Supplementary Information

Experimental Section:

Materials. Cell counting kit-8 was purchased from Dojindo Molecular Technologies (Tokyo, Japan). RMPI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Penicillin and streptomycin were provided by Sigma-Aldrich (St. Louis, MO, USA). The sequence of miR-203 is as follows: 5'-GUGAAAUGUUUAGGACCACUAG-3'. The mimic was synthesized and labeled Cy-5 by RiboBio Co. (Guangzhou, China). TAT peptide was synthesized by ChinaPeptide Co., LTD (Shanghai, China). All solvents used in this investigation were of HPLC grade. All other chemicals and solvents were analytical or reagent grade and used without further purifications. The water used was of ultrapure grade and was supplied by a Milli-Q purification system (Millipore Co., Billerica, MA, USA).

Synthesis of CMC. CMC was prepared based on the method described previously (Scheme S1).[1] Briefly, chitosan (2 g) and sodium hydroxide (NaOH) (5 g) were dispersed in isopropanol (30 mL). The mixture of chitosan and sodium hydroxide (CS) was left to swell at room temperature for 1 h and alkalized at −10 °C for 24 h. The solution of monochloroacetic acid (4 g) in isopropanol (20 mL) was then added to the frozen CS mixture. The mixture was left to react at 50 °C for 6 h. The reaction was stopped by adding 70% ethanol to the mixture and the product (sodium salt of CMC) was obtained by filtration and converted to CMC by dropping 32% hydrochloric acid solution until reaching neutral pH after removing 70% ethanol by rinsing. Finally, CMC was dried in a vacuum oven at 50 °C and stored in a desiccator. The degree of substitution of carboxymethylation was determined as 65% by potentiometric titration method.[2] Fourier transform infrared spectroscopy (FTIR) was used to analyze the CS and CMC.

Preparation of nanoparticles. All the samples were prepared in 20 mM PBS buffer aqueous solution (pH 7.4) containing 20 mM NaCl. Blank CMC-TAT nanoparticles (B-CTNs) were prepared by simply mixing CMC and TAT solutions. Briefly, 1 mL of aqueous solution containing TAT (1 mg mL^{-1}) was slowly added to 1 mL of solution containing CMC with the concentrations ranging from 0.25 to 4.0 mg mL^{-1} under constant magnetic stirring at room temperature. The mixed solution was then incubated at room temperature for 20 min to allow complete formation and stabilization of the complexes. After the formation of the nanoparticles, the solution became turbid. The 2 mL mixed solution was further transferred to eppendorf tube and the nanoparticless were precipited by centrifugation (12000 *g*, 10 min). Supernatants were discarded and the nanoparticles in the botton of the eppendorf tubes were then resuspended in nuclease free water by vortex mixture. For the preparation of miR-203 encapsulated CMC-TAT nanoparticles (M-CTNs), the needed amount of miR-203 was first mixed with the CMC solution phase and M-CTNs were formed using the same methods as described above. Briefly, an aqueous solution of miR-203 (0.02 mg) in 100 μL of RNase-free water was first mixed with 1 mL of 1.5 mg mL^{-1} CMC solution. Then, 1 mL of 1 mg mL^{-1} TAT solution was dropwise added into the miR-203/CMC solution under constant magnetic stirring at room temperature. As a control, bare miRNA-TAT nanoparticles (m-TNs) were prepared according to other literatures.[3,4] Briefly, miR-203 solution was added to TAT solution at a fixed N/P of 20 with repeated pipetting and then incubated for 30 min at room temperature.

Characterization of nanoparticless. The average size, polydispersity (PDI), and zeta potential of the different components of nanoparticles were determined by dynamic light scattering (DLS) using a ZetaSizer Nano series Nano-ZS (Malvern Instruments Ltd, Malvern, UK). Determinations were performed at 633 nm with a constant angle of 90° after the samples were appropriately diluted in distilled water. Each batch was analyzed in triplicate. TEM images were obtained using a JEOL model JEM-2010 transmission electron microscope at an acceleration voltage of 200 KV.

