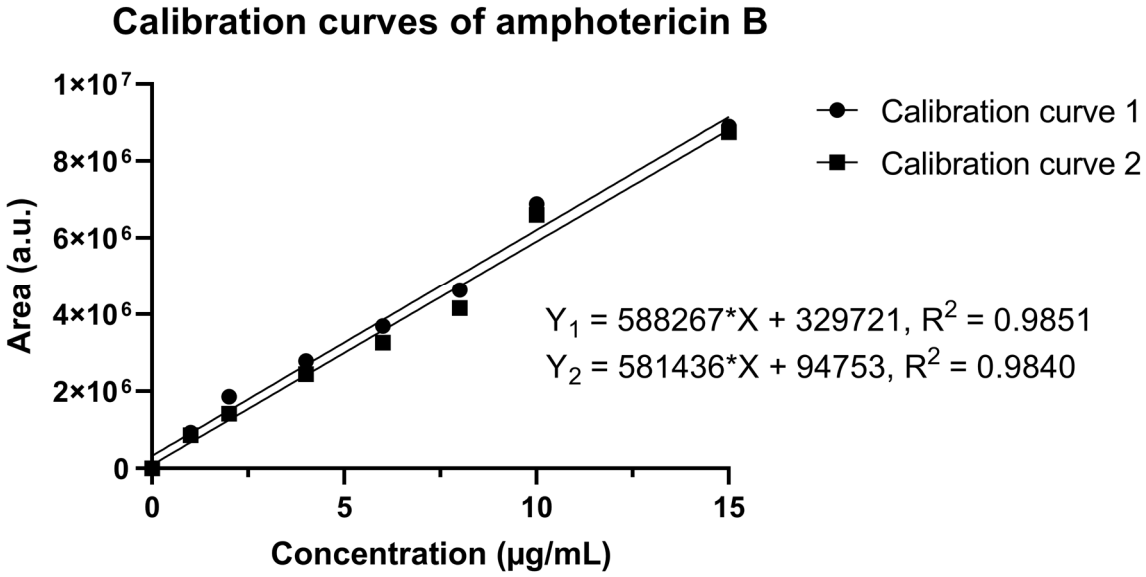

Figure S6 Calibration curves of free amphotericin B, generated through simple linear regression analysis (GraphPad Prism, v.8.4.3). The regression equations are displayed, illustrating the relationship between concentration and absorbance for accurate quantification.

