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Nanoparticulate System for Efficient Gene Transfer into Refractory Cell Targets†

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A biocompatible, nanoparticulate formulation has been designed to retain, protect, and deliver adenoviral gene constructs over an extended time course. Such devices can be administered locally or systemically with low toxicity. A multipolymeric nanoparticulate system, featuring very high stability in physiologic media, was designed to allow efficient in vitro gene transfer. The efficacy of nanoparticulate delivery is effective in cell systems that are normally refractory to gene transfer, such as pancreatic islets and antigen-presenting cells. The findings suggest a nonspecific uptake system that permits adenoviral particle release within the transfected cells. A comparison with literature data revealed that our system is efficient at much lower levels (at least three orders of magnitude) of infectious viral particles.

Introduction

Nanoparticles (NP) are structures that range from 1 to 1000 nm, and their preferred size is <300 nm for cellular uptake. NP can be formed from variety of materials, including synthetic polymers and biopolymers (proteins and polysaccharides), and they can be used as carriers for drugs and other biotechnology products, such as growth factors and genes. A pharmaceutical composition can also be prepared using a drug entrapped within the NP. This composition can comprise a drug (gene) in a suitable polymeric form and a biologically acceptable matrix. NP are particularly useful because of their small size and suitability for use in injectable formulations.

Polymer (particle)-enhanced gene transfer offers the potential of sustained release of gene/gene products within the transfected cells. We tested a multipolymeric nanoparticulate system for gene transfer with an adenoviral construct. The key to conventional gene transfer and infection with adenoviral gene constructs is the engagement of specific AdV receptors on the periphery of transfected cells. We hypothesized that polymer−NP-mediated gene transfer could bypass such a requirement and deliver genes via a different uptake mechanism that involves the subsequent release of adenoviral particles within the transfected cells. Hemopoietic islets and dendritic cells (DC) particularly are known to be quite resilient to gene transfer, and a high level of infecting adenoviral particles are needed to achieve the effect. We have validated the efficacy of this nanoparticulate delivery system in cells which are normally resilient to gene transfer. We suspect that extended delivery is achieved through polymeric delivery vehicle degradation rather than diffusion.

Development of an efficient method for introducing a therapeutic gene into target cells in vivo is the key issue in treating genetic and acquired diseases by gene therapy. To this end, various nonviral vectors have been designed and developed, and some of them are in clinical trials. The simplest approach is naked DNA injection into local tissues or systemic circulation. Physical (gene gun, electroporation)[1] and chemical (cationic lipid or polymer) approaches have also been utilized to improve the efficiency and target cell specificity of gene transfer by plasmid DNA.[2] After administration, however, nonviral vectors encounter many hurdles that result in diminished gene transfer into target cells. Cationic vectors sometimes attract serum proteins and blood cells when entering into blood circulation, which results in dynamic changes in their physicochemical properties. To reach target cells, nonviral vectors should pass through the capillaries, avoid recognition by mononuclear phagocytes, emerge from the blood vessels to the interstitium, and bind to the surface of the target cells. They then need to be internalized, escape from endosomes, and then find a way to the nucleus, avoiding cytoplasmic degradation. Many barriers in gene transfer and development of vectors exist. Viruses have evolved natural mechanisms to avoid degradation and to be effectively expressed.

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Retroviral vectors have been used with limited success. Their main feature is their ability to integrate into the host cell genome, leading to sustained or even permanent expression of the transgene. The limitations include the lower concentration attainable (number of infectious units) and the necessity of cell division for effective gene transfer.[3,4] Lentiviruses are able to circumvent this limitation. Adenoviral vectors provide advantages in that they can be produced in high concentrations, exhibit substantially higher stability, and can easily infect both dividing and nondividing cells. The nonintegration into the host chromosome obviates the fear of insertional oncogenesis, and transient expression avoids the problem of lingering transgenic proteins staying beyond their welcome. Adenoviral vectors account for nearly 27% of the current gene therapy trials.[5] These vectors have been used successfully for in vivo gene transfer in several therapeutic fields.[6−9]

Chemical approaches have been utilized to improve the efficiency and cell specificity of gene transfer. Polymer- and cationic lipid-enhanced adenoviral-mediated delivery has been reported extensively.[10−15] When the adenovirus vector is complexed with cationic lipids or polymers, the gene transfer is improved. It appears that the binding of cationic substances is dependent on an electrostatic interaction with the viral and cell surfaces. The entry does not require an interaction of adenovirus fiber protein with its cell surface receptor, and the complexes then enter cells via a pathway different from that utilized by adenovirus alone.[16] The enhanced adenoviral infection via polymeric components offers an advantage for cells that are not otherwise easily infected. Construction of a long-lasting gene expression system is also important for some forms of gene therapy.

