Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 298 (2005) 354–360
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Release of DNA from dendriplexes encapsulated in PLGA nanoparticles

Suzie Ribeiro [a], Nasir Hussain [b], Alexander T. Florence [a,*]

[a] *The School of Pharmacy, University of London, 29/39 Brunswick Square, London, WC 1N 1AX, UK*
[b] *Kingston University, Pharmaceutical Science, Kingston-upon-Thames, Surrey, KT1 2EE, UK*

Received 31 January 2005; received in revised form 26 February 2005; accepted 1 March 2005

Abstract

Biodegradable PLGA particles of less than 1 μm can encapsulate DNA and DNA–dendron complexes (dendriplexes) providing sustained DNA release for transfecting cells in gene delivery. Two polylysine-based dendrons prepared by solid state peptide synthesis were used to condense pRedN-1 DNA (7.5 kbp), a fluorescent protein vector. The dendrons had 16 free surface amino groups attached to seven lysine groups, bound to a lipid core, one containing three C_{18} chains and the other a single C_{10} chain. Increased lipophilicity and molar charge ratios are key factors in producing compact and reproducible dendriplexes, shown by the hydrodynamic diameter which is of the order of 800 nm (p.d. > 0.5) at a 2:1 molar charge ratio, a value which decreases to around 200 nm at a 5:1 charge ratio. At lower charge ratios the dendriplexes are negative and have a zeta potential in order of −18 mV. As the ratio increases (5:1, 10:1) the complexes bear a positive potential (13 ± 2 mV). This suggests that at the 2:1 ratio the DNA is not fully condensed. The DNA was radiolabelled with ^{35}S dCTP (deoxycytidinetriphosphate) with the removal of the un-incorporated radiolabelled nucleotides. The encapsulation efficiency of dendriplexes in PLGA particles is higher than that for uncomplexed DNA. When the results are normalised for DNA content and particle surface area, complexation of the DNA was found to decrease release rate.

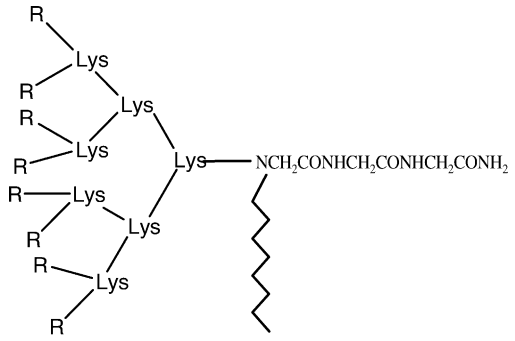
Keywords: Dendron; pDNA; Encapsulation; PLGA; Dendriplexes

Cationic polymers are routinely used as transfection agents for DNA delivery to cultured cells. These polycations interact with and condense DNA producing a complex with a small residual negative surface charge, and enhance cellular internalization of DNA, in part protecting the DNA from degradation (Panyam and Labhasetwar, 2003). Cationic dendrimers and partial dendrimers (dendrons) can also condense DNA. Important attributes of dendrons include the molecular weight, charge, hydrophobicity, flexibility, and geometry of the molecule (Sakthivel et al., 1998; Shah et al., 2000).

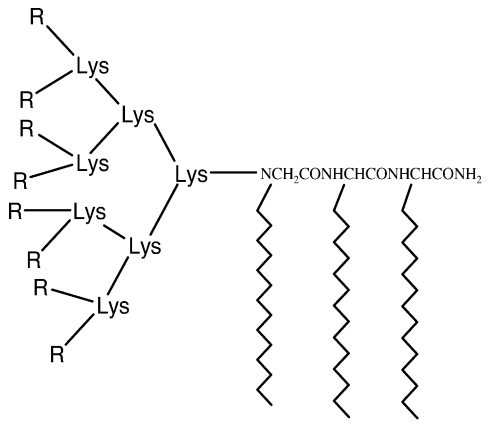
The two dendrons used in this work have a cationic branched asymmetrical polylysine dendritic

* Corresponding author.

0378-5173/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2005.03.036



$(C_{10})_1Lys_{15}(NH_2)_{16}$ **MW 2109**



$(C_{18})_3Lys_{15}(NH_2)_{16}$ **MW 2784**

Fig. 1. The simplified chemical structure of two cationic (lysine)-based dendrons (Lys = lysine): $(C_{10})_1Lys_{15}(NH_2)_{16}$, MW 2109 and $(C_{18})_3Lys_{15}(NH_2)_{16}$, MW 2784 both containing 16 amino groups and seven lysine groups attached to the core with one or three lengths of hydrocarbon chains.