Gel electrophoresis and serum stability. M-CTNs were analyzed by 2% agarose gel electrophoresis. The gels were prepared with 2% agarose in tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer containing 0.5 μg/ml GelRED™ (Biotium, USA). For electrophoretic mobility shift analysis, free miR-203 was used as control. Nanoparticles samples were loaded on a 2% agarose gel after

incubation at room temperature for 15 min, with or whithout 10% glycerine and 5 μL 2% SDS. Gel electrophoresis was carried out at 110 V for 10 min and the gel was subsequently photographed using Alpha Innotech gel imager system. Serum degradation assays were performed as fellows: Free miR-203 in aqueous solution and the nanoparticles-encapsulated miR-203 were mixed with fresh serum (Gibco, USA) in a 1:1 ratio to give 50% serum concentration, respectively. The mixtures were then incubated at 37 °C for the indicated times. Aliquots (15 μL) from each mixture samples were picked up and mixed with 5 μL 2% SDS and 2.5 μL 10% glycerine. The mixtures were further loaded onto a 2% agarose gel containing 0.5 μg/mL GelRED™ (Biotium, USA). Gel electrophoresis was carried out at 110 V for 10 min and the gel was subsequently photographed using Alpha Innotech gel imager system.

Colloidal stability assay. M-CTNs were suspended in deionized water, phosphate-buffered saline (PBS) of pH 7.4 and RMPI 1640 with 10% FBS, respectively, under mild stirring at 37°C. The M-CTNs sizes were determined at 0, 1 and 2 days to examine the stability of M-CTNs at different liquid environments by DLS.

MiRNA encapsulation efficiency. To evaluate the efficiency of miR-203 encapsulated in nanoparticless, the fluorescence of un-encapsulated Cy5-labeled miR-203 was quantified using fluorescence spectrophotometer (excitation at 646 nm, emission at 664 nm). The Cy5-miR-203 encapsulated M-CTNs were prepared as described above. After centrifugation at 12 000 g for 10 min, the fluorescence of the Cy5-miR-203 in the supernatant was detected using a fluorescence spectrophotometer (Hitachi, Japan). The encapsulation efficiency was calculated using the formula:
Encapsulation efficiency (%) = $(F_0-F_1)/F_0 \times 100\%$
where F_0 was the fluorescence of Cy5-miR-203 used for encapsulation,
F_1 was the fluorescence of Cy5-miR-203 in the supernatant.

In vitro release of miR-203. To measure the release kinetics of Cy-5-labeled miR-203, M-CTNs were incubated in PBS of pH 5.5 and pH 7.4, respectively, in a 96-well culture plate at 37 °C under moderate shaking. Samples were harvested from the plate at scheduled time points and centrifuged at 12 000 rpm for 10 min. Release values were normalized to 0% for day 0. The miR-203 in the supernatant and pellet were measured using a standard curve by measuring the fluorescent density of Cy-5 using a hybrid multi-mode microplate reader (Tecan, Durharm, USA).

Cell culture, cellular uptake and intracellular tracking assays. Cells were cultured in high-glucose DMEM supplemented with 10% (v/v) heat-inactivated FBS, 100 units/mL penicillin and 100 μg/mL streptomycin in a 5% CO2 incubator at 37 °C under 95% humidity.

Human esophageal cancer cells EC-109 were cultured in RPMI-1640 media supplemented with 10 % FBS (Gibco-BRL) and incubated at 37°C in a humidified atmosphere with 5% CO_2. The cell uptake and intracellular tracking Cy-5-labeled miR-203 in different formulations was assessed using confocal microscopy. For the uptake assay, EC-109 cells were seeded onto a borosilicate chambered cover glass (Nunc, USA) at a density of 1×10^5 per well at 37 °C and cultured for 12 h before transfection. The cells were then incubated with M-CTNs and m-TNs (100 nM miR-203) in 1 mL serum-free culture media for 4 h. Subsequently, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS) and fixed with 4% formaldehyde. Red fluorescence of Cy-5 was observed using a Zeiss LSM780 confocal microscopy (Zeiss Co., Germany) at excitation wavelength of 543 nm.

For the intracellular tracking assay, after different incubation times with M-CTNs and m-TNs, EC-109 cells were washed with PBS and stained with LysoTracker green for 20 min. The cells were washed twice with Dulbecco's phosphate buffered saline (DPBS) and then imaged following the operation mentioned above. The confocal laser scanning microscopic observation was performed at excitation wavelengths of 646 nm and 457 nm for Cy-5 (red) and LysoTracker Green (green), respectively. The colocalization ratio of Cy-5 miR-203 with LysoTracker was quantified as follows: colocalization ratio (%) = A/B × 100%, where A represent the number of Cy-5 pixels colocalization with LysoTracker Green, and B represent the number of all Cy-5 pixels. The results are presented as mean and standard deviation obtained from 10 cells. P value was calculated by Student's *t* test.