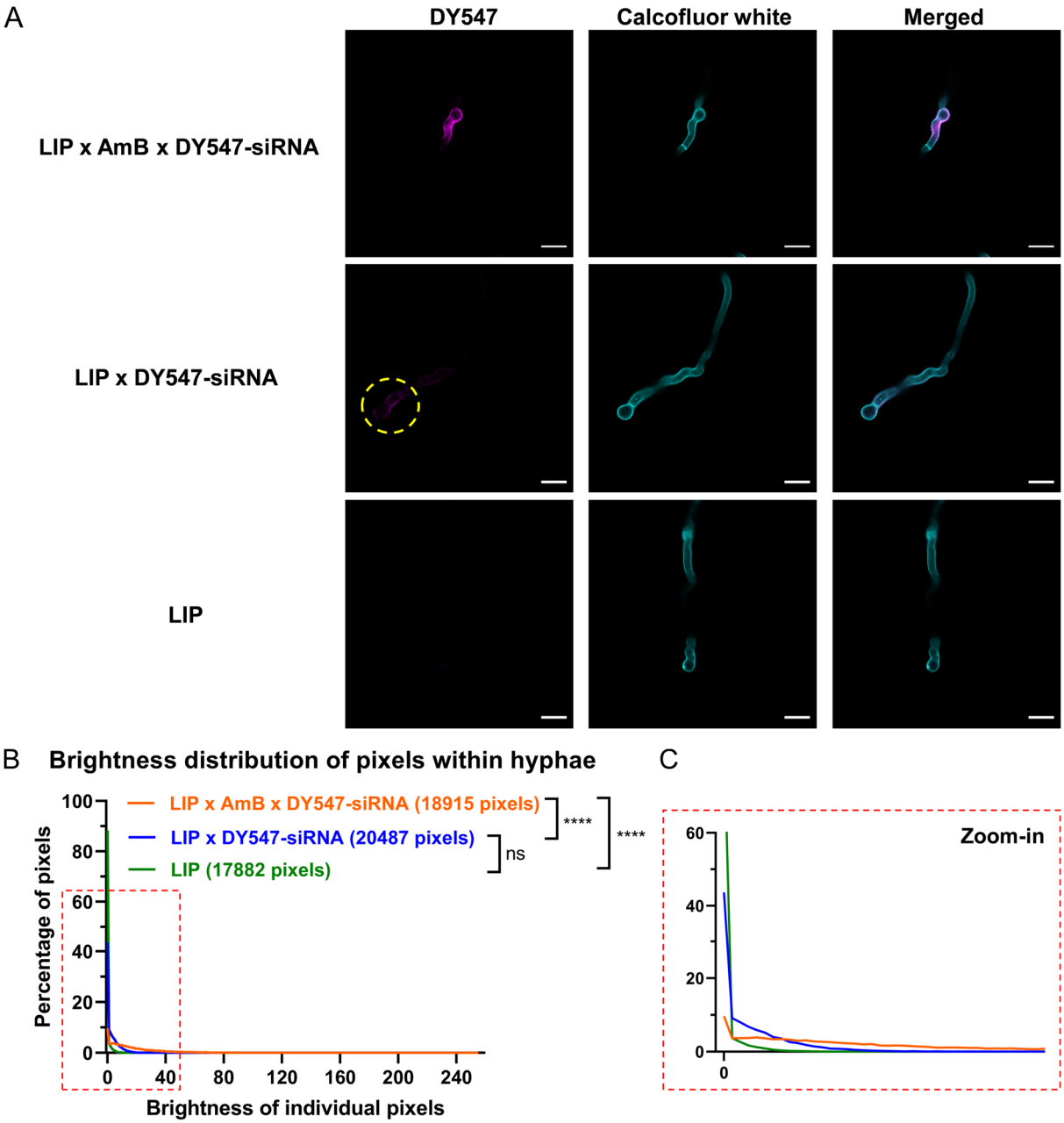

Figure S7 Enhanced interaction of DY547-siRNA with *A. fumigatus* when co-encapsulated with amphotericin B (AmB) in anionic LIP, compared to encapsulation without AmB. (A) Representative images following co-incubation of fungal spores with encapsulated DY547-siRNA (AmB concentration in the upper panel: 0.43 µg/mL) or LIP (negative control) for 16 h at 37°C in AMM. Scale bars = 10 µm. Yellow dashed circle highlights a weak signal of DY547 on the hyphal cell wall. (B) Quantification of DY547-siRNA uptake by fungal cells, based on pixel brightness distribution within hyphae (pooled from multiple images). The inner area of each hypha was defined by calcofluor white staining of the hyphal cell wall, with DY547 signals overlapping with the cell wall excluded from analysis. Pixel counts are indicated in parentheses in the legend. Statistical analysis using the Kruskal-Wallis test (multiple comparisons) showed significant enhance DY547-siRNA uptake with LIP x AmB x DY547-siRNA compared to LIP x DY547-siRNA (****: $p < 0.0001$) or LIP alone (****: $p < 0.0001$), while LIP x DY546-siRNA *vs.* LIP alone was not significant (ns: $p = 0.2451$). (C) Zoom in of (B), area marked by the red dashed box.

