Supporting Information for

Enhanced Delivery of siRNA to Triple Negative Breast Cancer in Vitro and in Vivo through Functionalizing Lipid-coated Calcium Phosphate Nanoparticles with Dual Target Ligands

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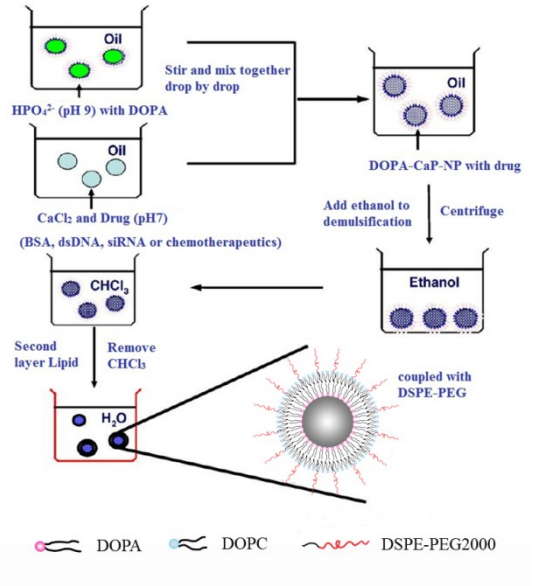

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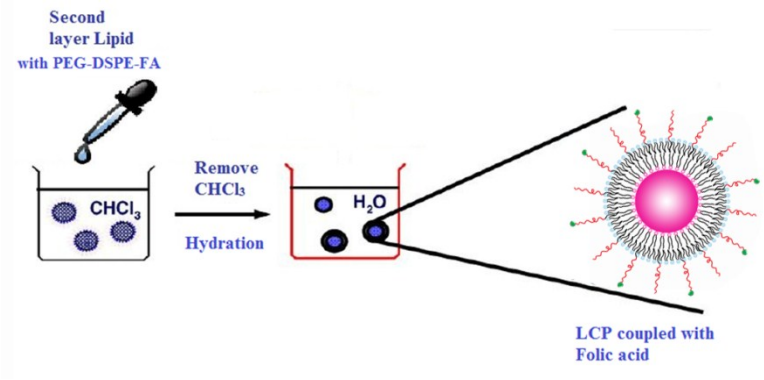
21 Figure S1. The outline for LCP NP preparation.



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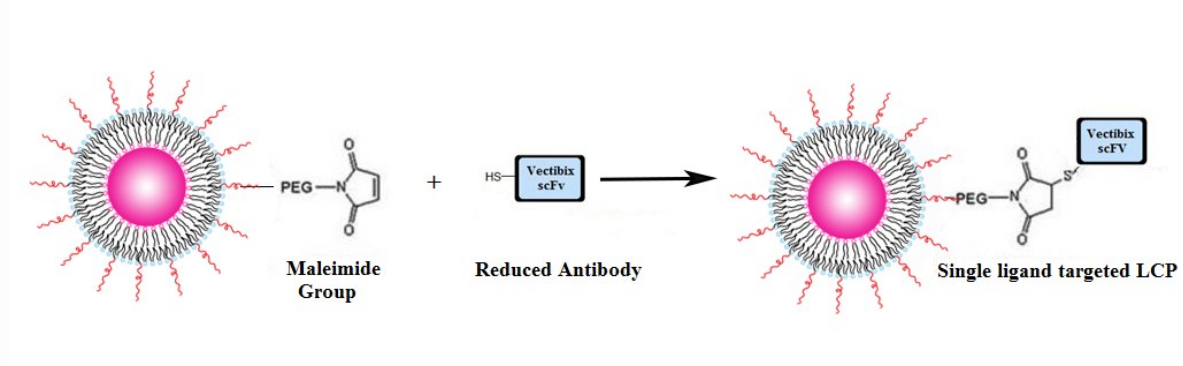
23 Figure S2. Folic acid ligand conjugation method to LCP NP.



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25 Figure S3. Reaction scheme for conjugating antibody and LCP NPs with maleimide group.

