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"Intelligent" nanoassembly for gene delivery: In vitro transfection and the possible mechanism

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ABSTRACT

A new "intelligent" nanoassembly (INA), consisting of a condensed core of pDNA with protamine sulfate (PS) and a dioleoylphosphatidyl ethanolamine (DOPE)-based lipid envelope containing poly(ethylene glycol)-disulfide-DOPE (PSD), was designed and investigated. The in vitro release experiment was carried out in solution containing 10 mM of Glutathione, which reflected the redox potential of the intracellular environment. The experimental result indicated that PSD possessed a good ability of self-dePEGylation and could result in efficient release of content in the reductive environment. INAs showed higher transfection efficiency and much lower cytotoxicity compared with Lipofectamine™ 2000 on HEK 293 cells. Cellular uptake and subcellular localization, as well as the quantitation of nuclear transfer demonstrated that the superior transfection efficiency of INAs could result from both enhanced cellular uptake mediated by DOPE and efficient nuclear delivery mediated by PS. The biodistribution of INAs in nude mice bearing tumor implied that this PSD-based nanoassembly loading PS/DNA could be a promising gene delivery system for tumor therapy.

1. Introduction

The nucleic acid therapeutics has recently emerged as powerful new drug entities for treatment of gene-related diseases. However, the absence of an efficient delivery system has been identified as the major hurdle for clinical application of genetic drugs (El-Aneed, 2004; Torchilin, 2005; Li and Huang, 2006; Kogure et al., 2008). Generally, there are two different gene delivery systems: virus-derived vectors and non-viral ones. The practical use of viral vectors is limited by their drawbacks such as the lack of specificity toward target cells, high cost of production, and safety concerns including the risk of potential immunogenecity and chromosomal insertion of viral genome. To avoid the safety issues, non-viral gene delivery systems have attracted more attentions in recent years. Unfortunately, most of non-viral vectors suffer from either low gene transfection efficiency or significant toxicity (De Smedt et al., 2000; Brown et al., 2001; Glover et al., 2005).

With the evolved infectious pathway in mind, the viruses might offer us some inspirations to create more efficient non-viral vectors. Enveloped viruses have a bilayer lipid membrane that protects the capsid and the genome, and can function as a 'transport vesicle'. They can be endocytosed and fuse with the endosomal membrane. Subsequently, the non-enveloped capsid is present in the cytoplasm. During cytoplasmic trafficking, uncoating and structural change occur, and expose new layers of the viral particles such as nuclear localization signals (NLS). These structural changes can occur in response to changes in pH, reductive environment, Ca^{2+} concentration or enzymatic activity. Nuclear entry is often the final step in the complex transport and uncoating program of viruses (Whittaker et al., 2000).

We are interested in designing an efficient non-viral gene vector that could mimic the virus during the transfection. We constructed a new intelligent nanoassembly (INA) with multifunctional components, which was composed of a condensed core of pDNA with protamine sulfate (PS) and a DOPE-based lipid envelope containing poly(ethylene glycol)-disulfide-DOPE (PSD). PEG shield was attached to INA to stabilize its structure and extend the systemic circulation time. DOPE was utilized to trigger the direct fusion into cytoplasm or the endosome disruption after endocytosis (Drummond et al., 2000; Hafez and Cullis, 2001). The dynamic linkage disulfide bond was supposed to be cleaved in the reductive environment in cytoplasm (Go and Jones, 2008; Meng et al., 2009), which would result in the release of PS/DNA complexes. PS was introduced to condense the pDNA and enhance the nuclear transfer (Sorgi et al., 1997; Masuda et al., 2005). In present work, the in vitro properties such as physicochemical characteristics, transfection efficiency and cytotoxicity of INAs (DOPE/PSD) were investigated, and the biodistribution of INAs in nude mice bearing tumor was evaluated. In addition, the intracellular fate of INAs and the possible mechanism of enhanced transfection were also discussed.