Flow cytometry measurements. EC-109 cells were plated in 6-well plates 6×10^5cells per square centimeter and treated 6 h later with Cy-5-labeled miR-203 formulations. After incubation, the cells were washed twice with PBS, trypsinized (750 mL trypsine 0.25% per well) and diluted with 7 mL cell culture medium. Following centrifugation (7 min, 1200 rpm), the cell pellet was resuspended in 400 mL flow buffer (PBS supplemented with 1% BSA and 0.1% sodium azide) and placed on ice until analysis. The cells were analyzed using a Beckman Coulter Cytomics FC500 flow cytometer. Generally, a minimum of ~10^4 cells were analyzed in each measurement. The data were analyzed using Beckman Coulter CXP analysis software.

RNA isolation and real-time quantitative PCR (qRT-PCR). The expression level of miR-203 was evaluated by quantitative real-time PCR (qRT-PCR). Trizol reagent (Invitrogen, USA) was applied for total RNA extraction from cultured cells using following the protocols of the manufacturer. For miRNA expression analysis, 1 μg of total RNA was used to synthesize cDNA of miRNA through reverse transcription using Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems). QRT-PCR analysis was carried out using TaqMan Universal Master Mix II, No AmpErase UNG (2×) (Invitrogen, USA) and special RT-primer designed for miR-203 (Taqman assay ID 000426, Invitrogen, USA) according to the manufactuer's instructions. 25 μL of the reverse transcription mix was amplified by PCR with the following program: 95 °C for 10 min, 55 °C for 2 min, 72 °C for 2 min, and 12 cycles at 95 °C for 15 s and 60 °C for 4 min. U6 was used as an endogenous control for normalization. Each sample was analyzed in triplicate. Primer sequences used were as follows:
MiR-203: 5'- GCGTGAAATGTTTAGGACCACTAG -3'
U6: 5'-GCTTCGGCAGCACATATACTAAAAT-3'

Western-blot analysis. 100 nM concentration of miR-203 were used to treat EC-109 cells. Untreated cells were used as control. At 48 h post-treatment, cells were collected and then were lyzed with RIPA lysis buffer by incubation on ice for 10 min. The supernatants were harvested after centrifugation at 12 000 *g* for 10 min and the concentrations of proteins were determined using Bradford's reagent (Bio-Rad laboratories, USA). After boiling for 10 min, the denatured protein samples were loaded onto SDS-PAGE gel for electrophoresis. The proteins were then incubated in the blocking solution (5% non-fat dried milk) at room temperature for 1 h after transferred onto PVDF membranes (Millipore, USA). The anti-E2F1 (1:2000, Abcam, USA), anti-Ran (1:2000, Abcam, USA) and anti-LASP-1 antibody (1:2000, Abcam, USA) was added into blocking solution and incubated with the membranes at 4 °C overnight. Goat anti-mouse antibody conjugated with fluorescent dyes: IRDye 800 CW (1:5000, KPL, USA) was subsequently applied for membrane incubation for 45 min. Protein expression was normalized against GAPDH expression (RD, USA). Blotting images were acquired with the Odyssey infrared imaging system (Li-COR Biosciences, USA) and analyzed by the software provided by the manufacturer.

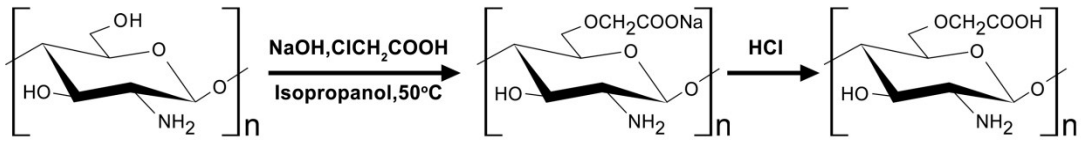
Cell proliferation assay. Cell viability was determined at indicated time points using CCK-8 assay according to manufacturer's instructions (Dojindo, Japan). The percentage of the viable cells was calculated using the following formula: Viability% = A-B/C-B × 100%, where A represents the absorbance of test, B represents the absorbance of blank (medium), C represents the absorbance of control (cells). Absorbance at 450 nm was detected with TECAN Infinite M200 microplate reader (Tecan, Durham, USA).

