Efficient intracellular delivery of functional proteins using cationic polymer core/shell nanoparticles

Lee, Ashlynn L. Z.; Wang, Yong; Ye, Wen-Hui; Yoon, Ho Sup; Chan, Sui Yung; Yang, Yi-Yan

2007

Lee, A. L., Wang, Y., Ye, W. H., Yoon, H. S., Chan, S. Y., & Yang, Y. Y. (2008). Efficient intracellular delivery of functional proteins using cationic polymer core/shell nanoparticles. Biomaterials, 29(9), 1224-1232.

https://hdl.handle.net/10356/95129

https://doi.org/10.1016/j.biomaterials.2007.11.021

Downloaded on 30 Mar 2023 09:51:30 SGT

Efficient intracellular delivery of functional proteins using cationic polymer core/shell nanoparticles

Ashlynn L.Z. Lee[a], Yong Wang[a], Wen-Hui Ye[b], Ho Sup Yoon[b], Sui Yung Chan[c], Yi-Yan Yang a,*

[a] Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, The Nanos 04-01, Singapore 138669, Singapore

[b] School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore

[c] Department of Pharmacy, National University of Singapore, Science Drive 4, Singapore 117543, Singapore

* Corresponding author. Tel.: þ65 68247106; fax: þ65 64789084. E-mail address: yyyang@ibn.a-star.edu.sg (Y.-Y. Yang).

Abstract

Cationic core/shell nanoparticles self-assembled from biodegradable, cationic and amphiphilic copolymer poly{*N*-methyldietheneamine sebacate)-*co*-[(cholesteryl oxocarbonylamido ethyl) methyl bis(ethylene) ammonium bromide] sebacate}, P(MDS-co-CES), were fabricated and employed to deliver lectin A-chain, an anticancer glycoprotein. Lectin A-chain was efficiently bound onto the surfaces of the nanoparticles at high mass ratios of nanoparticles to lectin A-chain. The nanoparticle/lectin A-chain complexes had an average size of approximately 150 nm with zeta potential of about +30 mV at the mass ratio of 50 or above while the BioPorter/lectin A-chain complexes had a larger particle size and relatively lower zeta potential (150 nm *vs*. 455 nm; +30 mV vs. +20 mV). Therefore, the cellular uptake of nanoparticle/lectin A-chain complexes was much greater than that of BioPorter/lectin A-chain complexes. The results obtained from cytotoxicity tests show that lectin A-chain delivered by the nanoparticles was significantly more toxic against MDA-MB-231, HeLa, HepG2 and 4T1 cell lines when compared to Bio-Porter, and IC50 of lectin A-chain delivered by the nanoparticles was 0.2, 0.5, 10 and 50 mg/l, respectively, while that of lectin A-chain delivered by BioPorter was higher than 100 mg/l in all cell lines tested. These nano-sized particles may provide an efficient approach for intracellular delivery of biologically active proteins.

Keywords: Cationic core/shell nanoparticles; Intracellular delivery; Proteins; Lectin

1. Introduction

Recombinant protein therapeutics has emerged as an important R&D sector for effective treatment against a broad range of human diseases, including cancer, autoimmune diseases and metabolic disorders [1,2]. The main obstacle to achieving *in vivo* efficacy of proteins is delivery. Lectins from *Viscum album coloratum* (Korean Mistletoe) are heterodimeric, consisting of the A-chain with cytotoxic activity and the B-chain with sugar-binding property. They are glycoproteins with rRNA N-glycosidase activity, which inactivates the ribosome, leading to the disruption of the translocation steps of cellular translation during protein biosynthesis and thus cell death [3-5]. Apoptosis induction may also occur through the down-regulation of Bcl-2 and telomerase activity and up-regulation of Bax [6,7]. Reports have shown that this lectin is cytotoxic to Molt4 cells [4,8], and is able to bring about apoptotic death of U937 cells via activation of caspase cascades [9]. The counterpart of A-chain, lectin B, serves to mediate the delivery of cytotoxic lectin A-chain by binding to the cell surface and facilitating the subsequent internalization of the A-chain via endocytosis [4,10]. The isolation of these biologically active lectins from plant extract and/or its production *via* recombinant methods encompasses difficulties such as extensive purification and scale-up problems.

A number of methods have been proposed to deliver biologically active proteins into cells, among which are physical methods such as microinjection [11] and electroporation [12,13]. These physical methods may be difficult to be applied *in vivo* [14]. The protein transduction domains (PTDs) have been conjugated to various active proteins for mediating the cellular uptake of the proteins [15,16]. Another approach in rational drug delivery research that is becoming increasingly popular involves cationic lipids and polymers [17,18]. For example, polyethylenimine (PEI)-conjugated proteins are able to enter cells based on ionic charge interactions [19]. The conjugation of proteins with PEI must be conducted under mild conditions to prevent proteins from denaturation. Moreover, cytotoxicity of PEI especially with high molecular weight also limits its *in vivo* applications. In this study, cationic core/shell nanoparticles were fabricated *via* a self-assembly process using biodegradable, cationic and amphiphilic copolymer poly{*N*-methyldietheneamine sebacate)-*co*-[(cholesteryl oxocarbonylamido ethyl) methyl bis(ethylene) ammonium bromide] sebacate}, P(MDS-*co*-CES) [20]. Lectin A-chain was bond with the cationic nanoparticles to form nano-sized complexes, and the lectin A-chain binding ability of the nano-particles was studied by native gel electrophoresis. The particle size and zeta potential of nanoparticle/lectin A-chain complexes were measured and compared to those of lectin A-chain complexed with a commercially available cationic lipid-based protein carrier, BioPorter [21,22]. The cellular uptake and distribution of nanoparticle or BioPorter/lectin A-chain complexes were studied in HeLa cervical cancer cell line by confocal microscopy. The cytotoxicity of lectin A-chain delivered by the nanoparticles was investigated against MDA-MB-231 human breast cancer cell line, HeLa cell line, HepG2 human hepatocellular liver carcinoma cell line and 4T1 mouse breast cancer cell line in comparison with BioPorter. Lectin A-chain delivered by the nanoparticles yielded significantly higher anticancer effectiveness when compared to BioPorter. These nano-sized particles may provide a platform for intracellular delivery of biologically active proteins.