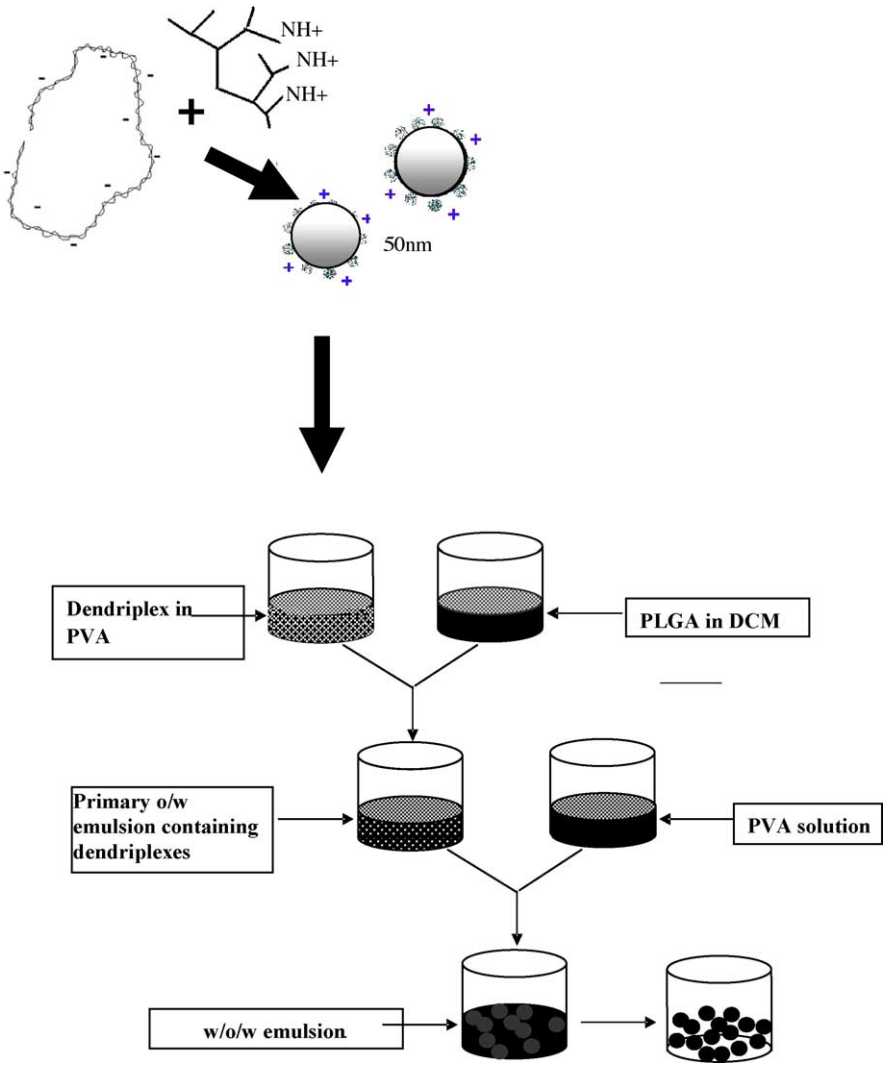
head group attached to a lipidic head group—one with a single C_{10} chain and the other with three C_{18} chains, as shown in Fig. 1. Both dendrons have 16 free amino groups on their dendritic surface attached to seven lysine groups. The dendrons were synthesized by stepwise solid-phase peptide synthesis from Boc-Lys(Boc)-OH and 2-amino octadecanoic acid (C_{18} dendron) and decanoic acid (C_{10} dendron), on 4-methyl benzhydrylamine (MBHA) resin using the Boc-strategy (Sakthivel et al., 1998).