Table S4 PCR primers used in this study (synthesized by Sigma Aldrich)

Gene name	Gene ID	Primer sequences
hapB	AFUA_2G14720	Forward: 5´-ACTGCACCAAGACTCGATGG-3´ Reverse: 5´-GTCTGGACATGTGGGCTACC-3´
hapX	AFUA_5G03920	Forward: 5´-GCATTGCCAGTGCATTGAGG-3´ Reverse: 5´-CTGAGGCTCGGAAAGACCAG-3´
sreA	AFUA_5G11260	Forward: 5´-AGACCTCGACATCGCCAAAG-3´ Reverse: 5´-CGGTGATCGTCGCCATAGAG-3
tubA (housekeeping)	AFUA_1G10910	Forward: 5´-GGACGTTACCTCACCTGCTC-3´ Reverse: 5´-CACGCTTGAACAACTCCTGA-3´

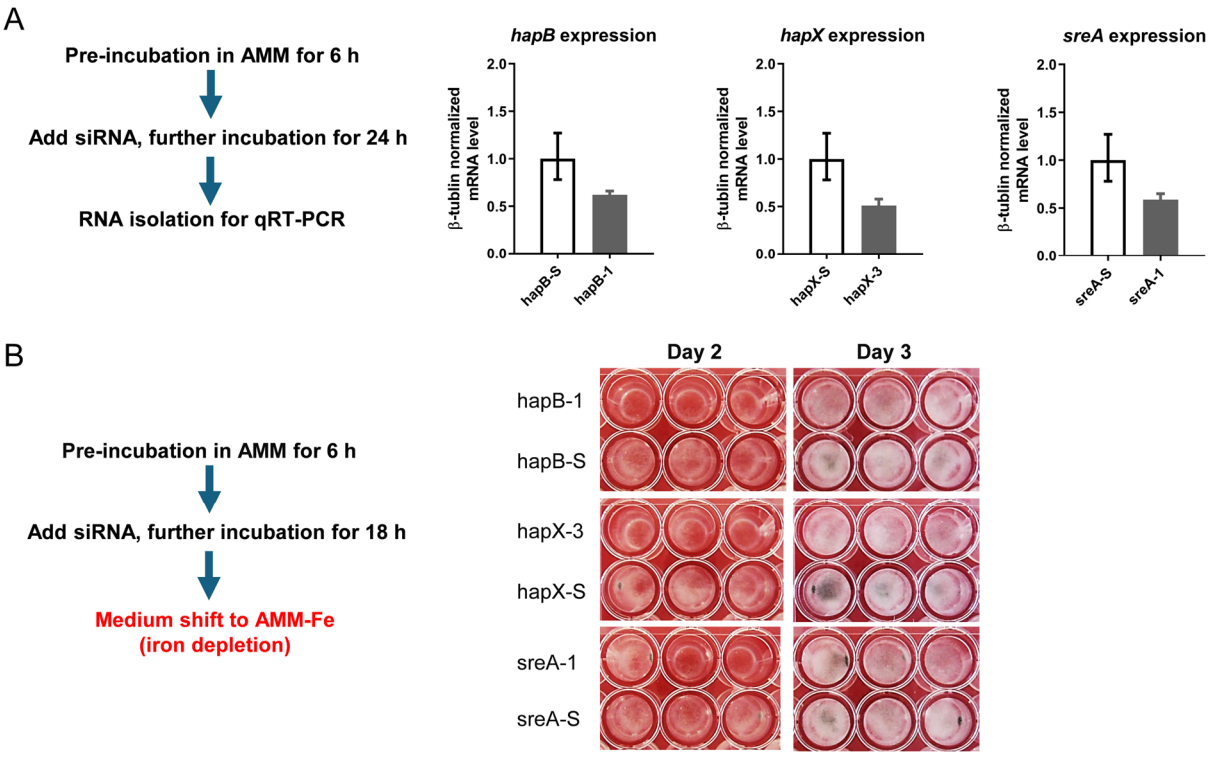

Figure S8 Functionality of candidate siRNAs. (A) Preliminary screening identified three candidate siRNAs (hapB-1, hapX-3, sreA-1) targeting mRNAs of the *hapB*, *hapX* and *sreA* genes, respectively, which showed knockdown effects in AMM via quantitative real-time PCR (qRT-PCR), compared to respective scrambled controls (hapB-S, hapX-S, sreA-S). The final siRNA concentrations used were 30 nM for hapB-1/hapB-S and sreA-1/sreA-S, and 20 nM for hapX-3/hapX-S. However, no inhibitory effects on fungal growth were observed in all cases. (B) After pre-incubation of *A. fumigatus* spores for 6 h at 37°C in AMM, specified siRNAs were added for an 18 h co-incubation. Subsequently, AMM was replaced with iron-depleted AMM (AMM-Fe). Temporary and ambiguous inhibitory effects on fungal growth were noted on day 2 and diminished by day 3.

