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Polyethyleneimine-coated iron oxide nanoparticles as a vehicle for delivery of small interfering RNA to macrophages in vitro and in vivo

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Dear Dr. Steindel,

Thank you for your time in editing our ms. Listed below are answers to your concerns and we have accordingly revised our ms. The changes in the revision are shown in red.

Sincerely,

Xiaohua Mao

1. Figure 1: Where do the siRNA-PEI-SPION complexes go in the gel, then? Also, what do you mean by a ‘value of 0’ -the Fe-siRNA ratio?

Authors’ response: The siRNA band can be either retarded or not detectable upon binding to PEI-SPIONs (stated in protocol 2.6). The latter case reflects a strong binding ability of PEI-SPIONs with siRNA, which results in an exclusion of EB to siRNA during staining. The explanation is now given in the legend to figure 1. Value of 0 has been defined in the legend.

2. Figure 2: What is M2 and PE-H?

Authors’ response: They have been defined in the legend to figure 2.

3. Figure 3: What are the percentages here?

Authors’ response: Now defined in the legend to figure 3.

TITLE:

Polyethyleneimine-coated Iron Oxide Nanoparticles as a Vehicle for the Delivery of Small Interfering RNA to Macrophages *In Vitro* and *In Vivo*

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KEYWORDS:

Macrophage, transfection, nanoparticle, siRNA delivery, polyethyleneimine, vehicle, gene silencing

SUMMARY:

We describe a method of using polyethyleneimine (PEI)-coated superparamagnetic iron oxide nanoparticles for transfecting macrophages with siRNA. These nanoparticles can efficiently deliver siRNA to macrophages *in vitro* and *in vivo* and silence target gene expression.

ABSTRACT:

Because of their critical role in regulating immune responses, macrophages have continuously been the subject of intensive research and represent a promising therapeutic target in many disorders, such as autoimmune diseases, atherosclerosis, and cancer. RNAi-mediated gene silencing is a valuable approach of choice to probe and manipulate macrophage function;

however, the transfection of macrophages with siRNA is often considered to be technically challenging, and, at present, few methodologies dedicated to the siRNA transfer to macrophages are available. Here, we present a protocol of using polyethyleneimine-coated superparamagnetic iron oxide nanoparticles (PEI-SPIONs) as a vehicle for the targeted delivery of siRNA to macrophages. PEI-SPIONs are capable of binding and completely condensing siRNA when the Fe:siRNA weight ratio reaches 4 and above. *In vitro*, these nanoparticles can efficiently deliver siRNA into primary macrophages, as well as into the macrophage-like RAW 264.7 cell line, without compromising cell viability at the optimal dose for transfection, and, ultimately, they induce siRNA-mediated target gene silencing. Apart from being used for *in vitro* siRNA transfection, PEI-SPIONs are also a promising tool for delivering siRNA to macrophages *in vivo*. In view of its combined features of magnetic property and gene-silencing ability, systemically administered PEI-SPION/siRNA particles are expected not only to modulate macrophage function but also to enable macrophages to be imaged and tracked. In essence, PEI-SPIONs represent a simple, safe, and effective nonviral platform for siRNA delivery to macrophages both *in vitro* and *in vivo*.

INTRODUCTION:

Macrophages are a type of innate immune cells distributed in all body tissues, albeit in different amounts. By producing a variety of cytokines and other mediators, they play critical roles in the host defense against invading microbial pathogens, in tissue repair following injury, and in maintaining tissue homeostasis¹. Due to their importance, macrophages have continuously been the subject of intensive research. However, despite its prevalence in gene regulation and function studies, siRNA-mediated gene silencing is less likely to succeed in macrophages because these cells—particularly, primary macrophages—are often difficult to transfect. This can be ascribed to a relatively high degree of toxicity associated with most well-established transfection approaches in which the cell membrane is chemically (*e.g.*, with polymers and lipids) or physically (*e.g.*, by electroporation and gene guns) disrupted to let siRNA molecules cross the membrane, thereby drastically reducing macrophages' viability^{2,3}. Furthermore, macrophages are dedicated phagocytes rich in degradative enzymes. These enzymes can damage the integrity of siRNA, weakening its silencing efficiency even if gene-specific siRNA has been delivered into the cell^{3,4}. Therefore, an effective macrophage-targeted siRNA delivery system needs to protect the integrity and stability of siRNA during delivery⁴.

It is increasingly evident that dysfunctional macrophages are implicated in the initiation and progression of certain common clinical disorders like autoimmune diseases, atherosclerosis, and cancer. For this reason, modulating macrophage function with, for instance, siRNA, has been emerging as an attractive methodology for treating these disorders⁵⁻⁷. Although much progress has been made, a major challenge of siRNA-based treatment strategy is the poor cell specificity of systemically administered siRNA and the insufficient siRNA uptake by macrophages, which consequently lead to undesired side effects. Compared with free nucleic acid therapeutics that usually lack optimal cell selectivity and often lead to off-target adverse effects, drug-loaded

nanoparticles (NPs), owing to their spontaneous propensity of being captured by the reticuloendothelial system, can be engineered for passive targeting to macrophages *in vivo*, allowing for improved therapeutic efficacy with minimal side effects⁸. Current NPs explored for the delivery of RNA molecules include inorganic nanocarriers, various liposomes, and polymers⁹. Among them, polyethyleneimine (PEI), a type of cationic polymers capable of binding and condensing nucleic acids into stabilized NPs, shows the highest RNA delivering capacity^{9,10}. PEI protects nucleic acids from enzymatic and nonenzymatic degradation, mediates their transfer across the cell membrane, and promotes their intracellular release. Although initially introduced as a DNA delivery reagent, PEI was subsequently demonstrated to be an attractive platform for *in vivo* siRNA delivery, either locally or systemically^{9,10}.

Superparamagnetic iron oxide nanoparticles (SPIONs) have shown great promise in biomedicine, owing to their magnetic properties, biocompatibility, comparable size to biologically important objects, high surface-area-to-volume ratio, and easily adaptable surface for bioagent attachment¹¹. For instance, because of their potential utility as a contrast agent and rapid uptake by macrophages, SPIONs have emerged as a favorite clinical tool to image tissue macrophages¹². While SPIONs have also been extensively studied as nucleic acid delivery vehicles^{11,13-15}, to our knowledge, the literature contains few reports of SPIONs as a carrier for macrophage-targeted siRNA delivery. For gene delivery by SPIONs, their surface is usually coated with a layer of hydrophilic cationic polymers onto which negatively charged nucleic acids can be electrostatically attracted and tethered. Here, we present a method for synthesizing SPIONs whose surface is modified with low-molecular-weight (10 kDa), branched PEI (PEI-SPIONs). These magnetic nanoplatforms are then employed to condense siRNA, forming PEI-SPION/siRNA complexes that enable siRNA transport into the cell. We reason that spontaneous phagocytosis of SPIONs by cells of the reticuloendothelial system¹⁶, coupled with the strong ability of binding and condensing nucleic acids by PEI, renders PEI-SPIONs suitable for the efficient transport of siRNA into macrophages. The data presented here support the feasibility of PEI-SPION/siRNA-mediated gene silencing in macrophages in culture as well as *in vivo*.

