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Assessment of a Modular Transfection System Based upon Cellular Localization of DNA

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Abstract: Delivery of plasmid DNA for protein production in mammalian cells is not an efficient process. In this study, the theory that cellular localization of plasmid DNA increases transfection efficiency is examined with an emphasis on the understanding of the cellular association of the components of a ternary transfection complex. Mammalian cells take up transfection reagent−DNA complexes primarily from their local environment. Via formation of a ternary complex consisting of the DNA−transfection reagent complex and a heavy particle, such as silica, the efficiency of transfection is substantially increased. We have analyzed cells transfected with the ternary complexes to determine if sedimentation alone affects the percentage of cells that contain the complexes or specific components of the complex. A significant fraction of cells associate with the ternary complexes, including silica nanoparticles. The percentage of cells that associate with DNA was not significantly influenced by the use of the ternary complex. This result suggests that the silica nanoparticles are more than just a sedimentation agent, being also a secondary transfection reagent. These data also confirm that cells may contain transfection reagent−DNA complex but do not express the protein of interest. This knowledge will be used in further research to better design transfection reagents that will increase the efficiency of protein production.

Keywords: Transfection; cellular drug delivery; gene therapy; dendrimer; nanoparticle

Introduction

Recombinant proteins and peptides have the potential for curing many inherited and acquired diseases. Two main hurdles remain for further utilization of recombinant proteins: identification of the malfunctioning protein and delivery of the protein. Every day, the first hurdle is being conquered, but the second hurdle still remains. Clinical delivery of protein therapeutics is quite challenging because of *in vitro* and *in vivo* instability. One possible method of protein delivery is using autologous cells to produce the necessary proteins; cellular factories are particularly interesting in light of the widespread identification of stem cells.[1] In this method of protein delivery, cells from either the patient (autologous) or another source (allogeneic or xenogeneic) are implanted in the patient after the proper genetic sequence has been introduced by viral infection or by nonviral transfection methods. Use of cellular factories could become a major factor in drug delivery in the future if the ability to safely and effectively modify the cells is developed.

Viral methods of infecting cells have safety issues that must be addressed prior to the creation of a viable product for use in humans.[2] Adenovirus, adenovirus-associated virus (AAV), and retrovirus are the primary methods of clinical cellular infection (gene therapy) today. Infection of cells has potential detrimental effects on cells, including possible genetic transformation. Even with newly developed techniques, such as AAV infection, there is still concern with possible infection of host cells with unaltered virus or of

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(1) Kaji, E. H.; Leiden, J. M. Gene and stem cell therapies. *JAMA, J. Am. Med. Assoc.* **2001**, *285*, 545−550.

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oncogene activation. To overcome some of the problems with viral infection for gene therapy, nonviral methods of transfection have been investigated.[3] Current transfection mechanisms have been designed with the primary focus of DNA complexation and lysosomal escape. These ideas have resulted in great increases in the efficiency of cellular transfection; however, the efficiency of transfection is still quite low compared to that of viral infection. Improved transfection efficiency is necessary for basic research and clinical therapies.

If *in vitro* or *ex vivo* transfection is being considered, there are four primary barriers to DNA expression: cellular entry, lysosomal escape, nuclear localization, and nuclear entry. Many recently developed ideas for improving transfection efficiency are based on biologic principles at work in viruses for overcoming these barriers. On the basis of previously published data, the primary barrier, cellular entry, can be overcome by using a modular approach to transfection.[4] Sedimentation of DNA with silica nanoparticles could be considered a rudimentary version of cellular docking. Other research groups have targeted each of the four barriers using viral-inspired methods. Specific "fusogenic" peptides have been designed that integrate into and disrupt the endosome when the pH becomes acidic.[5,6] Polymeric analogues have also been used to cause endosomal escape based upon the idea of the proton sponge.[7] By condensation of DNA with dendrimers, DNA is protected from nuclease cleavage, enters the cell, and escapes the endosome.[8] Fracturing the dendrimers in a specific manner can increase the efficiency of transfection.[9] Further improvement in transfection was shown by using a modular system composed of dendrimer, silica nanoparticles, and DNA.[4]

Several groups have increased the local DNA concentration at the surface of the cell, and increased transfection efficiency has resulted.[4,10] Unfortunately, little is known about the mechanism that increases transfection efficiency. Here, we present further evidence that a modular approach can increase the efficiency of transfection with little additional toxicity compared to that of the fractured dendrimer alone. Furthermore, we report evidence that the silica component of the modular system is interacting directly with the DNA−dendrimer complex. Surprisingly, the association of the silica nanoparticle component of the modular system with cells did not directly correlate with increased DNA production. This fact may, however, allow for more rational design of transfection reagents in the future.

