Supporting Information

Nanobody-displaying porous silicon nanoparticles for the co-delivery of siRNA and doxorubicin

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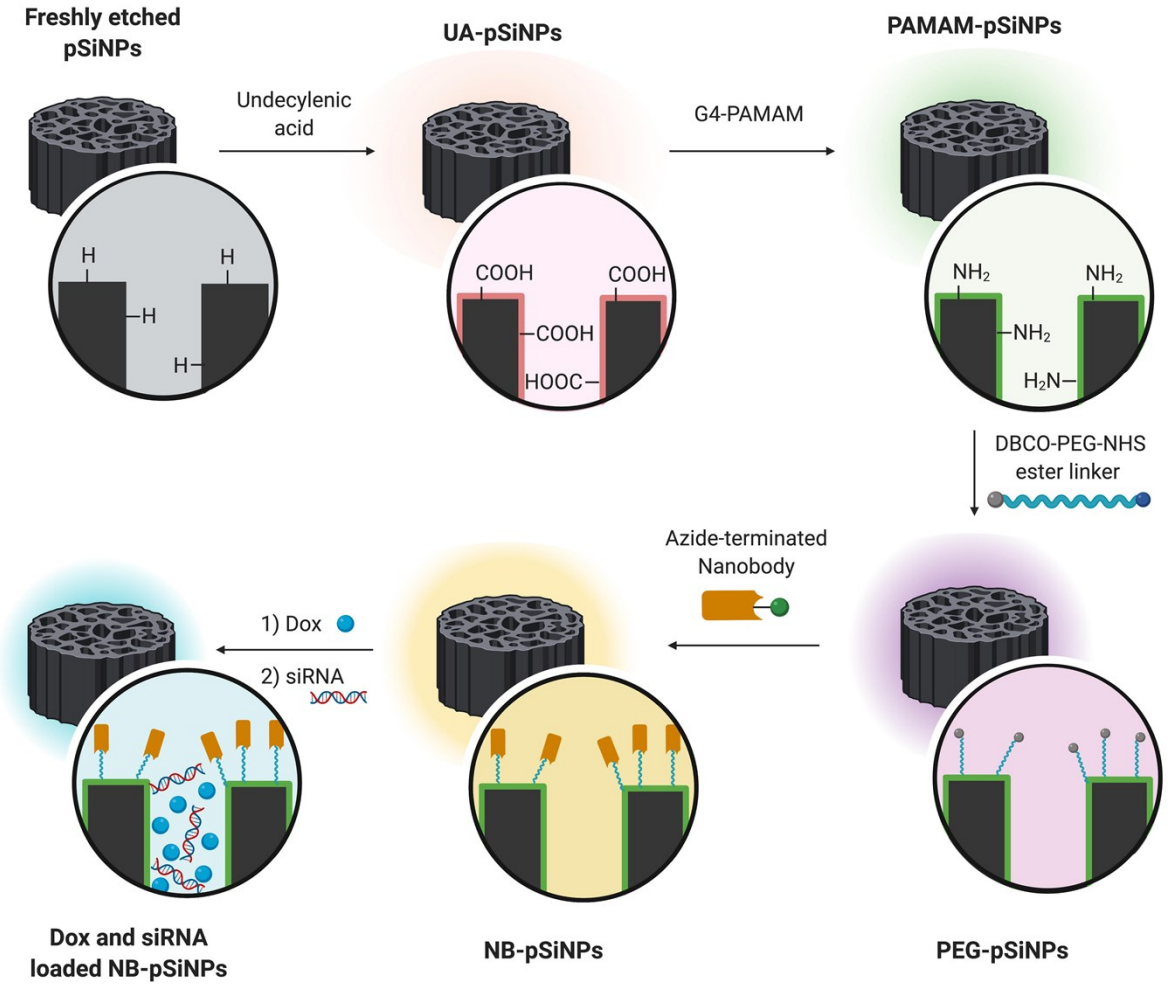

Scheme S1. Schematic representation of the engineered nanobody-displaying porous silicon nanoparticle and the loading of doxorubicin and siRNA. Briefly, freshly etched nanoparticles were immersed in neat undecylenic acid to stabilize and install carboxyl groups (UA-pSiNPs). The carboxyl groups were reacted with a 4th generation polyamidoamine dendrimer *via* carbodiimide chemistry (PAMAM-pSiNPs), providing a plethora of terminal amines to provide a cationic charged nanoparticle. PAMAM-pSiNPs were reacted with a PEG linker (PEG-pSiNPs) displaying an activated *N*-Hydroxysuccinimide (NHS) ester group and a Dibenzocyclooctyne (DBCO) group, where the NHS ester reacted with the terminal amines. PEG-pSiNPs were reacted with complementary azide-terminated nanobodies against EGFR and PSMA in excess *via* copper-free click chemistry in order to exhaust all DBCO groups present from the PEG linkers (NB-pSiNPs). Later on, the particles were sequentially loaded with doxorubicin (Dox) and siRNA targeting the protein, MRP1. Created using Biorender.

