Delivery of dual miRNA through CD44-targeted nanoparticle for enhanced and effective Triple-negative breast cancer therapy

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Synthesis Protocols

General procedure for synthesis of Mesoporous silica nanoparticles (MSNPs)

A solution containing 6.4 mL of Mili-Q H_2O, 1.14 mL of ethanol, 1.04 g of CTAB and 0.02 g of DEA was mixed and stirred in a water bath at 60 °C for 1h prior to drop wise addition of 0.73 mL TEOS within 2 min. Then the reaction mixture was stirred for 3 h at 60 °C. The solution was cooled to room temperature, and MSNPs were collected by centrifugation. Then it was washed several times with absolute ethanol and Mili-Q H_2O to obtain MSNPs. Finally it was dried in vacuum and calcinized at 400 °C for 5h to obtain mesoporous silica nanoparticles as white powder.

Synthesis of amine functionalised mesoporous silica nanoparticles: (MSN- NH_2):

130mg dried and calcinized mesoporous silica nanoparticles MSN was suspended in 40 ml of ethanol and sonicated in bath sonicator. Then, 130ml of water was added to the solution. After stirring for 15min, 3ml glacial acetic acid was added and allowed to stir for 4 hours. Then 0.5 ml

APTES (AminopropylTriethoxysilane) was added, then solution was stirred overnight. Next day, the solution was centrifuged, washed with cold ethanol and was dried in vacuum overnight to obtain amine functionalised mesoporous silica nanoparticles as white powder.

Synthesis of Activated PLGA:
500 mg of PLGA dissolved in anhydrous methylene chloride and stirred at room temperature. Then 80mg of N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and 100 mg of *N*-Hydroxysuccinimide (NHS) for 24 h at room temperature under nitrogen atmosphere. The reaction mixture was filtered through a 0.45-μm Teflon syringe filter. The filtrate was precipitated and washed with ice cold diethyl ether. The precipitate was dried over vacuum to obtain activated PLGA as white powder.

Conjugation of 2,2′-(Ethylenedioxy)bis(ethylamine) with activated PLGA:
Activated PLGA and 2,2′-(Ethylenedioxy)bis(ethylamine) were dissolved in anhydrous methylene chloride (1:1 molar ratio) in argon atmosphere. Then the reaction mixture was kept at room temparature for overnight. The reaction mixture was precipitated by adding cold diethyl ether and washed with ethyl acetate. The precipitate was then dried over vacuum to obtain PLGA-PEG-NH_2.

Synthesis of hyaluronic acid conjugated mesoporous silica nanoparticles: (MSN@HA):
HA (50 mg) was hydrated in water overnight and lyophilized. Then it was activated by using 4 mg of EDC and 4 mg of NHS in a 2-(N-morpholino) ethanesulfonicacid (MES) buffer (pH 6.0) for 1 h. After then, 20 mg of MSN-NH_2 was added at room temperature with continuous stirring for overnight h. The unreacted HA was removed by centrifugation. The resulting NPs was dried over vacuum. [1]

SupplementaryTables

Table S1: Primer list of miRNAs

Name	Sequence
U6 RT	5′-AAAATATGGAACGCTTCACGAATTTG-3′
Universal Reverse	5′-CCAGTGCAGGGTCCGAGGTA-3′
U6 Forward	5′-CTGGCTTCGGCAGCACATA-3′
U6 Reverse	5′-CACGAATTTGCGTGTCATCC-3′
Has-miR-34a-5p Stem	5′-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC ACA ACC-3′
Has-miR-34a-5p Forward	5′-CAG GCA TGG CAG TGT CTT AGC T-3′

Has-miR-10b Stem	5′-GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACCACAAA-3′
Has-miR-10b Forward	5′-CCCCCCCAAATACCCTGTAGAACC-3′