2. Materials and methods 2.1. Materials

2.1 Materials

The recombinant lectin A-chain of the heterodimeric lectin from Viscum album *coloratum* (Korean Mistletoe) was obtained as previously described [23]. Dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) were purchased from Sigma-Aldrich, USA. Dialysis buffer used for nanoparticle fabrication was self-prepared using sodium acetate and acetic acid (ACS grade, Merck, USA). Unless stated otherwise, all reagents and solvents were of commercial grade, and were used as received. MDA-MB-231 human breast cancer, HeLa human cervical cancer, HepG2 liver carcinoma and 4T1 mouse breast cancer cell lines were obtained from ATCC, USA, and cultured according to ATCC's recommendation. HepG2 and HeLa were grown in DMEM, MDA-MB-231 in Leibovitz L-15 and 4T1 in RPMI 1640. All the culture media were supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin (HyClone, USA). BioPorter was purchased from Genlantis, USA, and used according to the manufacturer's instructions. MTT and paraformaldehyde were obtained from Sigma, USA.

2.2. Synthesis of P(MDS-*co*-CES)

P(MDS-*co*-CES) was derived by a similar protocol reported previously (Scheme 1) [20]. In brief, the main chain, poly(*N*-methyldietheneamine sebacate) (PMDS), was first produced by condensation polymerization between *N*-methyldiethanolamine and sebacoyl chloride. Excess triethylamine was used to remove hydrochloride and limit protonation of the tertiary amine. Next, cholesteryl chloroformate was allowed to react with 2-bromoethylamine hydrobromide in an amidation reaction. The resulting hydrophobic *N*-(2- bromoethyl) carbarmoyl cholesterol was then grafted onto the relatively hydrophilic PMDS main chain through a quaternization reaction to obtain the final product. The copolymer consisted of a polyester main chain, and a pendant chain that contains hydrolytically labile urethano groups, rendering it degradable. P(MDS-*co*-CES) had a cholesterol grafting degree of ~29%, with a weight average molecular weight (M_w) of ~3.5 kDa and a polydispersity index of 1.7, as measured by gel permeation chromatography [Waters 2690, MA, USA, with a differential refractometer detector (Waters 410, MA, USA); Mobile phase: water of HPLC grade with a flow rate of 1 mL/ min; Polystyrene standards: molecular weight ranging from 1300 to 30,000].

2.3. Formation of core/shell nanoparticles and their lectin A-chain complexes

P(MDS-*co*-CES) was self-assembled into cationic core/shell nanoparticles in aqueous solutions. Briefly, 15 mg of P(MDS-*co*-CES) was dissolved in 5 mL of dimethylformamide (DMF), and dialysed against 500 mL of 20 mM sodium acetate/acetic acid buffer at pH 4.6.

Nanoparticle or BioPorter/lectin A-chain complexes was formed in sodium acetate/acetic acid buffer (20 mM, pH 6.0) by adding P(MDS-co-CES) nano-particles to lectin A-chain (8 μg) in varying polymer to protein mass ratios between 0.1 and 50. The complexes were then left to stand at room temperature for 30 min prior to further analyses.

2.4. Characterization of nanoparticles, BioPorter and their lectin A-chain complexes

The particle size and zeta potential of nanoparticles, BioPorter and their lectin A-chain complexes formed at various mass ratios were measured using a zeta potential analyzer (Malvern Instruments Zetasizer Nano ZS, UK) equipped with a He-Ne laser beam at 658 nm (scattering angle: 90$^{°}$) at 25 $^{°}$C. The cell was a capillary cell, and the current was 0.2 mA.

2.5. Native protein gel shift assay

Nanoparticle/lectin A-chain complexes were formed at various mass ratios as described in Section 2.3. Samples were loaded into 5% Tris glycine gel and ran at 200 V with Tris glycine running buffer (BioRad, USA) for 35 min. Bromophenol blue tracked the fronts and the gel was stained with SYPRO ruby protein gel stain (Invitrogen, USA). Destaining was carried out in water containing 10% methanol and 7% acetic acid, and the stained gel was imaged under ultraviolet excitation.

2.6. Intracellular distribution of fluorescence-labeled lectin A-chain, P(MDS-co-CES) nanoparticles and their nanocomplexes

Lectin A-chain was labeled using Alexa Fluor 647 protein labeling kit (Invitrogen, USA). In brief, Alexa Fluor 647 dye with a tetrafluorophenyl (TFP) ester moiety was added to lectin A-chain in a molar ratio of 15:1, and reacted at room temperature for 1 h. Purification of the fluorescent conjugate was carried out using spin columns provided in the labeling kit. The conjugate was then analyzed spectrophotometrically using the NanoDrop ND-1000 spec-trophotometer (NanoDrop Technologies, USA) and the degree of labeling was determined to be 1.7 mol Alexa Fluor 647 per mole of lectin A-chain.

