

produced a PEG-PEI with the aim of reducing toxicity whilst retaining transfection efficiency of higher molecular weight PEI (Ahn et al., 2002). Toxicity was reduced (80 % viability of control) compared to 25 kDa PEI (40 % viability of control) and transfection efficiency higher than that of the starting Mw PEI (1.8 kDa) (Ahn et al., 2002). However, no direct comparison to 25 kDa transfection efficiency was made but PEG-PEI co-polymers were acknowledged to be less efficient (Ahn et al., 2002).

The first clinical trial using a polymeric vector, PEI, was published in 2005 (Ohana et al., 2004). Bladder cancer (two human subjects) was treated through bladder installation of a transurethral catheter and a > 75 % reduction in tumour size with no adverse side effects was reported (Ohana et al., 2004).

In the studies reported in this thesis PEI has been used as a positive control. Its cytotoxicity and transfection efficiency compared with that of the chitosan derivatives prepared. PEI was also used in some studies as an additional component in the development of an improved non-viral gene delivery system.

1.5.2.1.2 Chitosans: Natural Origin Cationic Polymers as Non-viral Vectors

Chitosan is a naturally occurring polysaccharide of β 1-4 linked *N*-acetyl-D(+)-glucosamine and D(+)-glucosamine. It is produced through the deacetylation of chitin, the extent of which is usually 40-100 % (reviewed in Thanou & Junginger, 2004). Chitin is found in the cell walls of fungi; this was where it was first discovered in 1811 by H. Braconnot who named it fungine (Domard & Domard, 2002). It is also found in the exoskeleton of insects, and crustacea (crab, shrimps) and was termed chitin, after the Greek word for coat, chitos, by C. Odier in 1823 after the discovery in the elytrum of the cock chafer beetle (Domard & Domard, 2002).

The main commercial source of chitin is shell waste from the food industry. Chitin is processed by treatment with 3-5 % (w/v) aqueous alkali (NaOH) at 80-90 °C to remove protein, this is followed by treatment with 3-5 % (w/v) aqueous acid (HCl) to remove inorganic materials (i.e. calcium carbonate). The purified chitin is deacetylated, producing primary amines, using 40 % sodium hydroxide at 120 °C for 1-3 h (Kumar, 2000).

Chitosan is widely regarded as being a non-toxic biologically compatible polymer (Corsi et al., 2003). It is approved for dietary applications in Japan, Italy and Finland (Illum, 1998) and it has been approved by the FDA for use in wound dressings (McCue, 2003). Chitosans (<5 kDa, 5-10 kDa and >10 kDa) were found to display little

cytotoxicity against CCRF-CEM (human lymphoblastic leukaemia) and L132 (human embryonic lung cells) ($IC_{50} > 1$ mg/ml) (Richardson et al., 1999). In contrast to most reports Carreno-Gomez et al. (1997) found chitosan (hydrochloride salt) to be relatively toxic ($IC_{50} 0.21 \pm 0.04$ mg/ml) against B16F10 (murine melanoma) cells. However, this study appears to have used solutions of the chitosan salts at the pH when dissolved, for chitosan HCl in PBS (10 mg/ml) pH = 5.8 (Carreno-Gomez & Duncan, 1997). At this pH the MTT assay is not appropriate (Sgouras, 1990) and cell viability is sure to be compromised. However, an appreciation that the salt form of the polymer will have an effect on its interaction with cells and macromolecules should be acknowledged (Wan et al., 2004). The reported safety makes chitosan attractive for gene delivery as other cationic molecules have relatively high toxicities, e.g. PLL (Merdan et al., 2002), PEI (Florea et al., 2002a). Chitosan is soluble at acidic pH, and precipitates in neutral/alkaline solutions; it is therefore problematic in this unmodified form in the preparation of complexes and in their administration.

Chitosan has membrane perturbant properties making it an interesting polymer for gene delivery (Chan et al., 2001). Polyplexes of pDNA with chitosan were investigated by Erbacher et al. (1998) who found that pDNA was condensed to 50-100 nm sized particles having donut or rod-like structures (Erbacher et al., 1998).

Chitosan-mediated transfection has been studied by many groups (Corsi et al., 2003, Hejazi & Amiji, 2002, Ishii et al., 2001, Kiang et al., 2002, Koping-Hoggard et al., 2003, Koping-Hoggard et al., 2001, Mao et al., 2001, Roy et al., 1999). In summary, it has been suggested that transfection efficiency of chitosan polyplexes is related to the nitrogen/phosphorus (N/P) ratio, polymer chain length, salt concentration (and type) at complex formation, cell type, time prior to harvesting and complex size/shape. The fact that many parameters influence the final gene expression may be indicative of the multi-step process being studied. Several barriers exist that must be crossed and a number of processes performed in order to achieve protein expression (Fig. 1.12). The N/P ratio is based on the number of nitrogen residues in the polymer and the number of phosphate residues in the DNA, it is approximately the same as a monomer molar ratio. Investigation of N/P ratios has found 3:1 (N/P) to be the most efficient for 40 kDa chitosan (>85 % deacetylated) in SOJ cells (Ishii et al., 2001). Similar results were found in 293 cells with 190 kDa chitosan (85 % deacetylated), where a polymer : DNA ratio of 3:1 was the most efficient ratio tested (Koping-Hoggard et al., 2001). This was also true in HeLa cells with 70 kDa chitosan (Erbacher et al., 1998). As seen for other polymers, chitosan molecular weight influences transfection efficiency.