Experimental Protocol

Nanoparticle Labeling. Silica nanoparticles (radius of 225 nm; Polysciences, Inc., Warrington, PA) were concentrated by centrifugation at 3500 rpm for 5 min in a glass conical centrifuge tube, followed by a minimum of three washings with 100% ethanol. Silane solutions (United Chemical Technologies, Bristol, PA) of aminopropyl triethoxy silane (APTES) and methyl triethoxy silane (MTES) were added to the nanoparticles and the mixtures allowed to incubate at room temperature for at least 2 h. Nanoparticles were recovered and washed a minimum of three times each in ethanol followed by water. This resulted in nanoparticles with varying surface amino and methyl group content. Fluorescent labeling of the nanoparticles with Alexa Fluor 488-succinimide (AF488, Molecular Probes, Eugene, OR) was conducted using a slight modification of the manufacturer's protocol. AF488 was dissolved in deionized water, and nanoparticles were added. After 2 h, the dispersion was added to a conical glass centrifuge tube and recovered as previously described. Multiple washings with deionized water were used to remove unbound AF488 and succinimide. Nanoparticles were sterilized in 70% ethanol, resuspended in sterile phosphate-buffered saline, and maintained under sterile conditions.

Particle Size Determination and ζ Potential of Nanoparticles. Nanoparticles were examined following drying of an aqueous sample on appropriate sample stubs [Hitachi S-3000N scanning electron microscope and Digital Instruments Nanoscope III atomic force microscope (AFM)] to confirm size and shape. Diameters of a minimum of 500 particles were determined using Scion Image software. Dilute suspensions of nanoparticles in phosphate-buffered saline were sampled (Lazer Zee Meter, Pen Kem, Inc., Bedford Hills, NY) and compared to standard ζ potential samples (Laszlo Kovacs Consulting, Mohegan Lake, NY).

Cell Culture. Chinese hamster ovarian cells (CHO, ATCC catalog no. CCL-61, ATCC, Manassas, VA) were cultured at a density of 1×10^5 cells/mL in 96-well plates in F12-K medium (ATCC) with 10% fetal bovine serum (BioWhitaker, Walkersville, MD) at 37 °C in 5% CO_2. For subculture and flow cytometry, the cells were trypsinized,

(2) Wu, S. C.; Huang, G. Y. L.; Liu, J. H. Production of retrovirus and adenovirus vectors for gene therapy: A comparative study using microcarrier and stationary cell culture. *Biotechnol. Prog.* **2002**, *18*, 617−622.
(3) Luo, D.; Saltzman, W. M. Synthetic DNA delivery systems. *Nat. Biotechnol.* **2000**, *18*, 33−37.
(4) Luo, D.; Saltzman, W. M. Enhancement of transfection by physical concentration of DNA at the cell surface. *Nat. Biotechnol.* **2000**, *18*, 893−895.
(5) Plank, C.; Zauner, W.; Wagner, E. Application of membrane-active peptides for drug and gene delivery across cellular membranes. *Adv. Drug Delivery Rev.* **1998**, *34*, 21−35.
(6) Santos, A. F.; Murthy, N.; Stayton, P. S.; Press, O. W.; Tirrell, D.; Hoffman, A. S. Design of polymers to increase the efficiency of endosomal release of drugs. *J. Invest. Med.* **1998**, *46*, 91a.
(7) Boussif, O.; Lezoualch, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. A Versatile Vector for Gene and Oligonucleotide Transfer into Cells in Culture and in Vivo: Polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7297−7301.
(8) Haensler, J.; Szoka, F. C. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjugate Chem.* **1993**, *4*, 372−379.
(9) Tang, M. X.; Redemann, C. T.; Szoka, F. C. In vitro gene delivery by degraded polyamidoamine dendrimers. *Bioconjugate Chem.* **1996**, *7*, 703−714.
(10) Segura, T.; Shea, L. D. Surface-tethered DNA complexes for enhanced gene delivery. *Bioconjugate Chem.* **2002**, *13*, 621−629.

centrifuged, and suspended at appropriate concentrations in supplemented media or phosphate-buffered saline.

Transfection. Transfections were carried out according to manufacturer's protocols (SuperFect, Qiagen, Valencia, CA) with one additional step included to create silica nanoparticle complexes. Briefly, plasmid DNA (g-Wiz β-gal, Aldevron, Fargo, ND) was first mixed with transfection reagent for 5 min at room temperature. Then, the transfection reagent−DNA complexes were incubated with silica nanoparticles for at least 5 min and added to cells in log-growth phase. The complexation of transfection reagent−DNA complexes with nanoparticles was conducted in serum-free medium; however, the pH greatly influenced complexation. To assess this influence, nanoparticles were added to serum-free mediun and the pH was adjusted using 0.1 N HCl or 0.1 N NaOH. Immediately after complexation, the ternary complexes were added to an excess of well-buffered medium; thus, the pH of the culture was not significantly different from typical values. Following transfection, cells were grown at 37 °C in 5% CO_2 for 2 days in fresh growth medium. Cells were then lysed using MPER lysis buffer (Pierce, Rockford, IL) and split onto two plates. On the first plate, enzyme activity was assayed using the β-galactosidase (β-gal) assay kit (Promega, Madison, WI) and compared to β-gal protein standards. The second plate was used to determine the total protein content using the bicinchoninic acid (BCA, Pierce) technique[11] as described by the manufacturer. The transfection efficiency was determined using eq 1 where the control is SuperFect-DNA-transfected cells.

$$E = \frac{[\beta\text{-gal}]_{\text{sample}}}{[\text{protein}]_{\text{sample}}} \frac{[\text{protein}]_{\text{control}}}{[\beta\text{-gal}]_{\text{control}}} \quad (1)$$

Mobility Retardation Assay. Agarose gel electrophoresis mobility was used to assess the presence of the complex between the components of the complex. A low (0.5%) agarose concentration was utilized to allow migration of the dendrimer−DNA complex and DNA, but not the silica nanoparticles or the silica−DNA−dendrimer complex. Complexes were formed as described above, but instead of being added directly to cells, the complexes were added to the wells of the agarose gel with ethidium bromide for imaging DNA. The agarose gel was run at 100 V and was imaged using a Chemi-Doc system (Bio-Rad, Hercules, CA).