Table S1. Total amount of protein (nanobodies) attached by back calculating the amount of protein remaining in the residual supernatant solutions and quantified via a bicinchoninic (BCA) protein assay.

	EGFR-pSiNPs	PSMA-pSiNPs
Starting concentration (μg)	400	400
After reaction supernatant (μg)	285.0	282.0
Washing Step 1 in PBS (μg)	5.7	4.2
Washing Step 2 in PBS (μg)	2.3	1.8
Washing Step 3 in PBS (μg)	< 1	< 1

Quantification of the number of nanobody attached per EGFR-pSiNPs or PSMA-pSiNPs

For the quantification of the nanobody coverage density on the pSiNPs, we followed the method outlined in Cifuentes-Rius *et al.*[1] Briefly, 1 mg of PEG-pSiNPs was reacted with a nanobody solution of 0.4 mg/mL. By using the calculated amount of nanobodies as quantified via a BCA assay from the collected supernatants, the concentration of nanobodies attached to the PEG-pSiNPs was 0.107 mg/mL and 0.112 mg/mL for EGFR-pSiNPs and PSMA-pSiNPs, respectively. This was consistent with each batch of functionalized nanoparticles as (i) we used the same functionalized nanoparticles for each condition and (ii) we used the same initial nanobody concentration. However, we are under the assumption that all samples conjugated with the nanobodies will have similar densities and that the molecular weight of an average nanobody is approximately 15 kDa (15,000 g/mol). Using Avogadro's number, we determined that the number of individual nanobody molecules attached to EGFR-pSiNPs and PSMA-pSiNPs were 4.29×10^{18} and 4.50×10^{18} molecules, respectively. To calculate the number of nanoparticles, we estimated the volume of one nanoparticle by using the formula of the volume of a cylinder ($V = \pi r^2 h$, where r is 90 nm and h is 120 nm according to TEM measurements), resulting in 3.05×10^{-18} L. The density of pSiNPs was calculated to be 931.84 g/L by factoring the density of silicon (2,329.6 g/L) and the porosity of the pSiNPs (~60%). By knowing the volume of a single nanoparticle and its density, we calculated that the mass of a single nanoparticle was 2.84×10^{-15}. Thus, as our concentration of nanoparticles used in the reaction was 1 mg/mL (or 1 g/L) as determined via ICP-OES, the number of particles in 1 L of solution would be 3.52×10^{14}. By dividing the number molecules of nanobody in solution by the number

of nanoparticles in solution – we determined the amount of nanobody per EGFR-pSiNPs and PSMA-pSiNPs was 1.22×10^4 and 1.28×10^4, respectively.

Table S2. Doxorubicin and siRNA loading (w/w) percentage in nanobody-displaying pSiNPs. Data shown as the mean ± S.D. (n = 3).

	PAMAM-pSiNPs	PEG-pSiNPs	EGFR-pSiNPs	PSMA-pSiNPs
Dox (w/w)%	11 ± 1%	12 ± 1%	12 ± 1%	12 ± 1%
siRNA (w/w)%	21 ± 1%	15 ± 1%	14 ± 1%	14 ± 1%

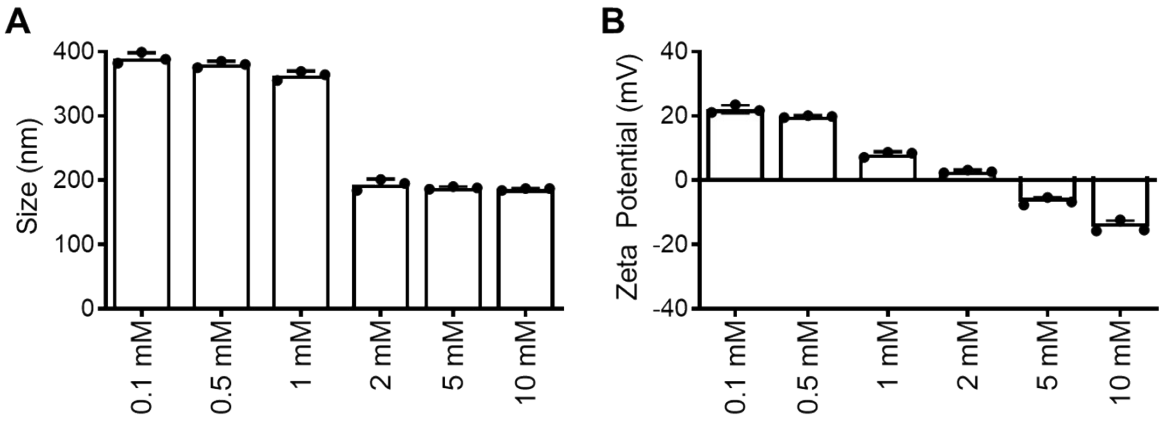

Figure S1. Measurement of (A) hydrodynamic size and (B) zeta potential of G4-pSiNPs with various molar concentrations of NHS-Ester-PEG5-DBCO to optimize the concentration of PEG linker, while maintaining sufficient free dendrimer amine terminals to promote electrostatic interaction with siRNA molecules. Data shown as a mean ± S.D. (n = 3).