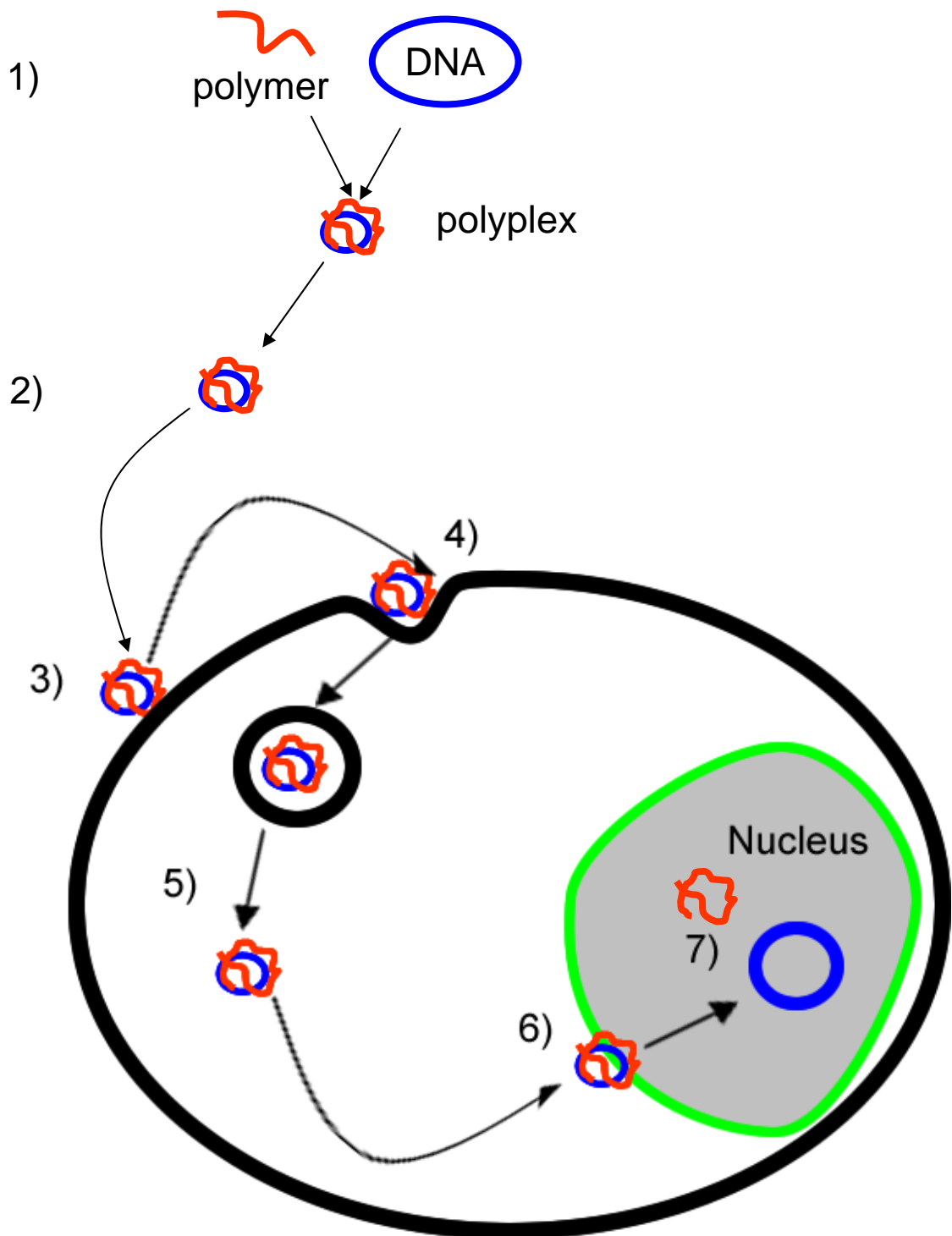


Figure 1.12 – Barriers to non-viral gene delivery

Key: 1) Achieving efficient plasmid condensation, 2) Protection of DNA from nucleases, 3) binding to cell, 4) endocytosis, 5) endosome escape, 6) nuclear entry, 7) release from vector (After Brown et al., 2001)