Until recently, the cells of hematopoietic origin (e.g., DC and monocytes) were not considered good adenovirus targets, primarily because they lack the specific adenovirus receptors required for productive and efficient adenovirus infections. In addition, because of limitations inherent in AdV infections, such as short-term expression and a nonintegrating nature, their application has been precluded from hematopoietic stem cell (HSC) and bone marrow transduction protocols where long-term expression has been required. With recent insights into the critical interactions between adenovirus and cells, new AdV-mediated gene transduction strategies have now been reported that may overcome these limitations. These new strategies include AdV possessing synthetic polymer coatings, genetically modified capsid proteins, or antibody-redirected fibers that can efficiently redirect and retarget adenovirus to transfer genes into HSC. Embedding the adenovirus within the polymeric NP is another strategy presented in this paper.

Materials and Methods

Adenoviral Gene-Loaded NP Production Process. Particles were generated using a droplet-forming polyanionic solution composed of 50 mg/100 mL sodium alginate, 50 mg/100 mL cellulose sulfate in water, 100 μL of 1×10^7 plaque-forming unit (pfu) adenoviral gene construct (below), and 2 g/100 mL NaCl (Sigma, St. Louis, MO) and a corona-forming polycationic solution composed of 50 mg/100 mL spermine hydrochloride, 50 mg/100 mL poly(methylene-*co*-guanidine) hydrochloride (PMCG) hydrochloride (see below), 50 mg/100 mL calcium chloride, and 1 g/100 mL F-68 in water. The polymers were high-viscosity sodium alginate from Kelco/Merck (San Diego, CA) of an average molecular weight of 460 000 g/mol; cellulose sulfate and sodium salt, from Janssen Chimica (Geel, Belgium), of an average molecular weight of 1 200 000 g/mol; PMCG from Scientific Polymer Products, Inc. (Ontario, NY), with an average molecular weight of 5000 g/mol; and spermine hydrochloride from Sigma, molecular weight 348.2 g/mol. Pluronic F-68, from Sigma, average molecular weight 5400 g/mol, is a water-soluble nonionic block polymer composed of poly(oxyethylene) and poly(oxypropylene) segments. PMCG is a synthetic oligomer which mimics peptide structure. It contains highly charged cationic guanidinium groups. Pluronic was used to provide a steric stabilization.[17]

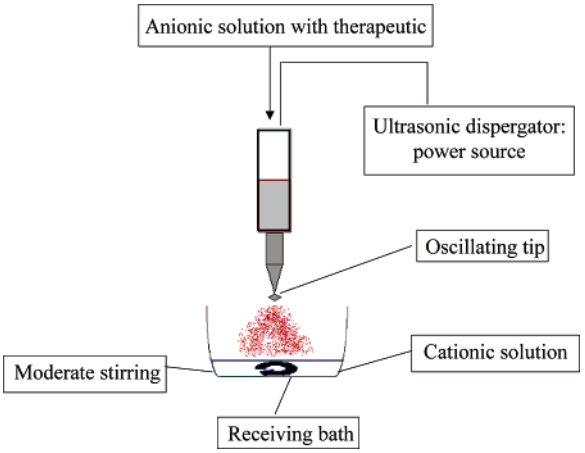

Figure 1. Schematic representation of NP production.

The particles were formed instantaneously via mixing 2 mL of the core solution, dispergated with help of a sonicator,[18,19] with 20 mL of the corona solution, and then allowed to react for 1 h under stirring. The NP size and charge were evaluated in the reaction mixture as determined with the Zetasizer (Malvern, U.K.). Particles were separated by centrifugation at 15 000*g*. A typical production scheme is depicted in Figure 1.

Virus Propagation and Cloning. Adenovirus transformed human embryo kidney cells (293T, ATCC CRL-11268) were grown in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal calf serum (Sigma) supplemented with 2 mM L-glutamine (Sigma). The XbaI/SmaI DNA fragment containing an internal ribosome entry site (IRES) and GFP (green fluorescent protein) was isolated from pIRES-GFP (Clonotech, Palo Alto, CA), and another XbaI/XhoI luciferase DNA fragment cut from pGL-Basic (Promega, Madison, WI) was separately subcloned into pShuttle-CMV vector (Quantum Biotechnologies, Montreal, Canada). The resulting plasmid was co-transformed into BJ5381 cells with pAdEasy-1 adenoviral DNA plasmid that was E1 and E3 deleted and replication-deficient. The recombinant adenoviral construct was linearized with Pac I and transfected into 293T cells in which E1 functions can be complemented, to produce viral particles. To achieve a large adenovirus preparation, AdV-

luc-IRES-GFP was amplified in 293T cells cultured in cell factories (Nalgene Nunc, Rochester, NY), purified by cesium chloride centrifugation, desalted with a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden), and stored at −80 °C. Viral titer was determined with the cytopathic effect assay ($TCDI_{50}$) on 293T cells, and calculation was done according to the manufacturer (Qbiogene, Irvine, CA).[20] The titer was expressed as multiplicity of infection, MOI/cell. For simplicity, AdV-luc-IRES-GFP is denoted below as AdV.

