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Direct Cytosolic Delivery of siRNA Using Nanoparticle-Stabilized Nanocapsules **

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Abstract

We report the use of nanoparticle-stabilized nanocapsules (NPSC) for the direct cytosolic delivery of siRNA. In this approach, siRNA is complexed with cationic arginine-functionalized gold nanoparticles via electrostatic interactions, with the resulting ensemble self-assembled onto the surface of fatty acid nanodroplets to form NPSC/siRNA nanocomplex. The complex rapidly delivers siRNA into cytosol via membrane fusion, a mechanism supported by cellular uptake studies. Using destabilized green fluorescent protein (deGFP) as a target, 90% knockdown was observed in HEK293 cells. Moreover, the delivery of siRNA targeting polo-like kinase 1 (siPLK1) efficiently silenced PLK1 expression in cancer cells with concomitant cytotoxicity.

Keywords

siRNA delivery; cytosolic delivery; nanocapsule; gold nanoparticle; cancer therapy

RNA interference (RNAi) is an endogenous pathway that silences gene expression at a post-translational level. The predominant strategy for RNAi uses small interfering RNA to target and cleave complementary mRNA, with concomitant inhibition of protein translation.^[1] Since the discovery of RNAi by Fire *et al.*^[2] and siRNA by Tuschl *et al.*,^[3] the application of RNAi to knock down the expression of tumor-specific proteins or anti-apoptotic pathways has emerged as a novel therapeutic approach for cancer treatments.^[4] siRNA is a double-strand RNA with a 21–23 base pair length, and hence possesses a high molecular weight and multiple negative charges.^[5] These physicochemical characteristics prevent passive diffusion across the membrane of most cell types for RNAi,^[6] necessitating vectors for delivery of siRNA into cytosol, where the incorporation of siRNA into RNAi machinery occurs.^[7]

Recently, nanocarriers have been developed for siRNA delivery, including polymers,^[8] liposomes,^[9] and inorganic nanoparticles.^[10] These siRNA vehicles generally enter cells

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through endocytic pathways, and are hence prone to entrapment within subcellular compartments.^[11] This entrapment requires increased dosage of siRNA, increasing the possibility of off-target effects.^[12] Polyamine polymers and dendrimers can facilitate the escape of siRNA from endosomes^[13] by taking advantage of the “proton sponge effect”.^[14] These highly cationic vehicles are, however, associated with cytotoxicity.^[15] Therefore, delivery of siRNA into cytosol remains a key challenge for the application of RNAi,^[16] with direct cytosolic delivery providing the optimal outcome.

In this study, we demonstrate direct cytosolic delivery of siRNA using nanoparticle-stabilized nanocapsules (NPSC). The stability of NPSC relies on the supramolecular guanidine-carboxylate interactions between the arginine-functionalized gold nanoparticles (Arg-AuNP) of the shell and the hydrophobic fatty acid “oil” components in the core (Figure 1a). This method produces nanocapsules capable of effective delivery of both small molecules^[17] and proteins.^[18] We hypothesized that electrostatic self-assembly of Arg-AuNP and siRNA on the surface of the “oil” droplet would generate stable nanocapsules for siRNA delivery applications. We report here the preparation of an siRNA e NPSC that provide cytosolic delivery of siRNA, as revealed through still and video microscopy of fluorescently labeled siRNA delivery. Moreover, we demonstrate that this cytosolic siRNA delivery process is mediated by direct fusion between NPSC/siRNA complex and the cell plasma membrane. The cytosolic delivery of siRNA resulted in highly efficient (90%) knockdown of a destabilized green fluorescence protein (deGFP) in deGFP-HEK293 cells. Moreover, the delivery of siRNA targeting polo-like kinase 1 (siPLK1) silenced PLK1 expression in cancer cells, resulting in pronounced toxicity. The effective siRNA gene silencing using NPSC suggests the high potency of NPSC-facilitated direct cytosolic siRNA delivery as a platform to knock down targeted genes for disease treatment.