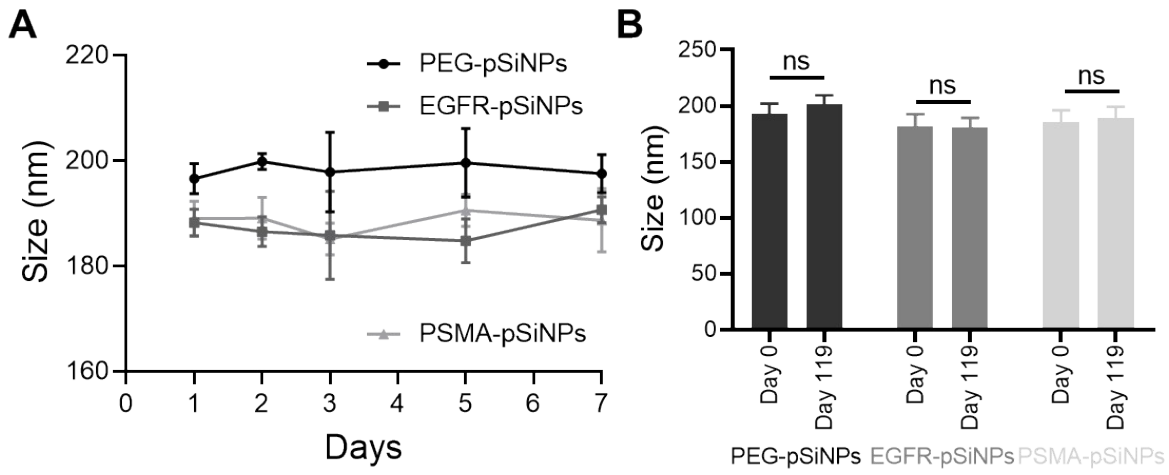

Figure S2. Colloidal stability of NB-pSiNPs at 4 °C (storage conditions) in phosphate buffered saline (PBS) as measured by DLS for the nanoparticles' hydrodynamic diameter. (A) Colloidal stability over 7 days. (B) Colloidal stability over a 4-month period (thankyou COVID-19 lockdown). Data shown as a mean ± S.D. (n = 3).

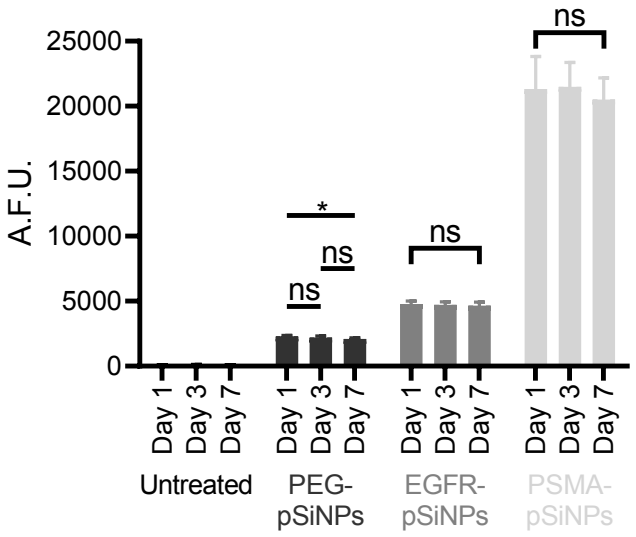

Figure S3. Cellular association studies of NB-pSiNPs towards C4-2B cells analyzed using a fluorescence plate reader (A.F.U. = Arbitrary fluorescence units). NB-pSiNPs were stored in PBS at 4 °C to assess the colloidal stability in physiological buffer and their ability to retain their receptor selectivity. Data shown as a mean ± S.D. (n = 4, *p = ≤ 0.05).