Formation of Cross-Linked NP. These particles were generated using the same solutions as described above, except the droplet-forming solution (core) contained additional polymer, PDA, and 1 g/100 mL calcium chloride instead of sodium chloride. PDA is dextran polyaldehyde from CarboMer (Westborough, MA), with an average molecular weight of 40 000 g/mol. The Schiff base product between the cationic groups of the corona polymers and aldehyde group of PDA is supposed to allow for an adjustment of release via increase of the polymer chain entanglement and for full retention, if necessary. The particles were instantaneously formed and allowed to react for 1 h, and their size and charge were evaluated in the reaction mixture. The particles were separated by centrifugation and incubated for 30 min in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer (pH 8.0; Sigma) to perform the cross-linking reaction between the polymer constituents and PDA. The PDA concentrations were 0 (no cross-linking), 10, 30, and 60 mg/100 mL. The in vitro AdV release was assessed via an external sink method—efflux method—with daily fluid replacement.[21] The quantification of the viral titer in the releasate was done by plaque assay. The particles were resuspended with different buffers (neutral pH 7, pH 1.85, and pH 8), and plasmid release was measured.

NP Characterization and Colloidal Stability. Colloidal stability (nonaggregation) following the centrifugation at physiologic ionic strength and pH (in buffers) defines the practical usefulness of NP. In addition, stability tests were also carried out in an animal serum. A change in size over time was employed as a measure of stability. A standard instrument was used to assess particle size and surface charge density (Zetasizer).[2,17] Both size and charge results are obtained automatically via proprietary software. In addition, particle morphology was evaluated via transmission electron microscopy (TEM). Samples were vortexed, and approximately 20 μL of suspension was spread[22] by a nitrogen stream onto parlodion and carbon-covered cooper grids (300 Mesh). The specimen was rotary-shadowed with platinum−palladium alloy (80:20) at an angle of 20°. Micrographs were taken in a JEOL JEM 1200-EX electron microscope.

Cell Studies. Cell lines tested for gene transfer using NP technology were UMR-106 (a clonal derivative of a transplantable rat osteosarcoma, kindly received from N. C. Partridge, St. Louis University), CT26 (mouse colon cancer, kindly received from A. Pozzi, Vanderbilt University), HT1080 (human dermal fibrosarcoma, ATCC, CCL-121), DC, and BC-1 (lymphoma cell line, kindly obtained from V. L. Shephard, Vanderbilt University) cells. The cells were treated either with free AdV or with NP (in 0.9% trehalose) loaded with the adenovirus encoding luciferase at the indicated times at 37 °C. Trehalose was employed to simulate physiologic osmotic pressure. Cells were then centrifuged, supernatant with NP was removed, cells were then washed once with phosphate-buffered saline (PBS; Sigma) to remove the unbound AdV, and new growth medium was added. Luciferase activity was measured every 24 h up to 4 days. The total protein concentration was determined by the bicinchonic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL) with bovine albumin as a standard.

Islet cells were isolated by a standard collagenase-based process.[23] They were infected at MOI = 10. The infections were carried out overnight using a complete growth medium used to grow the cells (200 μL of RPMI, Sigma, 1640/well). The following day islets were washed twice with PBS, and 500 μL of growth medium was added per well. At 24 and 48 h after infection, islets were washed with PBS, lysed using reporter lysis buffer (RLB by Promega Corp., Madison, WI), harvested, and analyzed for gene expression. To measure the luciferase activity, the cells were freeze−thawed once, spun down, and analyzed with a luciferase assay system kit (Promega, Madison, MI) according to the manufacturer's instructions (technical bulletin no. 281), using 20 μL of cell lysate with 100 μL of luciferase assay buffer. The light emitted during 10 s was measured by a luminometer (Pharmigen, San Diego, CA) set for single photon counting. The protein concentration was determined by the BCA method (Pierce).

DC were generated using peripheral blood obtained from healthy adults (obtained with previous informed consent). PBMCs (peripheral blood mononuclear cells) were separated using Ficoll gradient. PBMCs were plated in culture dishes in 10% FBS RPMI-1640 medium supplemented with IL-4 (Sigma) and GM-CSF (Sigma) at 37 °C in a humidified atmosphere flushed with 5% CO_2. The transduction of the DC was performed at day 4 by adding into each well (containing 3×10^5 cells) NP-AdV or free AdV at equivalent MOI and incubating for 6 h. After washing, a medium was replaced with 10% FBS RPMI-1640 medium supplemented with IL-4 and GM-CSF. DC were harvested at day 7 (60 h post-transfection with NP-AdV or infection with free AV), lysed with 1× RLB (Promega), and assayed for firefly luciferase assay. Values were normalized against the total protein quantified by BCA assay (Pierce).