Molar charge ratios of positively charged dendrons and negatively charged plasmid DNA were calculated according to the molecular weight and charge ratios of each dendron. A charge ratio 2:1 refers therefore to 2 positive charges (from the dendron) to 1 negative charge (from plasmid). DNA pRedN-1 (7.5 kbp) was used and purified using a Qiagen Endotoxin Free plasmid Mega kit according to the manufacturer's instructions. The DNA encodes far-red fluorescent proteins DsRed used for transient gene expression in mammalian cells. To label the plasmid DNA with ^{35}S dCTP, a modified nick-translation technique previously described (Ouahabi et al., 1997) was used. Briefly, plasmid DNA (10 μg/50 μl) was prepared from a stock solution (1 μg/μl) to be within the working range 20–200 ng/μl in TE (Tris–HCL, EDTA) buffer. The reaction mixtures of nucleotide/buffer containing 50 mM Tris–HCl pH 7.8, 10 mM $MgCl_2$, 2 mercaptoethanol, 100 mM of each dATP, dGTP, dTTP and enzyme solution (0.5 U/μl DNA polymerase I, 10 pg/μl DNaseI) and 250 μCi ^{35}S dCTP were added to pDNA. These conditions allow the incorporation of labelled nucleotide into the plasmid DNA without significant alteration of plasmid integrity, as assessed by agarose gel electrophoresis (data not shown). Unincorporated radiolabelled nucleotides are removed using gravity–flow chromatography with a maximum volume of 100 μl, prepacked with Sephadex G-50 DNA grade in distilled water with 0.15% Kathon CG/ICP Biocide.

A stock solution of 1 mg/ml of each dendron was made and subsequent volumes were removed depending on the concentration of DNA in this case 10 μg of DNA, and were separately diluted in 250 μl PBS mixed, and allowed to stand for 15 min.

The dendriplexes were characterized using a Philips CM 120 BioTwin transmission electron microscope; samples were stained with 1% uranyl acetate. Dendriplex hydrodynamic diameter and zeta potentials were measured using a Zetasizer 3000 (Malvern Instruments, Malvern, UK).

The dendriplexes were then separately encapsulated by a double emulsification method using a poly(vinyl)alcohol (PVA, 3% (w/v), Fisher, USA) solution added to PLGA (50:50) dissolved in DCM forming a primary emulsion after homogenisation as shown in Scheme 1. This o/w emulsion was added to a solution of PVA (1.25%, w/v) and further homogenised to form a w/o/w emulsion. The PLGA particles were harvested by centrifuging at 20,000 × *g* at 15 °C and



Scheme 1. Schematic diagram of methodology for dendriplex and dendriplex–PLGA particle formation. The dendriplexes formed by mixing the DNA and dendron, these particle are added to 3% (w/v) of PVA. The surfactant solution containing the dendriplexes are homogenised with PLGA (50:50) in DCM forming a primary o/w emulsion. A second concentration of 1.25% (w/v) PVA is homogenised with the primary o/w emulsion giving a w/o/w emulsion.

subsequently washed, then lyophilised to permit long-term stability.

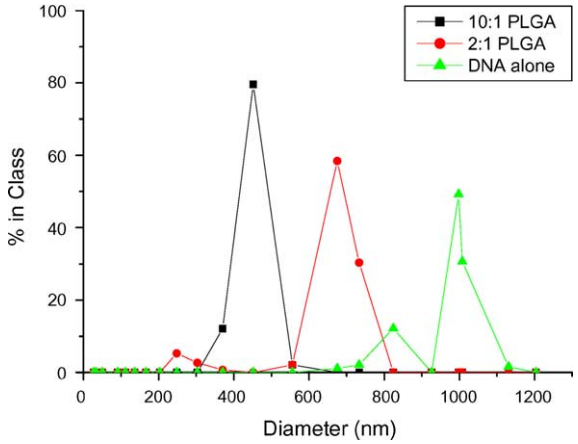
Radiolabelling pDNA allowed quantification of the encapsulation efficiency of the dendriplexes in PLGA particles. The particles were formulated as stated, they were harvested by centrifugation, washed to remove excess PVA and aliquots of the supernant were collected, and their radioactivity measured by scintillation counter. The particles were lyophilised and then dissolved in DCM and incubated for 30 min; distilled water was added forming organic-water phases and again aliquots taken and radioactivity measured (Table 1).

The nanoparticles (10 mg) were added to PBS (5 ml) (pH 7.4) at room temperature. Periodically, the supernatant was removed for testing and replaced with fresh solution. The DNA concentration in the supernant was quantified as in the encapsulation method.