PROTOCOL:

All methods involving live animals were performed in accordance with the animal care and use guidelines of Southeast University, China.

1. Preparation of PEI-SPIONs

1.1. Preparation of oleic acid-modified SPIONs

1.1.1. Dissolve $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in water under the protection of N_2 .

1.1.1.1. Add 28 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 20 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ into 80 mL of deionized water in a beaker. Introduce N_2 into the water through a glass conduit and stir until the solid matter has dissolved.

1.1.1.2. Heat the reaction mixture to 72 °C at a stirring rate of 800 rpm, followed by addition of 40 mL of ammonia water (28%). Stir for 5 min.

1.1.2. Add 9 mL of oleic acid dropwise into the above-mentioned solution and stir it at 72 °C for 3 h.

1.1.3. Cool the resulting solution to room temperature (RT). Precipitate the solution *via* magnetic separation.

1.1.4. Wash the precipitate containing SPIONs 3x with absolute ethyl alcohol and, then, disperse the precipitate in 100 mL of N-hexane.

1.2. Preparation of dimercaptosuccinic acid-modified SPIONs

1.2.1. Add 800 mg of oleic acid (OA)-modified SPIONs dispersed in 200 mL of N-hexane, and 400 mg of dimercaptosuccinic acid (DMSA) dispersed in 200 mL of acetone, into a three-neck flask in a water bath at 60 °C.

Note: To determine the mass of OA-modified SPIONs, volatilize the N-hexane and weigh the powder.

1.2.2. Add 200 µL of triethylamine dropwise into the above-mentioned solution with stirring at 1,000 rpm and refluxing.

1.2.3. After 5 h of stirring and refluxing, obtain a black precipitate by magnetic separation.

1.2.4. Disperse the hydrophilic SPIONs in deionized water homogeneously by adjusting the pH of the solution using tetramethylammonium hydroxide.

1.3. Preparation of PEI-SPIONs

1.3.1. Add DMSA-modified SPION colloidal solution dropwise into PEI solution (10 kDa) in a 500-mL three-neck flask under mechanical stirring at 1,000 rpm for 2 h ($W_{Fe}:W_{PEI} = 1:3$).

Note: The charge and size of PEI-SPIONs vary depending on the ratio of W_{Fe} to W_{PEI} . The $W_{Fe}:W_{PEI}$ ratio of 1:3 can be a good starting point for synthesizing PEI-SPIONs suitable for siRNA delivery.

1.3.2. Add the resultant solution into an ultrafiltration tube having a molecular weight cutoff of 100 kDa and a content of 15 mL; then, centrifuge at 5,400 x g for 10 min until the remaining solution is 1 mL. Add deionized water to the solution to make the volume 15 mL again and repeat

the above process 10x to obtain the final product. Then, filter the solution through a 0.22- μ m filter and store the final product at 4 °C.

1.3.3. Determine the Fe concentration of PEI-SPIONs by the colorimetric method using phenanthroline¹⁷. Dilute PEI-SPIONs with deionized sterile water to a concentration of 1 mg Fe/mL and store it at 4 °C.

1.3.4. Dilute 10 μ L of the PEI-SPION solution (1 mg Fe/mL) to 1 mL with deionized water; then, test its hydrodynamic size and zeta potential by a dynamic light-scattering device.

Note: Prepare PEI-SPIONs in the range of about 30 - 50 nm. In this size range, the effect of the NP size on siRNA binding and cellular uptake does not appear to be significant. PEI-SPIONs bearing an average zeta potential over +37 mV can be toxic in the dose range for transfection, and a cytotoxicity assay should be performed to ensure safety. The surface charge and hydrodynamic size of the nanoparticles can be controlled within a desired range by adjusting the PEI content.

2. Preparation and Agarose Gel Electrophoresis of PEI-SPION/siRNA NPs

2.1. Dilute siRNA with RNase-free water to yield a final concentration of 20 μ M (0.26 μ g/ μ L).

2.2. Prepare five RNase-free microcentrifuge tubes labeled 0, 1, 2, 4, and 8. The labels represent different Fe:siRNA weight ratios. Pipet 3 μ L of siRNA solution to all tubes (\sim 0.8 μ g of siRNA/tube).

2.3. Add 0, 0.8, 1.6, 3.2, and 6.4 μ g of Fe in the form of PEI-SPIONs to the tubes labeled 0, 1, 2, 4, and 8, respectively. Keep the total sample volume of each tube less than 20 μ L. Mix by gently pipetting up and down.

2.4. Incubate the mixtures at RT for 30 min to allow PEI-SPION/siRNA complex formation. During this period, make a 3% agarose gel with high-purity agarose.

Note: Additional PEI-SPION/siRNA complexes with other Fe:siRNA ratios (*e.g.*, 5 or 6) can be prepared and tested.

2.5. Add 1 μ L of 6x DNA-loading buffer per 5- μ L sample and mix cautiously. Load all samples and run electrophoresis at 5 V/cm until the bromophenol blue migrates as far as two-thirds of the length of the gel. Stain the gel with ethidium bromide (EB) for 15 - 20 min.

Note: Freshly prepared electrophoresis buffer and EB solution should be used.

2.6. Visualize siRNA bands under a UV imaging system. Check the Fe:siRNA ratios at which siRNA

forms complexes with PEI-SPIONs and, as a result, the bands representing free siRNA are retarded or not detectable.

3. Transfection of RAW264.7 Macrophages *In Vitro*

3.1. Culture mouse macrophage-like RAW264.7 cells in a 10-cm dish using DMEM complete medium containing 10% fetal bovine serum (FBS) per 100 U/mL penicillin per 100 µg/mL streptomycin at 37 °C in a 5% CO₂ incubator.

3.2. One day prior to the transfection, aspirate medium from the cells and rinse them with phosphate-buffered saline (pH 7.4). Add 1 mL of 0.25% trypsin to the 10-cm dish. Trypsinize RAW264.7 cells for about 5 - 10 min at 37 °C in a 5% CO₂ incubator.

3.3. When the majority of the cells have detached (after 5 - 10 min), add 5 mL of DMEM complete medium to the dish to inactivate the trypsin. Pipet up and down to disperse cell clusters into single cells.

3.4. Transfer the cell suspension to a sterile 15-mL conical tube. Centrifuge it at 300 x g for 3 min at RT. Remove the supernatant.

3.5. Resuspend the cells with 5 mL of fresh DMEM complete medium and count the cells.

3.6. Plate 9×10^4 cells per well in a 6-well plate with 2 mL of complete DMEM medium and incubate at 37 °C in a 5% CO₂ incubator for about 24 h.

Note: If using a plate of a different size, adjust the plated cell density in proportion to the relative surface area so that the cells reach 80% confluency at the time of transfection.

3.7. When cell confluency is 80%, remove the medium from the cells and replace it with 1 mL of DMEM complete medium per well. Return the plate to the incubator until PEI-SPION/siRNA complexes have been prepared and are ready for use (about 30 min).