Calculation of the number of phospholipid molecules and conjugated FA/scFv per LCP NP

The number of lipid molecules in the out lipid membrane and integrated ligand (scFv or FA) per LCP NP material was calculated as follows.[1]

(1) Equation 1 was conducted first to estimate **the total number of lipid molecules in the outer lipid membrane per LCP NP (*Nlip*),**

$$Nlip = \frac{4\pi(\frac{d}{2} + h)^2}{a} \quad \text{(Equation 1)}$$

where ***h*** is lipid bilayer thickness and taken as 5 nm, ***d*** is the diameter of a CaP core obtained under TEM observation (***d***=10 nm in our results), and ***a*** is polar head group. Average area per lipid molecule (***a***) for phospholipid molecules (DOPC) used in out lipid layer were 0.71 nm^2.[2-3] So in our work, ***Nlip***=3980.

(2) Number of LCP NP per mL (*Nnp*)

The number of LCP NP per mL (***Nnp***) for known concentrations of LCP NPs was calculated by using Equation 2:

$$Nnp = \frac{M(lip) \times NA}{Nlip \times 1000} \quad \text{(Equation 2)}$$

where ***NA*** is the Avogadro number and it is equal to 6.02E23, ***M(lip)*** is the molar concentration of DOPC which should only stay in the out-leaflet lipid layer (mol/L), and ***Nlip*** is the total number of DOPC molecules in the out-leaflet lipid layer per LCP NP.

(3) Number of ligand (scFv or FA) per LCP NP (*N(ligand)*)

Finally, number of ligand (***N(ligand)***) can be calculated by using Equation 3. M(ligand) is the molar concentration of conjugated ligand per mL of the sample.

$$N(ligand) = \frac{M(ligand) \times NA}{Nnp \times 1000}$$

$$N(ligand) = \frac{M(ligand) \times Nlip}{M(lip)}$$

(Equation 3)

For example, the molar concentration of folic acid (***M(FA)***) in the NP samples after removing the free DSPE-PEG_{2000}-FA was used to calculate the number of FA integrated into lipid layer per LCP NP (***N(FA)***). The concentration of ABX-EGF scFv conjugated per LCP (***M(scFv)***) was then calculated by subtracting the amount of free scFv from the initial scFv concentration in indirect method. In direct method (*i.e.* ELISA), the amount of accessible antibody present on LCP NPs can be directly quantified. This resultant molar concentration of ABX-EGF scFv was used for calculation of the number of scFv per LCP (***N(scFv)***).

Table S1. The mean size of LCP NPs with different number of scFv/FA ligand conjugated.

Sample name	Number mean size (nm)	Sample name	Number mean size (nm)	Sample name	Number mean size (nm)
LCP-200scFv	48.2±2.2	LCP-200FA	41.9±3.5	LCP-100FA-125scFv	47.8±3.4
LCP-125scFv	47.5±3.3	LCP-150FA	42.5±1.4	LCP-50FA-125scFv	47.2±4.3
LCP-75scFv	46.1±2.6	LCP-100FA	43.0±3.3	LCP-50FA-75scFv	43.5±4.2
LCP-50scFv	45.4±2.2	LCP-50FA	42.0±2.5	LCP-PEG	41.7±3.4
LCP-30scFv	42.3±1.4	LCP-20FA	43.7±3.2		