In vitro transwell migration assay. EC-109 cells were seeded at a density of 6×10^5 cells per well in 6-well plates and incubated for 12 h before experiments. The cultured cells were incubated with different formulations containing 100 nM of miR-203 in 1 mL of serum-free culture media for 4 h. All treated cells were then washed twice with PBS to remove the remaining NPs and cultured in RPMI-1640 medium with 10% FBS for additional 24 h. The migration assay was conducted using transwell insert chambers (Corning, USA). 1×10^5 transfected cells were harvested and plated into the upper chamber in serum-free medium. Medium containing 20% FBS in the lower chamber served as chemoattractant. After incubation for 24 h at 37 °C in a 5% CO_2 humidified incubator, cells in the upper chambers were removed by wiping with a cotton swab and cells migrated to the lower surface of filter were fixed in 70% ethanol for 30 min and stained with 0.2% crystal violet for 10 min. Cell migration was scored by counting five random fields per filter under a light microscope.

Cell migration rate was calculated by formula as follows: Relative rate of migration (%) =

migrating cells with treatment/ migrating cells without treatment ×100. The transwell assay was carried out in triplicate.

Statistical analysis. The mean ± SD was determined for each treatment group. Comparison of each group was evaluated by one-way analysis of variance (ANOVA). The differences were considered significant for *p < 0.05.