Cells were cultured up to 5 days in the presence of AdV-loaded NP. Qualitative assessment of cell physiology and their status was done at the end of the 5-day interval, by observing their morphology.

Results

Nanoparticulate Chemistry. We employed a rather complicated chemistry because of inherent instability of systems based on single pair interactions. A complex composed of one polyanion and one (poly)cation is typically very unstable in physiologic media (buffers, moderately strong salts, and serum) (e.g., ref 19). Our system consists of minimally two pairs of interacting principles, specifically alginate−calcium chloride (and spermine) and cellulose

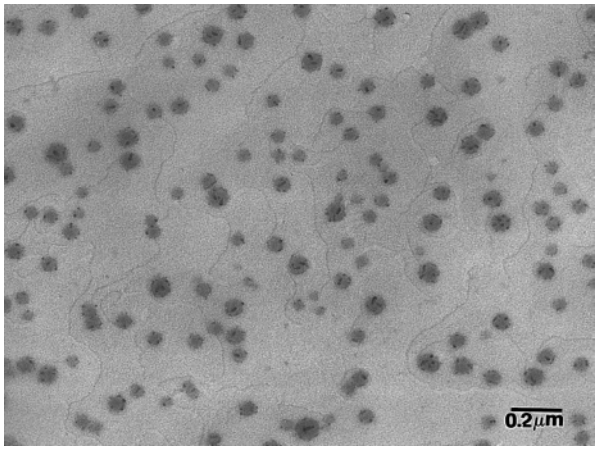

Figure 2. TEM of typical NP. Specimen was rotary-shadowed with platinum−palladium alloy (80:20) at an angle 20°. Micrographs were taken in a JEOL JEM 1200-EX electron microscope.

sulfate−PMCG.[24] The resulting complex is extremely stable in a variety of media and solvents. For example, the BCA reagent A for dissolution of biological material (a combination of bicarbonate, tartrate, and 0.1 M sodium hydroxide; see instructions for the BCA protein assay kit, Pierce) provides almost quantitative dissolution, enough for extraction of protein and its quantification; dimethyl sulfoxide under certain conditions leads to a complete digestion. We do not provide here comprehensive data on NP stability, however.

NP Size, Charge, Stability, and Morphology. The typical size of NP was on average 230 nm, and the average charge was +15 mV. The size of separated and resuspended particles was not different from the size of those obtained in the reaction mixture. The size of the NP was used to assess their stability over an extended period of time. NP were resuspended in 0.9% (w/w) NaCl, in buffers of different pHs (6−8), and tested following their separation via centrifugation. In addition, stability tests were also carried out in mouse animal serum. In all cases, NP retained their size over 1−5 days and no aggregation was noted (data not shown). A typical example of particle (not AdV-loaded) morphology is presented in Figure 2. AdV-loaded particles did not differ in morphology from nonloaded ones.

AdV Particle Retention. There was no evidence of release of AdV particles (plaque assay) from NP prepared in the absence or presence of cross-linking agent, PDA. DNA release was also negative when checked with the PicoGreen assay (Molecular Probes, Eugene, OR; data not shown). The most likely explanation was entrapment of AdV within the NP structure. We presume that the bioavailability requires the vehicle degradation in the presence of extracellular or intracellular hydrolases. During NP-AdV preparation, the second and third washings were tested for the presence of AdV particles by plaque assay. Neither showed any residual activity in vitro. The same applied for supernatants collected after several days of incubation in biological buffers.

Retention of Infectivity of Formulated AdV (NP-AdV) NP. We tested how the infectivity was retained within the formulated NP. Free AdV is known to be inactivated rather quickly. While following the MOI at different times, our results indicated that the stability was considerably improved

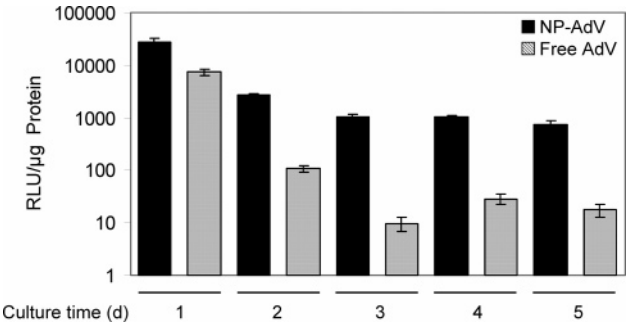

Figure 3. NP-mediated gene transfer into UMR-106 (rat osteosarcoma) cells. UMR cells were transduced with either NP loaded with adenovirus (NP-AdV, MOI = 100) or free adenovirus (AdV, MOI = 100). After 16 h of incubation at 37 °C, unbound NP/free AdV was removed by washing with PBS and cells were cultured for 5 additional days in complete growth medium. Every 24 h cells were harvested and assayed for luciferase activity. Luciferase values, expressed as relative luciferase units (RLU), were normalized on the basis of protein content. These cells are a clonal derivative of a transplantable rat osteosarcoma that had been induced by injection of radiophosphorus.

