Dendrosomes as novel gene porters-III

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Abstract

BACKGROUND: It was previously reported that dendrosomes, i.e. neutral, biodegradable, covalent or self-assembled, hyperbranched, spheroidal nano-particles with a size ranging from 15 to 100 nm, provide a convenient and efficient means of gene delivery into various kinds of cells such as human hepatoma and kidney cells as well as animal models.

RESULTS: New studies via circular dichroism show that hydrophilic and amphipathic dendrosomes either do not affect the DNA structure or moderately transform it from B- to A-conformation. Gene delivery into human liver, kidney, and endothelial cells as well as other animal cells like Bowes, U-937, Raw, CCRF-CEM, MOLT-4, K562, Huh-7 and VERO reveal that the genes are efficiently expressed and in comparison with other gene porters like Lipofectin or bacterial ghosts, do quite well. It is also shown that dendrosomes are able to deliver genes into cells like endothelials that are usually hard to transfect. Cell culture experiments as well as intraperitoneal/intradermal injections of dendrosomes into mice establish their nontoxicity (up to 2.5 mg kg^{-1} of animal weight in the latter case). Studies on immunization of BALB/c mice using conventional adjuvants such as aluminium phosphate, C_pG motif and one of the dendrosomes, indicate that the latter leads to the mildest initial response development while exceeding them afterwards.

CONCLUSION: CD studies reveal that, owing to the neutrality of dendrosomes, formation of Den/DNA complexes is accompanied by slight structural modifications of DNA cell culture, and animal studies reveal that dendrosomes are inert, non-toxic and highly efficient gene porters that perform at extremely low doses. In comparison with bacterial ghosts and some common porters, they are efficient in delivery of genes into animals and a variety of cells including those that are usually hard to transfect.

Keywords: Dendrosome; gene delivery; genetic manipulation; DNA vaccine; animal models; transfection efficiency; gene porter

INTRODUCTION

One of the principal steps in genetic manipulations is the safe and efficient delivery of extrinsic gene into the recipient cells and their integration into the host genomes. Obviously, the way in which the new gene is incorporated in, protected and translocated, by the gene porter, the place and mechanism of its release within the cell, are the prime determinants of the overall transfection/expression efficiency. Over the years a number of alternative gene delivery vehicles/techniques have been developed, among which the non-viral ones are probably the most convenient and inexpensive.[1] Although viral vectors are effective in gene delivery, the immune response elicited by viral proteins poses a major problem.[2] Therefore, several laboratories are involved in the development of non-viral DNA delivery vehicles. Polyamines, polycationic lipids, and neutral polymers, are capable of condensing DNA to nanoparticles with radii of 20–100 nm[3]. Although the structural and energetic forces involved in DNA condensation have been studied by physical biochemists for the past 25 years, this area has experienced a resurgence of interest in recent years because of the interests in developing novel gene porters.[4] However, the precise details of the energetic of DNA nanoparticle formation and their packing assembly are not known at present.[4,5] Concomitant with these developments significant progress has been made towards the use of cationic gene delivery systems. Polyelectrolyte nanoparticles (cationic

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polymers, cationic lipid mesophases), liposomes compact the DNA into rather large polymorphic particles with severely low translocation and efficiencies.[6] In contrast, oligocations like spermine, and cationic detergent micelles that have low binding cooperativity and fast exchange rates are capable of condensing the DNA into homogeneous populations of small particles.[7] Translocation efficiency is independent of the size of the vehicle but is affected by the size of the DNA–gene porter complex.[8] The condensed particles could also be formed from oligonucleotides using cationic polymers.[9–12] Polybutyl cyanoacrylate and polyisohexylcyanoacrylate are often used with antisense oligonucleotides. Because of the negative surface potential of the polymer particles, a cationic copolymer or a cationic hydrophobic detergent is combined with polyalkylcyanoacrylate (PACA) polymers. Oligonucleotide particles prepared in this manner have a size range of 50–500 nm.[10,12]

Cationic amphiphiles are widely used as effective tools in delivery of DNA into mammalian cells.[3] Block copolymers containing polyethyleneglycol (PEG) have been used in various drug delivery systems.[13] PEG has also been linearly conjugated to polycationic polymers (e.g. poly L-lysine and polyspermine) to improve solubility and gene transfection efficiency.[13] Among synthetic cationic polymers, amino-dendrimers have been attractive due to their defined structures and the large number of surface amino groups.[14–16] Polyethyleneimine (PEI)-DNA complexes exhibit remarkable transfection efficiencies, comparable with those of the best currently available synthetic vectors.[17] The high transfection efficiency of PEI is related to its buffering capacity. The addition of smaller PEI molecules to preformed PEI–DNA complexes strengthens the packing of amines around the DNA, yielding complexes with higher buffering capacity and and transfection efficiency.[18] Gene delivery has also been accomplished using a variety of positively charged dendrimers, including PAMAMS, to form DNA complexes and transfect cultured cells with lower toxicities and higher efficiencies than conventional polyamine transfection agents.[3] Some linear/dendritic block copolymers (containing amino/amide groups) self-organize with DNA under physiologic conditions, often forming water-soluble globular polycationic complexes of relatively narrow size distributions.[14] Large macromolecular assemblies with linear segments in their core and perfectly branched blocks in their periphery, called polymer micelles, have tremendous potential as extended drug delivery systems. These new materials represent the first examples of structure-controlled components: an interior core surrounded by an exterior shell and surface.[14]