Flow Cytometry. To determine the plasmid DNA content of cells, a platinum-based DNA labeling system was utilized. Prior to transfection, DNA was labeled with ULYSIS Alexa Fluor 647 (AF647, Molecular Probes) according to published procedures with slight modification. As high-molecular weight DNA will aggregate when efficiently labeled with this method, the DNA:AF647 ratio was decreased by a factor of 10. Using this modification, DNA labeling was sufficient for detection but did not cause aggregation. Following DNA labeling, transfection experiments were conducted as described above. Samples were harvested and diluted in DPBS and examined using either a FACS Calibur benchtop flow cytometer or a FACS Vantage cell sorter (Becton Dickinson). At least 10 000 cells were counted for each condition, and three independent runs of each sample were reviewed. Equivalent running conditions were used for each test to obtain quantitative data of the fluorescent activity of each run. Fluorescently labeled nanoparticles were run as a positive fluorescent control for each batch. CHO cells and unmodified silica nanoparticles have little intrinsic fluorescent activity and were used as negative controls.

Confocal Microscopy. To confirm the internalization of nanoparticles, confocal microscopy was utilized. CHO cells were seeded as described above except that chamber cover slips (Nalge-Nunc) were utilized. Cells were treated in a manner equivalent to that described for flow cytometry prior to harvest. Cover slips were examined without fixation using a Zeiss LSM-510 confocal microscope with a 63× objective illuminated with an argon laser (488 nm) and a helium−neon laser (633 nm).

Statistical Analysis. Data were analyzed by one-way analysis of variance (ANOVA) with Tukey's post-hoc test and presented as means ± the standard deviation of a minimum of three independent measurements.

Results

Nanoparticle Modification. Nanoparticle modification with the various silane compositions did not alter the size or shape of the silica spheres. No statistical difference in size was observed, and little loss of nanoparticles was observed. Nanoparticles that were labeled with fluorescent dye had 100% fluorescence as determined by flow cytometry (unpublished data), while nanoparticles that were modified with silane did not exhibit any fluorescence unless they were labeled with AF488. The size of all nanoparticles (Figure 1) was not altered significantly following silane or fluorescent labeling as determined by both AFM and SEM. The only observable difference between the unmodified and modified nanoparticles was a slight color that was apparent for samples that had been fluorescently labeled. This color became pronounced under irradiation with a hand-held UV lamp.

The ζ potential of the nanoparticles (Figure 2) was significantly altered from that of the unmodified nanoparticles. All modified nanoparticles retained a negative net charge which was somewhat unexpected due to the positive nature of the aminopropyl triethoxy silane, suggesting an incomplete surface coverage. A significant decrease in the ζ potential was observed with higher APTES coverage. Nanoparticles modified with only MTES had a slight increase in the ζ potential; however, this was not as significant as with APTES-treated samples. When the fluorescent dye (AF488) was conjugated to the APTES nanoparticles, an increase in the ζ potential of the nanoparticles was observed for all modified nanoparticles. A slight increase in the ζ potential for the 100% MTES-modified nanoparticles was unexpected since no amino groups were present to allow reaction with AF488. It is possible that a small fraction of

(11) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150*, 76−85.

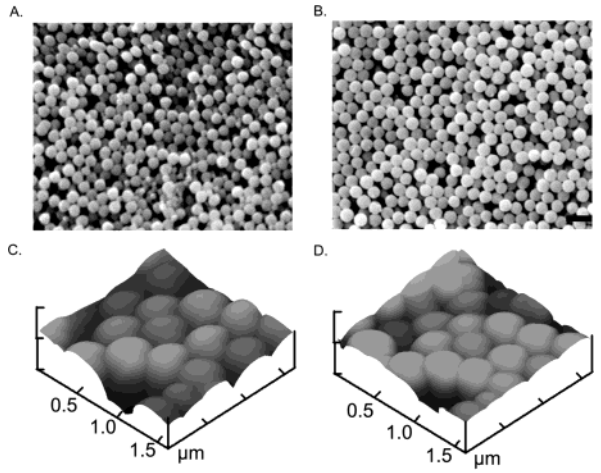

Figure 1. Representative (A and B) scanning electron micrographs and (C and D) atomic force micrographs of (A and C) unmodified silica nanoparticles and (B and D) 100% aminopropyl triethoxy silane, Alexa Fluor 488-modified silica nanoparticles. Similar images were obtained for all particle modifications with similar results for all. The scale bar is 1 μm long, and the two scanning electron micrographs are at the same magnification.