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2. Materials and methods

2.1. Materials

The plasmid DNA (pDNA, pGL3-control vector from Promega, Madison, WI, USA) was amplified in DH5α strain of *Escherichia coli* and prepared by EndoFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). PicoGreen® Kit and YOYO-1 were purchased from Molecular Probes (Eugene, OR, USA). Lipofectamine™ 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's Modified Eagle Medium (DMEM) and RPMI 1640 medium were purchased from Gibco BRL (Paisley, UK). mPEG-NH_2 (Mw = 2000), protamine sulfate (PS), dichloromethane (DCM) and the other reagents (analytical grade) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Poly(ethylene glycol)-disulfide-DOPE (PSD) was synthesized as previously described (Kirpotin et al., 1996; Longmuir et al., 2001), and confirmed by ^{1}H NMR spectra.

The epithelial cell line HEK 293 and hepatocellular carcinoma cell line BEL 7402 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and were grown in DMEM containing 10% fetal bovine serum (FBS) and RPMI 1640 medium containing 10% fetal bovine serum (FBS), respectively. Cells were maintained at 37 °C in a humidified and 5% CO_2 incubator.

Female nude mice were purchased from Shanghai Experimental Animal Center (Shanghai, China). All animal procedures were performed following the protocol approved by the Institutional Animal Care and Use Committee at Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

2.2. Preparation and characteristics of INAs loading PS/DNA

The PS/DNA complex (PS/DNA) was prepared in HEPES buffer (pH 7.4, 10 mM) according to the following process. Briefly, 100 μl of DNA solution (100 μg/ml in HEPES buffer, pH 7.4, 10 mM) was added drop-wise to 100 μl HEPES buffer (pH 7.4, 10 mM) containing PS under stirring at a moderate speed. The PS/DNA was allowed to sit at room temperature for 30 min to facilitate complexation. Experiments were performed to investigate whether PS form a complex with pDNA. The complex was prepared immediately prior to the experiment.

INAs loading PS/DNA were prepared by the lipid film hydration technique. Briefly, 400 μl of PS/DNA suspension containing DNA (20 μg) was added to the lipid film consisting of PSD and DOPE (total lipids, 15 mg), followed by incubation for 30 min to hydrate lipids. Then, the hydrated mixture was treated by sonification in an ice bath for 15 s × 2 times to construct INAs by coating PS/DNA with lipids. Finally, INAs were separated by Sephadex with HEPES buffer as eluent after heparin (0.1%, w/v) was added to dissociate the unentrapped PS/DNA. The optical density of the different collected fractions was measured at 365 nm. Subsequently, INAs were lyophilized.

Size distribution and ζ potential of INAs were measured by laser light scattering using a Nicomp 380/ZLS zeta potential analyzer (Particle Sizing System, USA) after resuspended in water. The DNA loading was obtained by measuring the amount of DNA that was encapsulated in INAs.

2.3. In vitro release experiment

INAs (10 mg) were suspended in 1.5 ml of 0.1 M PBS containing reducing agent, Glutathione at 37 °C under horizontal shaking (300 rpm, Thermomixer, Eppendorf). The final concentration of Glutathione was 10 mM, which reflected the redox potential of the intracellular environment. At predetermined time intervals, the suspension of INAs was ultra-centrifuged at 15,000 rpm for 15 min after heparin (0.1%, w/v) was added to dissociate the released PS/DNA and the supernatant was collected for further DNA analysis. The INAs were rinsed and resuspended in the same volume of fresh medium and incubated under the same condition. The amount of DNA released in each time interval was determined by the PicoGreen® assay.

2.4. In vitro transfection and cell viability

Cells (HEK 293) were seeded in 24-well plates at a density of 1×10^5 cells per well in 500 μl of complete medium (DMEM containing 10% FBS, supplemented with 40 μg/ml penicillin G and 40 μg/ml streptomysin) and incubated for 24 h prior to transfection. Medium was replaced, and naked pDNA, PS/DNA or INAs were added at equivalent to 1 μg DNA per well in 500 μl DMEM containing 10% FBS for 24 h. Cells were incubated at 37 °C for an additional 24 h after the medium was replaced by fresh complete medium. The transfection results were measured using Luciferase Assay System (Promega, Madison, WI, USA). As positive control, Lipofectamine™ 2000 was used according to the manufacture's protocol. All transfection experiments were performed in triplicate.