P(MDS-*co*-CES) nanoparticles were labeled by encapsulating FITC into the hydrophobic core of the nanoparticles. The polymer (15 mg) and 1 mg of fluorescent dye were dissolved in DMF. The mixture was placed in a dialysis membrane with a molecular weight cut-off of 2000 Da (Spectrum Laboratories, USA). The dialysis bag was then immersed in 500 mL of 20 mM sodium ace-tate/acetic acid buffer with pH 4.6 at 4 $^{°}$C for 2 days. The dialysis buffer was replaced three times per day with fresh buffer solution during the 2-day course. At the end of the dialysis process, the resulting nanoparticle solution was filtered through a 0.22μm filter in sterile environment to remove large aggregates.

HeLa cells were seeded onto borosilicate chambered coverglass (Nunc, USA) at a density of 2 x 10^5 cells per well, and cultivated in 500 μl of growth medium. The next day, spent growth medium was removed from each well and replaced with 500 μl of the pre-prepared complex solution 1 ppm lectin A-chain and 40 ppm P(MDS-*co*-CES) nanoparticles in serum-free or serum-containing medium. After 1 h of incubation at 37 $^{°}$C with the nanocomplexes, the growth medium was removed, washed with PBS and fixed with 4% paraformaldehyde. The samples were then imaged at 63x magnification using an LSM 510 DUO confocal unit (Carl Zeiss, USA). Each condition was performed in triplicates.

2.7. Cytotoxicity study using MTT assay

MDA-MB-231, HeLa, HepG2 and 4T1 cancer cells were seeded onto 96- well plates at a density of 1 x 10^4 cells per well and cultivated in 100 μl of growth medium. The plates were then returned to incubator for 24 h to reach 70-80% confluency before the administration of nanoparticle/lectin A-chain nanocomplexes. When the desired cell confluency was reached, the spent growth medium was removed from each well and replaced with 100 μl of the pre-prepared complex solution. After 4 h of incubation with the complexes, the culture medium was replaced with fresh ones. The plates were then returned to the incubator and maintained in 5% CO_2, at 37 $^{\circ}$C, for 2 days.

Each condition was tested in eight replicates. After 2 days of incubation, the culture medium was removed, and 20 μl of MTT solution was added with 100 μl of fresh medium. The plates were then returned to the incubator and maintained in 5% CO_2, at 37 $^{\circ}$C, for further 3 h. The growth medium and excess MTT in each well were removed. DMSO (200 μl) was added to each well to dissolve the internalised purple formazan crystals. An aliquot of 100 μl was taken from each well and transferred to a new 96-well plate. The plates were then assayed at 550 nm and reference wavelength of 690 nm using a microplate reader (PowerWave X, Bio-tek Instruments, USA). The absorbance readings of the formazan crystals were taken to be those at 550 nm subtracted by those at 690 nm. The results were expressed as a percentage of the absorbance of the blank.

3. Results and discussion

3.1. Particle size and zeta potential of nanoparticles, BioPorter and their lectin A-chain complexes

As shown in Fig. 1, the blank nanoparticles and BioPorter particles had an average size of ~140 nm and 334 nm with the zeta potential of approximately +28 mV and +22 mV, respectively. The size of the nanoparticle/lectin A-chain nano-complexes decreased but their zeta potential increased as increasing mass ratio of polymer to lectin A-chain (Fig. 1A). At the mass ratio of 50 or above, their size and zeta potential remained relatively constant at approximately 150 nm and +30 mV, respectively, indicating that at these mass ratios, lectin A-chain was well condensed and complexed with the nano-particles. The small size and positive zeta potential of the complexes rendered them to be suitable for endocytotic cellular uptake. Complexes formed using commercially available cationic lipid-based carrier, BioPorter were also characterized with respect to their size and zeta potential. In Fig. 1B, BioPorter/lectin A-chain complexes were observed to have even larger sizes (>455 nm) and less positive zeta potential (<20 mV) as compared to the blank P(MDS-*co*-CES) nano-particles and their lectin A-chain nanocomplexes. Particles of such large sizes were likely to be aggregates. It is expected that the resulting complexes would enter cells less efficiently than the P(MDS-*co*-CES) nanoparticle/lectin A-chain nanocomplexes.

3.2. Lectin A-chain binding of nanoparticles

The lectin A-chain binding ability of P(MDS-*co*-CES) nano-particles was studied by native polyacrylamide gel electrophoresis at varying mass ratios. In native PAGE, the mobility of lectin

A-chain will depend on both its charge and hydrodynamic size. The interactions between P(MDS-*co*-CES) nanoparticles and lectin A-chain will neutralize the protein's charge and decrease the intensity of protein gel bands. It was observed that electrophoretic mobility of lectin A-chain was increasingly reduced as more P(MDS-*co*-CES) was added for lectin A-chain binding, showing that stronger nanoparticle/ lectin A-chain interaction occurred at higher nanoparticles to protein mass ratios (Fig. 1C). In turn, this correlates to more effective lectin A-chain delivery and apoptosis induction with the use of more P(MDS-*co*-CES) to deliver the protein.