It has previously been reported that dendrosomes (low-cost, neutral, biodegradable. covalent or self-assembled, hyperbranched, spheroidal nano-particles) are capable of easily delivering genes into human hepatocytes (Huh7), kidney cells (Hek293) and animal models (production, more than 1 year, of antibody against the hepatitis B surface antigen).[16] A unique feature of dendrosomes is the ease with which the structure of their monomers can be varied in order to provide suitable inert gene porters for the various DNA sizes and target cells.

This paper summarizes recent studies on the interaction of dendrosomes with plasmid and genomic DNAs, their ability in delivery and expression of genes in Huh7, Bowes, Raw, U-937, CCRF-CEM, MOLT-4 and K562 cells; comparison of their performance with a commercial gene porter (Lipofectin) and bacterial ghosts; their non-toxicity against human cells and animal models and their performance as adjuvant in immunization of BALB/c mice against hepatitis C.

EXPERIMENTAL

Plasmids

Plasmids used in DNA/porter interactions were pGF68, 6.5kb (high GC content, kindly provided by F. Jenkin, Pittsburgh, USA) and pBasic-CAT, 3kb (low GC content). Those used in transfection/expression in Huh7 (human hepatocyte) cells were pON284 (β-galactosidase gene). To compare the performance of gene porter Den55 with Lipofectin, pC53-SN3 containing p53cDNA was used and to compare Den10 with bacterial (E.coli) ghosts, use was made of pEGFP and gαIgG-PE. In the case of DNA, vaccine against hepatitis C pcDNA3 was used as the recombinant plasmid encoding the hepatitis C virus core protein.

Dendrosomes (gene porters)

Dendrosomes used are designated as DenO4, DenO6, DenO1, DenS6, Den123 (all self- assembled nanoparticles made of neutral biodegradable amphipathic monomers), Den450 and Den700 (neutral covalent hydrophilic dendrimers), Den10 (neutral, covalent, hydophilic dendrimer with nitrogen nodes), and Den55, linear analogue of Den10. Among the commercial gene porters, Lipofectin (Life Technologies, Rockville, MD, USA) was chosen for comparative experiments via trypan blue exclusion and flow cytometry. Calcium phosphate was used as easy reference in transfection of Huh7 cells. Bacterial ghosts were prepared following the established procedures[25–30] and their performance assessed via flow cytometry. Aluminium phosphate was used as a reference adjuvant in animal studies.

CD spectroscopy

The CD spectra were recorded in the 200–320 nm region and results expressed in molar ellipticity $[\theta]$, deg cm^2 dmol^{-1}, which was determined from $[\theta] = (\theta \times 100\text{MRW})/Cl$, where C is the DNA concentration (mg mL^{-1}), l is the length of the light path (cm) and θ is the measured ellipticity (deg) at the wavelength. The instrument was calibrated with (+)-10-camphor sulfonic acid, assuming $[\theta] = 7920$ deg cm^2 dmol^{-1}.[20] The data were smoothed using the Jasco-J715 software

including the fast Fourier transform noise reduction routine, which allows the enhancement of most noisy spectra without distorting their peak shapes.

Size determination via AFM

Samples of dendrosomes dispersed in water (1%w/v) were applied on to a freshly cleaved mica surface and observed under atomic force microscope (AFM, Professor Stocker, University of Muenster, Berlin, Germany).

Cells

Cells examined were Huh7, human hepatocyte; U-937, histocytic lymphoma (macrophage); RAW, mouse macrophage; BOWES, melanoma (skin); CCRF-CEM, MOLT-4 (human T-lymphoma), and K562 (human erythroleukemia). Under sterile conditions 4 mL of the culture medium (DMEM) was pipetted into a 25 cm^3 flask (37 °C). An appropriate volume of each cell suspension was added into the flask and the contents thoroughly mixed by repeated pipetting and gentle shaking. The flask was then placed in a CO_2 incubator (5% CO_2, 95% air, 37 °C). The state of cells was monitored by observation under an inverted microscope.

Transfection/expression

Different ratios of gene porter/DNA were presented to Huh7 cells and their transfection/expression were examined by β-gal assay[24,28] and compared with that of calcium phosphate. In comparing the performance of dendrosomes with those of Lipofectin and bacterial ghosts, appropriate mixtures of DNA/dendrosome, or DNA/Lipofectin, DNA/bacterial ghost, DNA/(dendrosome+ghost), and DNA/reference gene porter (calcium or aluminium phosphate) were presented to the cells under investigation and their transfection/expression assessed as described below.