Table S2: Primer list of mRNAs

Name	Sequence
Human GAPDH Forward	5′-CTTTGGTATCGTGGAAGGACTC-3′
Human GAPDH Reverse	5′-GTAGAGGCAGGGATGATGTTC-3′
18S Forward	5′-GTAACCCGTTGAACCCCATTCGT-3′
18S Reverse	5′-CCATCCAATCGGTAGTAGCGACGG-3′
HOXD10 Forward	5′-CACCATGTCCTTTCCCAACAGCT-3′
HOXD10 Reverse	5′-CTAAGAAAACGTGAGGTTGGCGG-3′
RhoC Forward	5′-GGAGGTCTACGTCCCTACTGT-3′
RhoC Reverse	5′-TACCCGGACACTGATGTCATC-3′
Bax Forward	5′-GGGTGGTTGGGTGAGACTC-3′
Bax Reverse	5′-AGACACGTAAGGAAAACGCATTA-3′
Bcl-2 Forward	5′-GTGGATGACTGA0GTACCTGAAC-3′
Bcl-2 Reverse	5′-GCCAGGAGAAATCAAACAGAGG-3′
Human Alu Forward	5′- ACGCCTGTAATCCCAGCACTT -3′
Human Alu Reverse	5′- TCGCCCAGGCTGGAGTGCA- 3′
Chick GAPDH Forward	5′- GAGGAAAGGTCGCCTGGTGGATCG-3′
Chick GAPDH Reverse	5′- GGTGAGGACAAGCAGTGAGGAACG-3′

Table S3: Incorporation of RNAs in different form Mesoporous silica cation

Types	Sample	sorbance ($A_{260/280}$)	ncentration (ng/mL)
Non-functionalized Mesoporous silica nanoparticle	(MSN-NCH3)+I- +10b	1.95	129.01
	MSN +34a	1.90	132.85

(MSN)	MSN + 10b +34a	1.90	140.35
Cationic surface functionaliz mesoporous silica nanoparticle $(MSN\text{-}NCH_3)^+I^-$	(MSN-NCH3)+I- +10b	1.95	502.85
	MSN Cation+34a	1.90	578.01
	MSN Cation+ 10b +34a	1.90	511.35
Neutral surface functionalized Mesoporous silica nanoparticle (MSN- CH_2-CH_2-CH_3)	(MSN) +10b	1.95	110.01
	MSN +34a	1.90	98.85
	MSN + 10b +34a	1.90	105.35
Anionic surface functionalized mesoporous silica nanoparticle(MSN-SO_3H)	(MSN- SO_3H) +10b	1.95	53.80 ±0.15
	(MSN- SO_3H) +34a	1.90	58.0±0.15
	(MSN- SO_3H) + 10b +34a	1.90	55.3 ±0.15
PLGA coating on Cationic surface functionalized mesoporous silica nanoparticle $(MSN\text{-}NCH_3)^+I^-$	MSN Cation+ 10b @HA-PEG-PLGA	1.78	330.85
	MSN Cation+34a@HA-PEG-PLGA	1.77	339.85
	MSN Cation+ 10b +34a@	1.77	339.85
	HA-PEG-PLGA	1.08	19.994

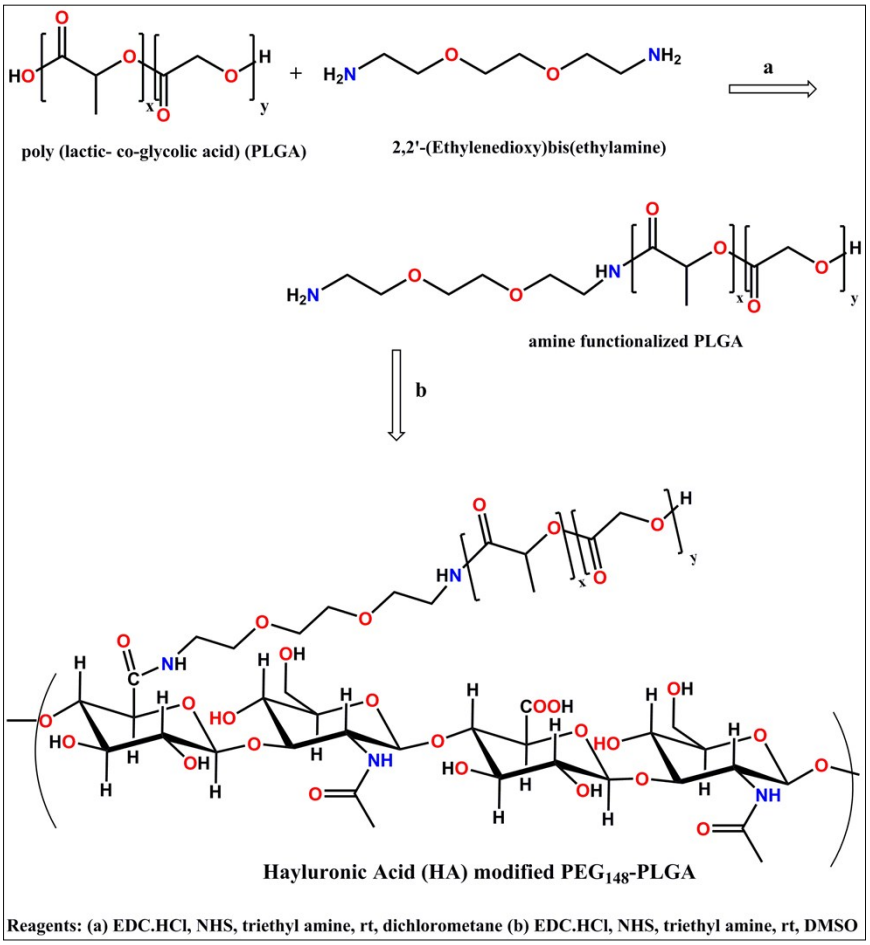
Table S4: Zeta Potential values of MSN Cation, MSN-NH_2 and PLGA MSN

