Supporting Information

Versatile Multiplex Endogenous RNA Detection with Simultaneous Signal Normalization Using Mesoporous Silica Nanoquenchers

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Table S1. Summary of different *q*MSNs used in this work

Name	ASO Capping	Cargo	MB Modification	CPD Coating	Study Goal
ASO21-FL-*q*MSN	ASO-21	FL	-	-	*In vitro* fluorescence "Turn-ON" release analysis for miR-21 detection in green output channel
CPD-ASO21-FL-*q*MSN	ASO-21	FL	-	Yes	Live cell detection of miR-21 in green output channel
^{FAM}ASO21-*q*MSN	^{FAM}ASO-21	-	-	-	*In vitro* fluorescence "Turn-ON" release analysis for miR-21 detection compared with ASO21-FL-*q*MSN
ASO21-RhB-*q*MSN	ASO-21	RhB	-	-	*In vitro* fluorescence "Turn-ON" release analysis for miR-21 detection in red output channel
ASO122-RhB-*q*MSN	ASO-122	RhB	-	-	*In vitro* fluorescence "Turn-ON" release analysis for miR-122 detection in red output channel
ASOsurvivin-RhB-*q*MSN	ASO-survivin	RhB	-	-	*In vitro* fluorescence "Turn-ON" release analysis for survivin mRNA detection in red output channel
ASO21-MSN^{FITC}	ASO-21	-	-	-	CPD-free Always-ON control for cellular uptake studies
CPD-ASO21-MSN^{FITC}	ASO-21	-	-	Yes	CPD-modified Always-ON control for cellular uptake studies
CPD-ASO21-RhB-*q*MSN	ASO-21	RhB	-	Yes	Live cell detection of miR-21 in red output channel
ASO21-CF405-*q*MSN	ASO-21	CF405	-	-	*In vitro* fluorescence "Turn-ON" release analysis for miR-21 detection in blue output channel
CPD-ASO21-CF405-*q*MSN	ASO-21	CF405	-	Yes	Live cell detection of miR-21 in blue output channel
ASO21-CF633-*q*MSN	ASO-21	CF633	-	-	*In vitro* fluorescence "Turn-ON" release analysis for miR-21 detection in purple output channel
CPD-ASO21-CF633-*q*MSN	ASO-21	CF633	-	Yes	Live cell detection of miR-21 in purple output channel
ASO122-RhB-*q*MSN	ASO-122	RhB	-	-	*In vitro* fluorescence "Turn-ON" release analysis for miR-122 detection in red output channel
CPD-ASO122-RhB-*q*MSN	ASO-122	RhB	-	Yes	Live cell detection of miR-122 in red output channel
ASOsurvivin-CF633-*q*MSN	ASO-survivin	CF633	-	-	*In vitro* fluorescence "Turn-ON" release analysis for survivin mRNA detection in purple output channel
CPD-ASOsurvivin-CF633-*q*MSN	ASO-survivin	CF633	-	Yes	Live cell detection of survivin mRNA in purple output channel
MB-*q*MSN	-	-	Yes	-	*In vitro* fluorescence "Turn-ON" analysis for GAPDH mRNA detection

CPD-MB-*q*MSN	-	-	Yes	Yes	Live cell imaging of GAPDH mRNA
MB-ASO21-RhB-*q*MSN	ASO-21	RhB	Yes	-	*In vitro* fluorescence "Turn-ON" analysis for miR-21 and GAPDH mRNA
MB-ASO122-RhB-*q*MSN	ASO-122	RhB	Yes	-	*In vitro* fluorescence "Turn-ON" analysis for miR-122 and GAPDH mRNA
MB-ASOsurvivin-RhB-*q*MSN	ASO-survivin	RhB	Yes	-	*In vitro* fluorescence "Turn-ON" analysis for survivin mRNA and GAPDH mRNA
CPD-MB-ASO21-RhB-*q*MSN	ASO-21	RhB	Yes	Yes	Live cell imaging of miR-21 and GAPDH mRNA with signal normalization analysis to eliminate cell-to-cell variation
CPD-MB-ASO122-RhB-*q*MSN	ASO-122	RhB	Yes	Yes	Live cell imaging of miR-122 and GAPDH mRNA with signal normalization analysis
CPD-MB-ASOsurvivin-RhB-*q*MSN	ASO-survivin	RhB	Yes	Yes	Live cell monitoring of survivin mRNA downregulation and GAPDH mRNA with signal normalization analysis
MB-ASO21-CF405-*q*MSN	ASO-21	CF405	Yes	-	*In vitro* fluorescence "Turn-ON" analysis for multiplex detection (Target: miR-21; Output: blue channel)
CPD-MB-ASO21-CF405-*q*MSN	ASO-21	CF405	Yes	Yes	Multiplex live cell imaging with signal normalization analysis (Target: miR-21; Output: blue channel)
MB-ASO122-RhB-*q*MSN	ASO122	RhB	Yes	-	*In vitro* fluorescence "Turn-ON" analysis for multiplex detection (Target: miR-122; Output: red channel)
CPD-MB-ASO122-RhB-*q*MSN	ASO122	RhB	Yes	Yes	Multiplex live cell imaging with signal normalization analysis (Target: miR-122; Output: red channel)
MB-ASOsurvivin-CF633-*q*MSN	ASO-survivin	CF633	Yes	-	*In vitro* fluorescence "Turn-ON" analysis for multiplex detection (Target: survivin mRNA; Output: purple channel)
CPD-MB-ASOsurvivin-CF633-*q*MSN	ASO-survivin	CF633	Yes	Yes	Multiplex live cell imaging with signal normalization analysis (Target: survivin mRNA; Output: purple channel)