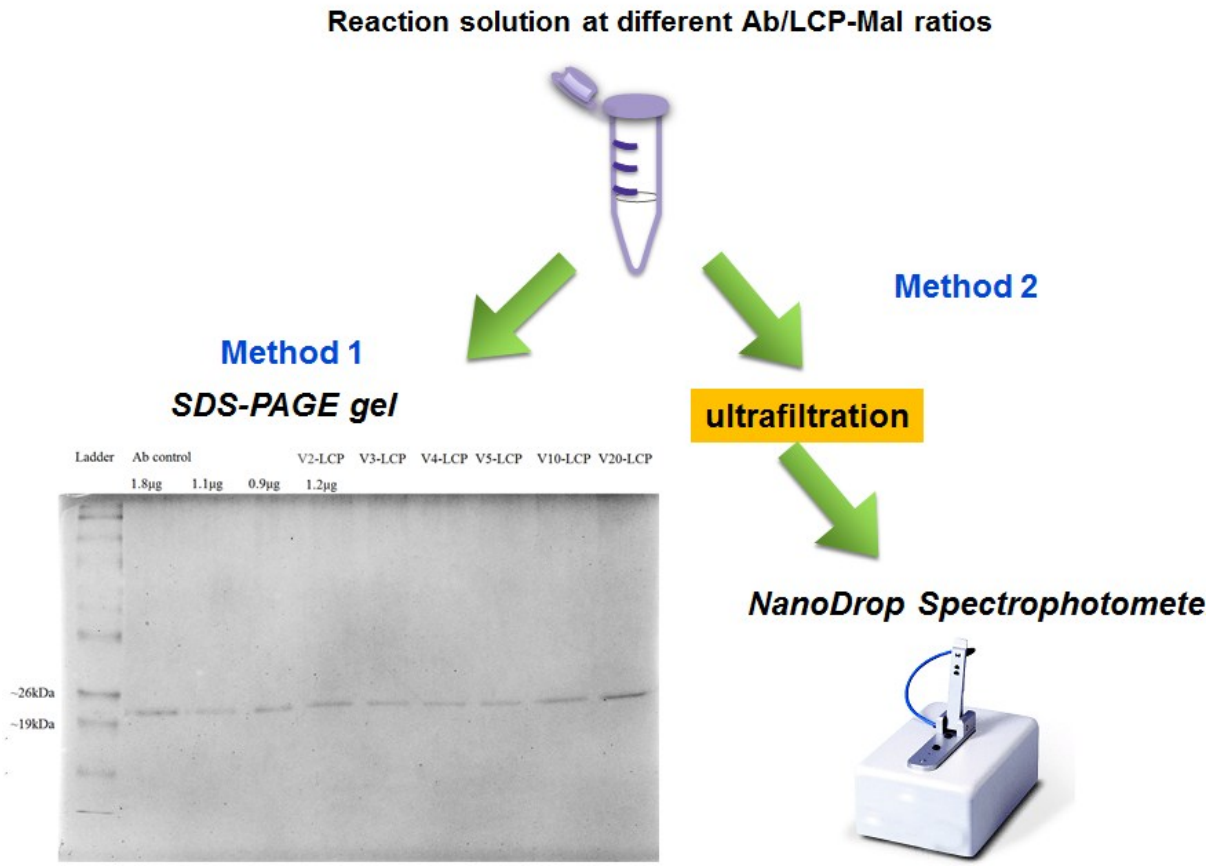


Figure S4. Indirect method to determine the scFv number per LCP NP. Electrophoresis of different Ab to maleimide group ratio (V2-LCP:0.5, V3-LCP:0.30, V4-LCP:0.25, V5-LCP:0.20, V10-LCP:0.10, V20-LCP:0.05).

In Method 1, the reaction solution containing LCP-scFv and free scFv was assessed by SDS-PAGE using native ABX-EGF scFv as control. The amount of unreacted antibody fragment was analyzed by analyzing the band intensity and comparing to antibody standards of known amounts. The number of ABX-EGF scFv per LCP was determined by a ligand to phospholipid ratio, assuming 3980 phospholipid molecules per LCP, yielding a bulk average for the number of ligands per LCP NP.

In Method 2, conjugation efficiency was assessed by absorbance at 280 nm (A280) protein concentration measurement with a NanoDrop ND-100 Spectrophotometer (Thermo Scientific) of free antibody in eluents after ultrafiltration. Conjugation efficiency was further assessed by absorbance at 280 nm (A280) protein concentration measurement with a NanoDrop ND-100 Spectrophotometer (Thermo Scientific) of free antibody in eluents after ultrafiltration. After conjugation of ABX-EGF scFV-SH to LCPs, added 70 μL of sample into 50 kDa molecular cut-off membrane centrifugal tube, centrifuged 4000 g for 15 min. Then collected the solution at the bottom, test the concentration of free antibody with Nanodrop at 280 nm. Dilute the ABX-EGF scFV-SH (0.2 μg) to gradient concentration and test their absorbance at 280 nm to get the calculating standard curve.