NPSCs were prepared according to our previous reports.^[18] Briefly, a template emulsion was prepared by homogenizing arginine functionalized gold nanoparticle (Arg-AuNP) with linoleic acid in 5 mM phosphate buffer (pH = 7.4). The template emulsion was transferred to Arg-AuNP solution and incubated for additional 10 min. to afford stabilized NPSC. The NPSC was then mixed with siRNA at different molar ratios and incubated at room temperature for 15 min., followed by gel electrophoresis assay and Ribogreen assay to measure siRNA encapsulation. As shown in Figure 1b, with NPSC to siRNA molar ratio increased from 1.1×10^{-4} to 11×10^{-4} , the migration of siRNA on the gel was gradually retarded. The binding at molar ratio of 5.5×10^{-4} completely retarded siRNA from migration, with $91 \pm 2\%$ of siRNA encapsulation, as determined by Ribogreen assay. This optimized NPSC to siRNA binding ratio was fixed for all subsequent intracellular delivery experiment. The encapsulation of siRNA had negligible effect on the morphology of NPSC, as revealed by transmission electron microscopy (TEM, Figure 1c). Dynamic light scattering (DLS) indicated NPSC and NPSC/siRNA formed nanoparticles with size at 149 ± 5 nm and 179 ± 8 nm in diameter, respectively (Table S1, SI). The slightly increased size of NPSC/siRNA compared to that of NPSC is consistent with swelling arising from the encapsulation of siRNA onto the NPSC surface. Additionally, the zeta potential of NPSC was made more negative from -25.5 mV to -39.4 mV for NPSC/siRNA complex, confirming the successful encapsulation of siRNA into NPSC.

The encapsulation of siRNA into NPSC efficiently protected siRNA against nuclease degradation, a prerequisite for the intracellular delivery of siRNA. As shown in Figure 1d, the treatment of free siRNA with Ribonuclease A (RNase A, 7 mU) for 1 h resulted in a complete degradation of siRNA.^[19] However, significant amounts of siRNA were still detected after the treatment of NPSC/siRNA complex even at a much higher RNase A amount (35 mU) and a longer incubation (2 h). Similarly, the NPSC/siRNA complex efficiently protected siRNA against serum degradation (Figure S1, in the supporting information). The enhanced enzymatic stability of siRNA with NPSC encapsulation may be ascribed to steric protection and neutralization of siRNA charge, which together decreased the susceptibility of siRNA toward nuclease degradation.

We prepared NPSC complex encapsulating fluorescently labeled siRNA (Cy3-siRNA) with a scrambled sequence to evaluate delivery of siRNA.^[20] The cellular uptake and subcellular localization of NPSC/siRNA complex was then monitored using confocal laser scanning microscopy (CLSM). Significant intracellular accumulation of red fluorescence occurred when the cells were treated with NPSC/Cy3-siRNA complex (Figure 2a). Significantly, the Cy3 fluorescence was evenly distributed within whole cell, and with little overlap with the endosome/lysosome by using LysoTracker@Green counter-staining. These CLSM studies suggest that the NPSC/siRNA complex was able to transport siRNA directly into cytosol without endosome/lysosome entrapment associated with polymer or lipid-based siRNA delivery vehicles.^[7] NPSC mediated siRNA uptake is siRNA concentration-dependent. With the Cy-3 siRNA increased from 12 nM to 36 nM, the Cy3 fluorescence intensity increased up to tenfold, and > 70% cells were Cy-3 fluorescence positive, as measured by flow cytometry analysis (Figure S2, SI).