Table 1
Encapsulation efficiency and mean size of PLGA particles containing C_{18} dendriplexes particles

Molar charge ratio of encapsulated species	DNA encapsulation (%, w/w)	Mean diameter (nm)	Polydispersity
10:1	15.6	451.6 ± 2.4	0.291
2:1	13.4	733.5 ± 7.2	0.391
DNA alone	9.9	998 ± 4.34	0.521

^{35}S radiolabelled DNA was mixed with varying ratios of C_{18} dendriplexes entrapped in PLGA, washed, and lyophilised. The particles were dissolved in DCM and incubated for 30 min; distilled water was added and mixed. Aliquots were taken measured in a scintillation counter. The mean size diameters of each set of particles were measured by re-suspending in distilled water (Zetasizer) as below.



The hydrodynamic sizes of both dendriplexes before and after encapsulation were measured (Fig. 2). The data shows that the molar ratio is important in the condensation process. A 10:1 C_{18} dendron:DNA ratio enabled complexes of less than 80 nm diameter are be formed (polydispersity, 0.26). The lipophilicity of the C_{18} dendron enhances the compaction and uniformity of the complexes. At a 2:1 ratio of both dendrons the apparent particle size of the dendriplexes are over 800 nm but this significantly decreases at the 5:1 molar ratio. The DNA is not fully condensed at the 2:1 ratio as evidenced by the size and the zeta potentials of -18 ± 5 and -12 ± 3 mV of the C_{18} dendron and the C_{10} dendron, respectively.

An agarose gel loaded with dendriplexes containing 10 μg of DNA at molar charge ratios of 0.5:1 to 10:1 and stained with ethidium bromide. DNA fragments were seen at the 0.5:1 but not at other ratios. As the ratios increased, the ethidium bromide was not able to intercalate with the DNA, confirming condensed structures had formed (data not shown).

The size of the dendriplex–PLGA particles (Fig. 2b) displays a similar trend, decreasing as dendriplexes with increasing charge ratio were encapsulated. The hydrodynamic size of PLGA–dendriplex particles are strongly related to the efficiency of DNA condensation. The 2:1 C_{10} dendriplex–PLGA particles are smaller in apparent size than its parent dendriplex. This is not the case for the C_{18} dendriplex where an increase in size may reveal higher encapsulation efficiencies. The C_{18} dendriplexes–PLGA particles were used for the encapsulation and release study. Cationic C_{18}-based dendriplexes are more efficiently encapsulated than native DNA having a size population of less than 1 μm and 10:1 ratio with mean size of 451.6 ± 2.4 nm. The particles were washed and 12% of DNA was removed from the systems containing DNA without complexation in comparison to 2% for the 10:1 molar ratio system. The release rate of radiolabelled DNA from the encapsulated dendriplexes at 2:1, 10:1, and native DNA particles was determined (Fig. 3a). An initial burst effect is seen in the first 24 h followed by a slower controlled release for all three sets of PLGA particles. Luu et al. (2003) measured the release of DNA from a porous scaffold using PLGA: an initial burst effect was also found, but between 18 and 36% DNA depend-