Table S2. Oligonucleotides name and sequence, written in the direction of 5' to 3'.

Probe	RNA or DNA Sequence (5' to 3')
Antisense Oligonucleotide of miR-21 (ASO-21)	TCA ACA TCA GTC TGA TAA GCT A
Antisense Oligonucleotide of miR-122 (ASO-122)	ACA AAC ACC ATT GTC ACA CTC CA
Antisense Oligonucleotide of survivin mRNA (ASO-survivin)	TGT GCT ATT CTG TGA ATT
Molecular beacon of GAPDH mRNA (MB)	FAM-ACG ACG GAG TCC TTC CAC GAT ACC ACG TCG-/Azide-dT/-BHQ1
Synthetic miR-21	TAG CTT ATC AGA CTG ATG TTG A
Synthetic miR-122	TGG AGT GTG ACA ATG GTG TTT GT
Synthetic survivin mRNA	AAT TCA CAG AAT AGC ACA
Synthetic GAPDH mRNA	ACT TTG GTA TCG TGG AAG GAC TCA TGA
1-base mismatched miR-21 (oligo 1)	TAG C**C**T ATC AGA CTG ATG TTG A
1-base mismatched miR-21 (oligo 2)	TAG CTT ATC AGA CTG AT**A** TTG A
1-base mismatched miR-21 (oligo 3)	TAG CTT ATC **G**GA CTG ATG TTG A
2-base mismatched miR-21 (oligo 4)	TAG C**C**T ATC **G**GA CTG ATG TTG A
2-base mismatched miR-21 (oligo 5)	TAG C**C**T ATC AGA CTG AT**A** TTG A
3-base mismatched miR-21 (oligo 6)	TAG C**C**T ATC **G**GA CTG AT**A** TTG A
1-base mismatched GAPDH (oligo 7)	TGG TAT CGT **A**GA AGG ACT C
18A Oligonucleotide	AAA AAA AAA AAA AAA AAA