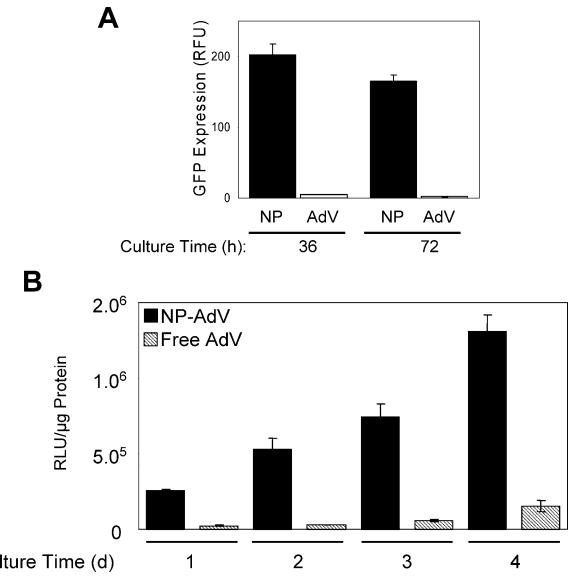

Figure 4. NP-mediated gene delivery to CT26 (rodent tumor) cells in vitro. (A) GFP expression in cultured CT26 cells following treatment with either NP loaded with adenovirus (NP-AdV) gene transfer or free adenovirus (AdV) infection. CT26 were incubated with AdV containing NP (NP-AdV) or free AdV for 4 and 36 h (MOI = 10). GFP fluorescence was expressed as relative fluorescence units (RFU) normalized on the basis of the protein content. (B) Sustained NP-mediated gene transfer to cells. In parallel to GFP analysis CT26 cells were collected every 24 h for 4 days and luciferase activity was assayed from their respective lysates.

and decay substantially reduced. The NP-AdV particles were aliquoted and maintained over a year at −80 °C without any loss of infectivity as tested by plaque assay.

Gene Transfer into Cells in Vitro. Several in vitro studies were performed. Figure 3 presents gene transfer into UMR cells, 1−5 days post-infection. Twenty-four hours after infection of UMR cells at MOI = 100, NP-AdV and free AdV elicited similar levels of luciferase activity. By day 3, the ratio of luciferase activity, normalized to protein, between the NP-AdV and free AdV was more than 100, and this differential was maintained up to day 5. The measurement of GFP expression (Figure 4A) at 36-h post-infection of CT26 colon cancer cells was much greater in the NP viral

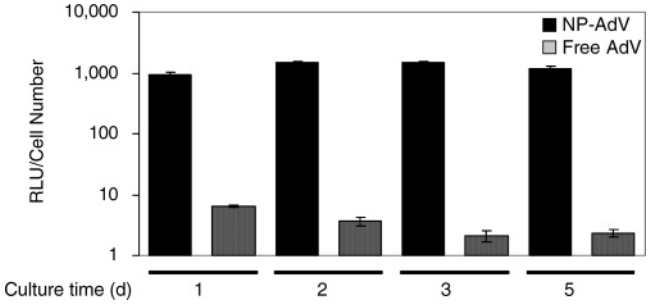

Figure 5. NP-mediated gene transfer into HT1080 (human dermal fibrosacroma) cells. Eighteen hours after plating, HT1080 cells were transduced with NP loaded with adenovirus (NP-AdV, MOI = 100) or infected with free adenovirus (AdV, MOI = 100) for 6 h in complete growth media. Starting from 24-h post-treatment, cells were assayed for luciferase activity. Luciferase values were normalized on the basis of cell number (viable) at the time of harvest. Cell viability was quantified by a trypan blue exclusion test. Results are the average ± SEM of three independent experiments.