To ensure that native PAGE was carried out in an accurate manner, the process was repeated with lectin A-chain together with bovine serum albumin (BSA). When bovine serum albumin (BSA) was used as a model protein, a clear band was observed (data not shown). Unlike BSA, a smear band pattern was observed for lectin A-chain possibly due to the hydrogen bonds formed between the glycoprotein and polyacrylamide gel matrix.

3.3. Intracellular uptake and distribution of nanoparticle/lectin A-chain complexes

To evaluate the efficiency of intracellular lectin A-chain delivery using P(MDS-*co*-CES) nanoparticles, the cellular distribution of protein and nanoparticles was investigated in HeLa cells using confocal microscopy in comparison with BioPorter. Labeled with Alexa Fluor 647, the cellular distribution of lectin A-chain shows up as red fluorescence in confocal images (Fig. 2A-C). Low fluorescence present in Fig. 2C illustrates that lectin A-chain alone was unable to enter cells efficiently without a transport carrier. Comparison between Fig. 2F and I shows the presence of higher amount of lectin A-chain in cells treated with P(MDS-*co*-CES) nanoparticle/lectin A-chain nanocomplexes as compared to those treated with BioPorter/ lectin A-chain complexes. Lectin A-chain delivery and internalization were significantly more efficient when delivered using P(MDS-*co*-CES) nanoparticles as compared to BioPorter. This is most likely due to the presence of quaternary ammonium groups in P(MDS-*co*-CES) designed for protein binding and tertiary amine groups for endosomal buffering [24] and endolysosomal release of nanocomplexes. Another possible reason is that P(MDS-co-CES) nanocomplexes were much smaller in size and of higher positive charge on the surfaces when compared to BioPorter/lectin A complexes, leading to greater cellular uptake. Fluorescein isothiocyanate (FITC) was loaded into the nanoparticles. Green and yellow regions in Fig. 2J and K represent the localization of the nanoparticles, and the co-localization of lectin A-chain and P(MDS-*co*-CES) nanocomplexes in the cytoplasm after 1 h of incubation, illustrating that the nanoparticles were able to bring lectin A-chain into the cells together as an associated complex. The nanoparticles, lectin A-chain and their nanocomplexes were also observed in the nuclei, suggesting that lectin A-chain is not only localized in the cytoplasm but also localized in the nucleus. This observation could provide one possible molecular basis of lectin A-chain in modulating telomerase activity in cancer cells as described previously [7].

3.4. Cytotoxicity and IC$_{50}$ of lectin A-chain

Intracellular release of lectin A-chain from the nanocomplexes is essential for it to function as a biologically active protein. To determine whether lectin A-chain retains its cytotoxic property after cellular internalization, cytotoxicity tests were carried out in MDA-MB-231, HeLa, HepG2 and 4T1 cancer cells. Cell viability after treatment was determined *via* the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. For all cell lines tested,

lectin A-chain alone did not exhibit significant cytotoxicity since the protein was unable to enter the cells and exert its cytotoxic properties without a transport carrier (Fig. 3). This correlates with the significantly lower cellular internalization of lectin A-chain seen in confocal images (Fig. 2A—C). At low P(MDS-*co*-CES) concentrations in the complexes, the cytotoxicity exhibited was insignificant, possibly due to insufficient binding between lectin A-chain and P(MDS-*co*-CES) nanoparticles (Fig. 1C). The size and zeta potential results also indicate that the nanoparticle/lectin A-chain nanocomplexes were too large for efficient cellular uptake at these concentrations (Fig. 1A). The cytotoxicity of blank nanoparticles at various concentrations was tested in all cell lines (Fig. 3). Blank nano-particles possessed non-selective cytotoxicity, which increased with increasing polymer concentration. This is probably due to electrostatic interactions with negatively charged glycocalyx of cell surface [25]. To reduce the effects of the non-selective cytotoxicity, polymer concentration for complex preparation was optimized for each cell line, being 20, 50, 40 and 100 ppm for MDA-MB-231, HeLa, HepG2 and 4T1, respectively, at which cell viability was greater than 80%.

To determine the IC_{50} value of lectin A-chain, the viability of each cell line was tested at varying lectin A-chain concentrations and the optimized polymer concentration, which was 20, 50, 40 and 100 ppm for MDA-MB-231, HeLa, HepG2 and 4T1 cells, respectively (Fig. 4). Differences in IC_{50} between the various cell lines revealed their different degrees of sensitivity to the cytotoxicity of nanoparticle/lectin A-chain nanocomplexes. MDA-MB-231 cells showed the least tolerance to the cytotoxic effects of the complexes followed by HeLa, HepG2 and 4T1 cells.

The cytotoxicity of nanoparticle/lectin A-chain nanocomplexes was compared to that induced by BioPorter/lectin A-chain complexes (Fig. 5). In all cell lines tested, Bioportermediated lower cytotoxic effects of lectin A-chain in the serum-containing cell culture medium than in the serum-free medium due to its instability in the presence of serum proteins. Comparing the two carriers, cytotoxicity exerted by lectin A-chain delivered via P(MDS-*co*-CES) nanoparticles in the serum-containing medium was significantly higher than that attained by BioPorter/lectin A-chain complexes. Under the same serum conditions, P(MDS-*co*-CES) nanoparticle/lectin A-chain resulted in 200 and 500 times lower IC_{50} of lectin A-chain in MDA-MB-231 and HeLa cells, respectively. This may be due to greater cellular uptake, stability and endosomal buffering capacity of P(MDS-co-CES) nanoparticle/lectin A-chain nanocomplexes.