3.8. Prepare PEI-SPION/siRNA complexes: calculate the amount of PEI-SPION/siRNA complexes needed for a transfection experiment. In a 1.5-mL RNase-free microcentrifuge tube, mix an appropriate amount of PEI-SPIONs with siRNA at a given Fe:siRNA ratio. For instance, to prepare PEI-SPION/siRNA NPs containing 100 µg of Fe at a Fe:siRNA ratio of 4, add 100 µL (1 mg Fe/mL) of PEI-SPIONs to 96 µL of siRNA (0.26 µg/µL), followed by mixing it gently with a micropipette. Incubate for 30 min at RT.

Note: Prepare a volume of PEI-SPION/siRNA complex that is 10% in excess of the total final mass to account for any incidental losses. Make PEI-SPION/siRNA complexes at low Fe:siRNA ratios,

under which siRNA molecules are completely loaded onto PEI-SPIONs and, hence, small amounts of PEI-SPIONs can be used to minimize potential cytotoxicity. Pilot experiments (gel retardation assay) for the optimization of the Fe:siRNA ratio are necessary.

3.9. Take out the 6-well plate from the incubator (step 3.7). Add a required volume of PEI-SPION/siRNA complex dropwise to each well and swirl the plate cautiously to ensure an even distribution. Return the plate to the incubator until the assessment of the cellular uptake or gene knockdown efficiency (1 - 3 d).

Note: Transfecting macrophages with PEI-SPION/siRNA at a concentration of ~15 µg Fe/mL may maximize transfection efficiency while minimizing potential cytotoxicity.

4. Systemic Delivery of siRNA to Macrophages in Rats with Experimental Arthritis

4.1. Obtain specific pathogen-free male Wistar rats that are 7 weeks old. Habituate the rats for 7 d prior to use and provide them with adequate food and water. Induce adjuvant arthritis (AA) in the rats as previously described¹⁸.

4.2. Prepare PEI-SPION/siRNA complexes as described in step 3.8.

4.3. Inject the PEI-SPION/siRNA NPs (0.3 mg of siRNA/kg) into the AA rats *via* the tail vein. Assess the cellular uptake *via*, for instance, flow cytometry, tissue biodistribution *via*, for instance, a real-time fluorescence imaging system, or therapeutic effects based on, for instance, clinical, histologic, and radiographic analyses at desired time points¹⁸.

Note: For cellular and tissue biodistribution studies, treat the rats with a single injection of the desired NPs; for therapeutic studies, inject the rats with the NPs to be tested 1x a week for three consecutive weeks.

REPRESENTATIVE RESULTS:

The size and zeta potential of PEI-SPIONs prepared with this protocol were in the range of 29 - 48 nm (polydispersity index: 0.12 - 0.23) and 30 - 48 mV, respectively. They were stable in water at 4 °C for over 12 months without obvious aggregation. To evaluate their siRNA binding ability, PEI-SPIONs were mixed with siRNA at various Fe:siRNA weight ratios. **Figure 1** shows that when the Fe:siRNA weight ratio reaches 4 and above, the band of free siRNA was completely missing, implying successful siRNA binding to PEI-SPIONs. A major concern of PEI in biomedical application is its toxicity, which is a result of the strong positive charge, particularly at high molecular weights and high doses. As shown in **Figure 2A**, PEI-SPIONs with a zeta potential of 30.5 and 37 mV did not exhibit apparent cytotoxicity at concentrations up to 30 µg Fe/mL, which is about twofold higher than the concentration (15 µg Fe/mL) normally used for cell transfection. However, PEI-SPIONs with a zeta potential of 48 mV were toxic even at the lowest dose examined (10 µg Fe/mL).

Therefore, PEI-SPIONs possess a charge-dependent toxicity. Since cationic charge is not important for the NP uptake by macrophages¹⁹, we suggest that PEI-SPIONs with an average zeta potential not higher than +37 mV are used for the siRNA transfer, although siRNA binding would decrease the charge to some extent and alleviate cytotoxicity¹⁸.

To test the potential application of PEI-SPIONs for siRNA delivery to macrophages, the *in vitro* transfection was performed with the murine macrophage cell line RAW 264.7. As analyzed by flow cytometry, more than 90% of the cells were transfected with fluorescently labeled PEI-SPION/siRNA complexes at 15 $\mu\text{g Fe/mL}$ (**Figure 2B**). With respect to transfection efficiency, there was actually no difference between PEI-SPION/siRNA NPs formed at Fe:siRNA weight ratios of 4 and 8, although, under the latter condition, the NPs that were formed were smaller in size and weaker in positive charge because a lesser amount of siRNA was loaded per particle. We also assessed the effect of PEI-SPION/siRNA concentration on cellular internalization by Prussian blue staining. As shown in **Figure 2C**, the blue spots within the transfected cells were minimally detectable at 7.5 $\mu\text{g Fe/mL}$, but clearly visible at 15 $\mu\text{g Fe/mL}$. Interestingly, increasing the PEI-SPION/siRNA concentration to 32 $\mu\text{g Fe/mL}$ did not increase the staining intensities, probably because the PEI-SPION/siRNA uptake was saturated at concentrations around 15 $\mu\text{g Fe/mL}$. In addition, the ability of PEI-SPIONs for mediating the siRNA transfer was corroborated in primary macrophages¹⁸, and the method presented here had a high siRNA transfection efficiency in rat peritoneal macrophages, equivalent to that in RAW264.7 cells. The peritoneal macrophages transfected with PEI-SPIONs harboring specific siRNA showed a significant decrease in the target mRNA level as compared with nonspecific siRNA (**Figure 2D**), implying that siRNA could escape from endocytosis vesicles into the cytoplasm and reach the RNAi machinery.

We previously also investigated the *in vivo* cellular uptake of systemically administered PEI-SPION/siRNA complexes in rats with adjuvant arthritis¹⁸. We analyzed PEI-SPION/siRNA transfection efficiency in phagocytic macrophages and nonphagocytic T lymphocytes. As shown in **Figure 3**, CD11b+ cells took up PEI-SPION/siRNA complexes more efficiently than CD3+ cells at any time point in all of the organs examined, indicating that PEI-SPION/siRNA NPs preferentially target macrophages¹⁸. Notably, a high-level accumulation of the NPs was observed in inflamed joints¹⁸, suggesting that the PEI-SPION can be an attractive platform for the systemic delivery of siRNA therapeutics in rheumatoid arthritis whose pathogenesis is linked to macrophage dysfunction and for which local siRNA administration is not a favorite choice due to the involvement of multiple organs of the disease.

FIGURE LEGENDS:

Figure 1: Agarose gel electrophoresis of PEI-SPION/siRNA complexes formed at various Fe:siRNA (w/w) ratios. A Fe:siRNA ratio of 0 represents free siRNA duplexes without PEI-SPIONs. The average size and zeta potential of the free PEI-SPIONs used here were 30 nm and 45 mV. siRNA could completely bind to PEI-SPIONs when the Fe:siRNA ratio reaches 4 and above, consistent

with the previous results using PEI-SPIONs with an average size of 48 nm and a zeta potential of 30.5 mV¹⁸. The absence of retarded bands (PEI-SPION/siRNA complexes) may reflect inaccessibility of siRNA to EB during staining, an indication of strong siRNA binding, and/or the condensing ability of PEI-SPIONs.