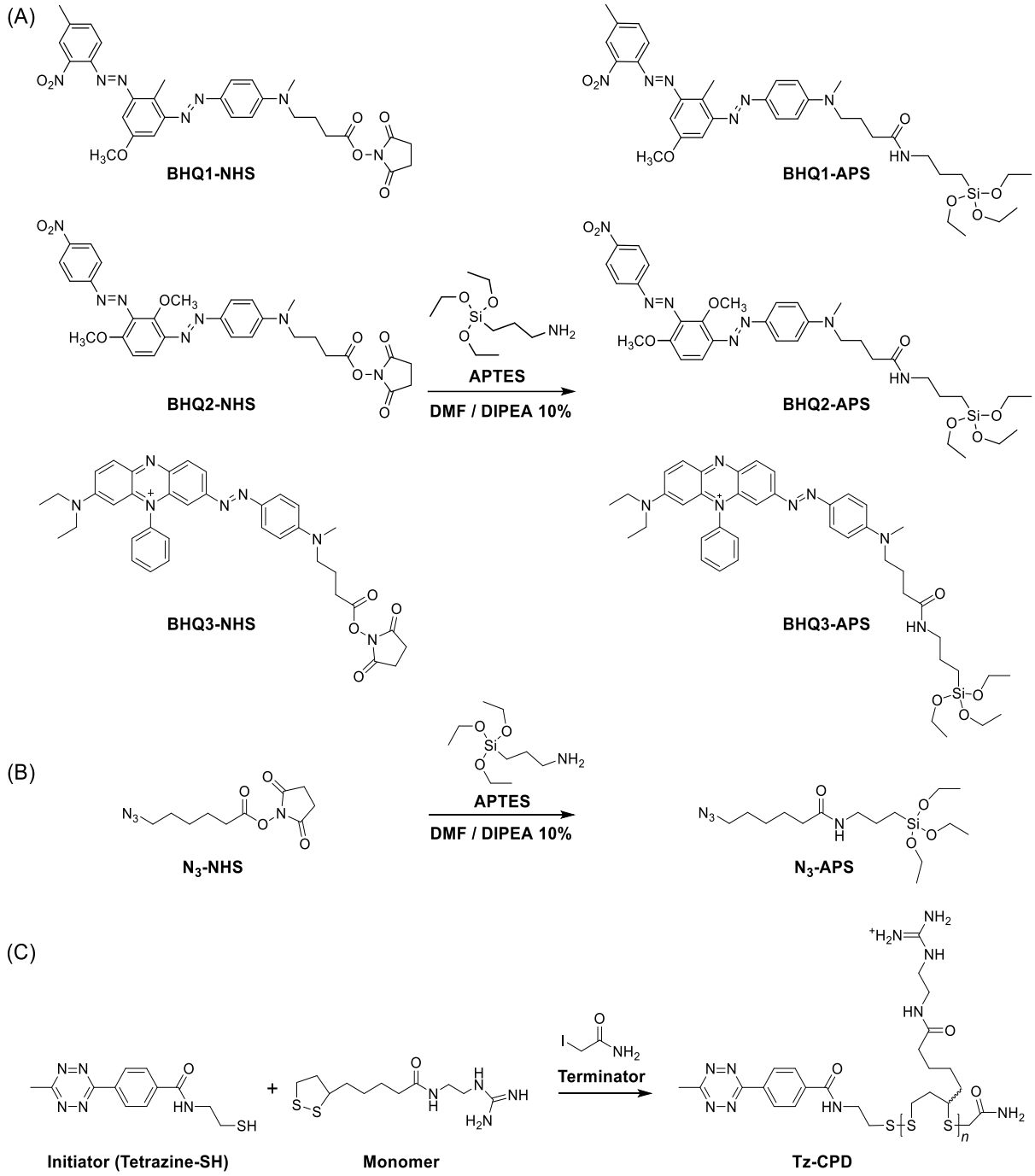

Figure S1. Synthetic schemes of (A) BHQ1/2/3-APS, (B) N_3-NHS, (C) Tetrazine-CPD (Tz-CPD) polymer.