Scheme S1 The synthesis procedure of CMC

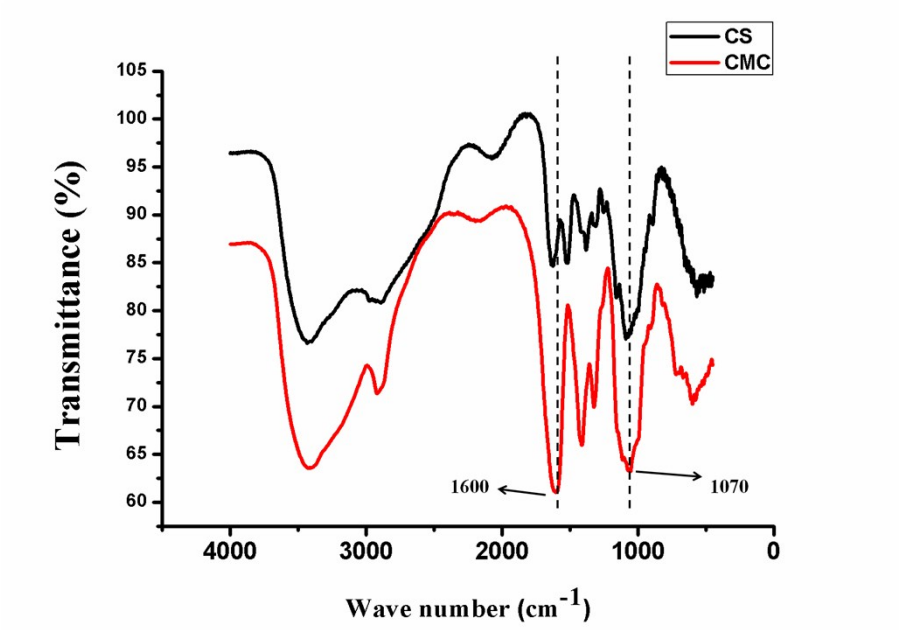


Figure S1 FTIR analysis of CS and CMC

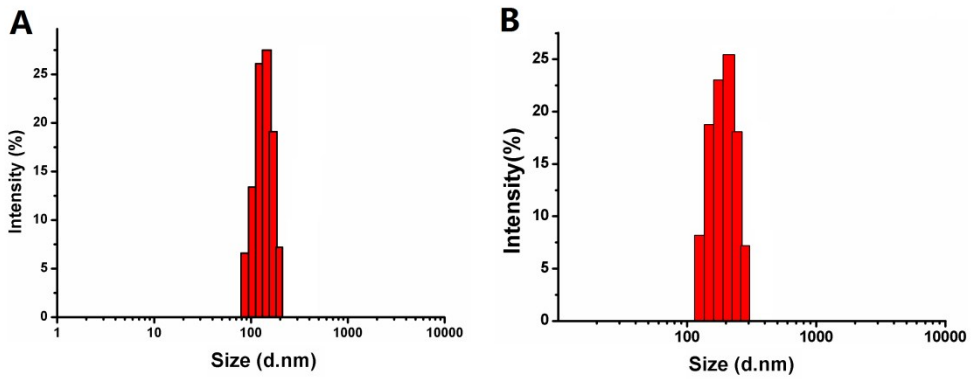


Figure S2 Number-averaged size histograms of B-CTNs (A) and M-CTNs (B) detected by DLS

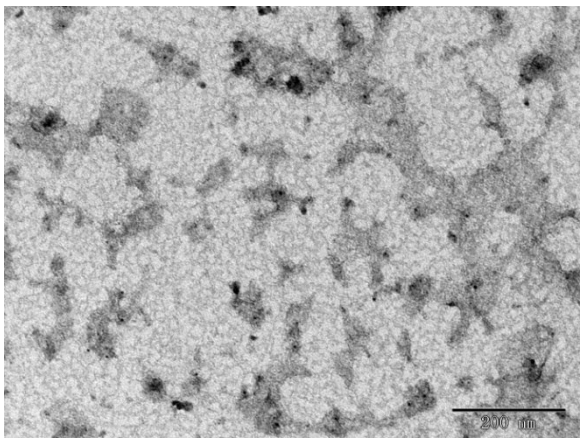


Figure S3 TEM image of M-CTNs after incubation at pH 5.5 for 2.5 hours.

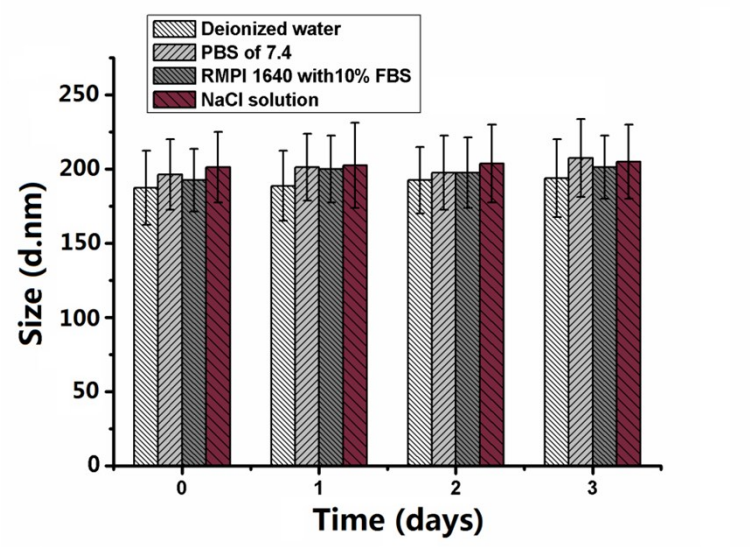


Figure S4 Post-formulation stability of M-CTNs in different solvent environments based on the average hydrodynamic diameters at 37 °C

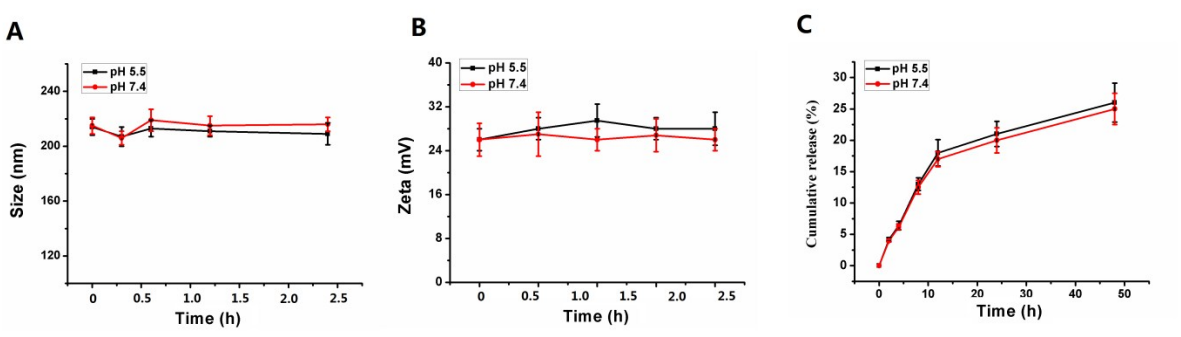


Figure S5 Particle size, zeta potential of m-TNs and miR-203 release from m-TNs were measured in PBS at pH 5.5 and pH 7.4, respectively,