Transfections

Calcium phosphate

In the case of Huh7 cells 5 μg of each plasmid was treated with 25 μL of 25 mol L^{-1} $CaCl_2$ in 250 μL of HEPES (2X), made up to 500 μL with ddH_2O. Once the mixture became turbid it was poured onto the cells in Petri dishes (10^6 cells per dish) and incubated under CO_2 (37 °C) for 24 h until reaching 80% confluency.

Comparing dendrosomes, Lipofectin, and bacterial ghosts

To verify transfections, a known amount of a given gene porter (dendrosome, Lipofectin or bacterial ghosts) was simply mixed for 5 min with a mixture of the desired plasmid. After applying the mixtures to the culture dishes, the dishes were gently shaken on a rotary shaker for 30 min and subsequently placed in a sterile hood and gently supplemented with fresh nutrients and placed in a CO_2 incubator at 37 °C until 80% confluency was reached. In the case of CCRF-CEM, MOLT-4 and K562 a mixture of 5DNA/1Den55 or DNA/Lipofectin was presented to each cell type in 35 mm culture dishes and placed in a CO_2 incubator at 37 °C. In the case of Bowes, RAW, THP-1 and U937 a mixture of DNA/Den10 or DNA/ghost, or DNA/(ghost+Den10) was applied to each cell type and their performance assessed via trypan blue exclusion counting and flow cytometry as described below.

It ought to be mentioned that fluorescence assay with the aid of confocal microscopy using labelled Den10 molecules proved that they do not adhere to the outside of the bacterial ghosts, rather about 400 molecules become entrapped in a single ghost.

Proof of transfection/integration

Transfection was assessed by two methods: trypan blue exclusion for assaying the cell viability (CCRFM-CEM, MOLT-4, and K562) and dig-labelled DNA probe followed by alkaline phosphatase detection.

In the first method cells were stained every 24 h with trypan blue (0.4% in PBS) and observed under optical microscope and counted for up to 5 consecutive days. In the second method, after an appropriate time (usually 24 h), total DNA of each cell type (treated and untreated reference) was extracted and appropriately digested with proper restriction enzyme following the established procedures, dissolved in sterile dd H_2O, placed in boiling water and quickly quenched on ice to obtain single stranded DNA.[28] Pieces of nitrocellulose membrane were soaked for 10 min in a buffer composed of 0.3 mol L^{-1} NaCl, 0.03 mol L^{-1} Na-citrate and 0.1 mol L^{-1} HCl. The membrane was air dried and 2 μL aliquots (total 20 μL) of each digested DNA sample were applied to a designated spot and allowed to dry for 1 h at 80 °C and subsequently exposed to the hybridization buffer containing an appropriate dig-DNA labelled probe following the manufacturer's recommended protocol. The presence of target DNA (in this case the gene delivered into cells) was confirmed by the appearance of a coloured ring (resulting from the reaction of alkaline phosphatase on its substrate) on the corresponding spot.

To assess the random integration of pC53-SN3 in MOLT-4 genome by Den55 or Lipofectin, use was made of neomycin selection protocol.[25] Cells that survived after 21 days confirmed the expression of neomycin resistance gene.

Proof of gene expression

This was assessed by four methods: β-gal assay, apoptosis assessment, flow cytometry and animal immunization.

β-gal assay. This was done following standard procedures.[26] Briefly, the monolayers of transfected Huh7 cells were thoroughly washed with PBS, collected, and pelleted at 5 °C and lysed (using 0.1 mol L^{-1} Tris-HCl, pH 7.8; 0.5%v/v Triton X100)

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for 15 min at 37 °C, centrifuged at 14000 rpm for 10 min and the supernatant recovered. The lysate was incubated for 40–60 min at 50 °C to inactivate endogenous β-galactosidase) and then mixed with a mixture of Mg^{2+}, ONPG, 0.1 mol L^{-1} Na-phosphate, pH 7.5, kept for 30 min at 37 °C (or until a faint yellow color develops). The reaction was stopped by adding 500 µL of 0.1 mol L^{-1} sodium carbonate and the optical density (OD) of samples measured at 420 nm and the enzyme activity calculated from 300 × OD/time (min).

Apoptosis assessment. To compare the p53 gene expression by Den55 and Lipofectin in CCRF-CEM, MOLT-4, and K562 use was made of an apoptosis detection kit based on fluorescence assay of Annexin V-FITC/PI (IQ Products, Houston, TX, USA) following the manufacturer's recommended procedure. Briefly, 72 h after transfection cells were harvested by centrifugation, and incubated with AnnexinV-FITC (10 µL per 1.5×10^5 cells) for 20 min at 4 °C. Thereafter, 10 µL PI was added to the mixture, incubated for 10 min at 4 °C and apoptotic assessment was made via flow cytometry.