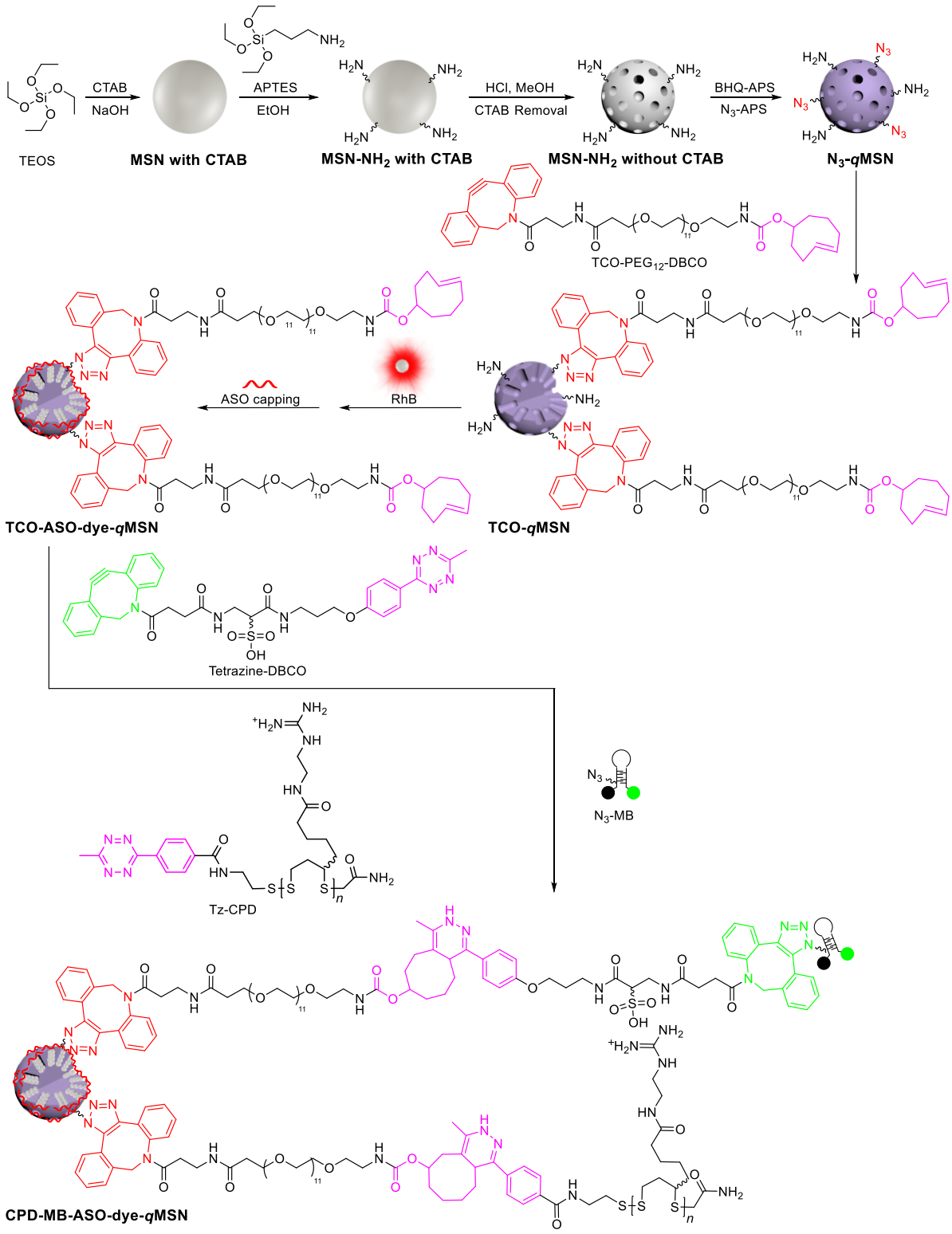

Figure S2. Scheme showing the preparation of CPD-MB-ASO-dye-*q*MSN. To obtain the final CPD-MB-ASO-dye-*q*MSN for further experiments, the preparation of MSN, amine surface modification, removal of CTAB template, BHQs surface modification, N_3 and TCO surface modification, dye loading, ASO capping, Tz surface modification and finally Tz-CPD and N_3-MB coupling *via* bio-orthogonal ligation reactions were done sequentially.

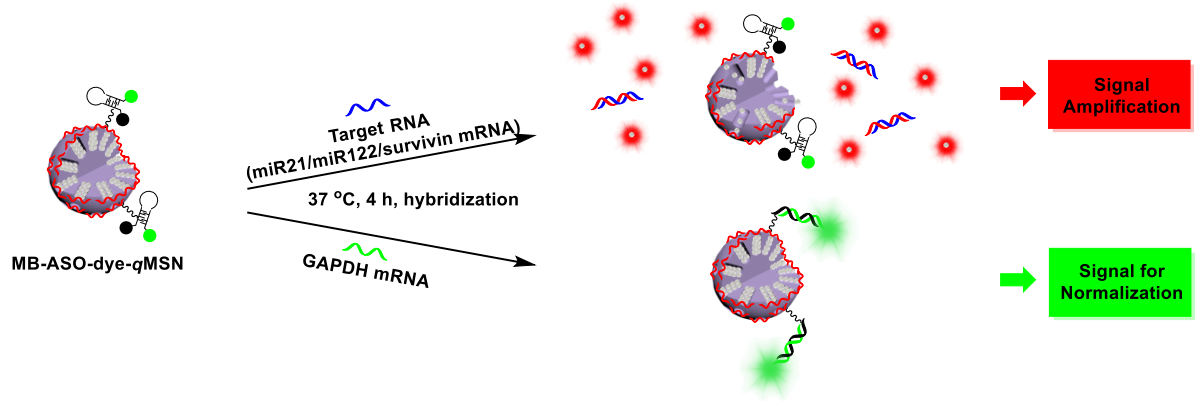

Figure S3. Scheme showing *in vitro* target detection with the corresponding fluorescence Turn-ON and detection signal amplification mechanisms.