The cytotoxicity was evaluated by MTT assay. Cells were seeded in 96-well plate at a density of 1×10^5 cells per well in 200 μl of complete medium and incubated for 24 h. Medium was replaced, and PS/DNA, INAs or Lipofectamine™ 2000 were added at equivalent to 1 μg DNA per well in DMEM containing 10% FBS. After 24 h, the media were replaced by 200 μl of fresh complete medium, and 20 μl of MTT solution (5 mg/ml) was added to each well. Cells were incubated for an additional 4 h. Then, medium containing MTT was removed, and 150 μl of DMSO was added to dissolve the crystals formed by living cells. Absorbance was measured at 490 nm using a microplate reader (Infinite F200, TECAN, Austria). The cell viability (%) was calculated and compared with the untreated control.

2.5. Cellular uptake and subcellular localization

The pDNA was labeled by the cell-impermeable fluorescent dyes YOYO-1 with a ratio of 1 dye molecule to 300 bp. Cells were seeded in 24-well plates with complete medium and incubated for 24 h. Then, PS/DNA, INAs, or Lipofectamine™ 2000 were added at equivalent to 1 μg labeled pDNA per well with 500 μl of DMEM containing 10% FBS and incubated for 2 h. Cells were collected, washed and resuspended in PBS (pH 7.4). The fluorescence was measured using a FACSCalibur. All experiments were performed in triplicate.

Cells were seeded on 10-mm^2 glass coverslips placed in 24-well plates with complete medium and incubated for 24 h. Then, PS/DNA, INAs or Lipofectamine™ 2000 were added at equivalent to 1 μg labeled pDNA per well with 500 μl of DMEM containing 10% FBS for 2 h. The cells were washed twice with PBS and fixed with 4% paraformaldehyde immediately or after another 24 h incubation with complete medium. The coverslips were mounted on glass slides with 3 μl MobiGlow (MoBiTec, Goettingen, Germany), an antifading substance to reduce photobleaching effects. Subcellular localization of labeled pDNA was determined using confocal microscopy (Leica Microsystems Inc).

2.6. Quantitation of nuclear transfer of pDNA

The pDNA was labeled by the cell-impermeable fluorescent dyes YOYO-1. Cells were seeded in 24-well plates with complete medium and incubated for 24 h. PS/DNA, INAs, SNAs or Lipofectamine™ 2000 were added at equivalent to 1 μg labeled pDNA per well with 500 μl of DMEM containing 10% FBS and incubated for 2 h. After incubation for another 24 h, cells were collected and the nuclear portion of cells was extracted using CHEMICON®'s Nuclear Extraction Kit (CHEMICON® international, Inc.). The fluorescence of the nuclear portion was measured using a microplate

reader (Infinite F200, TECAN, Austria). All experiments were performed in triplicate.

2.7. Biodistribution of INAs in nude mice

The pDNA was labeled by the cell-impermeable fluorescent dyes YOYO-1. Subcutaneous tumors on the left flank of female nude mice ($n = 30$) were initiated by injection of 1×10^6 viable cells (BEL 7402) in a volume of 0.1 ml. Tumors were allowed to grow to a volume of 100–200 mm^3. Female nude mice bearing hepatoma were randomly assigned to three groups ($n = 10$) and injected through the tail vein with saline, PS/DNA or INAs. In each treatment group, mice were sacrificed at 0.5 or 6 h after drug administration ($n = 5$ at each time point). Heart, liver, spleen, lung, kidney and tumor were collected. The tissues were homogenized and YOYO-1 was extracted with dimethyl sulfoxide (DMSO). The amount of YOYO-1 in each sample was determined using a microplate reader (Infinite F200, TECAN, Austria). The data were normalized to the tissue weight.

2.8. Statistical analysis

Statistical analysis was performed using a Student's *t*-test. The differences were considered significant for p value <0.05, and very significant for p value <0.01.