Flow cytometry. In comparing Den55 and Lipofectin against CCRF-CEM, MOLT-4 and K562, the level of gene expression was assessed by fluorescence assay of AnnexinV-FITC/PI, and in experiments comparing the performance of Den10 with bacterial ghosts against Bowes, U-937 and Raw cells, Green Fluorescent Protein (GFP) assay and antibody fluorescence measurement (IgG-PE) via flow cytometry were used. 2×10^5 cells incubated for 120 min with (Den+ pEGFP), washed twice with PBS supplemented with 2% FCS, fixed and permeabilized (FACs Perm solution), washed twice with PBS supplemented with 2% FCS. Cells suspended in 1.5% PFA in PBS and expression of GFP analysed by flow cytometry. Incubation with anti GFP monoclonal antibody for 45 min, washed three times, incubated with appropriate secondary antibodies conjugated with PE for 45 min, washed three times. Cells resuspended in 1.5% PFA in PBS and analysed by flow cytometry (Coulter EpicXL/MCL, Coulter Immunotech, Villepente, France).

Animal immunization. Five groups of BALB/c female mice ($n = 5–7$) 6–7 weeks old (18–20 g) were each immunized with 100 µL of different immunogens (aluminium phosphate, or Den123, each mixed with HCV core pcDNA3). Mice were injected three times after 9, 30, and 50 days in their quadriceps muscles. Blood samples were collected from the retro-orbital sinus at days 1, 17, 42 and 72 after injection and kept at −70 °C. HCV core kit (Biokit, SA, Spain) coated with core antigen was used to determine anti-core antibody response via serial dilution of individual blood sera, using the standard protocols and optical density determined at 450 nm.[29]

Proof of non-toxicity. Although it has previously been reported that dendrosomes (Den450 and Den700) are non-toxic against the aortic smooth muscle cells, A7r5 cells,[16] it was still necessary to establish their non-toxicity through further experiments against other human cells and animal models. This was done using two approaches. In the first, human cells, MOLT-4 and CCRF-CEM, and K562 were each separately exposed to Den55 or Lipofectin and assessed via trypan blue exclusion counting for 5 consecutive days after transfection. Cell samples were also taken for flow cytometry. In the second approach groups of four Suri mice were injected with 0.1, 0.2, 0.5, 1.0, 2.5 mg kg^{-1} (animal wt) of Den700 or Den138 in order to determine the toxic doses of the two types of dendrosomes following the standard LD_{50} protocols.[30] All utensils were sterilized by autoclaving at 132 °C for 30 min. Groups of four Suri mice each weighing 20 ± 2 g were injected intraperitoneally with sterilized, filtered (0.22 µm filter) aqueous solutions (dd H_2O) of the largest hydrophilic dendrimer, Den700, or sterilized solutions of Den138 in olive oil (# 037H6100, Sigma). Doses injected were slowly increased from 0.1 mg kg^{-1} up to 5mg kg^{-1} animal weight.

RESULTS

Size assay

AFM Results

Atomic force microscopy of dendrosomes showed that when a drop of their 1% dispersion is spread on a freshly spilt mica surface, a rough film morphology including holes, with a thickness of 25 to 70 nm is observed, depending on the type of dendrosome. Based on the chemical structure of dendrosomes used here, their radius is estimated at 30 to 100 nm, which is in the same range as their observed sizes, considering the subtleties involved in both approaches.

DNA–porter interactions

CD results

Figure 1 shows the variations in the CD spectra of calf thymus (ctDNA) and linear DNA when exposed to low levels (less than 10 µg µL^{-1} solvent) of Den123 (self-organized from amphipathic monomers). Although the effect seems minor, nevertheless the increase in the 270–275 nm peak, concomitant with a decrease in the 245 nm peak, clearly indicates a transition from B- to A-DNA.[11,12]

Figure 2 shows the CD spectral variations in ctDNA and linear DNA when exposed to higher levels of Den123 (more than 10 µg µL^{-1} solvent). As can be seen the effects indicate a mild transition from B- to A-DNA (intermediate between A and B-DNA structures).[20]

Sensitivity of the interactions to the GC content of DNA was studied using two types of DNA with different GC contents. Since Den123 and Den138 particles consist of a hydrophobic kernel surrounded

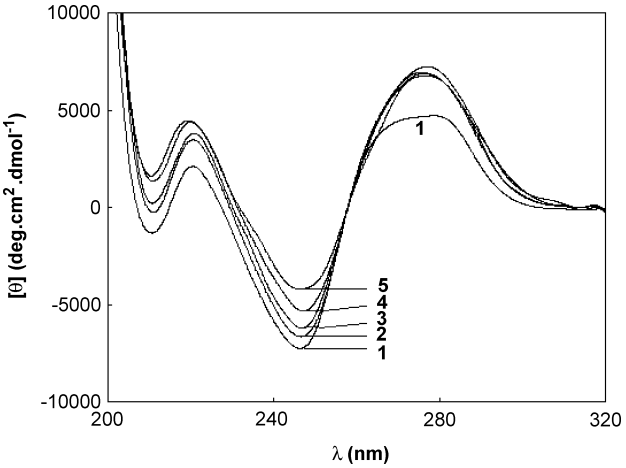

Figure 1. CD spectra of calf thymus DNA and its admixtures with Den123 (a self-assembled amphipath) at a low level of the latter. (1) DNA alone, (2) Den/DNA:1/17, (3) Den/DNA:1/10, (4) Den/DNA:1/5, (5) Den/DNA:1/1, (6) Den/DNA:5/1.