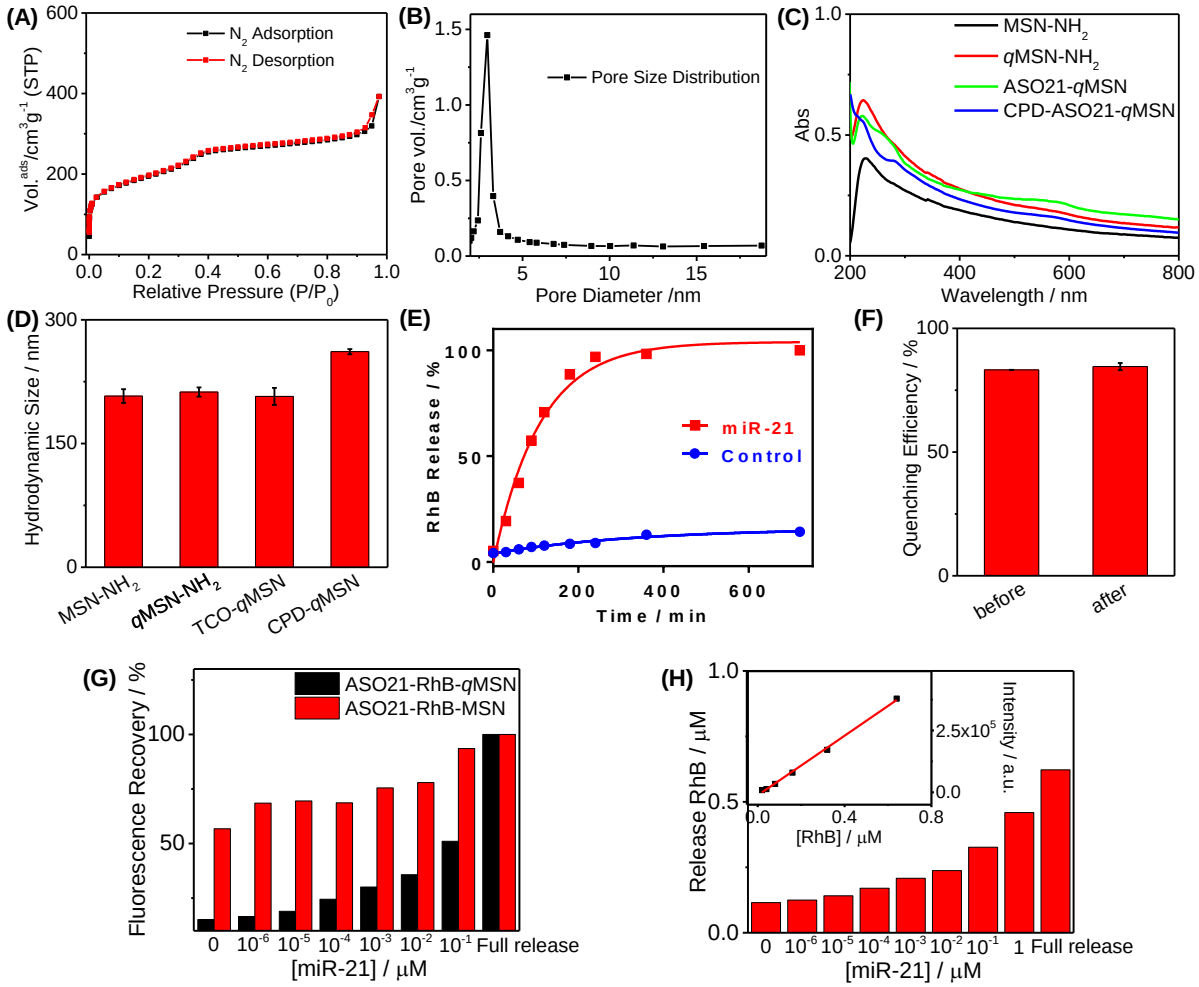

Figure S4. (A) BET nitrogen adsorption/desorption isotherms and (B) BJH pore size distribution of MSN-NH_2 without CTAB. The BET specific surface values, pore volumes, and pore sizes measured from the nitrogen adsorption-desorption isotherms were 695.041 m^2 g^{-1}, 0.706 cm^3 g^{-1} and 2.97 nm, respectively; (C) UV-Vis spectra of different MSNs (0.5 mg·mL^{-1} in PBS): black: MSN-NH_2; red: *q*MSN-NH_2; green: ASO21-*q*MSN; blue: CPD-ASO21-*q*MSN; (D) Hydrodynamic sizes of different MSNs (0.1 mg·mL^{-1} in water); The corresponding PDI were 0.225, 0.253, 0.152 and 0.153, respectively; (E) Time-dependent fluorescence profiles of RhB released from ASO21-RhB-*q*MSN (2 μg·mL^{-1}) upon incubation with miR-21 (10 μM, in PBS). ASO21-RhB-*q*MSN incubated with PBS instead of miR-21 was performed as control; (F) Quenching efficiencies of ASO21-*q*MSN (1 μg·mL^{-1} in PBS) on RhB with or without 4 h pre-incubation at 37 °C in PBS; The result indicates the quenching efficiencies has no obvious change before and after pre-incubation. (G) Comparison of in vitro target detection using ASO21-RhB-*q*MSN or ASO21-RhB-MSN (2 μg·mL^{-1} in PBS) upon incubation with different concentrations of miR-21 at 37 °C for 4 h; (H) The amount of RhB released from ASO21-RhB-*q*MSN (2 μg·mL^{-1} in PBS) after incubation with different concentrations of miR-21 for 4 h at 37 °C. Full release: ASO21-RhB-*q*MSN incubated with miR-21 (final concentration: 100 μM) and heated to 95 °C for 10 min. (Inset): Standard fluorescent calibration curve of RhB stock solutions in PBS buffer. RhB: λ_{ex}=545 nm, λ_{em}=580 nm.