Sample Id	Properties	Zeta values (mV)	Avg.±Std. Dev
MSN-NH_2	Temp: 30.4°C Specific conductance: 6.78mS/cm Concentration 8×10^{-4} pH=6.5	1. +33.4 2. +30.0 3. +48.9 4. +41.0 5. +43.8 6. +43.5	+40.10±7.07
Quaternized MSN/MSN-$NH_3^+I^-$	Temp: 30.0°C Specific conductance: 7.89mS/cm Concentration 8×10^{-4} pH=6.5	1. -47.5 2. -57.1 3. -58.6 4. -46.9 5. -57.4 6. -60.3	+54.62±5.85-
MSN-$NH_3^+I^-$ @HA-PEG-PLGA	Temp: 30.0°C Specific conductance: 1436.00mS/cm Concentration 8×10^{-4}	1. -20.9 2. -19.5 3. -25.9 4. -27.2	+24.20±3.76

	pH=6.5	5. -22.7 6. -29.7	

Supplementary Figures

Figure S1: Schematic representation of Coating of HA-PEG$_{148}$-PLGA on cationic MSN.



In addition, the synthesis of HA-PEG-PLGA copolymer was confirmed by ^{1}H-NMR. In D_2O, the occurrence of peaks at $\delta = 1.14$ ppm depict methyl group (–CH_3 groups of HA), and $\delta = 3.4$ ppm depict methylene proton peaks (–CH_2 groups of PEG). Moreover, an apparent N-acetylate proton peak ($NCOCH_3$) in HA was detected at 2.08 ppm. This study suggested

the formation of amide bonds between carboxylic groups of HA and one amine of bis-amine-PEG.