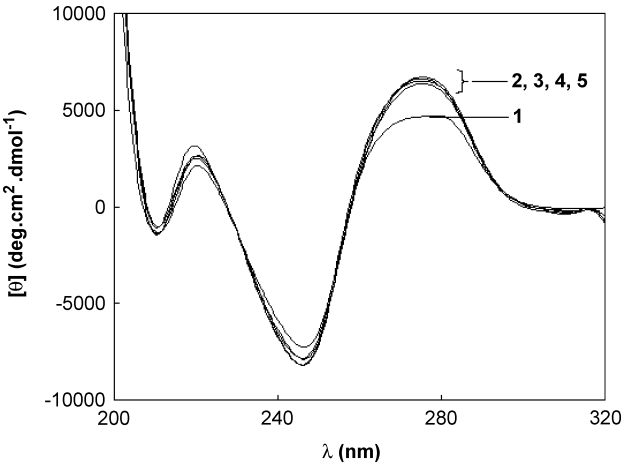

Figure 2. CD spectra of calf thymus DNA and its admixtures with Den123 at a high level of the latter. (1) DNA alone, (2) Den/DNA:1/17, (3) Den/DNA:1/10, (4) Den/DNA:1/5, (5) Den/DNA:1/1.

by a fuzzy coat of hydrophilic chains, it was decided to simulate the role of the exterior shell by examining the interactions of DNA with covalent hydrophilic dendrimers of the same construction, designated Den450 or Den700.

Figure 3 shows the variations in the CD spectra of a low GC plasmid DNA (pBasic-CAT, 3 kb) when exposed to Den123 and Den450 (porter/DNA: 17/1). As can be seen Den123 causes an increase in the positive peak at 275 nm (mild transition from B- to A- DNA), whereas Den450 causes a transition from a B-DNA with 10.4 bp/turn to another B-DNA with 10.2 bp/turn (note the disappearance of the positive peak at 275 nm, reminiscent of DNA wrapping around histones[21,22]).

Figure 4 shows variations in the CD spectra of DNA with a high GC content (pGF68, 6.5 kb) when exposed to Den123 and Den450 (porter/DNA: 17/1). As can be seen in both cases there is a transition from a normal B-DNA to a B-DNA with 10.2 bp per

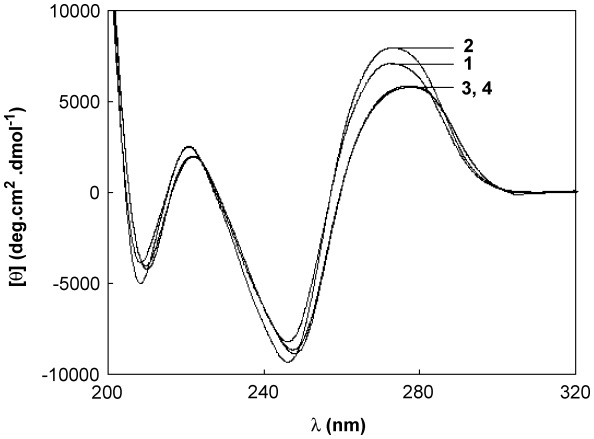

Figure 3. CD spectra of a low GC plasmid DNA (pBasic-CAT) and its admixtures (17/1) separately with Den123, Den450 (a neutral dendrimer), or a positively charged dendrimer. (1) DNA alone, (2) Den/DNA:1/1, (3) Den/DNA:5/1, (4) Den/DNA:10/1, (5) Den/DNA:20/1.

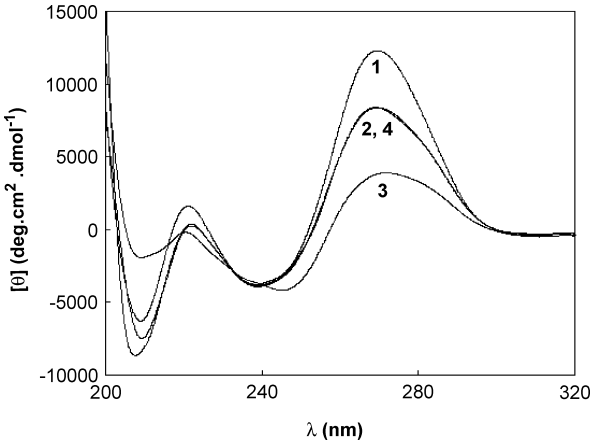

Figure 4. CD spectra of a high GC plasmid DNA (pGF68) and its admixtures (17/1) separately with: Den123, Den450, or a positively charged dendrimer. (1) DNA alone, (2) Den/DNA:1/17, (3) Den/DNA:1/10, (4) Den/DNA:1/5, (5) Den/DNA:1/1.