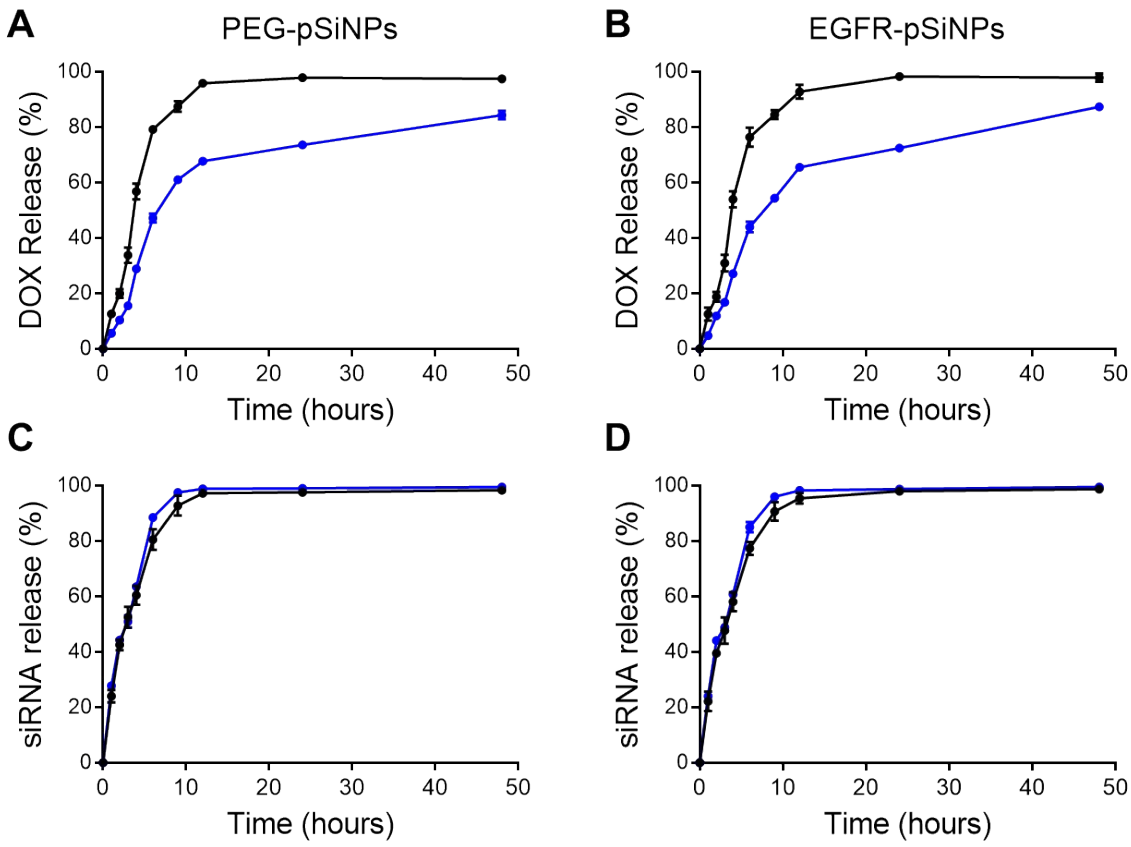

Figure S4. (A-B) Doxorubicin release profile from (A) PEG-pSiNPs and (B) EGFR-pSiNPs over 48 h in PBS at pH 5.2 (black) and pH 7.4 (red) under agitation at 37 °C. (C-D) siRNA release profile from (C) PEG-pSiNPs and (D) EGFR-pSiNPs over 48 h in PBS at pH 5.2 (black) and pH 7.4 (red) under agitation at 37 °C. Data shown as a mean ± S.D. (n = 4).

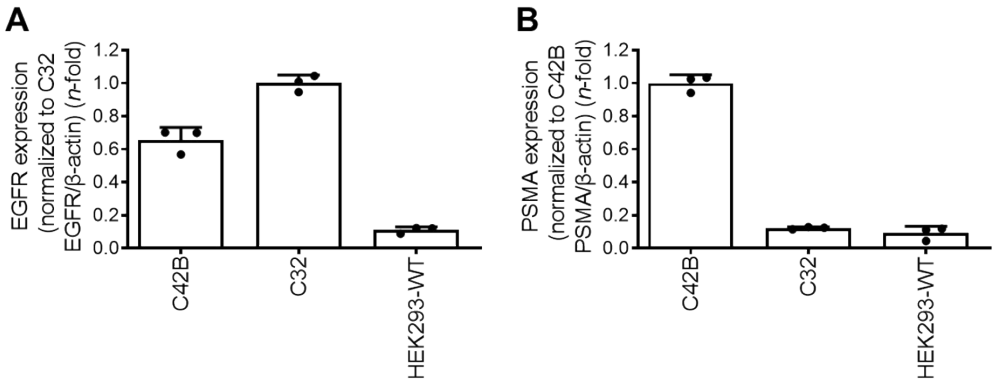

Figure S5. Quantification of EGFR and PSMA protein expression as determined via Western blotting. Ratio was determined by dividing the EGFR/PSMA band intensity by the housekeeping gene (β-actin) band intensity. (A) EGFR expression normalized to C32 cells, and (B) PSMA expression normalized to C42B cells. Data shown as a mean ± S.D. (n = 3).