4. Conclusion

Biodegradable and cationic P(MDS-*co*-CES) nanoparticles have been fabricated and utilized for efficient intracellular delivery of lectin A-chain to cancer cells. The nanoparticle/lectin A-chain nanocomplexes were small enough and of good positive charge for mediating cellular uptake. The nanoparticles also provided endosomal buffering capacity to induce intracellular release of lectin A-chain after being taken up by the cells. The cytotoxicity of lectin A-chain delivered by P(MDS-*co*-CES) nanoparticles was significantly higher even in the serum-containing medium than that induced by BioPorter due to smaller size and greater positive charge of P(MDS-*co*-CES) nanoparticles. These nano-sized particles show great potential to serve as an efficient carrier for intracellular delivery of biologically active proteins.

Acknowledgements

References

[1] Krejsa C, Rogge M, Sadee W. Protein therapeutics: new applications for pharmacogenetics. Nat Rev Drug Discov 2006;5:507-52.

[2] Pavlou AK, Reichert JM. Recombinant protein therapeutics—success rates, market trends and values to 2010. Nat Biotechnol 2004; 22:1513-9.

[3] Yoon TJ, Yoo YC, Kang TB, Shimazaki K, Song SK, Lee KH, et al. Lectins isolated from Korean mistletoe (*Viscum album coloratum*) induce apoptosis in tumor cells. Cancer Lett 1999;36:33-40.

[4] Vervecken W, Kleff S, Pfu ̈ller U, Bu ̈ssing A. Induction of apoptosis by mistletoe lectin I and its subunits. No evidence for cytotoxic effects caused by isolated A- and B-chains. Int J Biochem Cell Biol 2000;32:317-26.

[5] Endo Y, Tsurugi K, Franz H. The site of action of the A-chain of mistletoe lectin Ion eukaryotic ribosomes. The RNA N-glycosidase activity of the protein. FEBS Lett 1988;231:378-80.

[6] Büssing A, Multani AS, Pathak S, Pfüller U, Schietzel M. Induction of apoptosis by the *N*-acetyl-galactosamine-specific toxic lectin from *Viscum album* L. is associated with a decrease of nuclear p53 and Bcl-2 proteins and induction of telomeric associations. Cancer Lett 1998;130:57-68.

[7] Lyu SY, Choi SH, Park WB. Korean mistletoe lectin-induced apoptosis in hepatocarcinoma cells is associated with inhibition of telomerase via mitochondrial controlled pathway independent of p53. Arch Pharm Res 2002;25:93-101.

[8] Lee HS, Kim YS, Kim SB, Choi BE, Woo BH, Lee KC. Isolation and characterization of biologically active lectin from Korean mistletoe, *Viscum album var. coloratum*. Cell Mol Life Sci 1999;55:679-82.

[9] Kim MS, So HS, Lee KM, Park JS, Lee JH, Moon SK, et al. Activation of caspase cascades in Korean mistletoe (*Viscum album var. coloratum*) lectin-II-induced apoptosis of human myeloleukemic U937 cells. Gen Pharmacol 2000;34:349-55.

[10] Bantel H, Engels IH, Volter W, Schulze-Osthoff K, Wesselborg S. Mistletoe lectin activates caspase-8/FLICE independently of death receptor signaling and enhances anticancer drug-induced apoptosis. Cancer Res 1999;59:2083-90.

[11] Brustugun OT, Fladmark KE, Døskeland SO, Orrenius S, Zhivotovsky B. Apoptosis induced by microinjection of cytochrome c is caspasedependent and is inhibited by Bcl-2. Cell Death Differ 1998;5:660-8.

[12] Banga AK, Praunitz MR. Assessing the potential of skin electroporation for the delivery of protein- and gene-based drugs. Trends Biotechnol 1998;16:408-12.

[13] Fenton M, Bone N, Sinclair AJ. The efficient and rapid import of a peptide into primary B and T lymphocytes and a lymphoblastoid cell line. J Immunol Methods 1998;212:41-8.

[14] Luo D, Saltzman WM. Synthetic DNA delivery systems. Nat Biotechnol 2000;18:33-7.

[15] Ford KG, Souberbielle BE, Darling D, Farzaneh F. Protein transduction: an alternative to genetic intervention? Gene Ther 2001;8:1-4.

[16] Wadia JS, Dowdy SF. Protein transduction technology. Curr Opin Biotechnol 2002;13:52-6.

[17] Yoo HS, Park TG. Biodegradable nanoparticles containing protein-fatty acid complexes for oral delivery of salmon calcitonin. J Pharm Sci 2004;93:488-95.

[18] Kim SH, Jeong JH, Jeo CO, Park TG. Folate receptor mediated intracellular protein

delivery using PLL-PEG-FOL conjugate. J Control Release 2005;103:625-34.

[19]   Futami J, Kitazoe M, Maeda T, Nukui E, Sakaquchi M, Kosaka J, et al. Intracellular delivery of proteins into mammalian living cells by polyethylenimine-cationization. J Biosci Bioeng 2005;99:95-103.

[20]   Wang Y, Gao S, Ye WH, Yoon HS, Yang YY. Co-delivery of drugs and DNA from cationic core-shell nanoparticles self-assembled from a biodegradable copolymer. Nat Mater 2006;5:791-6.