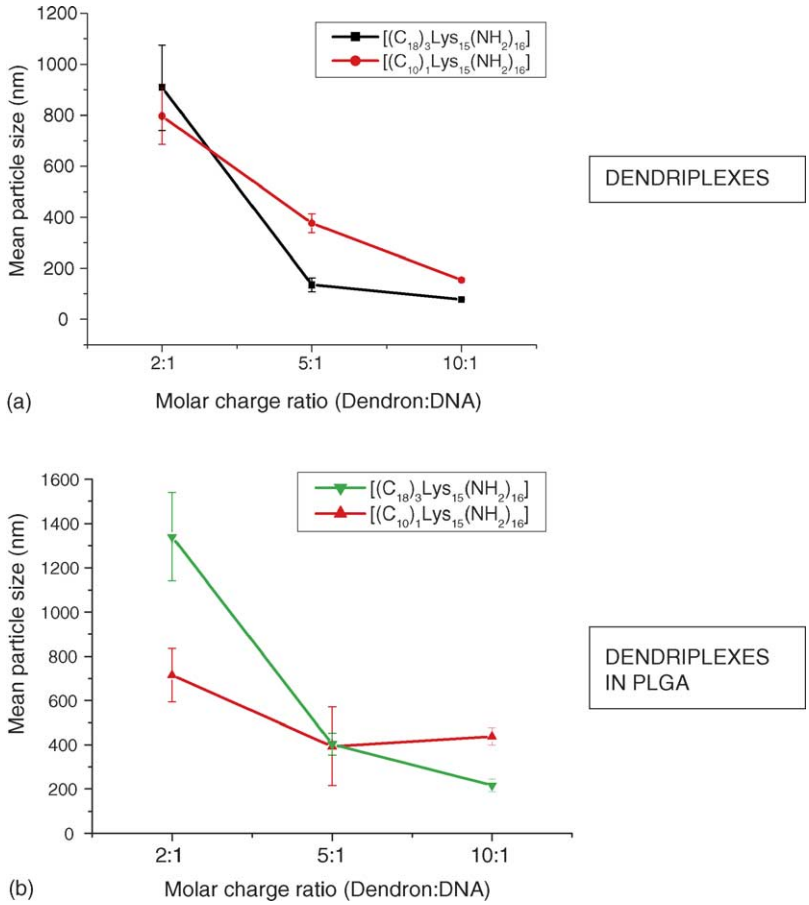


Fig. 2. (a) The relationship between the particle size of dendron/DNA complexes (dendriplexes) using the two different dendron structures and molar charge ratios (dendron:DNA) ($n = 3$) using a Zetasizer. (b) The relationship between particle sizes of PLGA particles containing 10 μg dendriplexes ($n = 3$) and molar charge ratios were measured after the nanoparticles were washed with distilled water, lyophilised and re-suspended prior to measurement.

ing on copolymer content was released after 15 min in aqueous suspension.

This study the particles containing DNA alone release 27% of DNA over 9 days, yet approximately 55% of DNA is released from particles containing a 10:1 dendriplex but when different DNA loads and particle sizes are taken into account as in Fig. 3b the particles containing the uncomplexed DNA have a higher rate of release compared to the dendriplexes. The morphological changes of the particles during dissolution were observed by SEM (Fig. 4). The 10:1 C_{18} dendriplex–PLGA particles which have a hydrodynamic diameter size of 510 ± 12 nm change shape and morphology after 24 h with a concave appearance, others "crumbling". The surface of the particles changes with increased porosity after 48 h, until finally they rupture. Gebrekidan et al. (2000) observed convective diffusion of pDNA through the porous structure of PLGA particles; and complexed pDNA/PLL was released in a more sustained fashion. The burst effect could be due to the porous nature of the PLGA around the dendron explaining the change in morphology. Similar morphological changes were also observed for the other of particle formulations.

The addition of lipidic dendrons condenses DNA, aiding efficient encapsulation into PLGA particles. The PLGA nanoparticles provide a system which protects DNA from degradation and provides sustained release

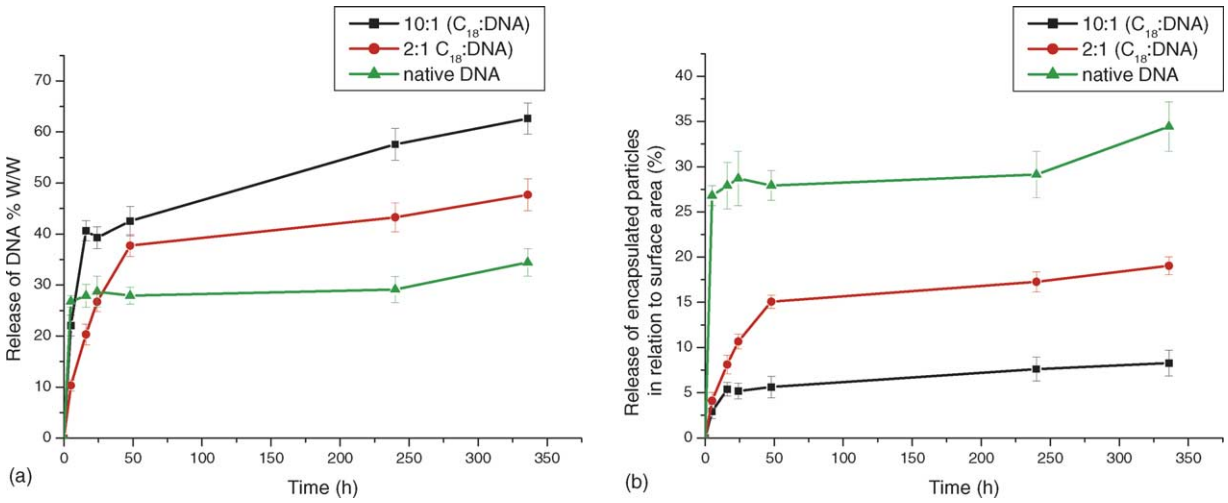


Fig. 3. (a) Cumulative release of radiolabelled DNA from PLGA particles containing entrapped dendriplexes with varying ratios of DNA/dendron of the isotonic PBS, pH 7.4. (b) The release of DNA normalised to a standard DNA content and particle size (that of the PLGA particles containing uncomplexed DNA).