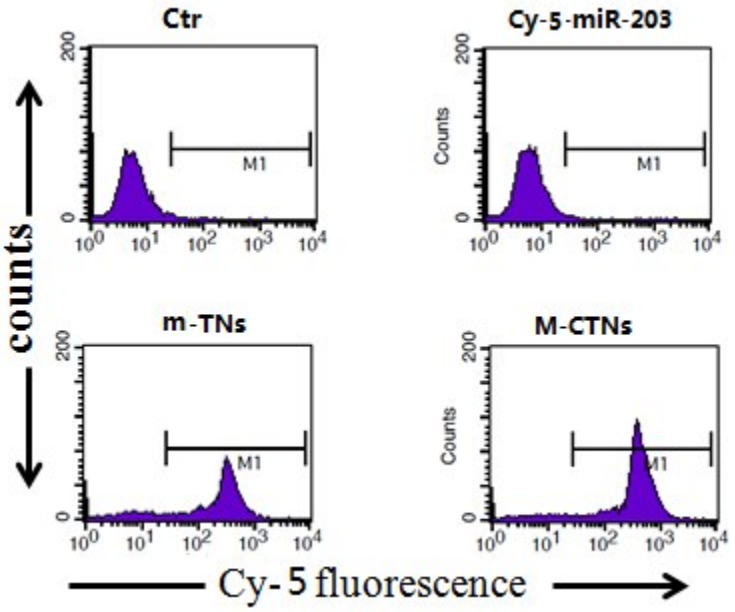


Figure S6 Flow cytometry analysis. MiR-203 was labeled by Cy-5. Cy-5 positive cells were quantitatively determined by flow cytometry.

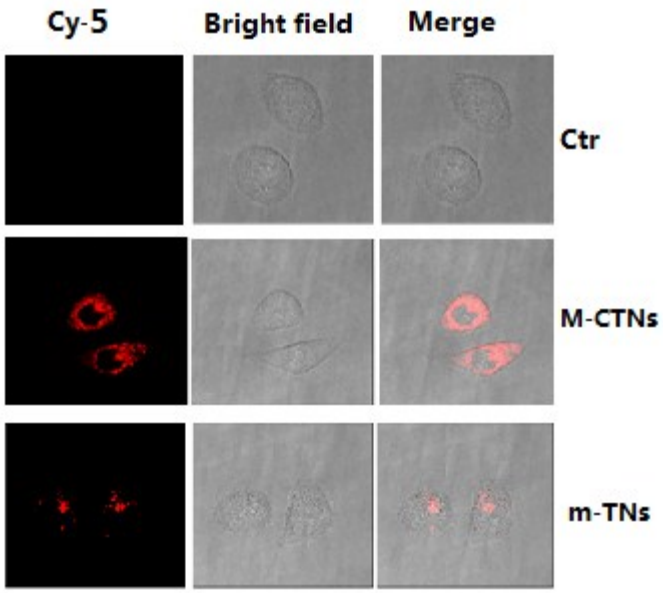


Figure S7 Laser confocal microscopic images of EC-109 cells incubated with M-CTNs and m-TNs for 4 h.

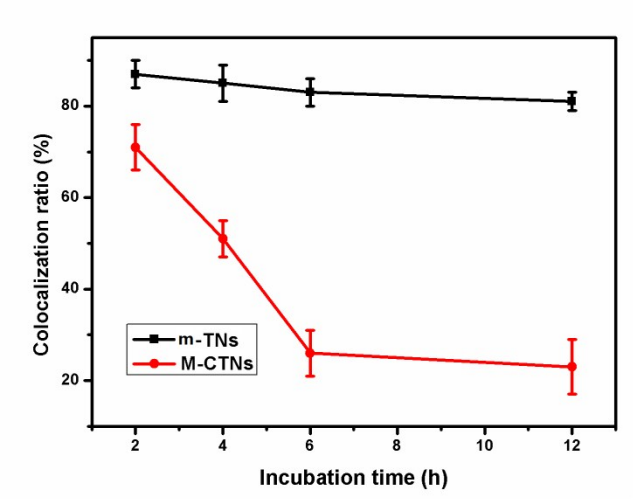


Figure S8 Time-course changes in the colocalization ratio of Cy-5-labeled miR-203 to late endosome/lysosome.