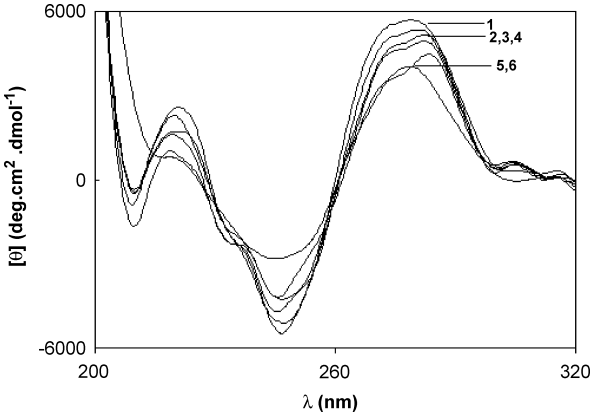

Figure 5. CD spectra of calf thymus DNA and its admixtures with Den10 at different ratios. (1) DNA alone, (2) Den/DNA:1/17, (3) Den/DNA:1/10, (4) Den/DNA:1/5, (5) Den/DNA:1/1, (6) Den/DNA:5/1.

turn, confirming the dominant role of the hydrophilic exterior of Den123.[22,23]

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Figure 5 shows the variations in the CD spectra of ctDNA when exposed to Den10 (dendrimeric analogue of Den55), which is expected to be slightly positively charged under experimental conditions.

Transfection/expression

As stated before, this was assessed by two methods: (a) expression of neomycin resistance gene in MOLT-4 cells using Lipofectin as a reference; and (b) β-gal assay using calcium phosphate as a reference. Figure 6 compares the performance of Den55 with that of Lipofectin in expression of neomycin gene in MOLT-4, where it is clearly seen that Den55 (5DNA/1P55) outperforms the commercial product. Figure 7 shows the activity of β-gal expressed in Huh7 cells via various gene porters. As can be seen, DenO2, DenO4 (self- assembled amphipath), Den10 (hydrophilic dendrimer) and Den55 (linear analogue of Den10) are able to safely deliver and express the genes in these cells. Interestingly almost all dendrosomes seem to perform best at very low levels and at weight ratios of Den/DNA $\ll 1$, having the least possible chance of undesirable side effects on the host organisms. Undoubtedly there exists an appropriate optimum ratio of porter/DNA for each cell type/dendrsome that leads to the highest transfection/expression efficiency.

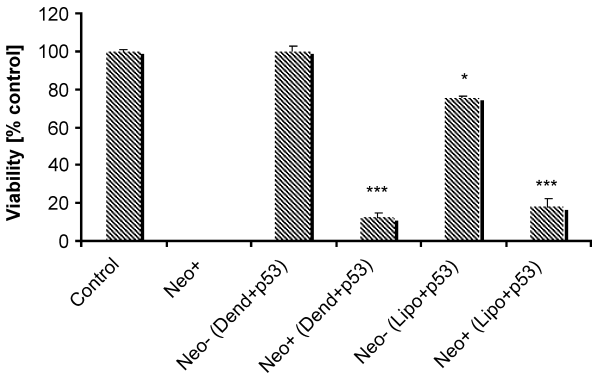

Figure 6. Comparison of transfection/integration performance of Den55 (linear analogue of Den10) against Lipofectin in delivery of the neomycin resistance gene into MOLT-4 cells.

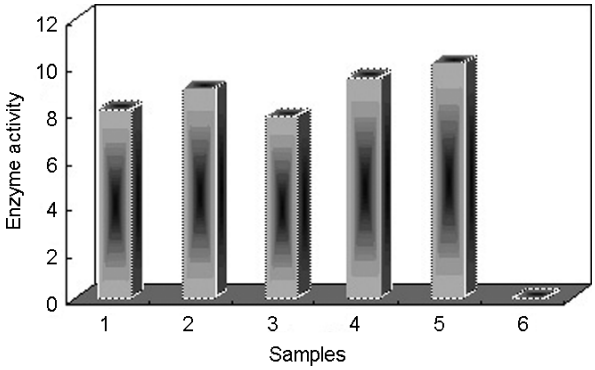

Figure 7. Comparison of activity of β-gal expressed in Huh7 cells employing a number of dendrosomes each at different proportions. (1) Neg.control, (2) Den10 (Den/DNA1/17), (3) DenO4 (Den/DNA1/17), (4) Den55 (Den/DNA1/5), (5) Den O2 (Den/DNA 1/17), (6) Ca phosphate.