Figure 2: Biological characterization of PEI-SPION and PEI-SPION/siRNA NPs. (A) This panel shows a cell viability assay. RAW 264.7 cells were treated for 16 h with the indicated doses of PEI-SPIONs bearing different zeta potential and, then, an MTS assay was performed. The cell viability was normalized against the control (no particle exposure). The data are the mean \pm SD of duplicate wells. (B) This panel shows a flow cytometric analysis of the PEI-SPION/Cy3-siRNA uptake by RAW 264.7 cells. The cells were incubated for 24 h with 15 μ g Fe/mL (upper panel) or 5 μ g Fe/mL (lower panel) of PEI-SPIONs complexed with Cy3-labeled siRNA at a Fe:siRNA (w/w) ratio of 4 and 8, respectively. Nonspecific (NC) siRNA represents non-fluorescent siRNA. M2: gated region; Pe-H: Cy3 fluorescence intensity. The siRNA transfection efficiency of PEI-SPIONs used here (37.8 nm, 48 mV) was similar to a previous study using PEI-SPIONs with an average size of 48 nm and a zeta potential of 30.5 mV¹⁸. (C) This panel shows an analysis of PEI-SPION/siRNA uptake by visualizing cellular iron deposits. RAW 264.7 cells were incubated with 7.5, 15, and 32 μ g Fe/mL PEI-SPIONs (48 nm, 30.5 mV) complexed with siRNA (Fe:siRNA = 8) and stained by Prussian blue. The scale bars are 20 μ m. (D) This panel shows an *in vitro* validation of the silencing efficiency of siRNA delivered by PEI-SPIONs. A specific siRNA-targeting rat IL-2/-15 receptor β chain was loaded onto PEI-SPIONs (48 nm, 30.5 mV) at Fe:siRNA = 8 and, then, transfected into rat peritoneal macrophages. An NC siRNA was used as control. The gene silencing effect was assessed by quantitative PCR. The cells were incubated with the complexes at 15 μ g Fe/mL. The data are the mean \pm the SD of triplicate wells. Panel D has been modified from Duan *et al.*¹⁸ with permission from the publisher.

Figure 3: *In vivo* cellular uptake of PEI-SPION/siRNA NPs. Three arthritic rats were injected intravenously with a single dose of 0.3 mg/kg Cy3-siRNA formulated with PEI-SPION (48 nm, 30.5 mV). A rat injected with PBS was used as a control. Blood, spleen, liver, kidney, and inflamed joints were collected at 2, 8, and 24 h after the injection. The cellular uptake of PEI-SPION/Cy3-siRNA NPs was assessed by flow cytometry using (A) anti-CD3 and (B) anti-CD11b monoclonal antibodies. The percentages are of Cy3-siRNA uptake within the gated CD3+ or CD11b+ cells. The results shown here are representative of two independent experiments. This figure has been modified from Duan *et al.*¹⁸ with permission from the publisher.

DISCUSSION:

Macrophages are refractory to transfect by commonly used nonviral approaches, such as electroporation, cationic liposomes, and lipid species. Here we described a reliable and efficient method to transfect macrophages with siRNA. Using the present protocol, over 90% of macrophage-like RAW 264.7 cells (Figure 2B) and rat peritoneal macrophages¹⁸ can be transfected with siRNA without significant impairment of the cell viability. This method depends

on the delivery platform PEI-SPION, which is a nanocarrier composed of a core of iron oxide and a shell of PEI. So, the first key step of the protocol is the synthesis of PEI-SPIONs suitable for siRNA delivery. Usually, PEI-coated SPIONs are prepared from oleic acid-coated SPIONs by a ligand-exchange method in which oleic acid is directly exchanged from the surface of SPIONs by PEI or its derivatives¹⁵, generating hydrophilic NPs with a positively charged surface. In the case presented here, the oleic acid capped on the surface of SPIONs was replaced by water-soluble dimercaptosuccinic acid, and then PEI was loaded onto SPION surfaces through electrostatic interactions. This method is gentle and easy to prepare in large quantities, and the synthesized NPs have excellent stability in water²⁰. It is well-known that PEI is cytotoxic, and the toxicity correlates strongly with its molecular weight²¹. To ensure safety, an important consideration when synthesizing PEI-SPIONs is that these particles are coated with low-molecular-weight PEI, which is 10 kDa in this protocol. The undesired toxic effect of PEI is mainly mediated by its positive charge; therefore, the measurement of the zeta potential of PEI-SPIONs is essential, and the value should be not higher than 37 mV. A decrease in positive charge can be achieved simply by reducing the PEI content. Another critical step for the successful application of this siRNA delivery system is the optimization of the Fe:siRNA ratio by gel retardation. It seems reasonable to make PEI-SPION/siRNA complexes at low Fe:siRNA ratios under which siRNA molecules are still capable of binding to PEI-SPIONs. In this circumstance, small amounts of PEI-SPIONs can be used, thus minimizing its potential cytotoxicity.

In case a desired silencing efficiency or therapeutic effect is not produced, check the transfection efficiency by flow cytometry or fluorescence microscopy using the carrier loaded with a fluorescently labeled siRNA. Alternatively, the PEI-SPION/siRNA uptake can be examined by conventional Prussian blue staining, which is sensitive enough to detect single granules of iron in cells. If the transfection efficiency is indeed low, it may be required to optimize transfection conditions such as cell density, transfection time, and the dose of PEI-SPION/siRNA particles. The cell passage number can also affect the efficiency of transfection²². In most cases, an insufficient silencing effect is not caused by an insufficient PEI-SPION/siRNA uptake, as the present PEI-SPION system has been demonstrated to facilitate an effective siRNA transfer to macrophages. Sometimes, combining several siRNAs targeting the same gene can be a good strategy to enhance knockdown efficiency. It is noteworthy that, although RNAi generally occurs within 24 h of transfection, the onset and duration of gene silencing depend on the turnover rate of the target, the rate of dilution and longevity of siRNA, and even the concentration of serum in the medium. Thus, time course experiments may be needed to accurately determine the time point of maximal effect^{2,22}. For *in vivo* application, the therapeutic efficacy also depends on whether, and to what extent, the siRNA target contributes to disease phenotypes; thus, the choice of an appropriate siRNA target is critical for expected results.