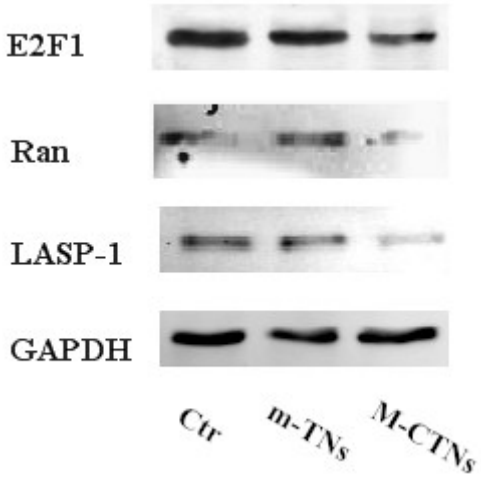


Figure S9 The expression of E2F1, LASP-2 and Ran detected by Western blotting.

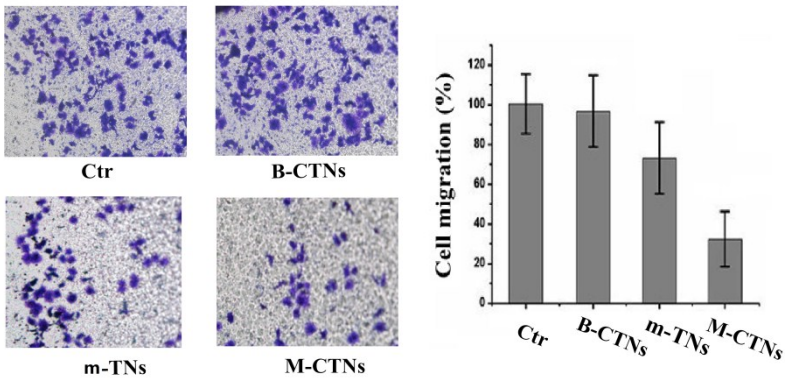


Figure S10 Photomicrographs of transwell filters showing the cells which across an 8-μm pore size membrane with matrigel. 200 x magnification. Quantitative determination of cell invasion was presented as means of 5 counts.

Table S1. Physicochemical properties of the B-CTNs with different mass ratios of CMS/TAT, which were evaluated in water (mean SD, n=3)

[CMC]/[Tat] Mass ratio	Size [nm]	Zeta potential [mV]	PDI	Yield (%)
0.25mg/1mg (1:4)	1112	+21	0.557	71
0.5mg/1mg (1:2)	979	+17	0.528	82
1mg/1mg (1:1)	N.A.	N.A.	N.A.	89
1.5mg/1mg (1.5:1)	186.8	-30.9	0.183	77
2mg/1mg (2:1)	174.4	-32.1	0.123	61
4mg/1mg (4:1)	169.6	-35.5	0.118	31

Table S2. Encapsulation efficiency of miR-203 was determined based on absorbance values. M-CTNs with the weight ratio of CMC and TAT of 1.5:1 was used for encapsulation.

MiR-203 (mg)	**E.E. (%)**
0.01	98.2
0.02	93.5
0.04	90.2
0.08	81.3
0.2	67.9

References

1. Patel, N.K. and Sinha, V.K, *Int. J. Polym Mater.* 2009, **58**, 548–560.
2. I. M. EI-Sherbiny and H. D. C. Smyth, *Carbohyd Res*. 2010, **345**, 2004-2012.
3. C. Rudolph, C. Planks, J. Lausier, U. Schillinger, R. H. Müller¶ and J. Rosenecker. *J Biol Chem*, 2003, **13**, 278.
4. W. J. Kim, L. V. Christensen, S. Jo, J. W. Yockman, J. H. Jeong, Y. H. Kim and W. Kim. *Mol Ther,* 2006, **14(3)**, 343-350.