3. Results and discussion

3.1. The characteristics of INAs loading PS/DNA

Protamine is a natural DNA condenser and possesses bifunctional advantages in intracellular delivery of plasmid DNA (pDNA): improvement in both nuclear targeting and intra-nuclear transcription (Sorgi et al., 1997; Masuda, 2005). When the ratio of PS/DNA was over 2:1 (w/w), satisfactory complexation (>95%) of pDNA could be achieved, and the size of complex was about 60 nm.

The physicochemical characteristics of INAs were investigated after resuspension. The INAs (DOPE:PSD = 90:10, molar ratio) loading PS/DNA (PS:DNA = 15:1, w/w) showed spherical in shape when observed by TEM (Fig. 1a). The particle size was about 180 nm with the zeta potential about 4 mV. The loading of DNA in INAs increased with the ratio of PS to DNA from 0.5:1 to 4:1 (w/w), and achieved 1.19 μg DNA/INA (mg) at the ratio of PS/DNA (5:1, w/w). The result of DNase I digestion experiment demonstrated that INAs provided a profound effect on protecting DNA from the enzymatic degradation, and DNA incorporated in INAs showed good stability and integrity (Fig. 1b).

3.2. In vitro release experiment

The release kinetics experiments of INAs (DOPE:PSD = 90:10, molar ratio) loading PS/DNA (PS:DNA = 15:1, w/w) were carried out in solution containing 10 mM of Glutathione at 37 °C, which reflected the redox potential of the intracellular environment. In vitro release profiles of DNA were obtained by representing the percentage of released DNA to the amount of DNA encapsulation in INAs (Fig. 2a). The nanoassemblies consisting of DOPE/non-degradable PEG-DOPE (90:10, molar ratio) were used as a control (Fig. 2b). To two nanoassemblies, no more than 10% of DNA was released in solution without Glutathione over 30 h, respectively, which indicated that both nanoassemblies were stable under physiological conditions. In solution with 10 mM Glutathione, over 50% of DNA in INAs was released within 12 h, while the control nanoassemblies did not show any significant difference compared with the result in solution without Glutathione.

DOPE can trigger the fusion with cell membrane or enhance the endosomal release because of its excellent fusogenic capacity.

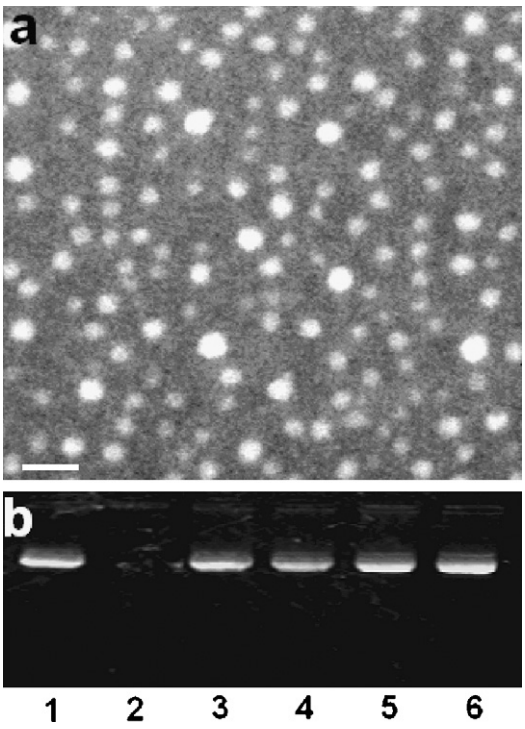

Fig. 1. Transmission electron micrograph of INAs (PSD:DOPE = 10:90, molar ratio) loading PS/DNA (PS:DNA = 15:1, w/w) (a), and agarose gel analysis of DNA in INAs after treatment with 2.5 U of DNase I/μg DNA (b). (a) Bar represents 300 nm. (b) Lane 1, pDNA control without treatment; line 2, naked DNA; lines 3–6, INAs loading PS/DNA (PS:DNA = 5:1, 10:1, 15:1, 20:1, w/w).