There are several advantages of this protocol for delivering siRNA to macrophages. (1) The method is easy to perform and is a cheap way to produce PEI-SPIONs in large quantities, and the NPs produced are stable in water for over 12 months if they are kept at 4 °C. (2) Spontaneous

phagocytosis of SPIONs by macrophages facilitates an effective PEI-SPION-mediated siRNA transfer, resulting in high transfection efficiency. It is expected that besides RAW 264.7 cells and rat peritoneal macrophages, this approach is applicable to other macrophage cell lines and primary macrophages, as long as the dose for transfection is optimized. (3) siRNA transfection is fast and easy to conduct compared with other macrophage transfection methods such as nucleofection, which is time-consuming and requires a Nucleofector device². (4) PEI-SPION can be an ideal vehicle for macrophage-targeted systemic siRNA delivery in certain disease models. Macrophages play critical roles in the development and progression of various chronic inflammatory disorders, as well as tumors; and one conspicuous histological feature of these diseases is the abnormal blood vessels with leaky endothelium. Hence, due to the enhanced permeability and retention effect, systemically administered drug-loaded NPs tend to accumulate in diseased tissues and are readily captured by local macrophages, leading to enhanced specificity, reduced side effects, and improved therapeutic efficacy. In a rat model of adjuvant arthritis, intravenously injected PEI-SPION/siRNA complexes were taken up by ~40% of the CD11b+ cells during the first 24 h after the injection¹⁸. In contrast, when cationic liposome was used as a carrier for systemic siRNA delivery in mice, less than 5% of the CD11b+ cells in the arthritic joints entrapped the siRNA lipoplexes²³. Moreover, owing to their magnetic properties, the application of an external magnetic field may further facilitate the accumulation of PEI-SPION/siRNA complexes in the target tissues and increase their cellular uptake. Also noteworthy is that such siRNA-loaded NPs can be used not only for modulating macrophage function but also for imaging macrophage to provide diagnostic information, monitor treatment efficacy, and predict patients' clinical outcomes¹².

However, limitations associated with this protocol do exist. The PEI-SPION system exhibits a narrow range of dosage for siRNA delivery. The maximum uptake occurred when RAW264.7 cells were exposed to PEI-SPION/siRNAs at a concentration of 15 µg Fe/mL (**Figures 2B and 2C**). Increasing the PEI-SPION/siRNA concentration to 32 µg Fe/mL did not result in an increase in cellular uptake (**Figure 2C**) but, on the contrary, might increase the risk of inducing cell death due to the intrinsic toxicity of PEI. On the other hand, decreasing PEI-SPION/siRNA to 5 or 7.5 µg Fe/mL obviously reduced its uptake by RAW264.7 cells (**Figures 2B and 2C**). Thus, we propose that the optimal PEI-SPION/siRNA concentration for *in vitro* macrophage transfection is ~15 µg Fe/mL (the final concentration in a well). Another limitation that needs to be taken into consideration is the possible effect of PEI-SPIONs on macrophage activity. Nanoparticles may induce the immune response²⁴, depending on their surface modification, surface charge, size, shape, and even on the methodology used to synthesize them. Mulens-Arias *et al.* recently reported that PEI-coated SPIONs trigger macrophage activation²⁵. The method of PEI-SPION synthesis presented here differs significantly from that of Mulens-Arias *et al.*, and therefore, whether PEI-SPIONs prepared based on the present protocol trigger macrophage activation awaits further investigation. However, to unambiguously address this concern, we suggest that, in addition to the PEI-SPION complexed with scramble siRNA, the vehicle itself (PEI-SPION only) can serve as another control when using the present protocol. Finally, this protocol is not suitable

for the delivery of DNA due to its relatively large size.