Figure S2: ^{1}H NMR spectra study of Hyaluronic acid appended PEG-PLGA

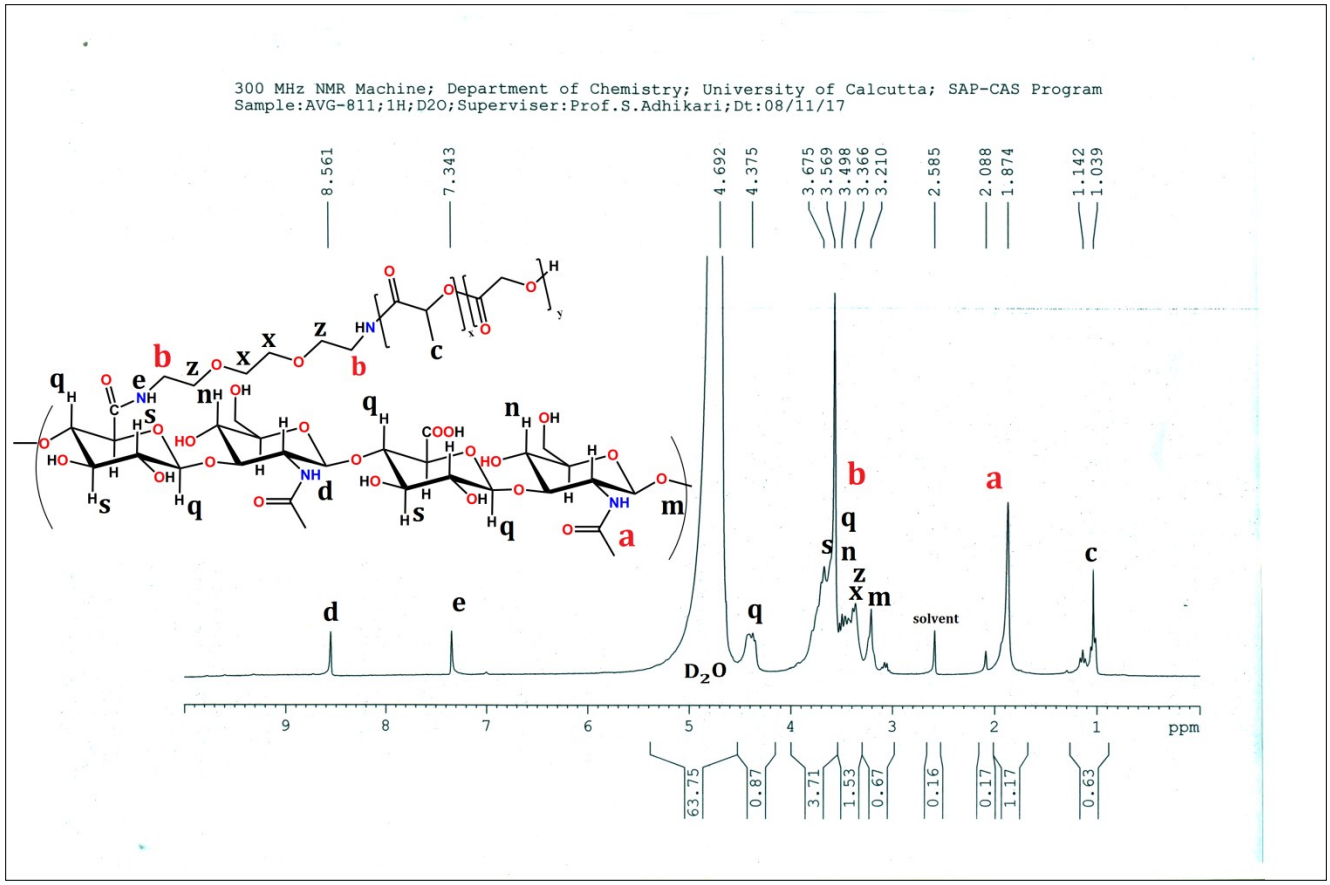


Figure S3: FT-IR spectrum study of different functionalized nanoparticles.