A molecular weight of 40 kDa was more efficient compared with 1 kDa and 84 kDa chitosans (Ishii et al., 2001). However, in other studies similar gene expression was found using 31 kDa and 170 kDa chitosans with 99 % deacetylation (Koping-Hoggard et al., 2001) indicating that molecular weight and degree of deacetylation must be taken into account. Roy et al. (1999) used 390 kDa chitosan to form nanoparticles (100-200 nm) that were used for oral gene delivery experiments. This indicates that, although molecular weight influences transfection, a wide range may be used (Koping-Hoggard et al., 2003, Roy et al., 1999). The buffer composition at complex formation also plays a key role in determining the morphology and subsequent transfection efficiency of complexes. Complexes prepared in water (pH 6.2) or 20 mM acetate buffer (pH 5.0) with increasing NaCl concentration were compared and more soluble globular particles were found in complexes prepared in acetate buffer (Koping-Hoggard et al., 2003). More aggregates were formed as NaCl concentration increased (Koping-Hoggard et al., 2003). Luciferase expression was found to be delayed, peaking at 72 h, in comparison with PEI polyplexes which peaked at 24 h post-transfection (Erbacher et al., 1998). It is important to note that different transfection efficiencies were observed in different cell lines using the same formulation (Ishii et al., 2001).

Modifications of chitosan have been made in an attempt to increase its transfection efficiency. Chitosan of 70 kDa (80 % deacetylated) was derivatised by Lee et al. (1998) with deoxycholic acid (a hydrophobic group) to give an amphiphilic molecule. This derivative formed pDNA complexes of 162 ± 18 nm diameter which successfully transfected COS-1 cells, although their efficiency was lower than lipofectamine (Lee et al., 1998). Chitosan modification with deoxycholic acid gave a self-aggregating derivative, the size and structure of polyplexes formed were dependent on the molecular weight of the chitosan used (Kim et al., 2001). A 40 kDa derivative produced the most efficient transfection of COS-1 cells (Kim et al., 2001), however, all these derivatives were highly affected by the inclusion of serum (Kim et al., 2001).

Domard et al. (1986) reported a new method for the quaternisation of chitosan and found that > 25 % quaternisation gave derivatives that were soluble at all pHs. Trimethylation of the amine groups to give quaternised amines gives a permanent positive charge and these derivatives have great potential for gene delivery applications (Murata et al., 1996). The application of trimethylation to oligomeric chitosan was first reported by Florea et al. (2002b) and Thanou et al. (2002). Trimethylated chitosan oligomer (TMO) was found to protect plasmid DNA from DNase 1 (Florea et al.,

2002b). It was also found that transfection efficiency was increased by an order of magnitude using TMO compared to chitosan in COS-1 (African green monkey kidney fibroblast) cells (Thanou et al., 2002). This favourable profile led to the study of trimethylated chitosans as the gene delivery vector in this thesis.

1.5.3 Targeted Non-viral Gene Delivery

Non-viral vectors are poorly efficient transfection systems when compared to viruses. It has been reported that at best, PEI polyplexes were taken up by 90 % of cells (30 mM) compared with 1 virus per cell being effective (Zaric et al., 2004). This has prompted many to investigate the possibility of incorporating targeting ligands into non-viral vectors with the aim of improving transfection efficiency.

1.5.3.1 Targeted Lipoplexes

Stealth liposomes are formed through the coating of the liposome with PEG, although this increases residence time in the body, cell uptake and therefore transfection efficiency is often lost. In an attempt to restore the activity of these stealth liposomes ligands are attached to them. When pegylated immunoliposomes carrying β -galactosidase (β -gal) were prepared containing a humanised anti-transferrin antibody and administered intravenously in Sprague Dawley rats, β -gal expression was found in the blood brain barrier, liver and spleen with little expression elsewhere (Shi et al., 2001). Although specific β -gal expression was claimed, the multiple sites at which expression was observed highlights the broad expression of transferrin receptor and raises questions over its value as a target.

Asialofetuin (a glycoprotein having tri-antennary galactose terminated sugar chains that targets ASGR) was included in a lipoplex containing the chloramphenicol acetyltransferase (CAT) gene and an increase (almost double) in CAT activity was observed in HepG2 cells (Hara et al., 1995). Similarly an anti-transferrin single chain antibody fragment bound to liposomes led to a 2-6 fold increase in transfection in H358, DU145, Hep3B and HT29 cells (Xu et al., 2002). Subsequent *in vivo* studies with p53 gene lipoplexes delivered via tail vein injection into tumour bearing mice and transfection assessed by Western blotting showed markedly enhanced p53 expression in DU145 tumours and low p53 expression found elsewhere (Xu et al., 2002). Integrin receptors were efficiently targeted using a 1,2-disteraoyl-sn-glycero-3-