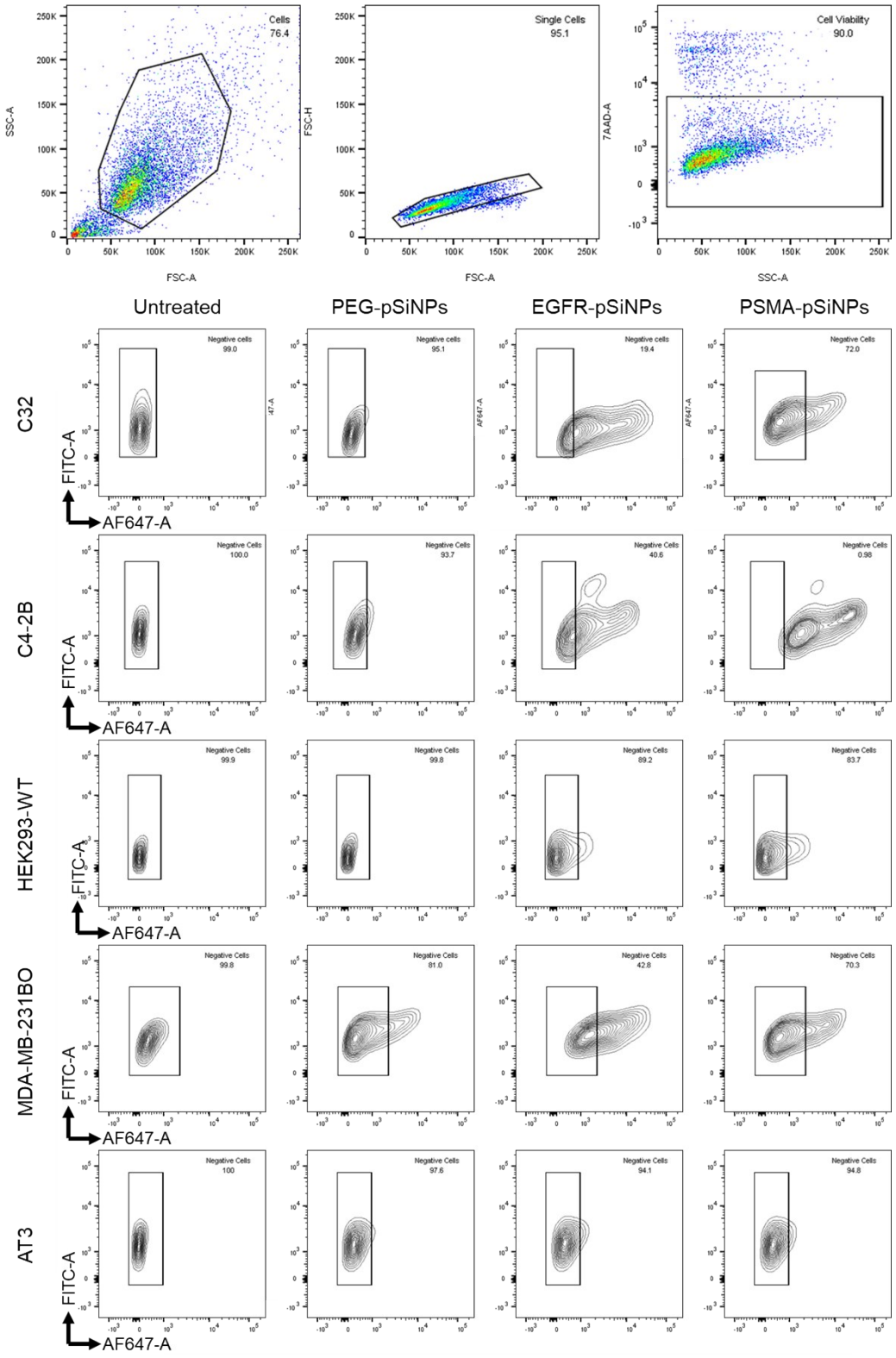

Figure S6. Flow cytometry gating for cellular association of NB-pSiNPs treated against multiple cell lines. Cellular association was compared to a negative control of cells without nanoparticle treatment.