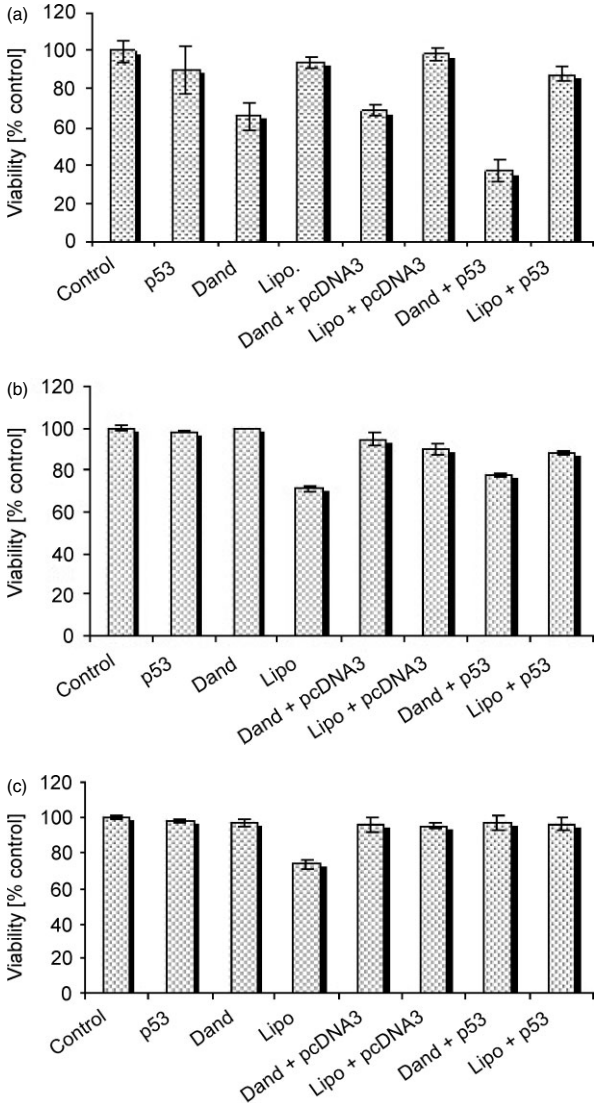

Figure 8. Effect of p53 gene expression on viability of: (a) K562, (b) CCRF-CEM, (c) MOLT-4 cells. Control: untransfected cells; p53: cells treated only with pC53-SN3; Den55: cells treated only with Den55 ; Lipo: cells treated only with Lipofectin; Den55+pcDNA3 and Lipo+pcDNA3: cells transfected with Den55 plus pcDNA3 (control, without p53 cDNA), and cells transfected with Lipofectin plus pcDNA3, respectively; Den55+p53 and Lipo+p53: cells transfected with Den55 plus pC53-SN3 and cells transfected with Lipofectin plus pC53-SN3, respectively (all 72 h after transfection).

This has yet to be fully investigated. It is worth mentioning that linear analogues of Den450, Den700 and Den10 are able to deliver genes albeit at much higher ratios of Den/DNA (data not shown).

Gene expression

Figure 8 compares the performance of Den55 with that of Lipofectin in delivery and expression of p53 gene in K562, CCRF-CEM and MOLT-4 cells, where superiority of Den55 is clearly demonstrated. Figure 9 compares the performance of Den10 (dendrimeric analogue of Den55) with that of the bacterial ghosts against Bowes, RAW, and U937 cells under identical conditions. It is seen that although the conditions are not yet optimized for dendrosomes, Den10 is as effective as the bacterial ghosts in the case of U-937

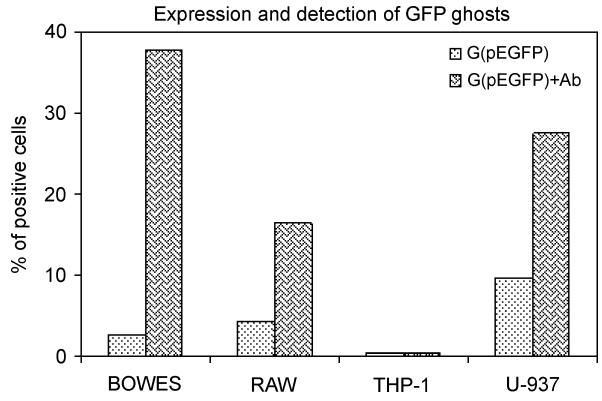

Figure 9. Comparing the expression performance of Den10 with that of bacterial (E.coli) ghost in BOWES, RAW and U397 cells.

cells. Interestingly, the smallness of Den10 allows its easy penetration through the pore of the bacterial ghost (up to ~400 per ghost), but the loaded ghosts do not perform any better than either the ghost or the dendrosome alone, indicating that the entrapped dendrosomes are, as expected, ineffective.

Non-toxicity

Cells exposed to Den55 did not show any signs of toxicity whereas those exposed to Lipofectin revealed severe toxicity (Fig. 10). None of the animals injected with Den138 (self-assembled amphipath) or Den700 (largest covalent hydrophilic dendrimer) showed any signs of discomfort or toxicity. In the case of Den700 at the very high dose of 5 mg kg^{-1}, however, initially some swelling developed under one arm and the animal died after 3 weeks. Obviously these levels are many orders of magnitude higher than those actually required to achieve transfection.