[21]   Zelphati O, Wang Y, Kitada S, Reeds JC, Felgner PL, Corbeil J. Intracellular delivery of proteins with a new lipid-mediated delivery system. J Biol Chem 2001;276:35103-10.

[22]   Zheng X, Lunberg M, Karlsson A, Johansson M. Lipid-mediated protein delivery of suicide nucleoside kinases. Cancer Res 2003;63:6909-13.

[23]   Ye W, Prakash Reddy Nanga R, Kang CB, Song JH, Song SK, Yoon HS. Molecular characterization of a recombinant lectin from *Viscum album coloratum* and its structural basis on ribosome-inactivating activity and sugar binding properties. J Biochem Mol Biol 2006;39:560-70.

[24]   Remy JS, Abdallah B, Zanta MA, Bousiff O, Behr JP, Demeneix BA. Gene transfer with lipospermines and polyethylenimines. Adv Drug Deliv Rev 1998;30:85-95.

[25]   Fischer D, Li Y, Ahlemyer B, Krieglstein J, Kissel T. In vitro cytotoxicity testing of polycations: Influence of polymer structure on cell viability and hemolysis. Biomaterials 2003;24:1121-31.
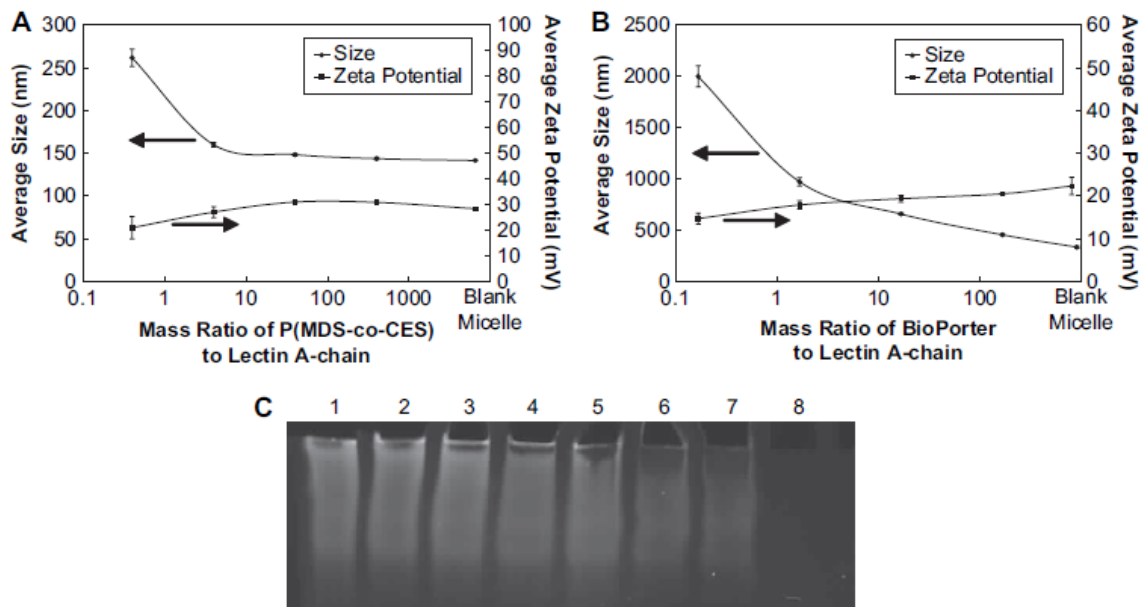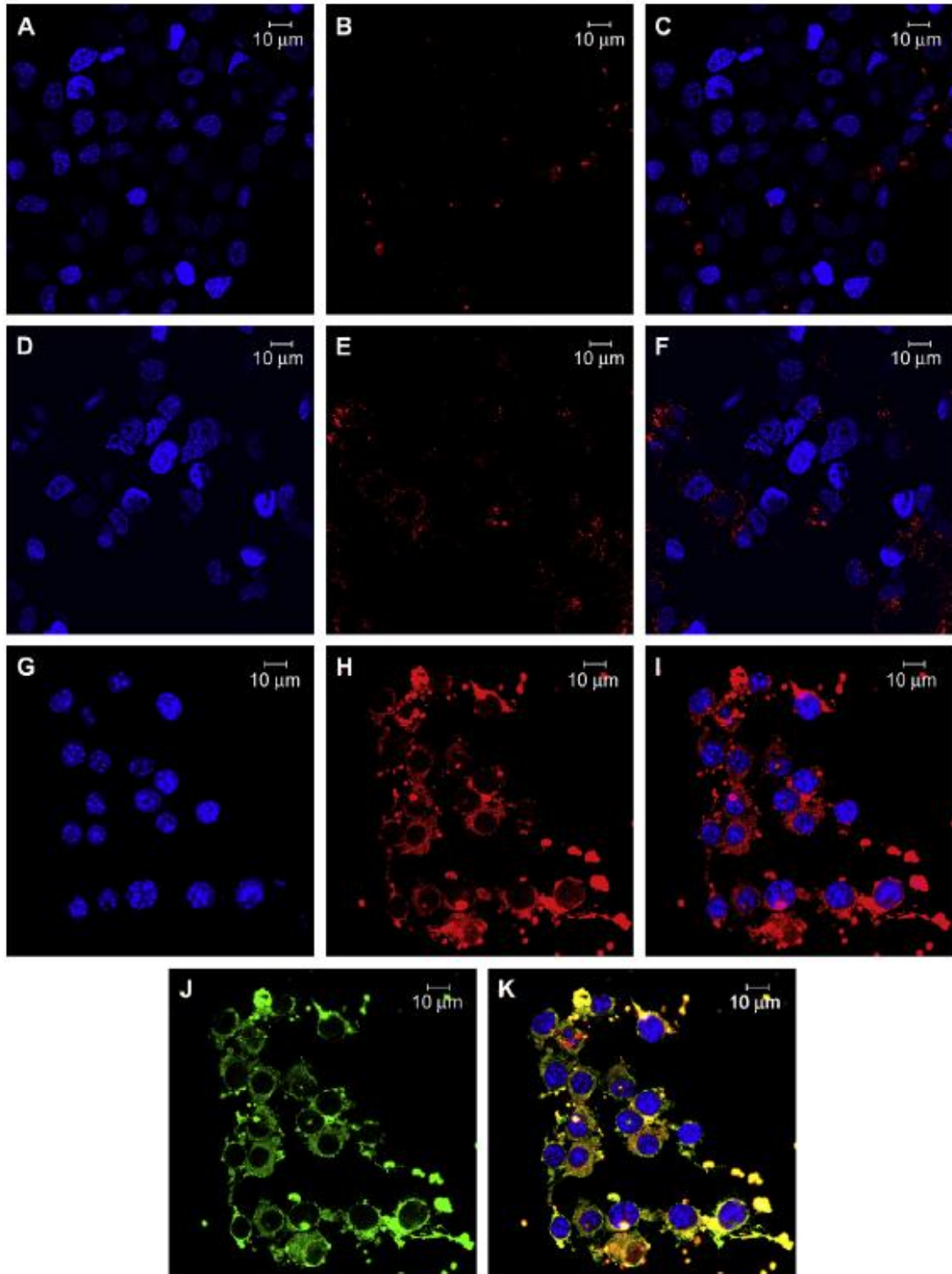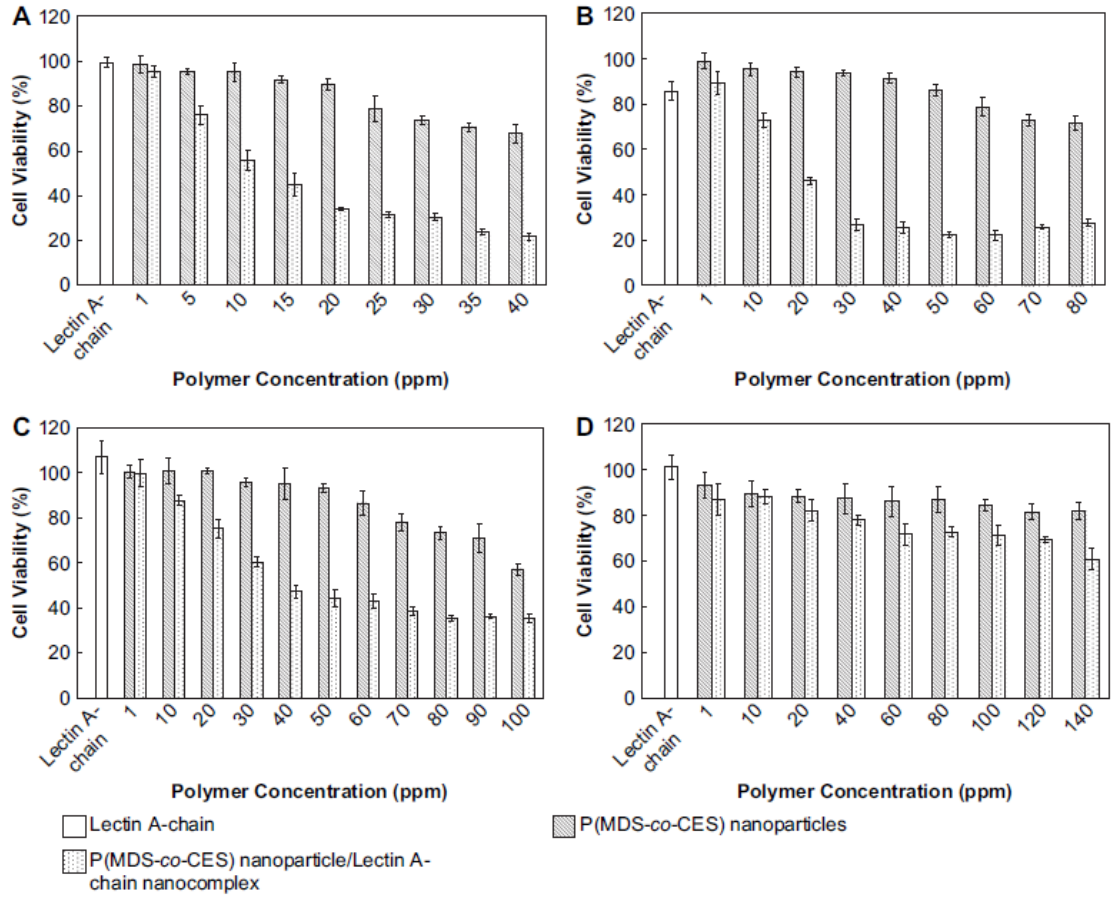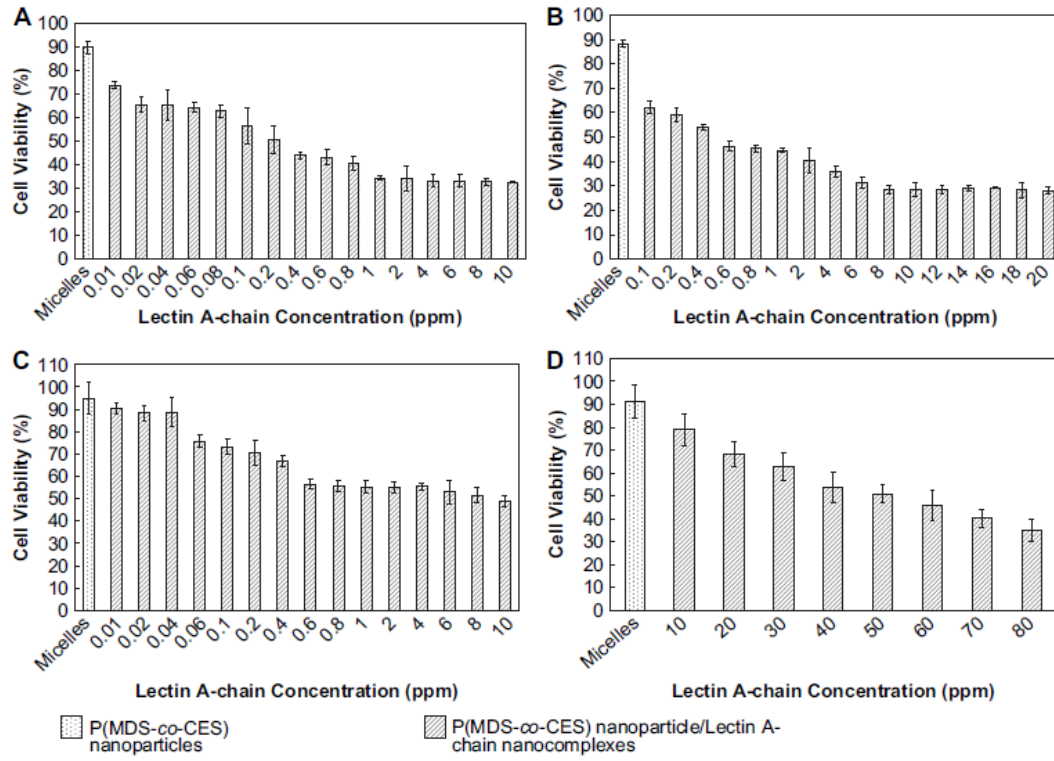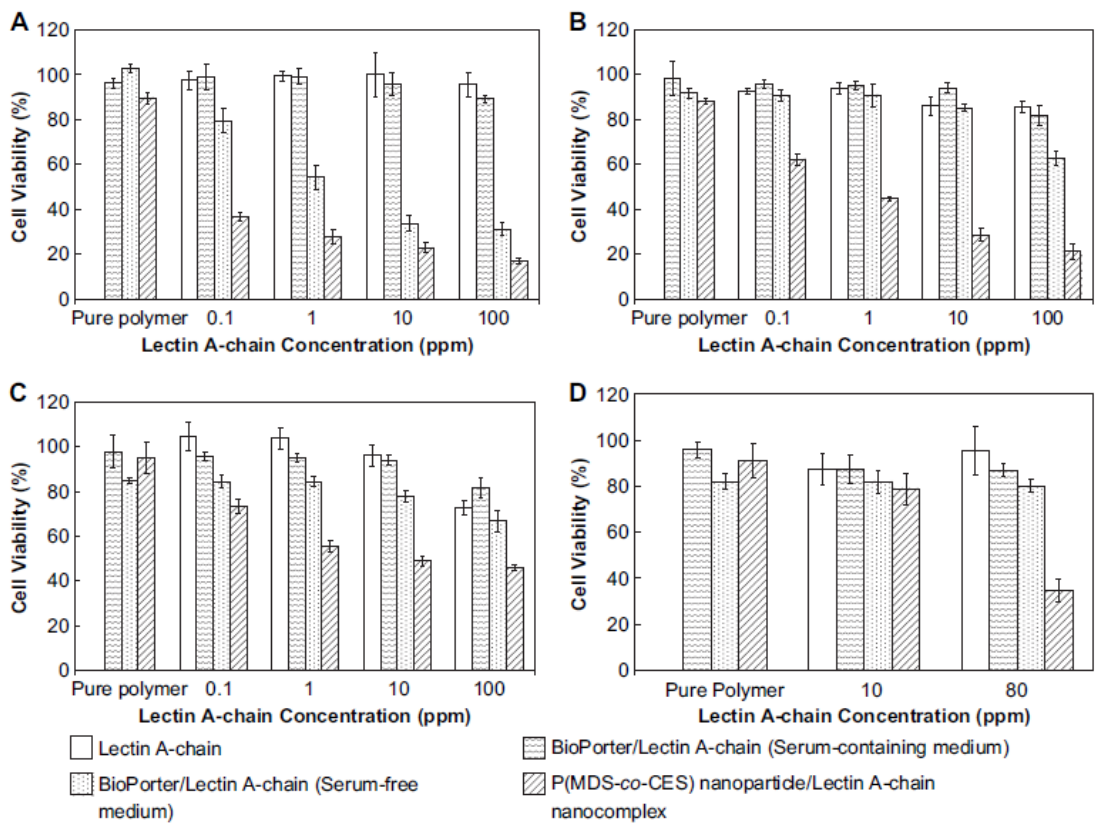
List of Figures

List of Scheme

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Scheme 1