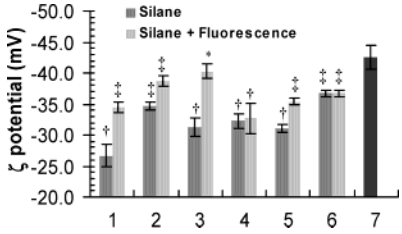

Figure 2. ζ potentials of the modified silica nanoparticles. The dark gray bars represent data for silica nanoparticles that were modified with varying amounts of aminopropyl triethoxy silane and methyl triethoxy silane. The light gray bars represent data for nanoparticles that were modified with varying amounts of aminopropyl triethoxy silane and methyl triethoxy silane followed by Alexa Flour 488-succinimide modification. The silane compositions were (1) 100, (2) 75, (3) 50, (4) 25, and (5) 0% aminopropyl triethoxy silane with the remainder being methyl triethoxy silane and (6) treated (no silane modification) control nanoparticles. An equivalent total molar content of silane was used for all studies. Untreated control nanoparticles have the greatest net negative ζ potential (7). All treated samples had a decreased ζ potential compared to that of the untreated control ($p < 0.01$) unless noted (∗); modified samples were statistically different from the untreated control (‡) or treated and untreated (†) control groups ($p < 0.01$).

silanol groups could be reactive with the AF488 reagent since succinimide-activated compounds are somewhat reactive toward alcohols;[12] however, the fluorescence of 100% MTES-modified nanoparticles could not be detected by either epifluorescent microscopy or flow cytometry (unpublished data).

Transfection. The transfection of mammalian cells using the partially degraded dendrimer (SuperFect) was found to

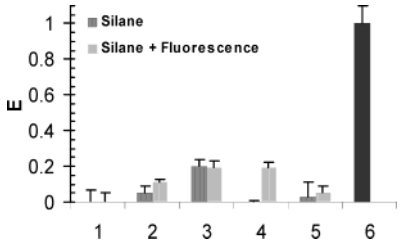

Figure 3. Enhancement of β-galactosidase activity following transfection of CHO cells with various modified silica nanoparticles. The dark gray bars represent data for silica nanoparticles that were modified with varying amounts of aminopropyl triethoxy silane and methyl triethoxy silane. The light gray bars represent data for the nanoparticles that were modified with varying amounts of aminopropyl triethoxy silane and methyl triethoxy silane and then with Alexa Flour 488-succinimide. The mixtures of silane were (1) 100, (2) 75, (3) 50, (4) 25, and (5) 0% aminopropyl triethoxy silane with the remainder being methyl triethoxy silane. No SuperFect was present in groups 1−5. The efficiency of SuperFect without silica nanoparticles is presented (6). An equivalent total molar content of silane was used for all studies. Enhancement is the comparison of the amount of β-galactosidase produced per total cellular protein for the sample to the amount of β-galactosidase produced per total cellular protein for the positive control of the DNA−transfection reagent complex.

be one of the most effective methods of transient transfection in preliminary studies. By comparing all results to this standard, we can determine the efficiency of transfection. Modified nanoparticles were used to transfect cells without any addition of transfection reagent, SuperFect (Figure 3); however, the efficiency of transfection was significantly lower than the efficiency of transfection of the reagent−DNA complex. When cells were treated with the ternary complexes, the transfection efficiency was altered in a pH-dependent manner. If the modified silica nanoparticles were included in the ternary complexes, the transfection efficiency was greatest at a pH slightly below that of normal culture media (Figure 4). The pH needed to be maintained only during complexation, and not when the complex was exposed to cells. Similar results were obtained for each of the modified nanoparticle complexes.

Mobility Retardation Assay. The mobility of DNA was utilized to confirm complexation between the three components of the modular system: DNA, nanoparticles, and transfection reagent. When DNA was complexed with the dendrimer without nanoparticles, migration was possible in the gels (unpublished data). The mobility was actually toward the negative pole of the chamber as would be expected for an amino content higher than that of phosphate and a net positively charged complex. A small fraction of the DNA remained free of the complex and migrated. Upon complexation of the silica nanoparticles with the DNA−transfection reagent complex, the mobility of the ternary complex was

(12) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, 1996.

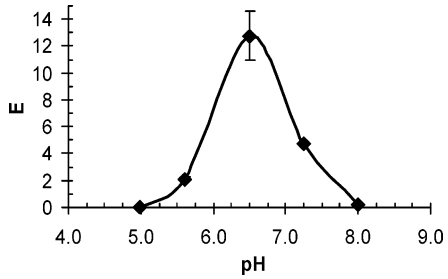

Figure 4. Transfection efficiency of 50% aminopropyl triethoxy silane- and 50% methyl triethoxy silane-modified nanoparticles with SuperFect as a part of the modular system. Similar graphs were obtained with each of the modified silica nanoparticles with and without fluorescent labeling. The enhancement is the comparison of the amount of β-galactosidase produced per total cellular protein for the sample to the amount of β-galactosidase produced per total cellular protein for the positive control of the DNA−transfection reagent complex.