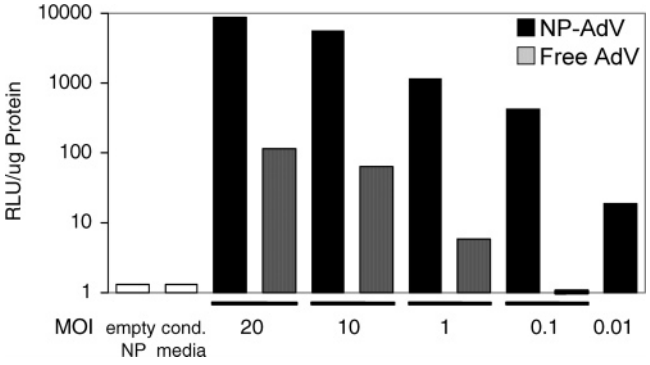

Figure 6. NP-mediated gene transfer into DC. PBMCs (2.5×10^6/mL) were cultured for 5 days in RPMI-1640 supplemented with 25 ng/mL GM-CSF and 100 ng/mL IL-4. Immature DC were harvested and transduced with either AdV loaded into NP (NP-AdV) or with free AdV (at the indicated MOI). Cells were also treated with empty NP (empty NP) or with conditioned media harvested during adenovirus expansion (cond. media) as negative controls. Transduction was maintained for 6 h, and then cells were washed with PBS and further cultured for 48 h in RPMI-1640 supplemented medium with GM-CSF and IL-4. Luciferase activity was assayed and normalized on the basis of protein content.

delivery experiment compared with that of free AdV. Figure 4B represents luciferase gene transfer into the same cells, 1−4 days post-infection. On a relative basis, luciferase activity was nearly four orders of magnitude larger, compared to that of free AdV, and this differential persisted from days 1−4. The GFP expression as visualized by fluorescence microscopy at 36 h was markedly better compared with free AdV (data not shown). Gene transfer into HT1080 cells, 1−5 days post-infection, is again compared on the basis of luciferase expression normalized per initial cell number at the same MOI (Figure 5). Luciferase activity for the NP-AdV vehicle was persistently enhanced by two orders of magnitude with no evidence of decay. DC were exposed to a NP-AdV-luciferase formulation and evaluated 60-h post-infection (Figure 6) at different MOI. At MOI = 1, the NP-AdV formulation was two orders of magnitude more active as compared to that of free AdV. Figure 7 shows viral luciferase gene transfer at two different temperatures into BC-1 (B-cell lymphoma) cells, followed by 1−4 days of incubation. Free AdV showed a somewhat smaller temperature dependency. A significant potentiation of luciferase

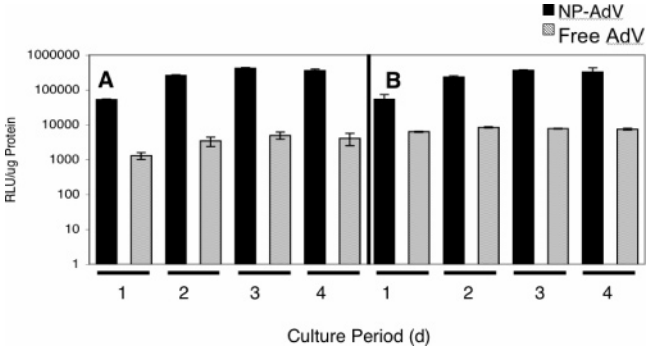

Figure 7. NP-mediated gene transfer into lymphoma cell line BC-1 cells. The cells were treated either with free adenovirus (AdV, MOI = 5) or formulated NP containing the same adenovirus (NP-AdV, MOI = 5) for 2 h under gentle agitation at room temperature (A) or at 37 °C (B). Cells were washed once with PBS to remove unbound adenovirus or NP and cultured with new growth medium. Luciferase activity was measured every 24 h up to 4 days. Luciferase activity was normalized on the basis of protein content.

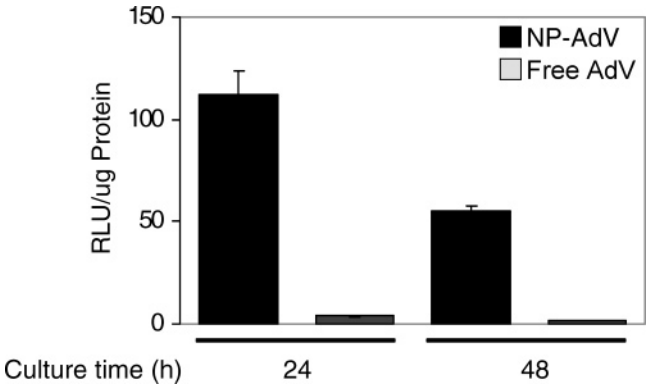

Figure 8. NP-mediated gene transfer into islets. Freshly isolated murine islet cells were transduced with either adenovirus containing NP (NP-AdV, MOI = 10) or free adenovirus (AdV, MOI = 10) in complete growth media. After 16 h, cells were washed twice with PBS and cultured with growth medium. At 24 and 48 h, cells were harvested and assayed for luciferase expression. Luciferase values were normalized on the basis of protein content.

expression (normalized per protein) was achieved with the NP-AdV formulation. Figure 8 presents results of viral gene transfer (luciferase normalized per protein) into pancreatic mouse islet cells, 1−2 days post-infection. NP-based gene transfer was 15−20 times more efficient as compared to free AdV infection.