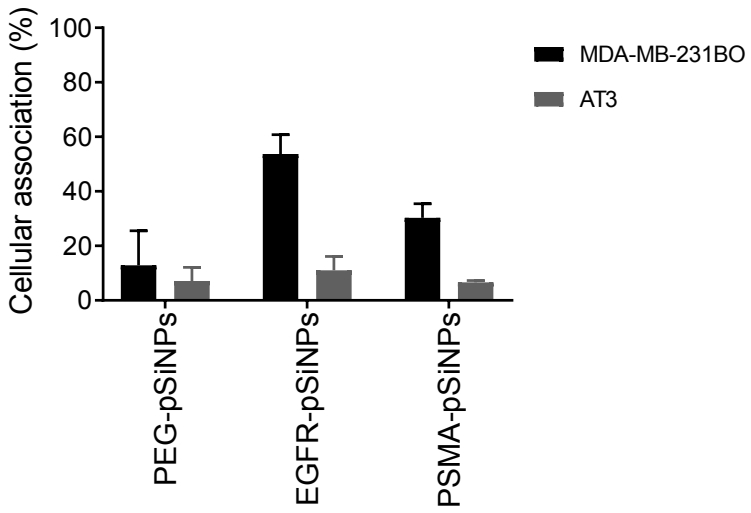

Figure S7. Further cellular association studies of NB-pSiNPs analyzed via flow cytometry where cellular association was compared to a negative control of untreated cells. Data shown as a mean ± S.D; (n = 3).

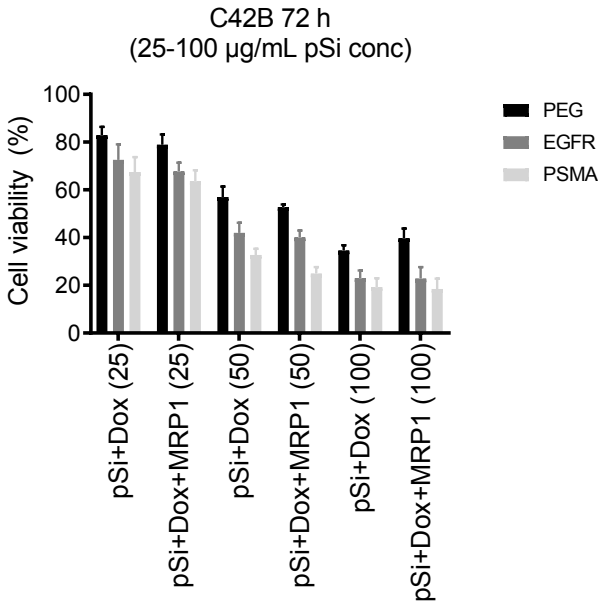

Figure S8. Cell viability as evaluated using an ATP-based luminescent cell viability assay. Different concentrations of pSiNPs loaded with Dox and/or siMRP1 were incubated with cells for 1 h, and then washed away with copious amounts of PBS. Cell viability was assessed after 72 h against untreated cells and cells treated with 10% DMSO which represented the negative and positive control, respectively. Data shown as a mean ± S.D. (n = 4).