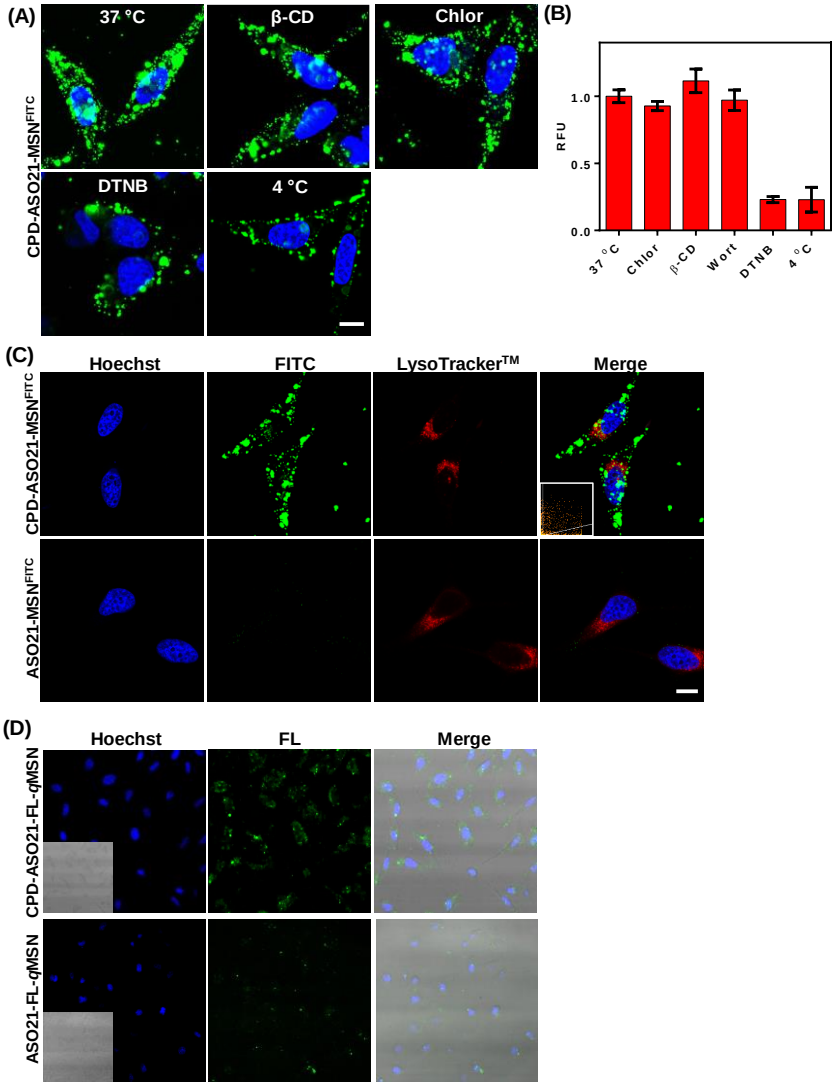

Figure S5. (A) CLSM imaging and (B) Flow cytometric analysis (FACS, fluorescence quantification based on analysis of at least 10000 cells) of HeLa cells incubated with CPD-ASO21-FITC labeled MSN (CPD-ASO21-MSN^{FITC}, 10 μg·mL^{-1}). Cells were pre-treated with different endocytosis inhibitors (chlorpromazine (10 μg·mL^{-1}, Chlor), wortmannin (50 nM, Wort), methyl-β-cyclodextrin (50 μM, M-β-CD)), or thiol-mediated pathway inhibitor 5,5'-dithioobis-2- nitrobenzoic acid (4.8 mM DTNB) at 37 °C for 30 min, or without inhibitor at 4 °C for 30 min before incubation with CPD-ASO21-MSN^{FITC} (10 μg·mL^{-1}) for 2 h. (C) CLSM imaging of HeLa cells incubated with CPD-ASO21-MSN^{FITC} and ASO21-MSN^{FITC} (10 μg·mL^{-1}) for 2 h, followed by washing with PBS buffer twice and staining with LysoTracker^{TM} (DND-99) and Hoechst (33342) for 10 min. (Blue: Hoechst, λ_{ex} = 405 nm, λ_{em} = 415-475 nm; Green: FITC, λ_{ex} = 488 nm, λ_{em} = 500-560 nm; Red: LysoTracker^{TM}, λ_{ex} = 561 nm, λ_{em} = 570-620 nm). (Inset): Corresponding scatter plot showing the co-localization between FITC (green) and LysoTracker^{TM} (red) channel. The Global Pearson's R value between FITC and LysoTracker^{TM} obtained from ImageJ software was -0.264. (D) CLSM imaging of HeLa cells incubated with CPD-ASO21-FL-*q*MSN or ASO21-FL-*q*MSN (10 μg·mL^{-1}, 2 h incubation for cell uptake and additional 4 h incubation for dye release) before staining with Hoechst for 10 min. (Inset): Bright field images. Scale bar = 20 μm.