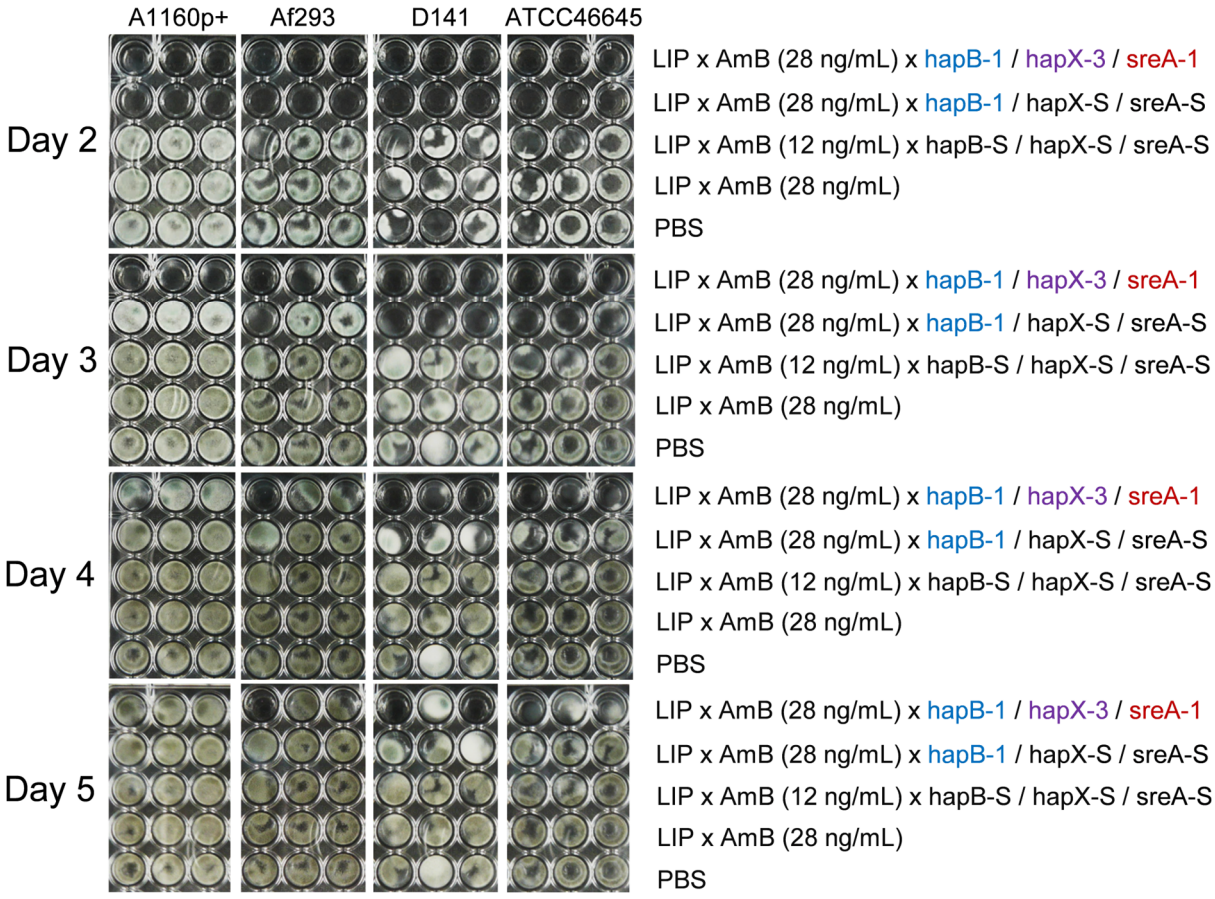

Figure S9 Antifungal effect of LIP x AmB x siRNA formulations under iron-replete conditions across four different *A. fumigatus* strains, revealing variations in effectiveness but demonstrating antifungal effects on all tested strains.

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