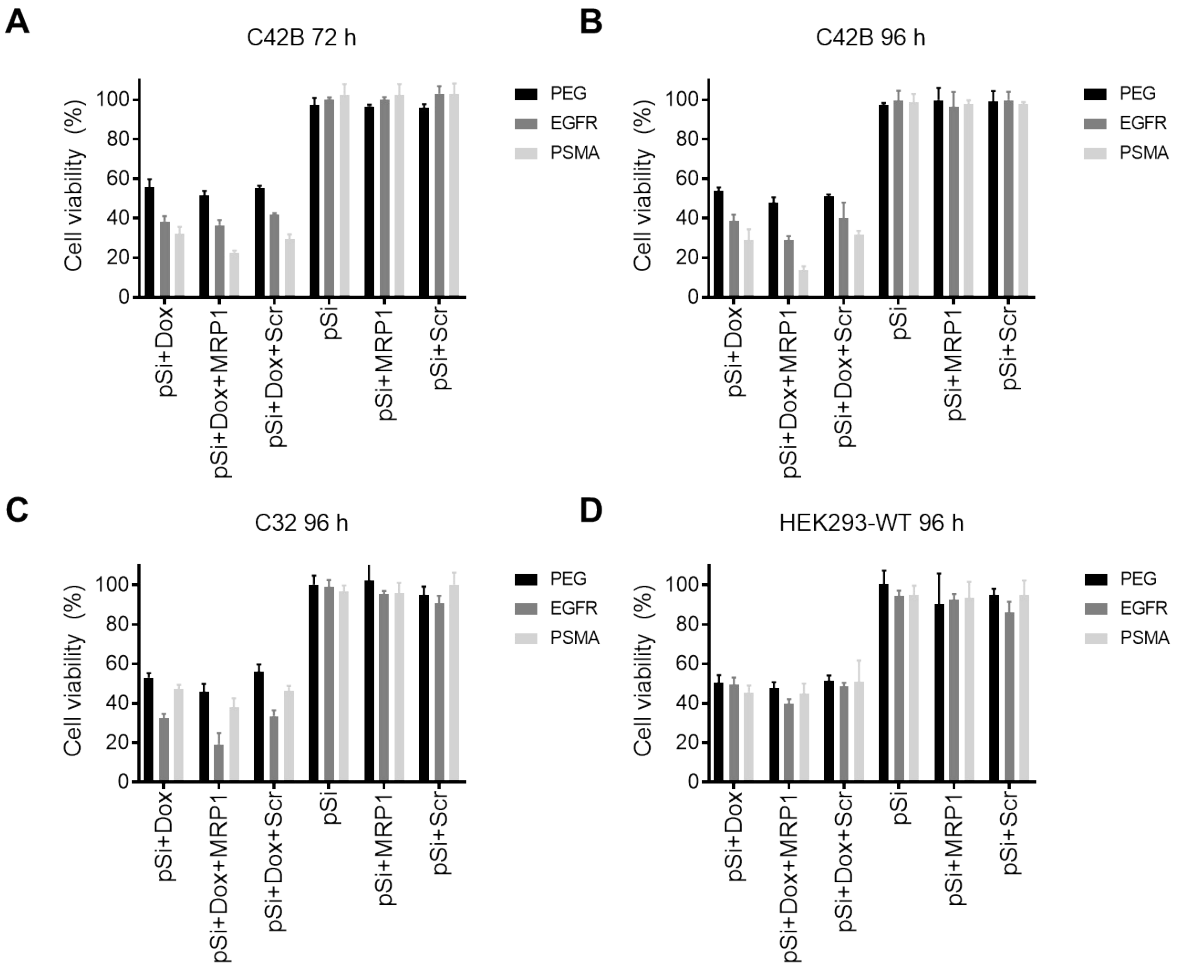

Figure S9. Cell viability as evaluated using an ATP-based luminescent cell viability assay. Different formulations of pSiNPs (50 μg/mL) loaded with Dox and/or siRNA (siMRP1 or siScr) were incubated with cells for 1 h, and then washed away with copious amounts of PBS. (A) 72 h and (B) 96 h after particle incubation in C4-2B cells. (C) 96 h after particle incubation in C32 cells. (D) 96 h after particle incubation in HEK293-WT cells. Cell viability was compared with untreated cells and cells treated with 10% DMSO which represented the negative and positive control, respectively. Data shown as a mean ± S.D. (n = 4).

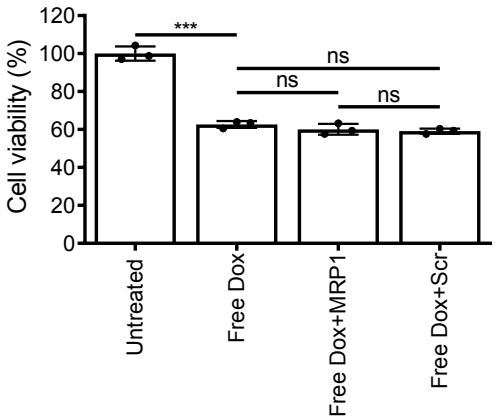

Figure S10. Cell viability as evaluated using an ATP-based luminescent cell viability assay. A matching concentration of free Dox (~2.4 μM) with or without siMRP1 or a scrambled sequence (~5 μM) were added to C4-2B cells for 1 h, washed copiously with PBS and further incubated for a total incubation time of 96 h. Cell viability was compared with untreated cells and cells treated with 10% DMSO which represented the negative and positive control, respectively. Data shown as a mean ± S.D. (n = 3, ***p = ≤ 0.001).