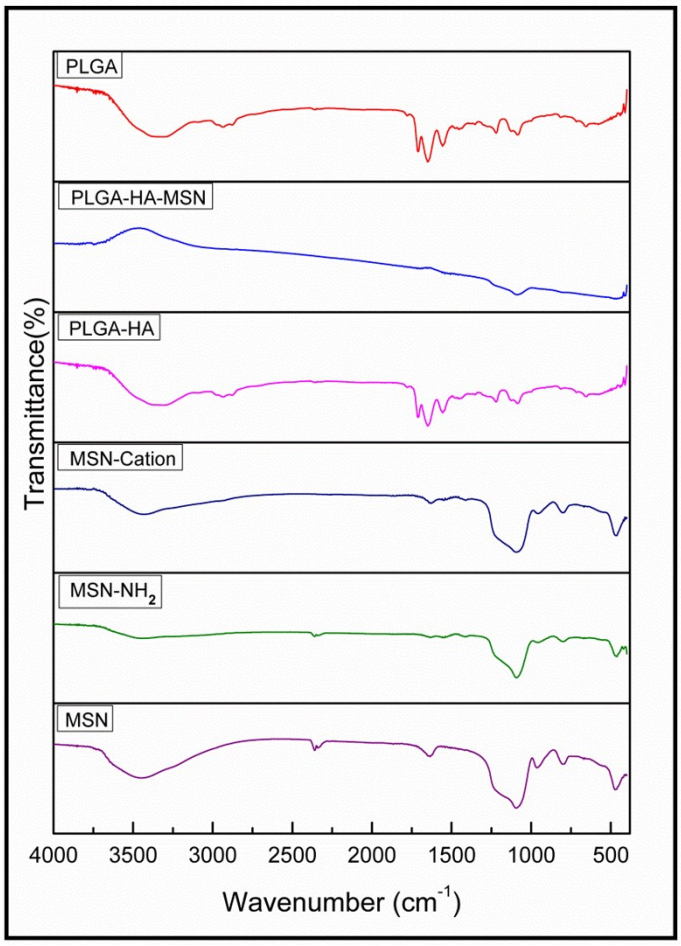
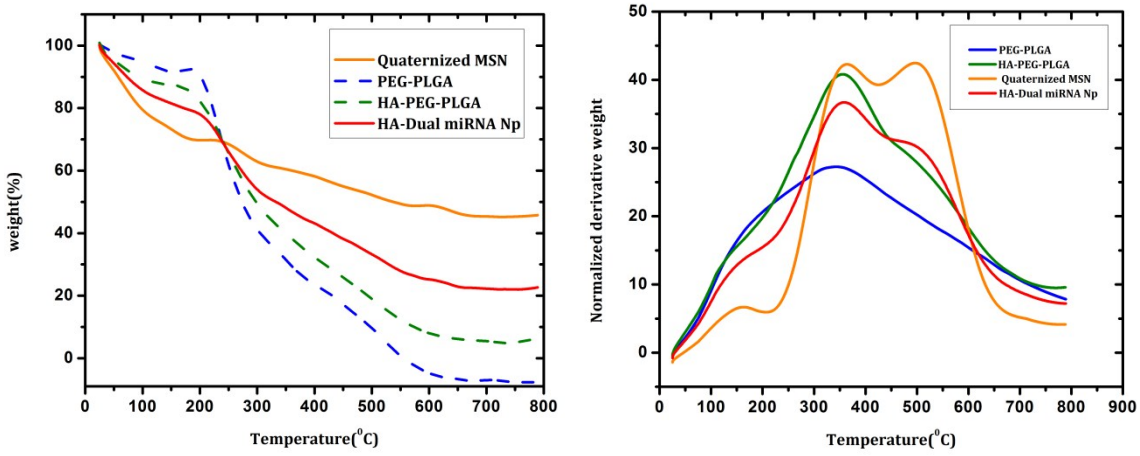


Figure S4: Thermogravemetric Analysis (TGA) and Derivative Thermogravemetric (DTGA) study of synthesized nanoparticles to understand Thermal Stability



TGA **DTGA**

Thermal stability and coating amount of polymer was analyzed by TGA and DTGA (first derivative) curves. Figure S4 clearly shows multiple weight loss steps for HA-PEG-PLGA. The first step is a characteristic for water loss of approx. 10% up to 200°C. The second steps are characteristic of two-stage polysaccharide degradation, starting at 180°C and 300 °C as shown by a weight loss close to 40%. For quaternized MSN, the weight loss between 20°C and 180°C was about 24%, corresponding to the decomposition of quaternized amine groups of MSN. The weight loss increased to 22% for HA-PEG-PLGA coated quaternized MSN. These results indicate that about 12 weight percent of coating was achieved by HA-PEG-PLGA on surface of quaternized MSN. The individual first derivative peaks of all samples are shown in Figure S4. The peak temperatures refer to the point whereby the sample degraded to half of its original weight. HA-PEG-PLGA coated samples had the good thermal stability.

Figure S5: DAPI Fluorescence images showing nuclei condensation(marked with arrows) in different nanoparticles treated sets in both MDAMB 231 and MDAMB 468 cell lines.