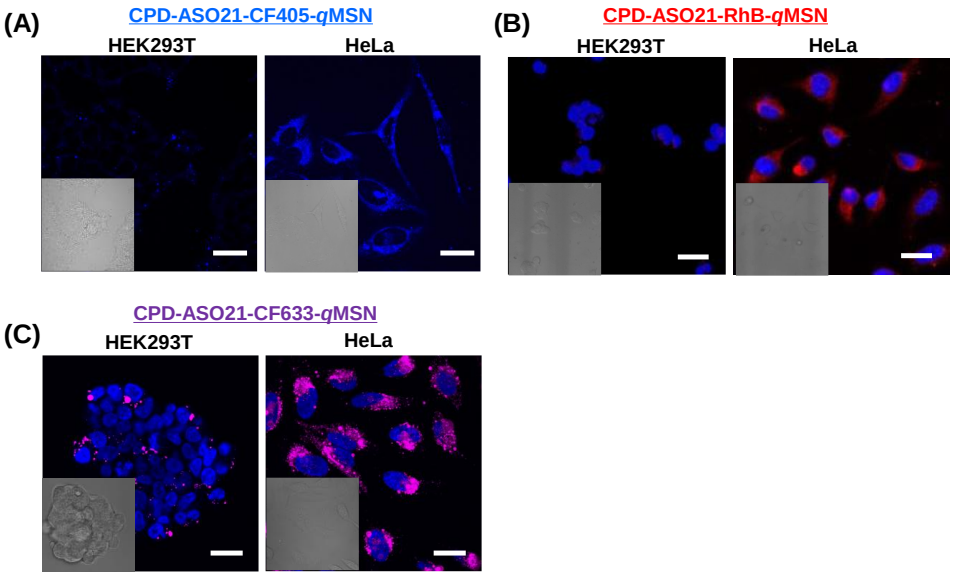

Figure S6. CLSM images of cells treated with (A) CPD-ASO21-CF405-*q*MSN, (B) CPD-ASO21-RhB-*q*MSN and (C) CPD-ASO21-CF633-*q*MSN (10 μg·mL^{-1}) for 2 h to uptake and incubated for additional 4 h to release dye. After that, cells were stained with Hoechst for 10 min prior to imaging acquisition. (Blue: Hoechst or CF405, λ_{ex} = 405 nm, λ_{em} = 415-475 nm; Red: RhB, λ_{ex} = 561 nm, λ_{em} = 570-620 nm; Purple: CF633, λ_{ex} = 640 nm, λ_{em} = 655-755 nm) Insets: bright field images. Scale bar = 20 μm.