Animal immunization experiments also reveal that mice injected only once with a mixture of the plasmid carrying the gene of the Hepetatis B surface antigen and Den123 (Den/DNA :1/120) or those injected with Den123/HCV core pcDNA3 developed long-term immunization (up to 8 months so far, data to be published) without any signs of discomfort or toxicity. Thus it may be safely concluded that dendrosomes are

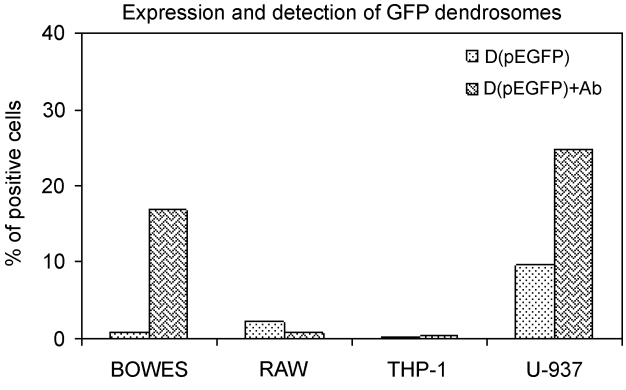

Figure 10. Evidence for nontoxicity of Den55 compared with commercial gene porter (Lipofectin) against CCRF-CEM, MOLT-4 cells and K562.

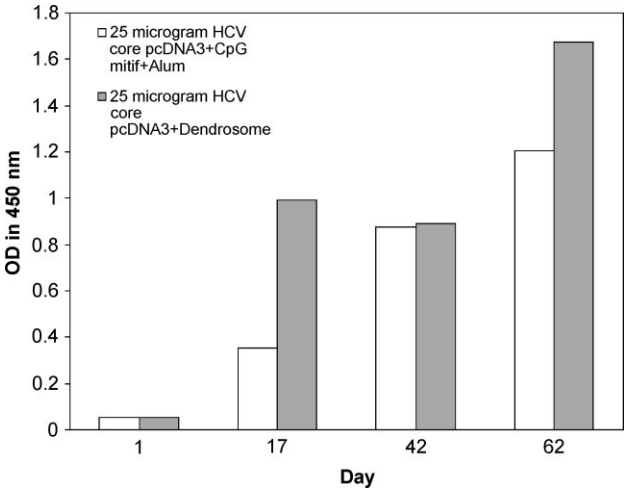

Figure 11. Time course of core-specific antibody development in BALB/c mice after injection with 10 μg HCV core pcDNA3+dendrsome (Den/DNA: 1/150) versus 50 μg HCV core pcDNA3 +CpG motif+ aluminium phosphate, assayed via ELISA.

inert and safe vehicles for gene (drug) delivery into cells/animals.

Animal immunization

Figure 11 shows the time course of imunostimulatory response in mice after injection with an adjuvant employing a ratio of 1/150 of Den123/HCV core pcDNA3. As can be seen there is an initial mild response followed by a rapid rise after 45 days reaching close to the performance of conventional adjuvants such as aluminium phosphate in 70 days.

It is also worth mentioning that exploratory experiments on animals have proven that dendrsomes are excellent carriers for oral drug delivery.

DISCUSSION

Currently a number of non-viral gene porters are available or being developed. The most important requirements for a suitable gene porter include ease of preparation, smallness of size, positive electrical charge, biodegradability, non-toxicity, shell-life stability, and ease of application. The experimental data outlined above demonstrate that dendrosomes are uniqe in meeting the above requirements.

AFM and confocal microscopy results show that dendrosomes are (as expected from their chemical construction) nano-sized (10–100 nm) neutral particles, in fact so small that nearly 100–400 of them fit inside one bacterial ghost.

Whereas the positive charge is considered favourable for DNA–porter interaction, one cannot ignore its unfavourable effect on dissociation of the DNA–porter complex where it is needed. The marked efficiency of dendrosomes as neutral entities in gene delivery to various cells supports this notion. Another concern with positively charged carriers is that even after dissociation of their complex with DNA, they may attach to other negatively charged components of the

cell thereby leading to undesirable consequences. This may explain the inertness of dendrosomes compared to Lipofectin, for example.

As expected, the CD data prove that dendrosomes, as neutral entities, interact very mildly with DNA and are thus expected to release them more easily.

The toxicity results in cell culture studies and animal models show that dendrosomes are inert, in fact, so much so that even at ridiculously high concentrations they show no deleterious effects.

Transfection/expression results clearly demonstrate that dendrosomes are quite efficient and able to function at extremely low quantities, Den/DNA ratios ranging from 1/120 to 1/10. This, besides their biodegradability, makes dendrosomes very unique and attractive gene porters.

It is therefore concluded that dendrosomes (neutral, biodegradable, covalent or self- assembled, hyper-branched, spheroidal nanoparticles), as a new generation of gene porters, offer the following advantages in delivery of genes/drugs into host cells and animal models: (a) they are biodegradable, low-cost, inert, non-toxic, and user friendly; (b) their complexation with DNA is accompanied by slight structural modifications, and thereby easily dissociate and readily release DNA, favouring their genomic integration and subsequent expression; (c) they are highly efficient and perform at extremely low doses (ratio Den/DNA); (d) in comparison with some commercial products and bacterial ghosts, perform quite well and are probably the simplest and most versatile of the gene porters available; (e) animals injected only once with simple mixtures involving very low ratios of Den/DNA develop long- term immunization; (f) although not specifically investigated here, five years of experimentation have proven that despite their biodegradability, dendrosomes are quite stable, not only in dry state but in solutions at low tempratures.

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