The siRNA uptake and trafficking dynamics of NPSC siRNA delivery was investigated using time-lapse fluorescence imaging of Cy3-siRNA (Figure 2b and Movie S1, SI). With the addition of NPSC/Cy3-siRNA complex into HeLa cells, the fluorescence images of cells post-transfection were immediately captured at 1 min. interval. Time-lapse imaging analysis revealed that cytosolic siRNA fluorescence could be recorded within 5 min. exposure of NPSC/siRNA complex to cells, and the siRNA fluorescence was saturated after 20 min. of incubation, demonstrating extraordinary fast and efficient siRNA delivery using NPSCs. The siRNA fluorescence was evenly distributed within whole cell cytosol further demonstrating that NPSC mediated siRNA delivery enter cells bypassing endo/lysosomes.^[21]

In our previous study on protein delivery we observed a similar even cytosolic distribution of GFP, prompting us to hypothesize that a membrane fusion process was operative.^[17] While there is circumstantial evidence of this mechanism, further studies were performed to test this hypothesis. The efficiency of membrane fusion-mediated uptake is dependent on the cholesterol level in cell membrane.^[22] To explore whether NPSC delivered siRNA in a membrane fusion pathway, HeLa cells were pre-treated with nystatin (100 µg/mL) an inhibitor that has been used to deplete cholesterol from the plasma membrane,^[23] prior to siRNA transfection. Meanwhile, FITC-labeled dextran, known to enter cells via endocytic pathways,^[24] was co-incubated with cells to exclude the potential effect of nystatin treatment on endocytosis. Nystatin has no effect on the stability of NPSC/siRNA complex,

as revealed by the DLS size of NPSC/siRNA complex in the presence and absence of nystatin (Figure S3, SI). The siRNA and FITC-dextran uptake was then monitored by CLSM imaging. As shown in Figure 2c, both dextran and NPSC/siRNA efficiently enter HeLa cells in the absence of nystatin, with low co-localization observed between dextran and siRNA, confirming non-endocytic uptake of NPSC/siRNA complex. The treatment of nystatin significantly blocked siRNA uptake, however, no uptake inhibition was observed for FITC-dextran. The siRNA uptake inhibition by nystatin treatment was further confirmed and quantified by flow cytometry analysis (Figure S4, SI). Nystatin pre-treatment significantly decreased the siRNA fluorescence intensity of HeLa cells; the weak Cy3 fluorescence could be ascribed to the non-specific binding of NPSC/siRNA complex on cell surface, as confirmed by z-stack CLSM imaging of NPSC/siRNA uptake (Figure S4 (SI)). The above results indicated that nystatin treatment had not blocked the endocytosis of FITC-dextran, but inhibited the membrane fusion of siRNA by depleting cell membrane cholesterol.

We further investigated the siRNA uptake efficiency by studying uptake at reduced temperatures and by inhibiting dynamin. We pre-treated HeLa cells at 4 °C for 30 min. before the exposure of NPSC/Cy3-siRNA, the siRNA uptake was quantified by flow cytometry analysis and compared to that at 37 °C. As shown in Figure 2d, Cy3 fluorescence arising from siRNA uptake was significantly decreased for cells with 4 °C pre-treatment, similar to previous reports that the membrane fusion process is a temperature dependent process.^[25] Meanwhile, the treatment of HeLa cells with dynasore (80 μM), an inhibitor of dynamin that regulates membrane fusion by expanding the fusion pores,^[26] similarly reduced the cellular uptake of siRNA (Figure 2d). Taken together, the above cellular uptake studies indicate that NPSC facilitated cytosolic siRNA delivery is a cholesterol dependent membrane fusion process.

We next evaluated the biocompatibility of the NPSC/siRNA delivery platform by treating destabilized green fluorescent protein expressing deGFP-HEK293 cells with varied concentrations of NPSC/siRNA complexes, followed by cell viability assay. With siRNA concentrations (complexed with NPSC) from 10 nM to 60 nM the cells retained viabilities > 80% (Figure S5, SI), with the biocompatibility of NPSCs comparable to that of a commercial gene transfection reagent, Lipofectamine 2000 (Figure S5, in the supporting information).