In summary, we presented here a method of using PEI-coated SPIONs as a vehicle for siRNA transfection in macrophages. These NPs can efficiently deliver siRNA into immortalized macrophage cell lines, as well as into primary macrophages *in vitro*, and functionally induce gene silencing without affecting cell viability at the optimal dose for transfection. Moreover, PEI-SPIONs may be used for *in vivo* siRNA delivery to macrophages, making it possible to image, as well as modulate, macrophages whose dysfunction contributes to the development and progression of many chronic inflammatory disorders and cancers.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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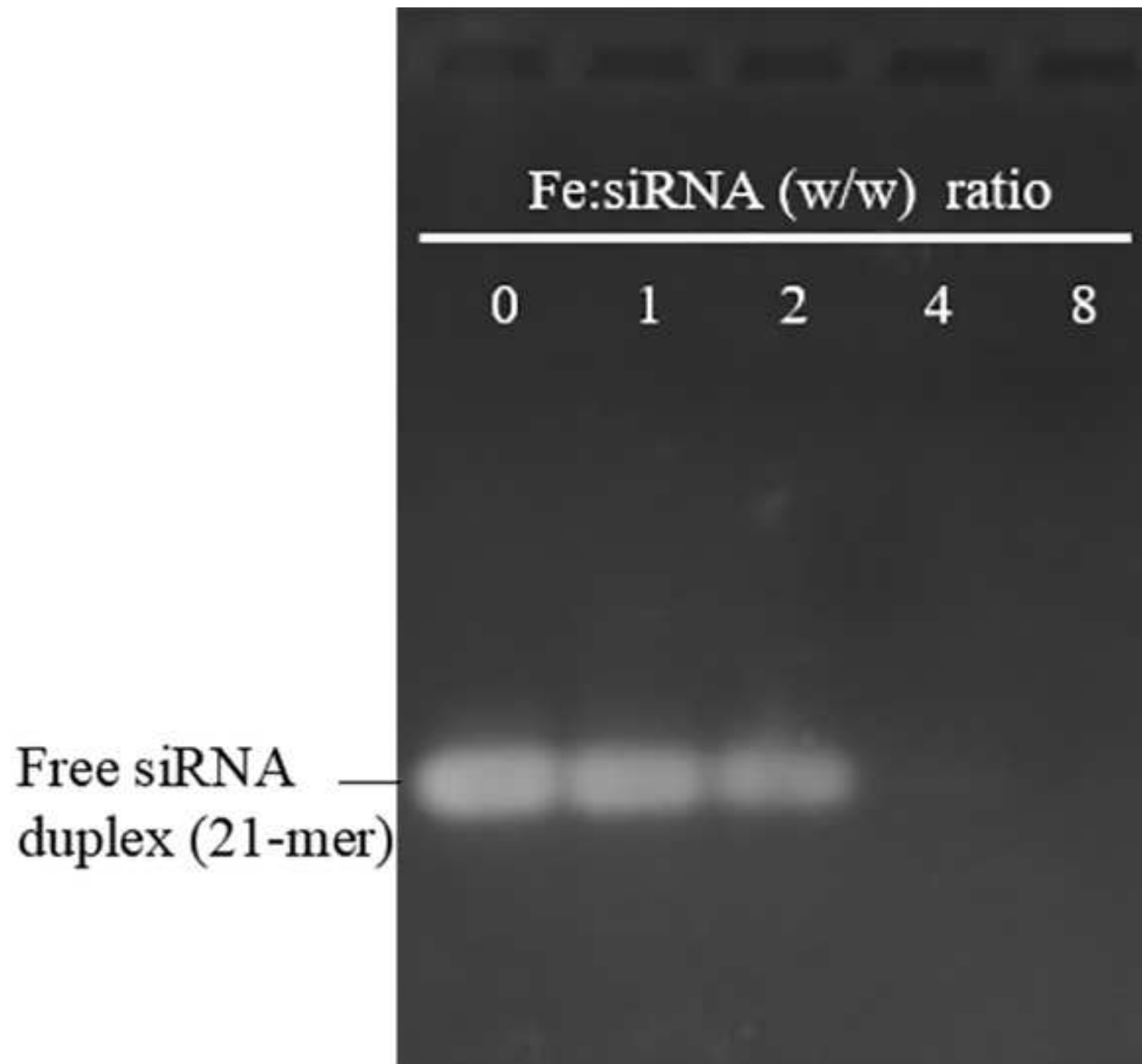
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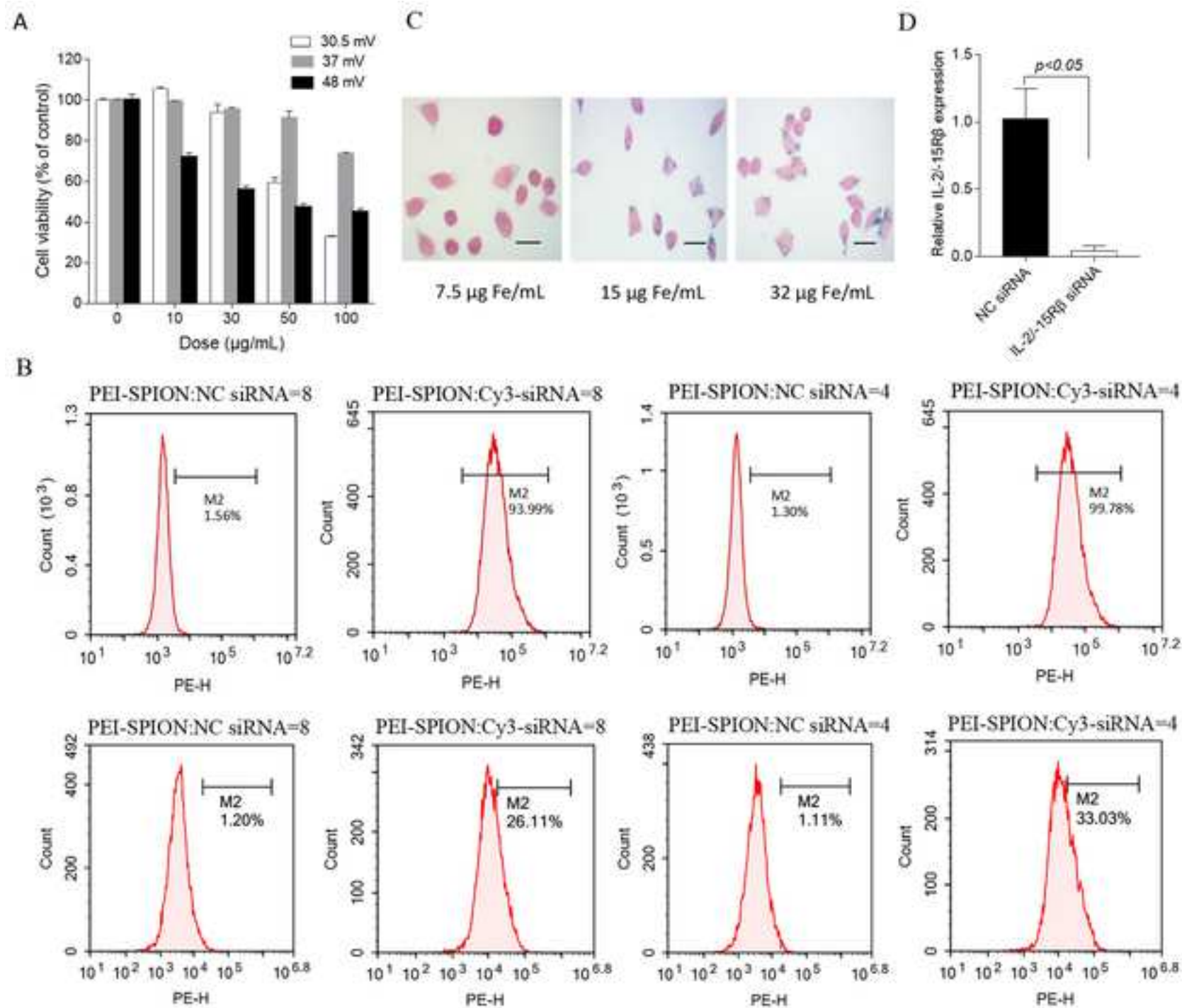
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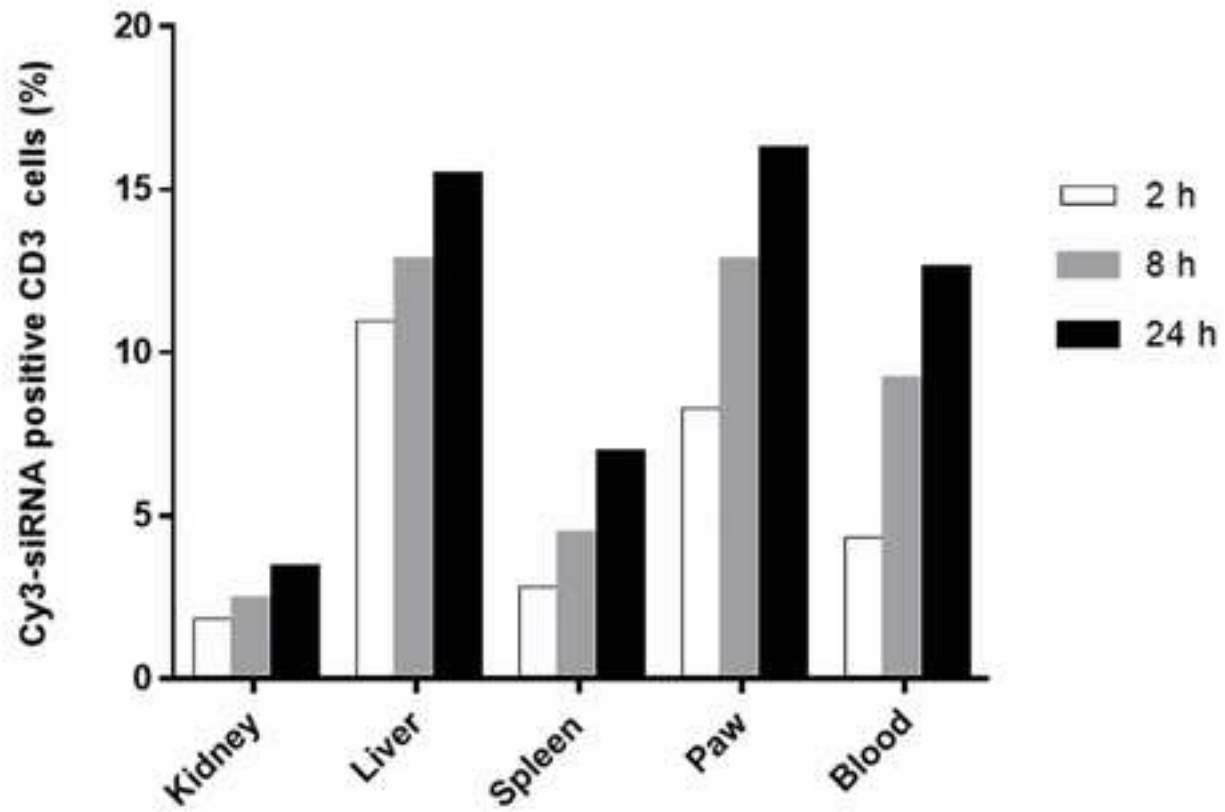
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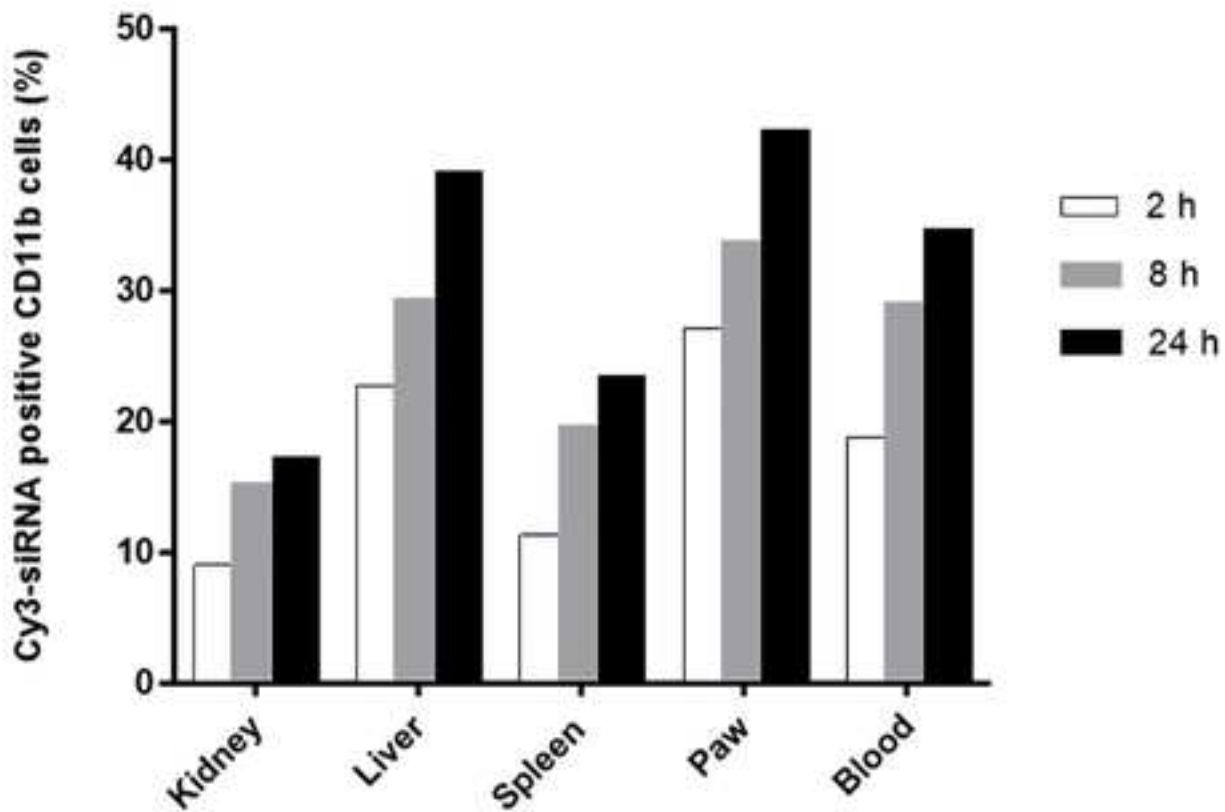