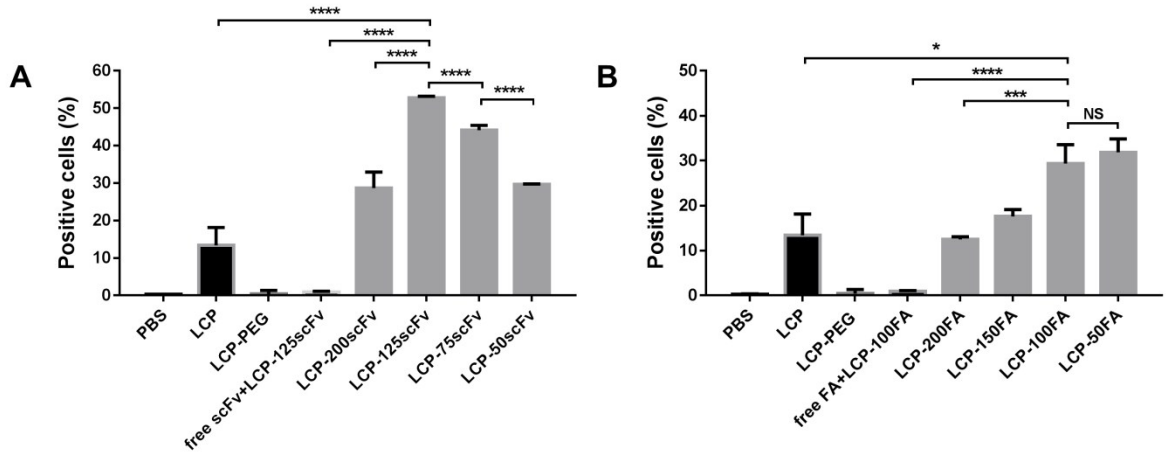
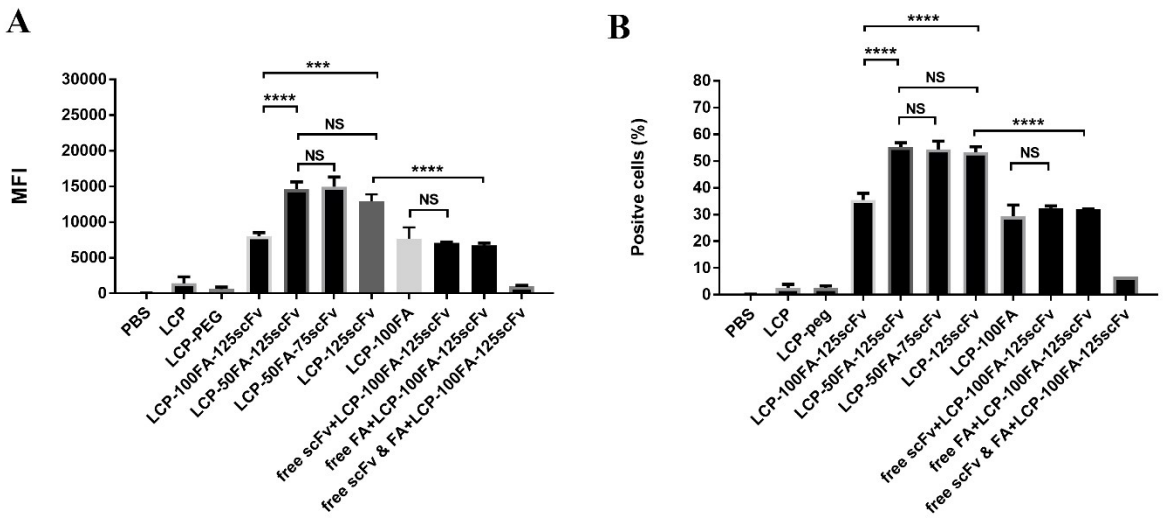


Figure S5. The effect of single ligand modification of LCP NPs (LCP-scFv (A) and LCP-FA (B)) on cellular uptake by MDA-MB-468 cells, represented by the positive cell percentage.



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88 Figure S6. The effect of dual ligand modification of LCP NPs on cellular uptake by MDA-
89 MB-468 cells, represented by (A) quantitative analysis of the mean fluorescent intensity (MFI)
90 as measured by flow cytometry and (B) the positive cell percentage (n = 3) (****, $p < 0.0001$;
91 ***, $p < 0.001$; NS, not significant).