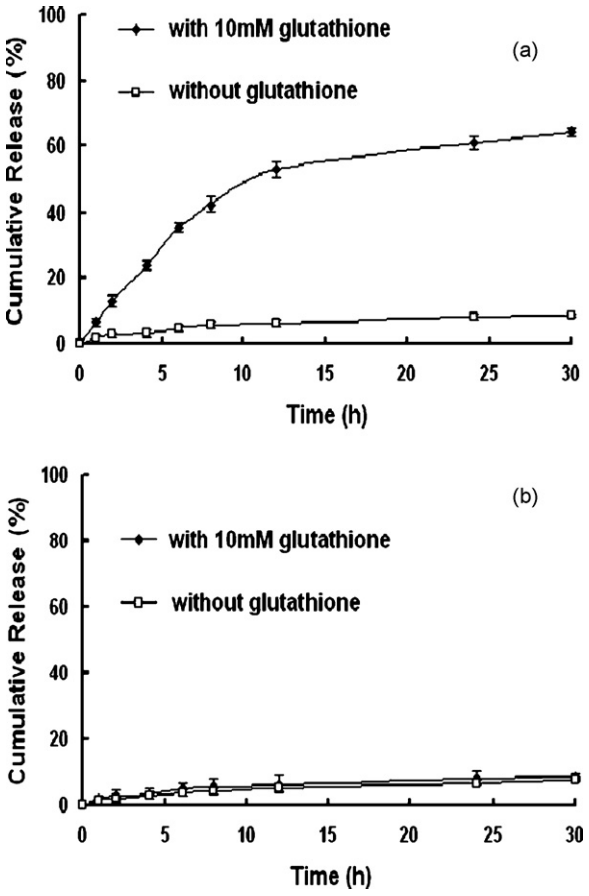

Fig. 2. Release profiles of INAs (PSD:DOPE = 10:90, molar ratio) loading PS/DNA (PS:DNA = 15:1, w/w) (a) and nanoassemblies with non-degradable PEG-DOPE (b).

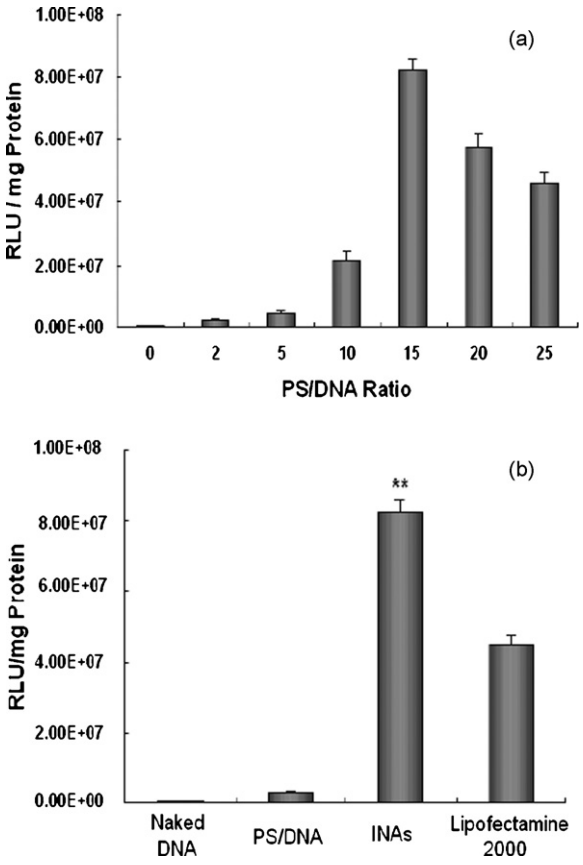

Fig. 3. The influence of the ratio of PS/DNA incorporated in INAs on transfection efficiency on HEK 293 cell line (a), and transfection efficiency of different vectors (b). Data were given as mean ± SD ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with Lipofectamine™ 2000 group.

Different from the majority of the phospholipids, DOPE presents a minimally hydrated and small headgroup which occupies a lower volume compared with the hydrocarbon chains, exhibiting a cone shape as opposed to the cylinder shape of bilayer-stabilizing phospholipids (Simões et al., 2004). Thus DOPE alone cannot form liposomes at physiological temperature and pH. PEGylated DOPE such as PSD, which exhibits a reversed cone shape, can form sterically stabilized liposomes with DOPE as a stabilizer. When PSD is de-PEGylated under proper condition, its capacity of stabilization shall vanish. The in vitro release profiles of INAs under different conditions can be presented as the evidence of dePEGylation of PSD. The above results indicated that disulfide bond would be dynamic in the intracellular environment, and PSD possessed a good ability of self-dePEGylation.