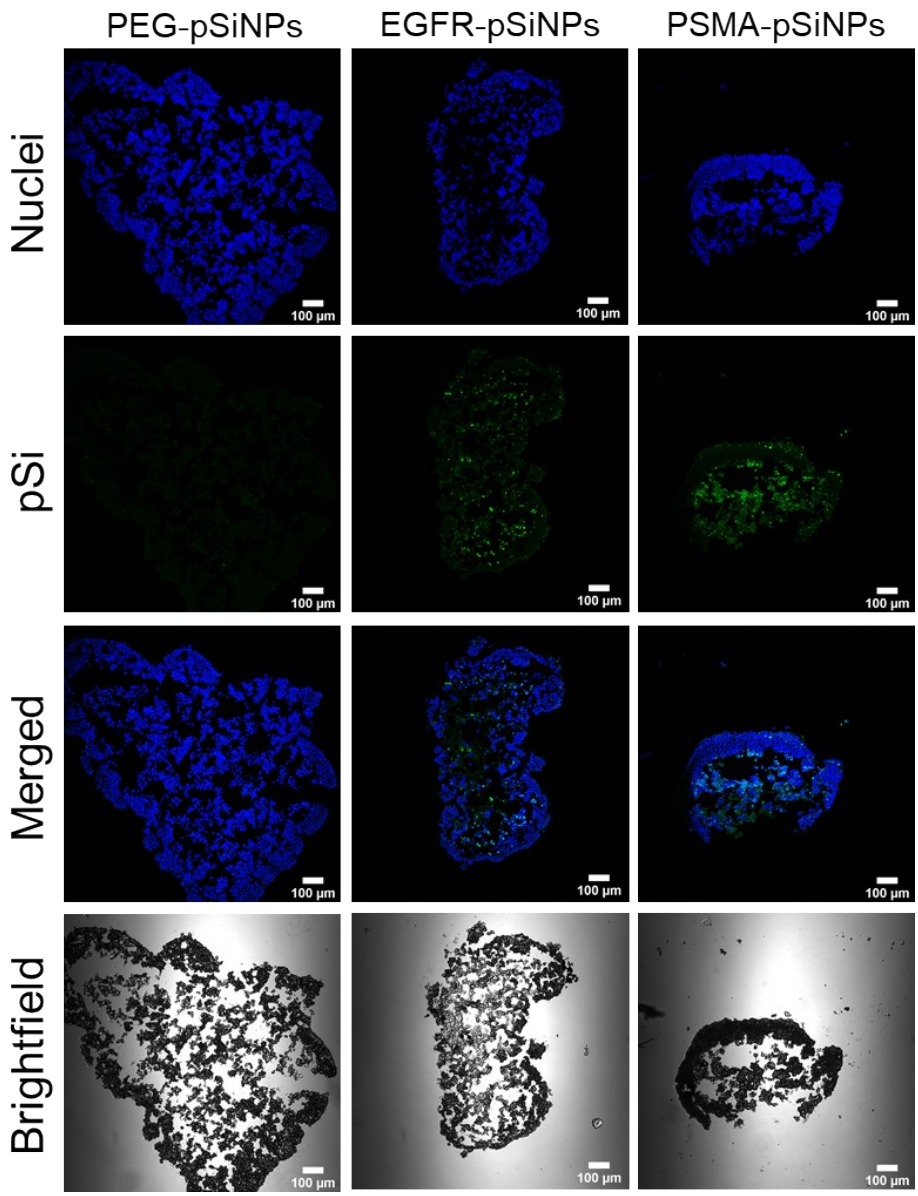

Figure S11. Confocal microscopy images of the middle slice of C4-2B spheroids treated for 1 h with NB-pSiNPs, washed and then fixed after 24 h, where the nuclei were stained with Hoechst 33342 (blue), and pSiNPs labeled with Cy5 (green) (scale bar = 100 µm).

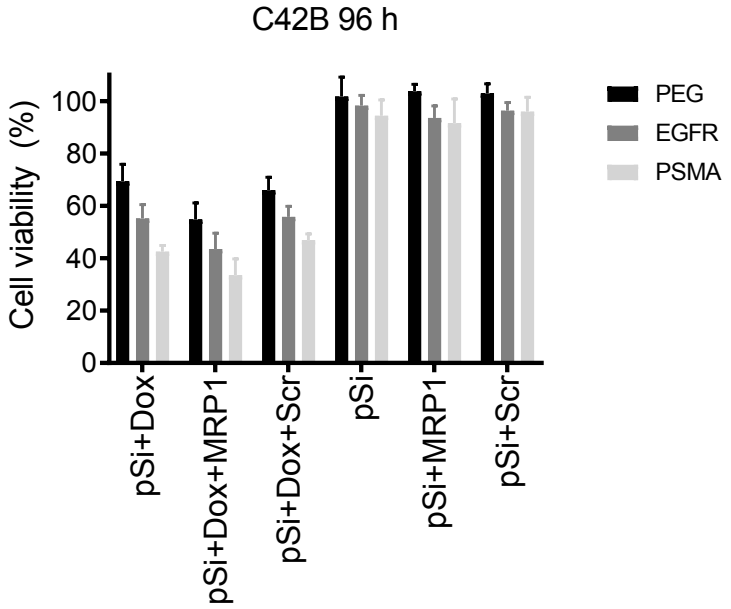

Figure S12. Cell viability as evaluated using an ATP-based luminescent cell viability assay. Different formulations of pSiNPs (50 μg/mL) loaded with Dox and/or siRNA (siMRP1 or siScr) were incubated with C42B cells for 1 h, then washed away with profuse amounts of PBS, and further incubated until the 96 h timepoint. Cell viability was compared with untreated cells and cells treated with 10% DMSO which represented the negative and positive control, respectively. Data shown as a mean ± S.D. (n = 4).

References

1. A. Cifuentes-Rius, A. Ivask, E. Sporleder, I. Kaur, Y. Assan, S. Rao, D. Warther, C. A. Prestidge, J.-O. Durand and N. H. Voelcker, *Small*, 2017, **13**, 1701201.