Discussion

This paper describes a new method of assembling NP using a combination of multicomponent water-soluble polymers that enable a high product stability in biological fluids and application of this method in viral gene transfer. Specifically, we provide a description of polymeric complexes[18,19] in which a gelling polymer and oppositely charged polymer create a stable NP. The gene construct is released from the particles, probably by an enzymatic degradation of the delivery vehicles. The method for preparing the gene construct and multipolymeric NP and microparticles is described. A stream of uniformly sized drops of a charged polymer solution in which the particle size of the drops is submicrometer or at most only a few micrometers is

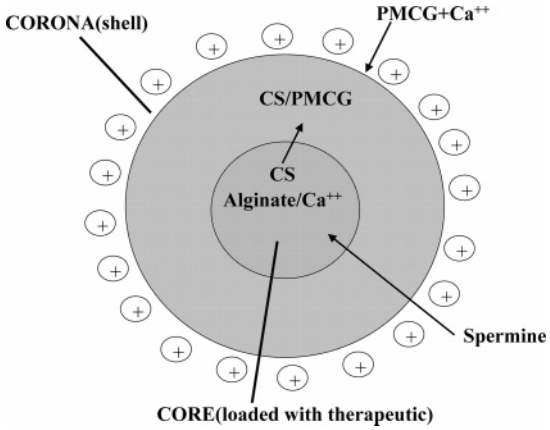

Figure 9. Schematic of the core−shell morphology. The core is made primarily from calcium alginate gel (small calcium ion can diffuse rapidly inside the preformed NP), the corona of NP from cellulose sulfate (diffusing from within the core outward), and PMCG (oligomer) diffusing from the outside receiving bath. Spermine, also a small cation, preferentially reacts with alginate as well.

generated and collected in a stirred reactor provided with a polymeric solution of opposite charge. The droplets and the solution react to form the particles. When the drops of polymer are polyanionic (as is the case here) and the receiving polymer solution is cationic, then the particles have a polyanionic core and a shell or corona is composed of a polyanionic/polycationic complex. The periphery of the particle has an excess positive charge. Cationically charged NP are well suited for gene transfer. They have a natural affinity to anionically charged cell surfaces. In addition, such NP surfaces can be easily functionalized to allow for targeted gene delivery. Our nanotechnology is water-based and is designed on the basis of natural polymers, as opposed to synthetic-based technologies that suffer from the presence of organic solvents needed for their assembly.

Particle Characteristics. On the basis of the assembly process we visualize NP as possessing a core−shell morphology (Figure 9). The core is composed of cross-linked anionic solution (by means of polycations or calcium chloride), perhaps in a microgel state, and the corona is visualized as being composed of cross-linked cellulose sulfate (by means of PMCG and spermine), providing some natural permeability barrier. Further cross-linking could immobilize all components, including the entrapped drug, and retard its exit from NP. TEM of the NP shows some heterogeneity in terms of more electron dense areas (Figure 2). Such denser areas also appear in AdV-free NP.

NP size and charge are both critical for delivery and uptake by cells. In the absence of specific ligands, there may be electrostatic or other modes of interaction of particles with the cell surface. It is generally understood that particles larger than 1 μm are precluded from entering cells, unless a pathological status is observed.[25] The size reported in this paper is around 230 nm; however, we have been able to scale down the product size to <100 nm. Smaller NP could permit more efficient uptake and internalization.

The surface properties of colloidal systems are critical for determining their drug carrier potential, because they will control their interaction with plasma proteins. Zeta potential measurement represents the overall surface charge of the particles in the presence of changes in the environment (e.g., pH, presence of counterions, adsorption of proteins). Charge-shielding by poly(ethylene glycol) (PEG) or other hydrophilic groups can be used to predict the effectiveness of the barrier function against coagulation and opsonization in vivo. We did not, however, measure the surface density of PEG molecules (F-68), co-entrapped during the assembly process.

Adenoviral-Mediated Gene Transfer: NP and Other Delivery Modes. Our data indicate that the nanoparticulate formulation is capable of very efficient gene transfer. We have illustrated several examples of NP-enhanced delivery of adenoviral gene reporters to cell lines, some of which are refractory to conventional viral gene therapy. We present efficacy on the basis of MOI units needed to achieve an efficient transfer, normalized per microgram of protein. However, there is a need to develop better normalization strategies. Infectivity may not be the same as gene expression. Effort to normalize data on the basis of DNA content have been hampered by interference from other NP components. The literature data are diverse in terms of normalization strategies and not readily compared.