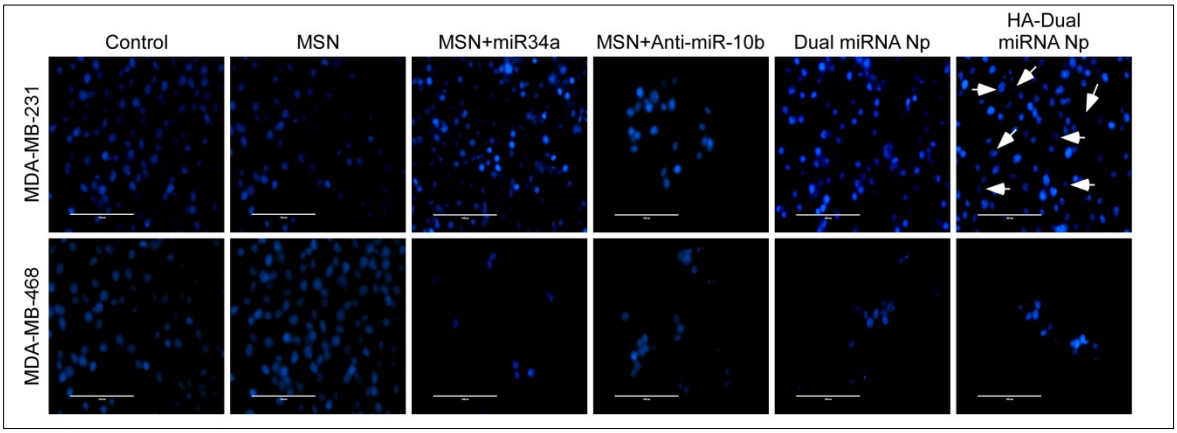


Figure S6: Relative expression of miR-34a and miR-10b in MDAMB-231 cells after transfection of control sequence, miR-34a mimics and miR-10b inhibitor. (B) Bar Graph representation of cell death caused in MDAMB-231 cells by the aforementioned sets. (C) Effect of miR-34a mimics and miR-10b inhibitor on cell migration depicted as bar graph representing change in cell migration . Values are mean ±SEM of three independent experiments in each of representative of typical experiment. **p< 0.01, and ***p< 0.001.

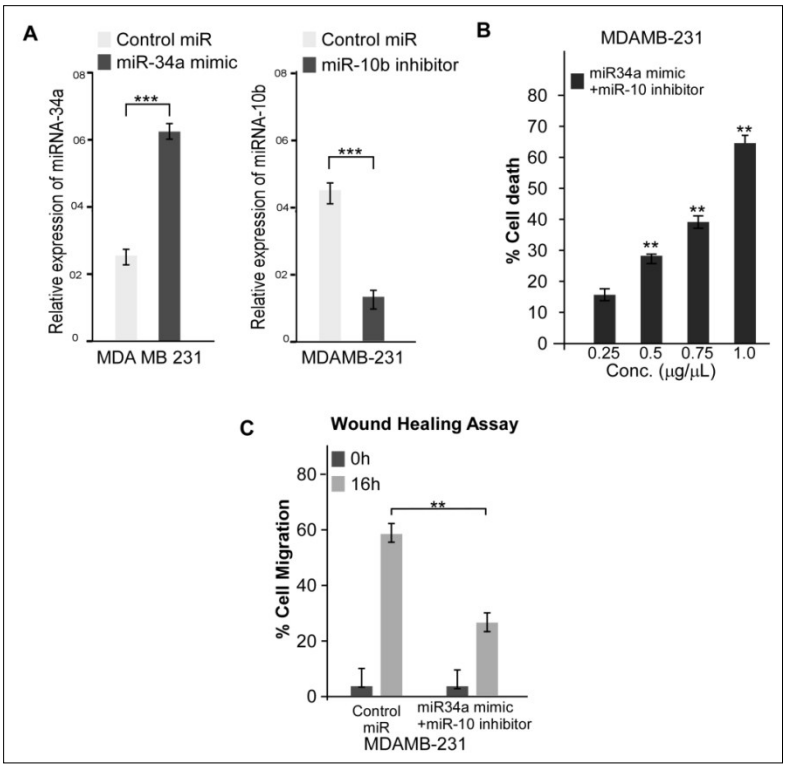


Figure S7: Phase contrast images showing unidirection wound healing assay in different nanoparticles treated sets in both MDAMB 231 and 4T1 cell lines.