Having demonstrated the efficient yet safe cytosolic delivery of siRNA using NPSC, we next investigated the efficacy of NPSC-facilitated siRNA delivery to knock down targeted genes. As many disease-related proteins have short half-lives inside cells, an efficient siRNA delivery to silence the gene of short half-life would have high therapeutic index.^[27] In this study, destabilized GFP (deGFP) with a half-life around 2 h was chosen as a testbed target gene. Stable-expressing deGFP-HEK293 cells were treated with NPSC/siRNA complex targeting deGFP (NPSC/si_deGFP) or a scrambled siRNA (NPSC/siScr), the deGFP expression profiles were monitored by CLSM imaging and flow cytometry analysis. As shown in Figure 4a, only very faint fluorescence signal was observed in the CLSM images when the cells were treated with NPSC/si_deGFP complex, indicating the high efficiency of NPSC mediated deGFP knockdown. Flow cytometry analysis revealed that the NPSC/siRNA complex treatment (with 60 nM of si_deGFP) silenced the deGFP expression

down to a level below 10% of blank control (Figure 3b and Figure S6a, SI). This gene silencing efficiency is substantially superior to the commercial lipid-based gene transfection reagents Lipofectamine 2000 (LPF2K, Figure 3b) and RNAi Max (Figure S7, SI). The NPSC/si_deGFP complex silenced deGFP genes in a siRNA concentration dependent manner. With siRNA concentration increased from 20 nM to 60 nM the deGFP expression was gradually suppressed from 90% down to 10% of blank controls (Figure S6b, SI). As expected, no gene silencing was observed when the cells were treated with either naked si_deGFP or an NPSC/siRNA complex with a scrambled sequence (Figure 3b). As NPSC delivered siRNA via a membrane fusion pathway, we hypothesized that the pre-treatment of HEK cells with nystatin to deplete cholesterol could block siRNA uptake and gene silencing. The GFP gene knockdown efficiency of HEK cells with nystatin or dynasore pre-treatment, followed by NPSC/si_deGFP treatment, was significantly decreased, as indicated by the strong fluorescence in CLSM images (Figure 3a) and minor GFP gene knockdown in the flow cytometry analysis (Figure 3b and Figure S8). The GFP gene silencing results again confirmed that NPSC facilitated siRNA is a cholesterol-dependent membrane fusion process, and the cytosolic siRNA delivery is superior to commercial reagents in terms of gene silencing efficiency.

Finally, we investigated the delivery of therapeutic siRNA. Polo-like kinase 1 (PLK1) was selected as a model therapeutic target. PLK1 is a key regulator of mitotic progression of cells, and is up-regulated in many types of cancer cells.^[28] Inhibition of PLK1 activity or the depletion of PLK1 protein can induce mitotic arrest and prevent tumor cell proliferation.^[29] Treatment of MDA-MB-231 cells with 40 nM NPSC/siPLK1 reduced cell viability to 30% whereas no change in viability was observed with naked or NPSC/siRNA scrambled controls (Figure 4a). Significantly, NPSC/siPLK1 inhibited cell proliferation with a higher efficiency than that of LPF2000. Knockdown of intracellular PLK1 protein was determined by western blot analysis. Essentially complete knockdown was observed when MDA-MB-231 cells were treated with NPSC/siPLK1 (40 nM siRNA) (Figure 4b), with protein expression decreased by ~95% (Figure S8, SI), significantly greater knockdown than LPF2000/siPLK1 complexes.

In summary, we have demonstrated that NPSC/siRNA complexes provide a highly effective siRNA transfection strategy. Microscopy studies showed that these systems delivered siRNA directly to the cytosol, providing efficient utilization of the siRNA payload by avoiding endosomal sequestration. In addition, we proved that such a direct cytosolic siRNA delivery was a temperature-dependent membrane fusion process. Extremely efficient (>90% GFP gene silencing) and effective PLK1 silencing for cancer therapy was achieved with this vehicle, substantially better than commercial available systems. Taken together, NPSC-siRNA complexes provide effective tools for *in vitro* applications, and promising platforms for biomedical delivery.