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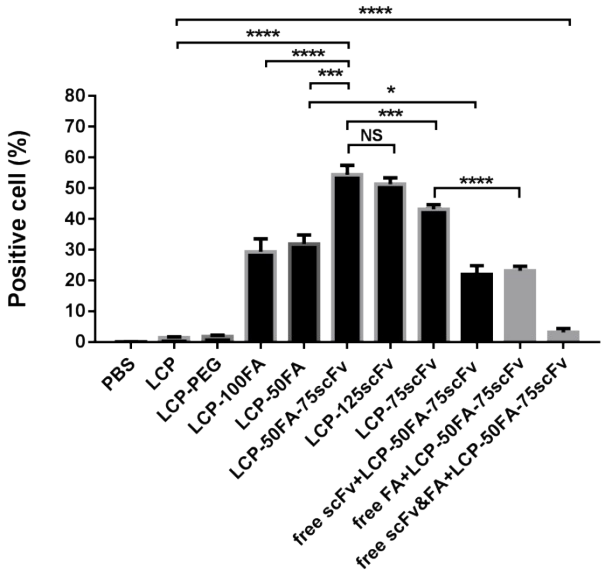

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95 Figure S7. The effect of dual ligand modification of LCP NPs on the cellular uptake by
96 MDA-MB-468 cells, represented by the positive cell percentage.

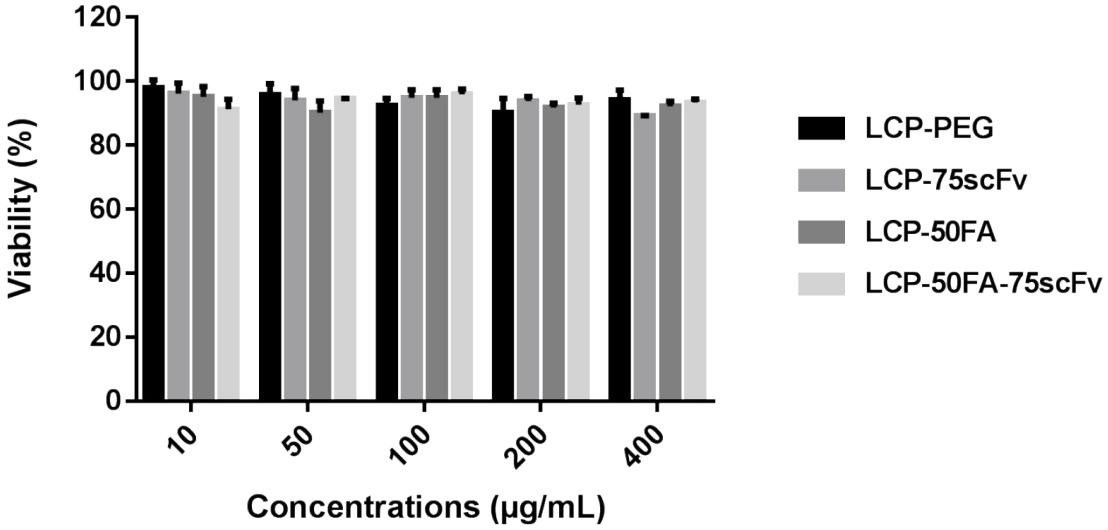
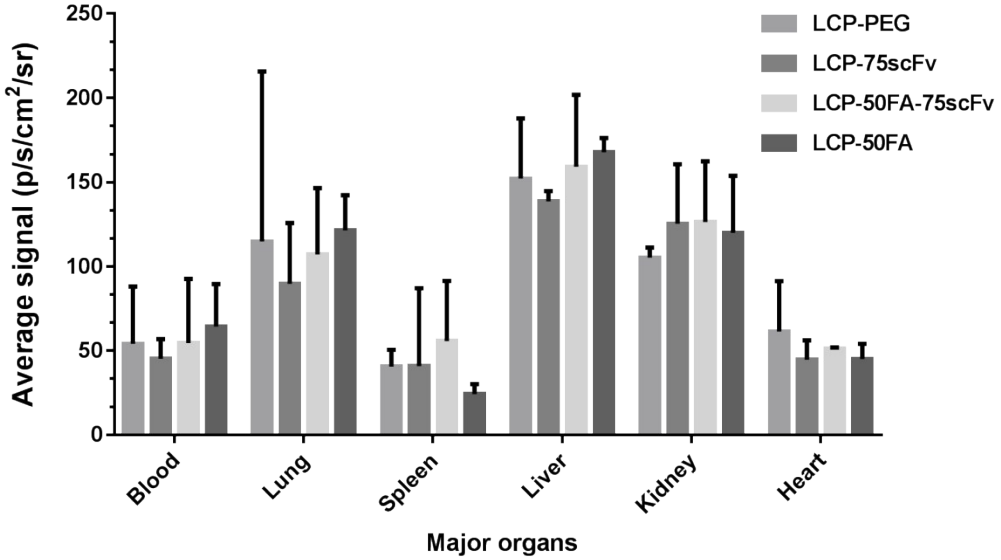


Figure S8. In vitro cytotoxicity of LCP-PEG, LCP-75scFv, LCP-50FA and LCP-50FA-75scFv incubated with HUVEC cells for 48 h at 37 °C. If 40 nM dsDNA or siRNA was used for cell culture, the corresponding concentration of LCP NPs was 10 μg/mL.