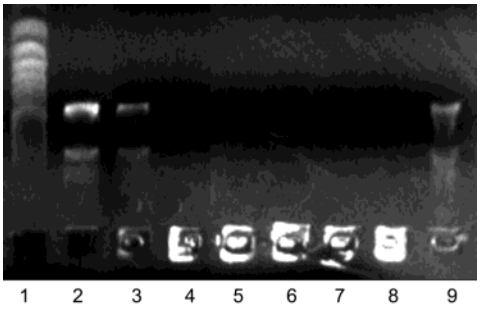

Figure 5. Agarose electrophoresis mobility retardation assays of β-galactosidase plasmid DNA (lane 2) compared to a ladder (lane 1). In lanes 3−8, modified silica nanoparticles complexed with the DNA−transfection reagent complex were present. Lanes 3−8 contained silica nanoparticles with increasing aminopropyl triethoxy silane content: (3) 0, (4) 25, (5) 50, (6) 75, (7) 100, and (8) 100% without Alexa Fluor 488 modification. The molar amount of silane for modification was equivalent in all cases with the amount of methyl triethoxy silane used for the remainder of the silane. The final lane (9) contained unmodified silica nanoparticles complexed with DNA without the transfection agent.

completely retarded (Figure 5). The complexation is sufficiently strong to retain the DNA in the well. When the unmodified (no APTES, MTES, or AF488 labeling) nanoparticles are complexed directly with DNA, only a small amount of DNA remains complexed.

Flow Cytometry. Flow cytometry data confirm that the nanoparticle−DNA−transfection reagent complexes are indeed associated with cells and internalized. Washing successfully removed a small portion of the adherent complex but could not remove the remainder of the complexes. Scatter diagrams of CHO cells (Figure 6A) or CHO cells transfected with unmodified nanoparticles or with silane-treated, but not fluorescently labeled, nanoparticles had little scatter and showed little base fluorescence. When fluorescently labeled nanoparticles (Figure 6B−E) were incubated with CHO cells, there was a significant increase in the number of cells

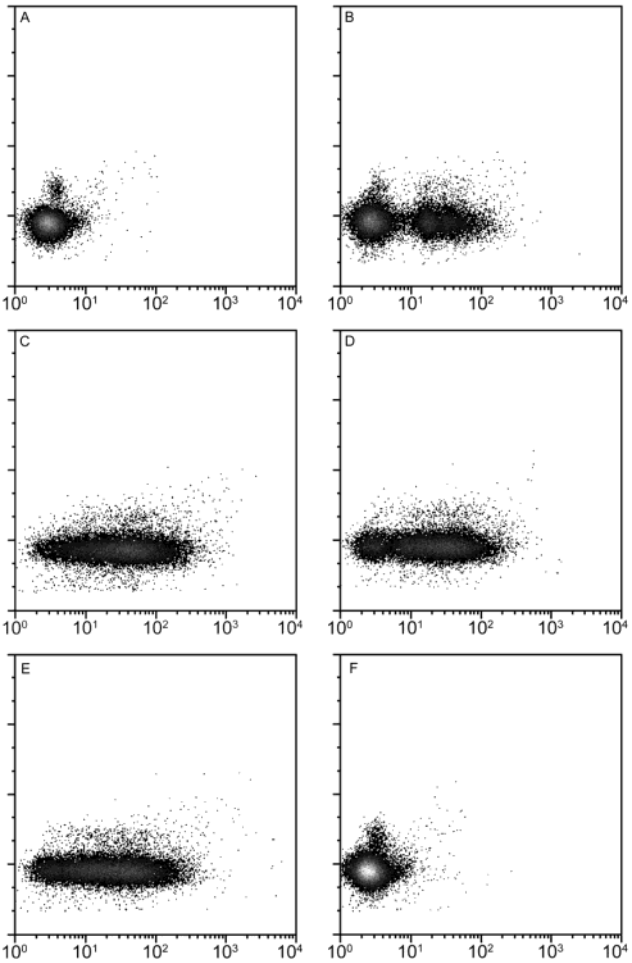

Figure 6. Representative scatter diagrams of (A) CHO cells and CHO cells transfected with plasmid DNA, transfection reagent, and nanoparticles modified with (B) 25, (C) 50, (D) 75, and (E) 100% aminoethyl triethoxy silane (remainder methyl triethoxy silane) and Alexa Fluor 488. (F) When no AF488 conjugated to the nanoparticles, the scatter is similar to the control with only cells (A). These scatter diagrams of forward scatter vs fluorescence intensity were created under equivalent conditions. Each silica particle modification was examined using flow cytometry at least three times, with similar results obtained for each run; a single run is represented in each diagram.

containing fluorescence. These results have been reproduced a minimum of three times with all experimental conditions. When no silane or 100% methyl triethoxy silane was used to modify the nanoparticles followed by fluorescent labeling, no fluorescence was observed in any case, indicating that the amino groups were necessary for incorporation of fluorescence. The relative number of cells containing fluorescence for the 25, 50, 75, and 100% aminopropyl triethoxy silane nanoparticles was approximately 50 $\pm$ 10% in all cases.