3.3. In vitro transfection and cell viability

In order to evaluate the transfection capability of INAs, in vitro transfection experiment was performed on HEK 293 cell line. The transfection efficiency was measured by Luciferase Assay System. The data were normalized to the amount of protein. To INAs (DOPE:PSD = 90:10, molar ratio), the transfection efficiency increased with the ratio of PS to DNA from 1:1 to 15:1 (w/w) and approached the maximum at 15:1 (w/w) (Fig. 3a), which was consistent with the report that transfection activity of PS/DNA would be enhanced drastically when the charge ratio increased (Masuda, 2005). In naked (non-encapsulated) pDNA group, only neglectable luciferase expression (relative to background level) was detected after 24 h. The expression of PS/DNA complex was only slightly higher than the naked pDNA. It was impressive that INAs

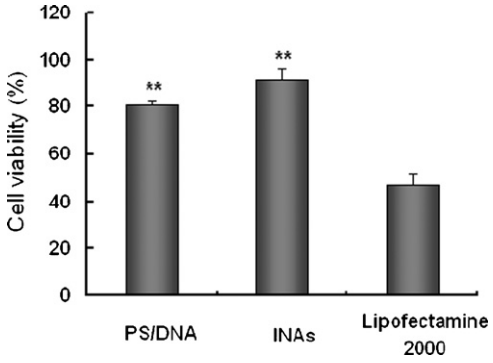

Fig. 4. Cell viability of PS/DNA and INAs on HEK 293 cell line. Data were given as mean ± SD ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with Lipofectamine™ 2000 group.

(DOPE:PSD = 90:10, molar ratio) group showed the highest relative light units (RLU) with over 80×10^6/mg protein among several formulations (Fig. 3b). As a positive control, Lipofectamine™ 2000 showed RLU with about 40×10^6/mg protein.

The cytotoxicity of the INAs loading PS/DNA was estimated by MTT assay. Both of the PS/DNA complex and INAs showed relative low cell cytotoxicity, and the cell viability in INAs group was about 90% (Fig. 4). On contrast, the cell viability in Lipofectamine™ 2000 group was only about 40%. According to these results, the INAs appeared to be a safer carrier than Lipofectamine™ 2000.

3.4. Cellular uptake and subcellular localization

The investigation on the intracellular fate of gene delivery system is of great importance to the rational design of non-viral vector with high transfection efficiency. In order to gain more details about the intracellular trafficking of INAs, cellular uptake and intracellular distribution were detected. The cellular uptake of PS/DNA (PS:DNA = 10:1, w/w), INAs (DOPE:PSD = 90:10, molar ratio) or Lipofectamine™ 2000 is shown in Fig. 5. The fluorescence of cells treated with INAs was much higher than that of PS/DNA, which demonstrated that encapsulation of PS/DNA complex in DOPE-based lipid envelope containing PSD could obviously enhance the entry into cells. The results of cellular uptake did not show any significant difference between the INAs and Lipofectamine™2000.

Confocal microscopy was used to observe the intracellular distribution of the internalized pDNA. To INAs group, the fluorescence distributed into the nuclear after 24 h incubation (Fig. 6c), while in Lipofectamine™ 2000 group, the fluorescence mainly remained in cytoplasm (Fig. 6b).