A



B



Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
DMEM	Gibco	C11995500BT	Warm in 37°C water bath before use
Fetal bovine serum	Gibco	A31608-02	
Penicillin/streptomycin (1.5 ml)	Gibco	15140122	
Tetrazolium-based MTS assay kit	Promega	G3582	For cytotoxicity analysis
	Cell Bank of Chinese Academy of Sciences, Shanghai, China	TCM13	
RAW 264.7 cell line	Corning	3516	
Tissue culture plates (6-well)	Corning	430167	
Tissue culture dishes (10 cm)			
RNase-free tubes (1.5 ml)	AXYGEN	MCT-150-C	
Centrifuge tubes (15 ml)	Corning	430791	
Trypsin	Gibco	25200-056	
	Shanghai Experimental Animal Center of Chinese Academy of Sciences		
Wistar rats	National Institutes for Food and Drug Control, China		
Bacillus Calmette–Guérin freeze-dried powder			for inducing adjuvant arthritis in rats

siRNA	GenePhar ma (Shanghai, China)	
Cy3-siRNA	RiboBio (Guangzho u, China)	
Polyethyleneimine (10 kDa)	Aladdin Chemical Reagent Co., Ltd.	E107079
Ammonia water	Aladdin Chemical Reagent Co., Ltd.	A112077
Oleic acid	Aladdin Chemical Reagent Co., Ltd.	O108484
Dimethylsulfoxide	Aladdin Chemical Reagent Co., Ltd.	D103272
FeSO ₄ •7H ₂ O	Sinopharm Chemical Reagent Co., Ltd	10012118
FeCl ₃ •6H ₂ O	Sinopharm Chemical Reagent Co., Ltd	10011918

Dimercaptosuccinic acid ultrafiltration tube	Aladdin Chemical Reagent	
	Co., Ltd.	D107254
	Millipore	UFC910096
Tetramethylammonium hydroxide solution	Aladdin Chemical Reagent	
	Co., Ltd.	T100882
Particle size and zeta potential analyzer	Malvern, England	Nano ZS90



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Author(s): Na Jia, Haoan Wu, Juanli Duan, Chunxue Wei, Yu Zhang, Xiaohua Mao

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3. Figure 1: Please label the molecular weight of the bands.

Authors' response: Revised as requested.

4. Figure 2: Please combine all panels of one figure into a single image file or split it into several figures.

Authors' response: Fig. 2 is now a single image.

5. Figure 2A: Please change ml to mL. Please include a space between 30 and mV (i.e., 30 mV).

Authors' response: Corrected as requested.

6. Figure 2B: Please line up the panels better. Some panels are off-set in the Figure.

Authors' response: Revised as requested.

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Authors' response: Revised.

13. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Authors' response: Ethics statement has been included in the revised ms.

14. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Authors' response: Revised as requested.

15. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Authors' response: Revised as requested.

16. 1.1.1: What type of water is used? How to dissolve under the protection of N₂? Where is this step performed?

Authors' response: Revised as requested. No special environment is needed to perform this step.

17. 1.3.2: How to perform purification by using 100k ultrafiltration tube? Does 100k mean 100 kDa?

Authors' response: Revised.

18. 1.3.3: Please describe how this is done.

Authors' response: Revised.

19. 2.2: Please specify the source of siRNA. Is it purchased or isolated in lab?

Authors' response: The source of siRNA used in this manuscript can be found in the Table of Materials. We don't think it is necessary to specify the source of siRNA at this step because viewers should, depending on their experimental purposes, use siRNA which may differ from those used in this manuscript. Our protocol should be suitable for delivery of any siRNAs, whatever their sequences.

20. 2.4: Please break up into sub-steps.

Authors' response: Done as requested.

21. 3.3: How long does it take for the majority of the cells to detach?