This approach to gene delivery compares favorably with published methods (see below). In the present report we found no toxicity up to 36 h; furthermore, longer exposure (up to 6 days; not shown) indicated no toxicity effects. This contrasts with the typical 2-h exposure of cells with lipo- or cation-condensed reagents (e.g., Lipofectamine or polyplex) required because of their inherent toxicity. Adenovirus is inherently more potent then plasmid delivery.

There are very few literature references on the formulation of polymeric gene delivery vehicles containing virus. A retroviral polybrene−chondroitin sulfate complex increased in vitro gene transfer into NIH 3T3 murine fibroblasts and primary human fibroblasts 10- to 20-fold.[26] The mechanism of polymer-based gene enhancement is not understood. For AdV−polymer complexes, charged polymers are thought to act on early, receptor-independent steps of infection.[27,28] Another modality for enhancing the AdV gene transfer is the formulation of nanoparticulate (or microparticulate) delivery vehicles. In the case of NP, the mechanism of enhancement involves intracellular uptake of NP and rapid escape from the endo-lysosomal compartment into the cytosol.[29−33] DNA entrapment is enhanced by condensation with polymers prior to its entrapment into NP or by synthesizing novel polymers with cationic groups that can condense DNA directly into the NP.[29,30] Sustained gene delivery has been shown for microparticles,[32,33] a technology limited to cells with high phagocytic activity, particularly macrophages and DC.[34]

Gene Transfer into Hematopoietic and Nonhematopoietic Cells. Entry of AdV into target cells occurs by two serial steps: the binding of Ad-fiber knob to the coxsackie/adenovirus receptor (CAR) receptor on the cell surface, and the subsequent interaction between the Arg-Gly-Asp (RGD) motif located in Ad-penton base and the α_v-integrins. Many cell types, including lymphocytes, are refractory to adenovirus infection because they lack the CAR needed for virus attachment. The technologies for transfer of exogenous genes into primary T and B cells have been limited until re-

cently.[35,36] These cells express low levels of CAR receptor as well as $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. A heparan sulfate proteoglycan may represent another primary binding site of adenoviral entry into cells.[37] Our data on gene transfer into lymphocytic cells are limited to transformed cell lines. Gene transfer into primary cells is in progress.

Gene Transfer into DC. The nanoparticulate formulation as described in this paper compares favorably with previous reports demonstrating that (i) human DC can be successfully transduced by retroviral or adenoviral vectors ex vivo, resulting in 40−90% transgene expression,[38−44] and that (ii) DC are relatively refractory to adenoviral-mediated gene transfer at low MOI, but the transduction rate increases substantially at MOI of 100−1000.[41−43] In addition, the selection of the specific stage of development of the DC is also critical. Mature DC isolated from mouse spleen have been reported to be refractory to adenoviral infection.[44] On the other hand, only immature DC are capable of uptake of particulate antigen.

Gene Transfer into Islet Cells. Adenoviral and adeno-associated viral vectors have shown some promise in abrogation of islet inactivation due to alloimmune rejection or autoimmune destruction[45−47] following transplantation.[48,49] However, a high MOI is often required, leading to fibrosis and matrix formation. Therefore, there is a growing need to develop gene carrier expression systems which do not elicit counterproductive immune response or cytotoxicity.

The pancreatic islets do not belong to the gene transfer resilient cell category. Experiments with neutralizing anti-CAR antibodies provided evidence that CAR was expressed by alpha and beta cells and that it played a role in the infection of these cells.[50] Also, the low transfection efficiency may be due to the islets being a cluster of approximately 1000 nondividing cells. Although polymer-based formulations can transfect genes into human islets, the efficiency is low and requires prolonged incubation.[51] Our data on NP-AdV-mediated gene transfer into mouse islets indicate that efficient gene transfer can be effected with help of NP.

Possible Mechanism of Uptake of NP. Our technology of introducing gene material into the above cells is straightforward and may be due to a new mechanism, a nonspecific entry because NP-AdV can enter cells shortly after proteolytic stripping; thus, it appears that the interaction of the NP with the cell is primarily electrostatic and does not involve receptor specificity of the binding site for the cationic moiety. Free AdV was much slower to enter, consistent with the time required for expression of newly synthesized CAR and other surface receptors.[52] Other properties of the NP such as size, stability, net surface charge, or charge density may be important. The polycationic nature of the NP periphery, particularly in terms of polyamine- and guanidinium-type chemistry, may also facilitate their cellular uptake and transport via cell surface heparan sulfate proteoglycan[53] or polyamine-based transporters.[54,55] A recent reference disputes the polyamine-based transport.[56] In addition to nonspecific receptor-mediated endocytosis, NP could be transported through a nonspecific receptor-independent fluid-phase endocytosis (macropinocytosis).[57] Our data on internalization (not shown) indicate clearly an entry of NP into cells. More conclusive results on NP uptake can only be obtained with the help of a microscopic technique. The molecular mechanism of entry has not yet been identified.

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