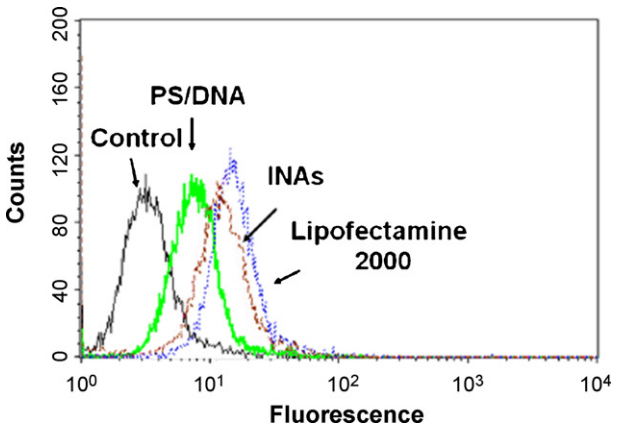

Fig. 5. Cellular uptake experiment. The pDNA was labeled by the cell-impermeable fluorescent dyes YOYO-1.

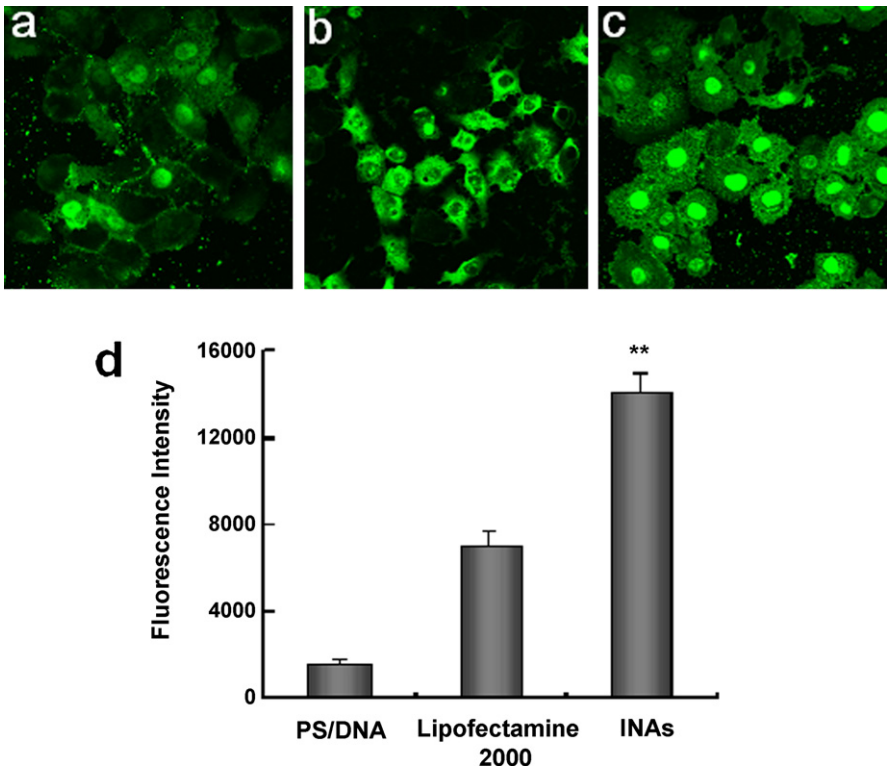

Fig. 6. Confocal images of HEK 293 cells, 24 h after the treatment with PS/DNA (a), Lipofectamine™ 2000 (b) or INAs (c), and the quantitation of the nuclear transfer (d). The pDNA was labeled by the cell-impermeable fluorescent dyes YOYO-1.

The quantitation of nuclear transfer was also performed using YOYO-1-labeled pDNA. After 24 h incubation, the nuclear portions of cells were extracted and the fluorescence was detected. As shown in Fig. 6d, the nuclear transfer of INAs was much higher than that of Lipofectamine™ 2000. Since the cellular uptake did not show obvious difference, the enhanced nuclear transfer could be one of the reasons that INAs showed superior transfection efficiency compared with Lipofectamine™ 2000.

3.5. Biodistribution of INAs in nude mice

The biodistribution of INAs in nude mice bearing hepatoma was determined using INAs loading YOYO-1 labeled pDNA. PS/DNA complex was used as control. As shown in Fig. 7, PS/DNA complex was eliminated immediately from the circulation after administration and distributed mainly into liver, spleen and lung at 6 h. The accumulation of the complex in tumor was neglectable. The INAs were eliminated from the systemic circulation much more slowly compared with PS/DNA, and the accumulation in liver and lung was less than that of PS/DNA complex. Enhanced distribution of INAs in tumor tissue was observed.