Experimental Section

See supplementary information for experimental details.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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a) Schematic presentation of cytosolic siRNA delivery

b) Gel retardation assay of NPSC/siRNA

c) TEM images of NPSC/siRNA

d) RNase stability of NPSC/siRNA

- 1) NPSC/siRNA
- 2) siRNA + RNase A 7 mU
- 3) NPSC/siRNA + RNase A 7 mU
- 4) siRNA + RNase A 14 mU
- 5) NPSC/siRNA + RNase A 14 mU
- 6) siRNA + RNase A 35 mU
- 7) NPSC/siRNA + RNase A 35 mU

Figure 1.

Preparation and characterization of NPSC/siRNA. a) NPSC/siRNA components and schematic presentation of NPSC mediated cytosolic siRNA delivery. b) Gel electrophoresis study of NPSC/siRNA complexation at molar ratios ranging from 1.1×10^{-4} to 11×10^{-4} . c) TEM images of NPSC/siRNA. d) Protection of siRNA from RNase A digestion as evaluated by electrophoresis. 5 pmol of siRNA alone or complexed with NPSC were incubated with 7, 14, and 35 mU RNase A at 37 °C for indicated times.

Figure 2.

Cytosolic delivery of Cy3-labeled siRNA into HeLa cells. a) Confocal microscopy images of HeLa cells after 1 hour treatment with 40 nM of NPSC/Cy3-siRNA complex. siRNA are not colocalize with endolysosomes. Endosome/lysosome was stained with LysoTracker @Green. Scale bars: 20 μm . b) Live cell imaging of rapid Cy3-siRNA release into the cytosol of HeLa cell by NPSC. “0 min” label represents the starting point of release. Scale bars: 20 μm . c) FITC-dextran and NPSC/Cy3-siRNA were co-incubated with HeLa cells in the absence and presence of nystatin (100 $\mu\text{g/mL}$). d) Flow cytometry analysis of HeLa cells

with NPSC/Cy3-siRNA treatment at 37 °C, or at 4 °C, or with a pre-treatment of dynasore (80 μ M). The error bars represent the standard deviations of three parallel measurements.

Figure 3.

NPSC/siRNA delivery silenced the deGFP expression of deGFP-HEK293 cells. a) Confocal microscopy images of deGFP-HEK 293 cells without siRNA transfection (top left), with 60 nM of NPSC/siScr (top right), with 60 nM of NPSC/si_deGFP (bottom left) and with 60 nM of NPSC/si_deGFP with nystatin pre-treatment (bottom right). Scale bars: 40 μ m. b) Quantification of deGFP expression of deGFP-HEK 293 cells without treatment, with naked si_deGFP, NPSC/siScr, NPSC/si_deGFP, Lipofectamine 2000 (LPF2K)/si_deGFP, and NPSC/si_deGFP with nystatin pretreatment. 60 nM of si_deGFP or scrambled siRNA was used for transfection. The fluorescence intensity was measured by flow cytometry analysis and normalized to cells without treatment. The error bars represent the standard deviations of three parallel measurements.

Figure 4.

NPSC mediated siPLK1 delivery in MDA-MB-231 cells. a) Cell viability of MDA-MB-231 cells treated with NPSC/siPLK1 or scrambled siRNA (40 nM). For the Lipofectamine 2000 control, 40 nM of siPLK1 was complexed with LPF2000 according to manufacturer's instructions. b) Representative PLK1 protein expression of MDA-MB-231 cells determined by Western blot analysis after incubation with 40 nM siPLK1 in NPSC/siPLK1 and controls. GAPDH expression was measured in all the samples to serve as an internal control.