When fluorescently labeled DNA was utilized, results similar to those of the fluorescently labeled nanoparticles were observed. When treated with unlabeled DNA, few CHO cells had background fluorescence in the range of the covalently attached dye (Figure 7A). When cells were not

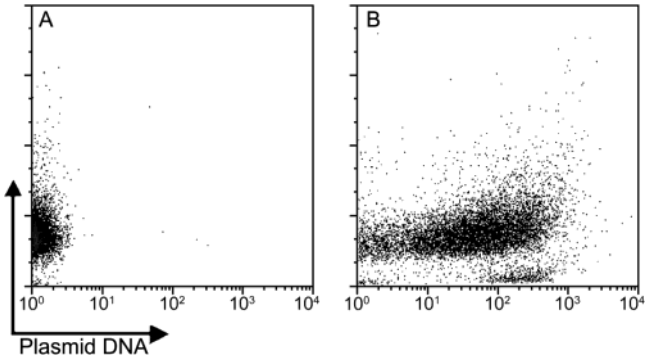

Figure 7. Representative scatter diagrams of CHO cells transfected with (A) unlabeled plasmid DNA and (B) labeled plasmid DNA. In both diagrams, the transfection reagent and unmodified silica nanoparticles were used. These scatter diagrams of forward scatter vs fluorescence intensity were created under equivalent conditions. Each silica particle modification was examined using flow cytometry at least three times, with similar results obtained for each run; a single run is represented in each diagram.

transfected, similar scattering was observed. When fluorescently labeled DNA was complexed with the nanoparticles and transfection reagent, a great increase in fluorescence was observed in all cases (Figure 7B). Surprisingly, no increase in the percentage of cells associated with labeled DNA was observed for any of the nanoparticle−DNA−transfection reagent complexes when compared to the percentage for the DNA−transfection reagent complex alone (Figure 8). All complexes, DNA−transfection reagent or nanoparticle−DNA−transfection reagent, had a similar extent of cellular association, approximately 80−85% of the cells.

Confocal Microscopy. Confocal imaging confirmed that nanoparticles and DNA are internalized in cells and are also present on the surface of cells (Figure 9). Qualitatively, the majority of complexes appear to be within cells. Serial sections of all samples containing fluorescently labeled DNA (red) or nanoparticles (green) exhibited results similar to those shown. DNA is present near nanoparticles as well as unassociated with particles as represented by yellow in the "merged" column. This is expected since the DNA−transfection reagent complexes were formed prior to complexation with nanoparticles. Not all cells have DNA or nanoparticles associated, but the majority of cells do have visual DNA and/or nanoparticles present either on the cell membrane or within the cell.

Discussion

Low-efficiency uptake of DNA by mammalian cells is one of the major drawbacks of nonviral DNA delivery. These studies confirm that it is possible to increase the local concentration of DNA at or near the cells under tissue culture conditions.[4] The reason for this is that gravitational force increases DNA levels near the cell. An effective theoretical model was used to describe this phenomenon, but further experiments have suggested that this theory is not complete.[13] When this theory was first proposed, two mechanisms for

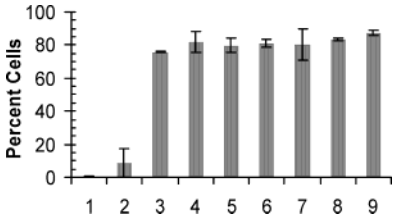

Figure 8. Flow cytometric determination of the percent of cells containing labeled plasmid DNA. (1) Cells without any DNA were run as a negative control. Cells transfected with (2) naked DNA, (3) DNA complexed with the transfection reagent, and (4−9) DNA−transfection reagent−silica nanoparticle ternary complexes. Nanoparticles were modified with (4) 0, (5) 25, (6) 50, (7) 75, and (8) 100% aminopropyl triethoxy silane with the remainder methyl triethoxy silane; unmodified nanoparticles were also used (9).

increased protein production were considered: (A) more cells were transfected or (B) each transfected cell was more efficiently transfected. To date, little evidence has identified one of these two mechanisms as the predominant mechanism for increasing transfection efficiency. The theory does not differentiate between the two mechanisms since either could be true when DNA is localized at the cell surface. With the help of flow cytometry and confocal microscopy, the second mechanism is beginning to become accepted, and this is the first report confirming this fact.

It has been established that cells do associate with nanoparticles regardless of the content of amino groups on the surface. The amino content may increase the uptake of nanoparticles.[14,15] The large size of the nanoparticles (r = 225 nm) compared to cells would suggest that little uptake would take place, but we have shown that these large nanoparticles are taken up by cells. No uptake of nanoparticles is mandated by this theory, but uptake does take place and is suggested to play a major role in transfection. The localization of silica in cells needs to be confirmed by electron microscopy with and without amino and fluorescent modification, but confocal imaging has confirmed that there appears to be uptake of the nanoparticles and complexes including nanoparticles. The original intent of this project was to determine cellular localization of nanoparticles, but it was quickly observed that amine and fluorescent modification of particles had a significant influence on transfection.[16]