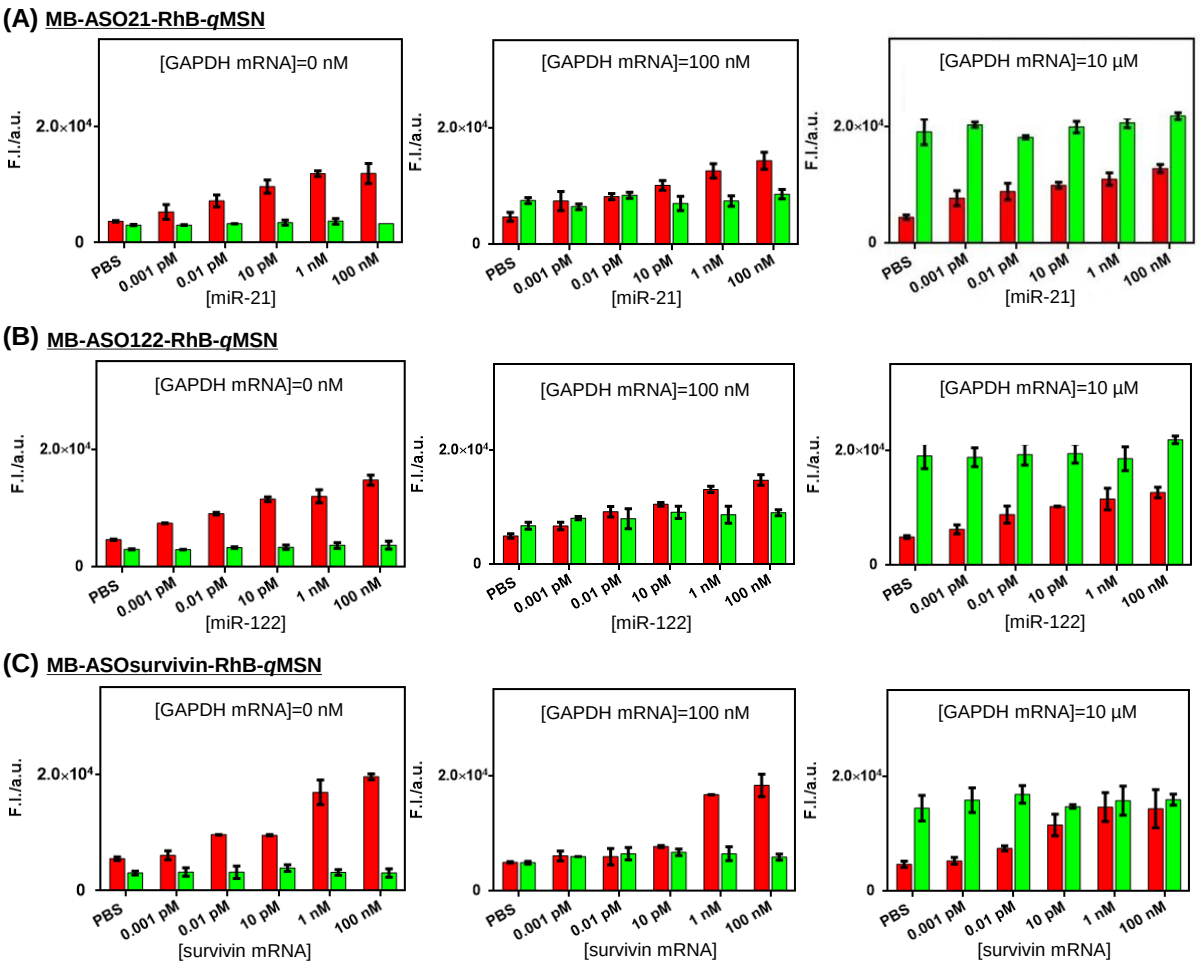

Figure S7. (A) Fluorescence intensity of MB-ASO21-RhB-*q*MSN, (B) MB-ASO122-RhB-*q*MSN, and (C) MB-ASOsurvivin-RhB-*q*MSN (2 μg·mL^{-1} in PBS) vs target concentrations after incubation with different amounts of respective targets (synthetic miR-21, miR-122 or survivin mRNA) and synthetic GAPDH combinations at 37 °C for 4 h. (Green bar: FAM for MB, λ_{ex} = 488 nm, λ_{em} = 510 nm; Red bar: RhB, λ_{ex} = 545 nm, λ_{em} = 585 nm.)

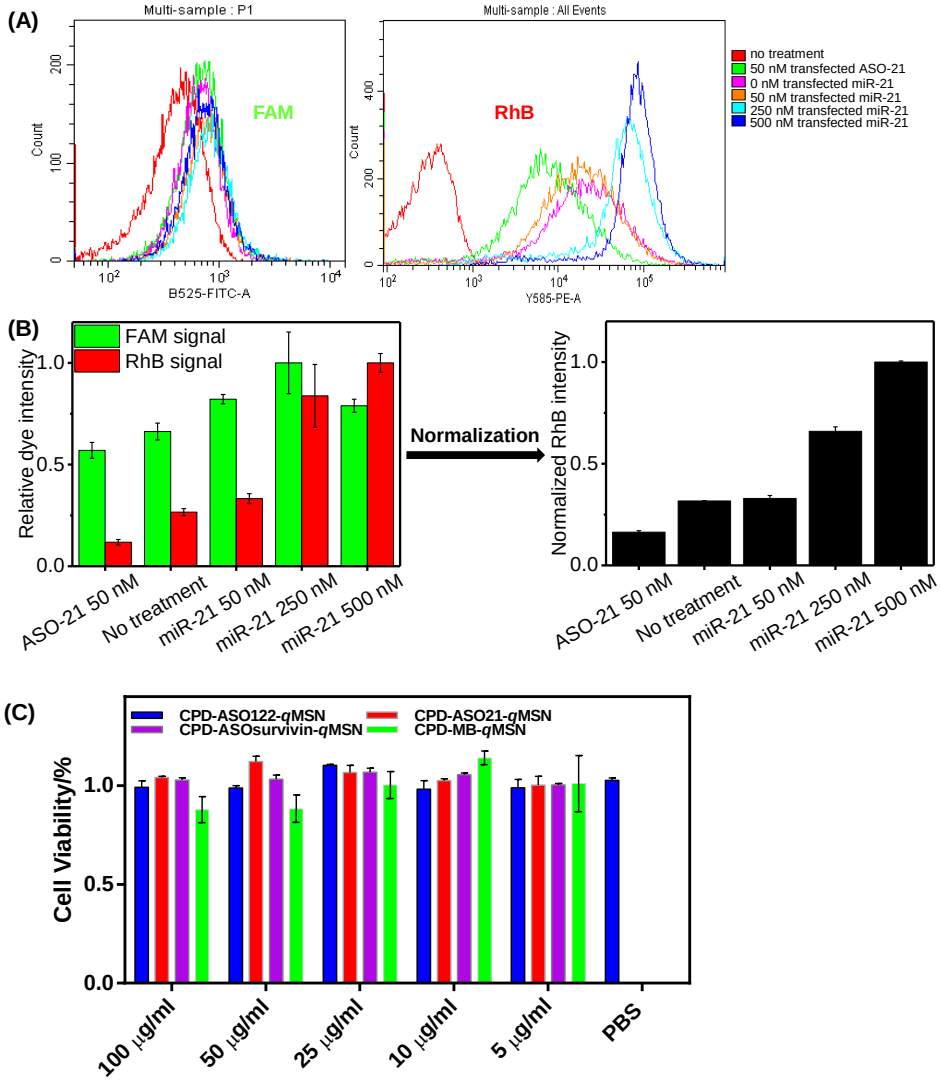

Figure S8. (A) Fluorescence quantification of HeLa cells transfected and treated with different concentrations of synthetic miR-21 and ASO-21 by FACS analysis (min. 10000 cells were analyzed). Left: FAM signal from MB; Right: RhB signal from CPD-MB-ASO21-RhB-*q*MSN. (B) RhB fluorescence intensity with normalized FAM signal shown in (A). Partial results shown in Figure 5E. (C) Cell viability of HeLa cells incubated with different types of *q*MSNs for 24 h. Error bars were obtained by analysis of triplicate tests.