The in vitro cytotoxicity of the LCP-PEG, LCP-75scFv, LCP-50FA and LCP-50FA-75scFv were detected using HUVEC cells after 24 h incubation using MTT (Fig. S8). At every studied concentration, the viability of HUVEC cells was above 90% for all four nanoparticles, and no significant difference was found between the formulations, indicating they had uniformly low cytotoxicity. Because calcium phosphate and PEG polymers are safe materials with FDA approval, the LCP-PEG nanoparticles modified with ABX-EGF scFv and folic acid might be promising drug carriers with little cytotoxicity.



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111 Figure S9. The measured fluorescent intensity of the major organs collected from sacrificed
112 mice treated with Cy5 dsDNA-loaded LCP NPs for 24 h.

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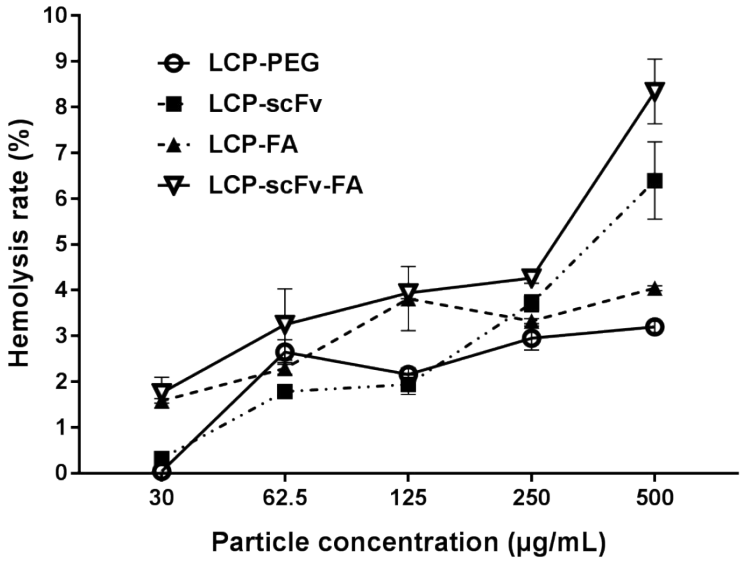

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115 Figure S10. Hemolysis of various LCP NPs at different concentrations, where the hemolysis
116 percentage of the positive control (water) and negative control (PBS saline) were 100% and
117 0%, respectively.

Hemolysis occurs when cells swell to the critical bulk to break up the cell membranes. The released adenosine diphosphate from broken red blood cells (RBCs) can intensify the assembly of blood platelets, which accelerates the formation of clotting and thrombus. Thus, hemolysis of the blood cells is another problem associated with the bio-incompatibility of a delivery system.[4] It is well known that red blood cells hemolyze when they come in contact with water. This problem may be aggravated in the presence of an implant material. Fig. S10 showed the hemolysis test results of the pegylated LCP NPs and LCP NPs with single and dual ligand modification at different concentrations. LCP-FA surfaces exhibited similar hemolysis degrees with the LCP-PEG sample. In comparison with that (about 3%) of the LCP-PEG NPs sample at the higher concentration of 500 μg mL^{-1}, the hemolysis degrees of folic acid modified LCP-NPs (LCP-FA) were about 4%. Moreover, the hemolysis degree doubled as the 75 scFv were added to the surface of nanoparticles (6%), and further increased to about 8% when LCP NPs were modified with both FA and scFv, because scFv would produce more interaction with the RBC membrane on the LCP surface. When the concentration of nanoparticles was below 250 μg mL^{-1}, all four tested LCP NPs surfaces exhibited much lower hemolysis degrees (<4.5%) than at high nanoparticle concentration. It was reported that up to 5% hemolysis is permissible for biomaterials.[5] The dual ligand modified LCP NPs samples can be used as biomaterials without causing any hemolysis.

References

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