(13) Luo, D.; Han, E.; Belcheva, N.; Saltzman, W. M. A self-assembled, modular DNA delivery system mediated by silica nanoparticles. *J. Controlled Release* **2004**, *95*, 333−341.
(14) Kneuer, C.; Sameti, M.; Haltner, E. G.; Schiestel, T.; Schirra, H.; Schmidt, H.; Lehr, C. M. Silica nanoparticles modified with aminosilanes as carriers for plasmid DNA. *Int. J. Pharm.* **2000**, *196*, 257−261.
(15) Kneuer, C.; Sameti, M.; Bakowsky, U.; Schiestel, T.; Schirra, H.; Schmidt, H.; Lehr, C. M. A nonviral DNA delivery system based on surface modified silica-nanoparticles can efficiently transfect cells in vitro. *Bioconjugate Chem.* **2000**, *11*, 926−932.
(16) Gemeinhart, R. A.; Luo, D.; Saltzman, W. M. Cellular Fate of a Modular DNA Delivery System Mediated by Silica Nanoparticles, manuscript in preparation.

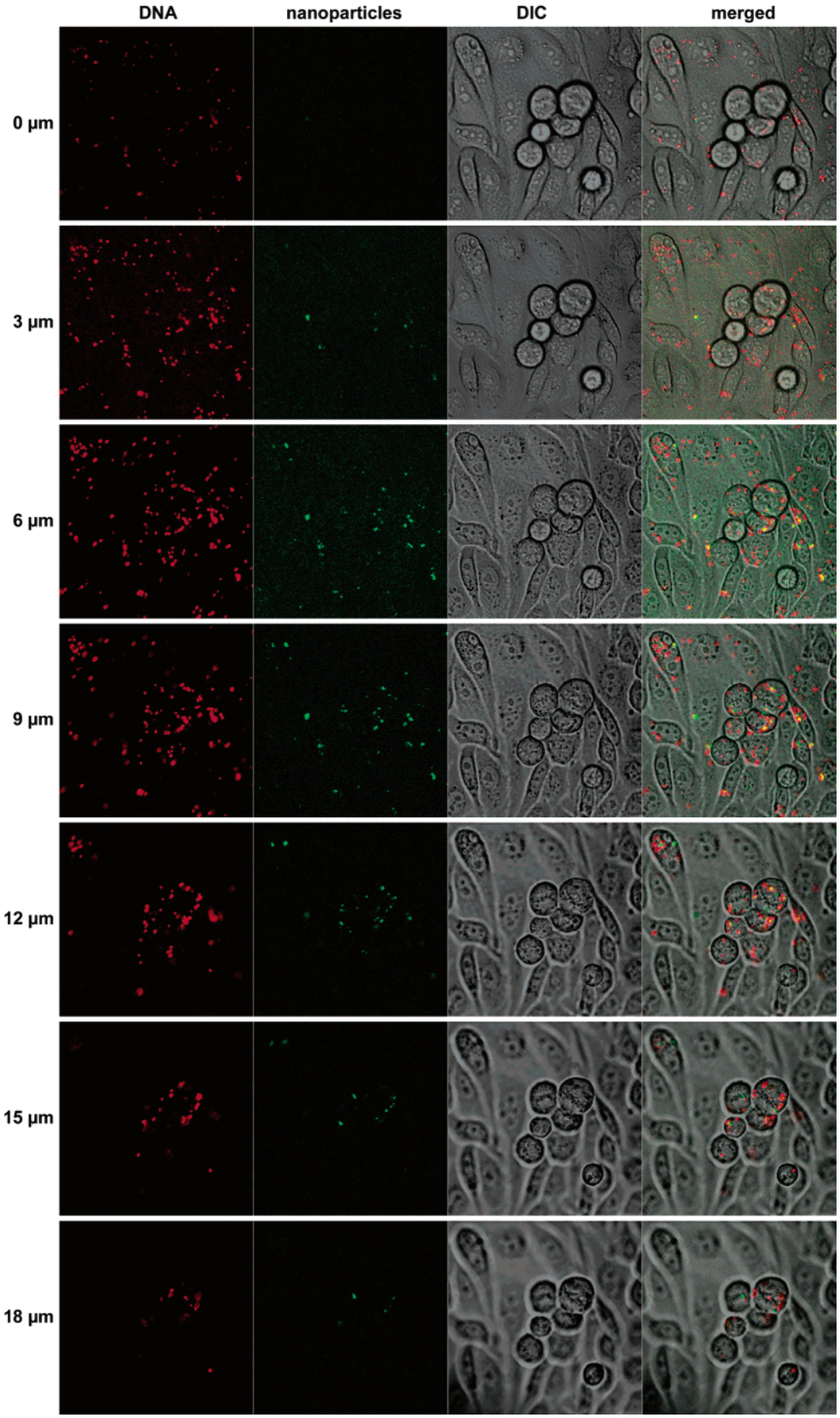

Figure 9. Confocal micrographs of living CHO cells incubated with ULYSIS Alexa Fluor 647-labeled DNA-modified nanoparticles (red in columns 1 and 4) and 50% aminopropyl triethoxy silane and Alexa Fluor 488-modified nanoparticles (green in columns 2 and 4). The columns of images are the color fluorescent (columns 1 and 2), differential interference contrast (DIC, column 3), and merged (column 4) images of confocal sections. To create this figure, a stack of 25 images was taken at 1 μm increments, and every third image is presented.