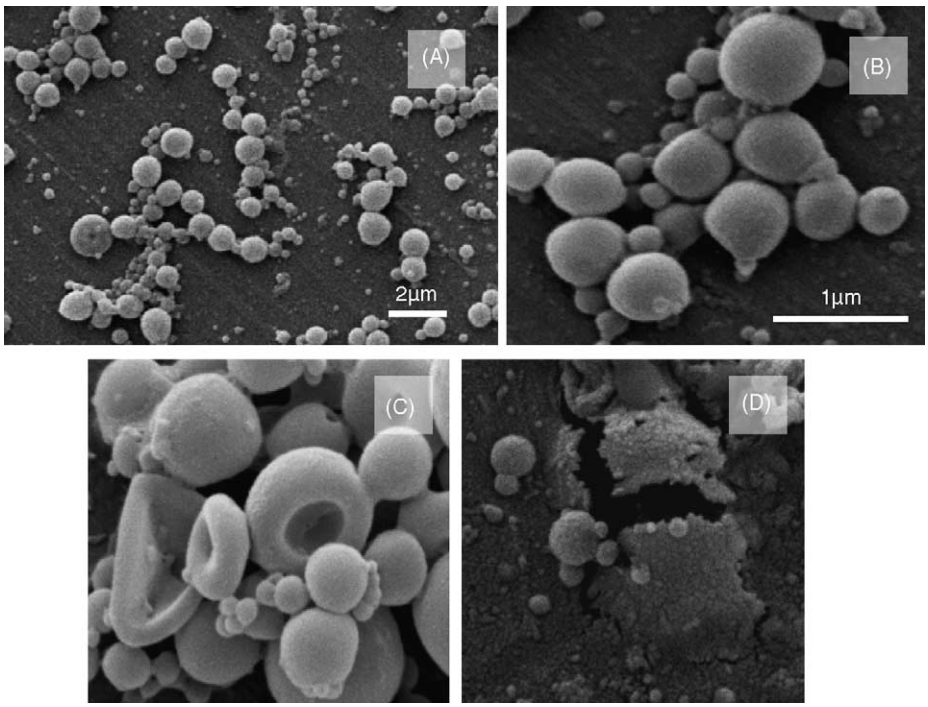


Fig. 4. Scanning electron micrographs showing a gradual degradation of PLGA particles containing dendriplexes. (A) and (B) 10:1 C_{18} dendriplex–PLGA particles at the initial time point, (C) after 24 h in PBS, while (D) is seen after 48 h.

of DNA. Studies are in progress using these formulations in animals.

Acknowledgements

SR received funding support for a Ph.D. studentship from The School of Pharmacy. Mr David McCarthy is thanked for assistance with the electron microscopy.

References

Gebrekidan, S., Woo, B.H., DeLuca, P.P., 2000. Formulation and in vitro transfection efficiency of poly(D,L-lactide-*co*-glycolide) microspheres containing plasmid DNA for gene delivery. AAPS PharmSciTech, 1 (article 28).

Luu, Y.K., Kim, K., Hsiao, B.S., Chu, B., Hadjiargyrou, M., 2003. Development of nanostructured DNA delivery scaffold via electrospinning of PLGA and PLA-PEG block copolymers. J. Contr. Release 95, 341–353.

Ouahabi, A.El., Thiry, M., Pector, V., Fuks, R., Ruysschaert, J.M., Vandenbranden, M., 1997. The role of endosome destabilizing activity in the gene transfer process mediated by cationic lipids. FEBS Lett. 414, 187–192.

Panyam, J., Labhasetwar, V., 2003. Biodegradable nanoparticle for drug and gene delivery to cells and tissue. Adv. Drug Delivery Rev. 55, 329–347.

Sakthivel, T., Toth, I., Florence, A.T., 1998. Synthesis and physicochemical properties of lipidic polyamide dendrimers. Pharm. Res. 15, 776–785.

Shah, D.S., Sakthivel, T., Toth, I., Florence, A.T., Wilderspin, A.F., 2000. DNA transfection and transfected cell viability using amphipathic asymmetric dendrimers. Int. J. Pharm. 208, 41–48.