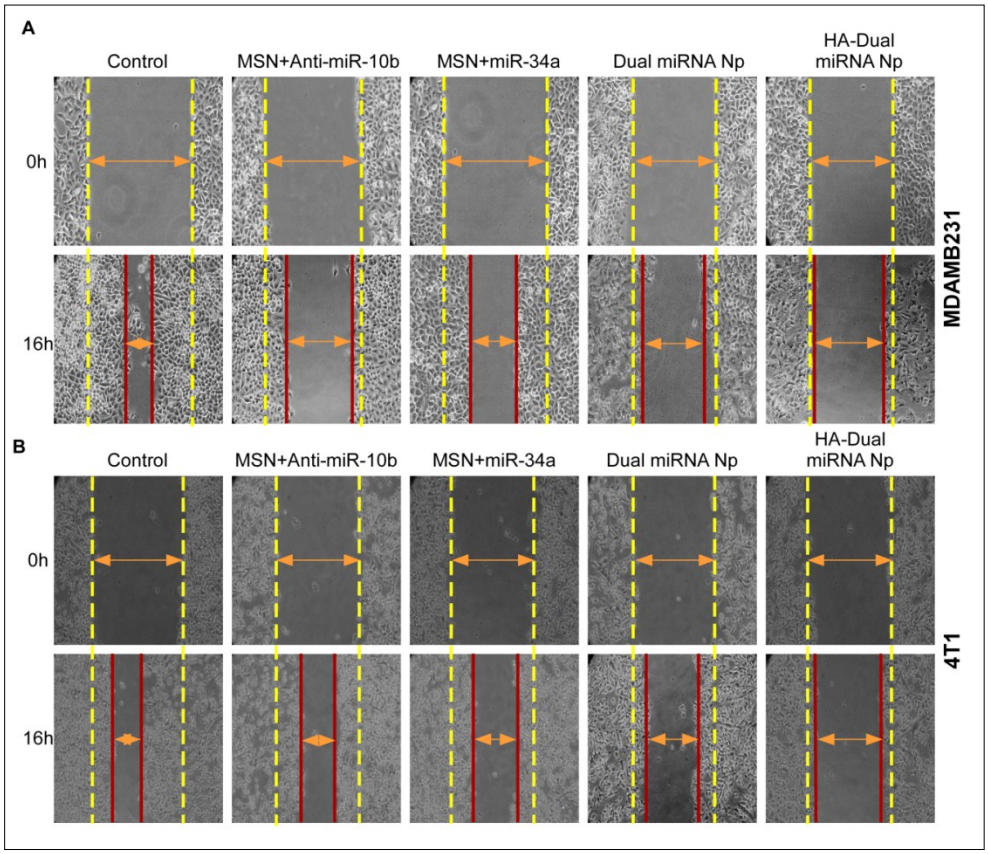
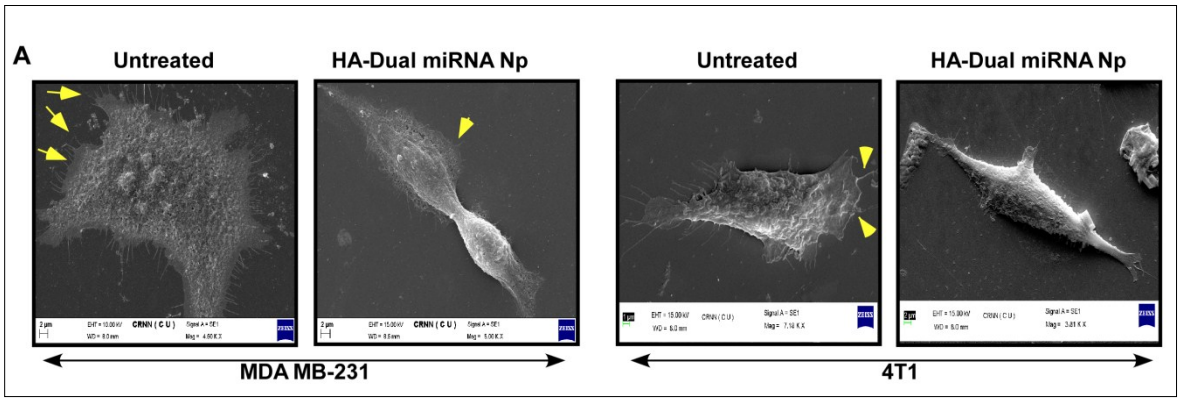


Figure S8: Bio-SEM images showing morphological changes in HA-Dual miRNA Np treated in both MDAMB 231 and 4T1 cell lines with respect to control set.



Reference:

1. Zhaowei Chen, Zhenhua Li, Youhui Lin, Meili Yin, Jinsong Ren, and Xiaogang Qu, Bioresponsive Hyaluronic Acid-Capped Mesoporous Silica Nanoparticles for Targeted Drug Delivery, Chem. Eur. J. 2013, 19, 1778 – 1783.