Amino-modified particles have been used as a transfection reagent, but the interaction between the DNA and modified silica nanoparticles was not effective for transfection (Figure 3). Other researchers have had success with transfection using silica nanoparticles that were significantly smaller than those discussed here.[14,15] In these cases, a different amino headgroup was utilized that may have a more favorable interaction with the DNA. It has been well established that use of appropriate cationic groups can greatly affect transfection efficiency, but our purpose for using amino-modified silica was for labeling, and not for increasing efficiency. The smaller size of the nanoparticles ($r = 5-50$ nm vs $r = 225$ nm) used in the earlier study greatly reduced the sedimentation effect in their system,[14,15] and thus, greater transfection efficiency would be expected with larger nanoparticles.

The efficiency of transfection with the nanoparticle−DNA−transfection reagent ternary complexes is related to the pH at complexation due to the ionic complexation mechanisms (Figure 4). The optimal pH for complexation differs for each modified silica sample; however, the pH in all cases is slightly below physiological conditions (pH ∼7.4). A complex was formed between all modified nanoparticles and the DNA−transfection reagent complex, but the complexes created under standard conditions (pH ∼7.4) were not as effective at transfection as unmodified silica complexes.[13] Gel migration retardation studies suggest that there is a strong ionic interaction between the DNA transfection reagent and the silica nanoparticle. Since all nanoparticles in this study retain a negative ζ potential, the transfection reagent may in fact be the bridge creating the complex. The transfection reagent−nanoparticle interaction plays the major role in complexation, but a secondary interaction between DNA and the amino-modified nanoparticles cannot be ignored.

The number of cells that receive nanoparticles is not significantly different for the various modifications, but slight differences in transfection efficiency are present for each of the ternary complexes. Most interesting is the fact that the number of cells containing DNA does not increase significantly with increased transfection efficiency. Production of the target protein (Figures 3 and 4) was greater with the silica−DNA−transfection reagent system, but there was no increase in cells containing DNA compared to cells with the DNA−transfection reagent complex. These results suggest that the silica nanoparticles are, in fact, increasing the production of protein in each cell, not that more cells are actually receiving DNA. Earlier studies have shown that the order of addition of complex components was ultimately important to enhancement and that silica nanoparticles alone do not increase protein production.[4] If the mechanism of increased transfection efficiency was due to more cells receiving DNA, the number of cells that receive DNA in the DNA−transfection reagent case (Figure 8) would be expected to be significantly fewer than that of any of the ternary complexes. This finding is paramount to further investigation with modular transfection systems; many of the currently available transfection reagents obtain DNA from many cells, but not at sufficient concentrations or with appropriate localization to have efficient protein production without causing toxicity. Further developments should focus on cellular targeting, cellular uptake, and nuclear targeting. Recent advances have established efficient lysosomal escape mechanisms,[7] but these studies suggest that other hurdles, which include cellular localization, may be overcome by modular approaches.

Even with optimized transfection efficiency, transfection may not be optimal for cellular therapeutics as production of proteins by cells is transient. Transient protein production may be advantageous for gene therapy in that not all gene therapy products need to be expressed for extended periods. For applications such as diabetes, where insulin is necessary for the life of the patient, this method would not be appropriate, but for situations such as tissue engineering, only transient protein production is necessary to induce further cellular integration with the tissue engineering material.[17,18] Under these conditions, continued growth factor delivery could result in overgrowth of the tissue. What would be needed in these cases is a precise understanding of the time scale for continued maintenance of transfection.

Conclusion

We have shown that model mammalian cells can have increased protein production when a ternary transfection complex is utilized compared to the case in which the binary DNA−transfection reagent complex is used. The ternary complex utilizes the mechanisms of the transfection reagent to increase the extent of lysosomal escape and nuclear targeting. Contrary to prior thinking, silica nanoparticles do more than simply localize the DNA at the cell. Further study is necessary to determine the exact mechanisms involved in increasing the efficiency of transfection, but it is clear that silica nanoparticles do not increase the number of cells that contain DNA. The data did not support the theory that a greater number of cells receive DNA when silica nanoparticles are utilized; thus, another mechanism is suggested. By choosing to create modular transfection reagents that mimic viral mechanisms, we may yet create a synthetic mechanism of protein production that will compete with viral infection methods in terms of efficiency, but careful choice of modules is necessary since intricate physical−chemical interactions may play a greater role than originally anticipated.

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(17) Gittens, S. A.; Uludag, H. Growth factor delivery for bone tissue engineering. *J. Drug Targeting* **2001**, *9*, 407−429.

(18) Saltzman, W. M.; Mak, M. W.; Mahoney, M. J.; Duenas, E. T.; Cleland, J. L. Intracranial delivery of recombinant nerve growth factor: Release kinetics and protein distribution for three delivery systems. *Pharm. Res.* **1999**, *16*, 232−240.