Authors' response: Specified.

22. 3.9: Does step 7 refer to step 3.7?

Authors' response: We thank the editor for careful reading of our ms. This has been corrected.

23. 4.1: Please specify the age, sex, and strain of rats.

Authors' response: Done as requested.

24. 4.3: Are rats constrained before injection? Please describe how to assess biodistribution or therapeutic effect.

Authors' response: Relevant info has been provided in the revised ms.

25. What happens to rats at the end of experiment?

Authors' response: In Section 4 of the protocol text, we describe how to deliver siRNA to macrophages in vivo using rats with adjuvant arthritis as a disease model. Because the sequence of siRNA used in this section is not provided, we don't think it is suitable here to mention what happens to rats at the end of experiment. Moreover, the therapeutic effect may vary, depending on siRNA specificity, its silencing activity, the choice of an appropriate target, dose, etc. Failure in obtaining expected therapeutic benefits does not necessarily mean infeasibility of the PEI-SPION-based siRNA transfection method.

26. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

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28. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Authors' response: Done as requested.

29. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

- a) Critical steps within the protocol
- b) Any modifications and troubleshooting of the technique
- c) Any limitations of the technique
- d) The significance with respect to existing methods
- e) Any future applications of the technique

Authors' response: We have completely rephrased the Discussion following these instructions.

Reviewer #1:

Comments:

The authors introduced many reported researches. Some errors and inappropriate statements (including English writings and experimental data) should be corrected. Specifically, the authors should address the following points:

1. The authors reported that "we presented a method for synthesizing SPIONs whose surface was modified with low-molecular-weight, branched PEI (PEI-SPION)". However, the molecular weight of PEI used in this research was not mentioned in the manuscript, although in section of preparation of PEI-SPIONs, the resultant solution was purified by 100K ultrafiltration tube, which seems like the cut-off molecular weight of free PEI was by less than 100kDa;

Authors' response: In our initial submission the molecular weight of PEI had been described in the Table of Materials. Anyway, in the revised manuscript we also indicate the molecular weight of PEI at step 1.3.1.

2. Mulens-Arias.V et al (2015) reported that Polyethylenimine-coated SPIONs activate macrophage through TLR-4 signaling and ROS production and modulate

podosome dynamics. Then, is PEI-SPION for siRNA delivery to macrophage suitable?

Authors' response: We thank the reviewer for pointing out the potential effect of PEI-SPION on macrophage activity. Nanoparticles may induce immune response, depending on their surface modification, surface charge, size, shape and even the methodology used to synthesize them. For instance, according to the paper of Mulens-Arias et al (2015), PMag 25Br-Zonyl FSA nanoparticles (a kind of PEI-SPION) synthesized at 90 °C dose-dependently increased IL-12 secretion when incubated with THP1 macrophages, while those synthesized at room temperature did not. Our method of PEI-SPION synthesis differs significantly from that of Mulens-Arias et al., and therefore whether PEI-SPIONs prepared based on our protocol could trigger macrophage activation awaits further investigation. However, since we previously demonstrated that PEI-SPION-mediated in vivo delivery of IL-2/IL-15R siRNA to macrophages was efficacious for treating experimental arthritis, a disease model in which abnormal activation of macrophages is a key pathological event, our PEI-SPIONs might have a negligible effect on macrophage activation. To address the referee's concern, in the Discussion of the revised manuscript we advocate incorporation of PEI-SPION (vehicle only) treated cells or animals as a control group.

3. For siRNA binding assay, Fe:siRNA ratio was calculated. This referee suggests the authors provide exact nitrogen:phosphate (N/P) ratio, which is the more accurate information in terms of reproducibility;

Authors' response: The PEI-SPION we used is not a commercial product. The person who would like to try this protocol has to synthesize the nanoparticle de novo. Even if vehicle:siRNA ratio is given in the form of N/P, pilot experiments for optimizing N/P ratio remain necessary. Due to its simplicity Fe:siRNA weight ratio was used to measure the amount of PEI-SPIONs needed for siRNA binding.

4. The author said that zeta potential of PEI-SPION over +37 mV can be toxic and suggested that PEI-SPION with an average zeta potential not higher than +37 mV be used for siRNA delivery. In additionally, the authors noted that the surface charge and hydrodynamic size of nanoparticles can be controlled within a desired range by adjusting the PEI content. But PEI-SPION was prepared at only W_{Fe}: W_{PEI} =1:3 ratio. Is this the actual ratio for siRNA delivery in this manuscript? This referee suggests that the authors mention the zeta potential and the size of this ratio, with and without siRNA loading;

Authors' response: The W_{Fe} to W_{PEI} ratio of 1:3 can serve as a starting point for synthesizing PEI-SPION suitable for siRNA delivery. We found that even for a given W_{Fe} to W_{PEI} ratio the size and charge of PEI-SPIONs prepared in independent experiments may vary to some extent. The PEI-SPIONs used in this manuscript were synthesized in separate experiments and the actual W_{Fe} to W_{PEI} ratio fluctuated about 1:3, depending on the charge and size wanted. Although the physicochemical properties of PEI-SPIONs in this manuscript differ to certain extent, these particles

showed similar siRNA binding ability and transfection efficiency except that highly positively charged particles can be toxic.

At the suggestion of the referee we provided the zeta potential and size of PEI-SPIONs in the revised figure legends. We did not provide the charge and size of the particles loaded with siRNA. We recommend performing gel retardation assay to determine the ratio of PEI-SPION (Fe content) to siRNA because using this assay siRNA binding to PEI-SPIONs can be presented in a straightforward, clear manner. Measurement of charge and size of PEI-SPION/siRNA is optional, as increase in size and decrease in zeta potential upon siRNA binding do not indicate to what extent siRNA is loaded onto PEI-SPIONs.

5. In line 271-272 and 278-279, the authors said that this figure was modified from ref. 17 and the authors are awaiting permission from the publisher. But ref.17 is not related to the present protocol. This referee suggests that the authors double check the references used in the manuscript;

Authors' response: We thank the referee for pointing out the mistakes. The two mistakes have been corrected.

Reviewer #2:

Manuscript Summary:

With the main objective of using macrophages as target for autoimmune diseases, the authors describe an interesting protocol in which PEI-SPIONs are synthesized for targeted delivery of siRNA to macrophages. In general, the manuscript is clear and copes with the aim of the journal. However, there are some points to be addressed before its acceptance.

Major Concerns:

1. There is a lack of information about the physicochemistry characteristics of the nanoparticles, mainly data about size and size distribution.

Authors' response: In the Results section we rephrased the opening sentence and provided the size and charge range of PEI-SPIONs used in this manuscript.

2. Figure legends are not clear. In addition, Fig 2 and 3, need permission from the Publisher but they correspond to an old reference. Why do not construct these figures with the results obtained by the authors?

Authors' response: In the revised figure legends we indicated the size and charge of PEI-SPIONs for each experiment. Fig. 2D and Fig. 3 were modified from our previous publication because these experiments, particularly Fig. 3, are time consuming and expensive.

Minor Concerns:

References should be revised.

Authors' response: Checked and mistakes corrected.

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