The limited pore size of the endothelial wall in tissue is the primary delivery barrier for nanoparticles but also allows selective accumulation in certain tissues. Tissues with a leaky endothelial wall such as tumor, liver, spleen and bone marrow usually contribute significant uptake of nanoparticles. The enhanced uptake in liver, spleen and bone marrow is largely attributed to the macrophages residing in the tissue, which are responsible for clearing particles and macromolecules circulating in blood. When nanoparticles are intravenously administrated, a variety of serum proteins would bind to the surface of the nanoparticles, which are recognized by the scavenger receptor on the macrophage cell surface and internalized, leading to a significant loss of nanoparticles from the circulation. The serum proteins binding on the nanoparti-

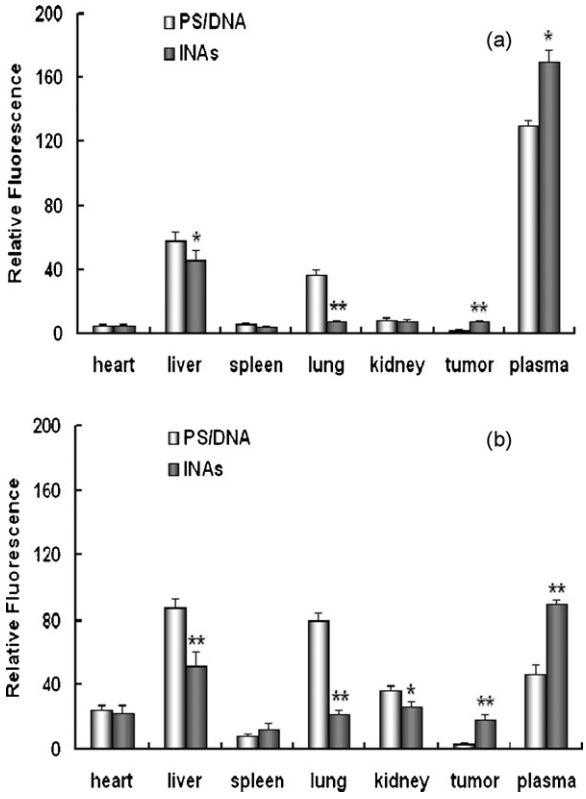

Fig. 7. The biodistribution of pDNA in female nude mice bearing hepatoma at 0.5 h (a) and 6 h (b) after INAs were injected through the tail vein. Data were given as mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with PS/DNA group.

cles are also termed as "opsonins". Considering the positive charge of PS/DNA complex, opsonization might be the major reason that PS/DNA complex distributed mainly into liver, spleen and lung. To minimize opsonization, the most commonly used strategy is to conjugate PEG onto the surface of nanoparticles, which is a relatively inert hydrophilic polymer that provides good steric hindrance for preventing protein binding. PEGylation would reduce the uptake of the macrophage cell and increase the circulation half-life for various types of nanoparticles (Woodle and Lasic, 1992; Owens and Peppas, 2006; Li et al., 2008). In present experiment, the reduced biodistribution of INAs in liver, spleen and lung compared with PS/DNA complex, was probably due to the PEGylation of the nanoassemblies.

The increased rate of tumoral uptake of nanoparticles is based on a phenomenon termed as "enhanced permeability and retention" (EPR) effect due to the increased capillary permeability in tumor (Brannon-Peppas and Blanchette, 2004). Prolonged circulation times may lead to passive targeting of INAs via EPR effect. These results indicated that INAs could be a promising gene delivery system for tumor therapy in future.

4. Conclusion

The particle size of INAs was about 180 nm with zeta potential about 4 mV. In vitro release experiment showed that PSD possessed the good ability of self-dePEGylation that could result in efficient release of the content in the intracellular environment. INAs showed higher transfection efficiency and much lower cytotoxicity compared with Lipofectamine™ 2000 on HEK 293 cells. The biodistribution in nude mice bearing tumor implied that this PSD-based nanoassembly loading PS/DNA could be a promising gene delivery system for tumor therapy in future.

Acknowledgments

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