Gene Ther Mol Biol Vol 8, 369-384, 2004

Transfection pathways of nonspecific and targeted PEI-polyplexes

Review Article

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Key words: PEI-polyplexes, transfection, DNase degradation, Interactions, cell surface, cell culture medium, specificity, efficacy, cell internalization, Endosome trafficking, proton-sponge effect, Cytoplasm transport, nuclear accession, dissociation

Abbreviations: Polyethyleneimine, (PEI); polylysine, (PLL); polyamidoamine dendrimers, (PAMAM dendrimers); epithelial growth factor, (EGF); basic fibroblast growth factor, (bFGF); 2-(dimethylamino)ethylmethacrylate, (pDMAEMA); transferrin-polylysine polyplexes, (Tf-pLL); poly-[N-(2-hydroxypropyl)methacrylamide], (pHPMA)

Received: 30 April 2004; Accepted: 24 June 2004; electronically published: September 2004

Summary

Polyethyleneimine (PEI) based vectors have become in an important vehicle for nonviral gene transfer. However, despite their extensive use and efficacy in the transfection of several cellular models both *in vitro* and *in vivo*, the mechanism by which they transfect cells has not been fully elucidated, and controversy remains over the interpretation of some apparently contradictory findings. A review is made of the studies on PEI polyplexes, focusing on PEI polyplex transfection properties (as physico-chemical characteristics important for transfection) and the mechanistic findings of PEI polyplex transfection comprising cell membrane binding with nonspecific and targeted–PEI polyplexes, the putative internalization pathways (such as the proton sponge hypothesis), the nuclear bioavailability of the transported nucleic acid, and other relevant issues such as the influence of polyplex size *in vitro* upon transfection activity.

I. Introduction

Specific and efficient delivery of nucleic acid into targeted cells is a priority objective of gene therapy. To achieve successful modification of the gene expression pattern, the exogenous nucleic acid must overcome a series of obstacles to gain access first to the cell and posteriorly to the intracellular compartments, where the nucleic acid exerts its function. Since nucleic acid uptake by cells is an inefficient process, it has been necessary to develop several strategies to increase nucleic acid delivery. One of the approaches is based on the use of nonviral vehicles such as liposomes (Wong et al, 1980; Alino et al, 1993), lipoplexes or nucleic acid-cationic lipid complexes (Felgner et al, 1997), and polyplexes (Gebhart and Kabanov 2001) - complexes of nucleic acids and cationic polymers such as polyethyleneimine (PEI) (Boussif et al, 1995). Due to its intrinsic transfection properties, PEI has been used to conform the backbone of a great number of vector formulations. Despite their widespread use and demonstrated efficacy in the transfection of several cellular models both *in vitro* and *in vivo*, the mechanism by which they transfect cells has not been fully elucidated, and controversy remains over the interpretation of some apparently contradictory findings. The present review discusses the hypothetical transfection pathways of PEI-polyplexes - from vector binding to the cell membrane to nucleic acid arrival in the nucleus, the influence of physico-chemical properties of PEI in transfection activity and other relevant issues such as the influence of polyplex size and cell type upon transfection activity, and the most relevant differences or similarities between PEI and other polymers used in transfection (fundamentally polylysine polyplexes).

II. Characteristics of PEI-polyplexes

A. PEI physico-chemical properties of importance for transfection

PEI is a synthetic polymer with a nitrogen-carbon base (32.5% nitrogen). Ethanolamine, the monomeric unit of PEI (CH_2-CH_2-NH-), confers great PEI solubility in

water and most polar solvents. The most prominent characteristic of PEI is its high positive charge density (20-25 mEq/g), which facilitates ionic interaction with negatively charged molecules such as nucleic acids, via the protonation of amine groups taken from the surrounding medium. This implies the existence of a correlation between PEI positive charge density and the pH of the medium, which (as we will see) largely accounts for the transfection properties of PEI. Two types of polyethyleneimine are used in transfection: branched PEI (mainly of molecular weights 25 and 800 KDa) (Boussif et al, 1995) and linear PEI (22 kDa) (Ferrari et al, 1997). Branched PEI has three kinds of amine groups – primary, secondary and tertiary - with an amine ratio of 1:2:1, respectively, while linear PEI amines are exclusively secondary. Thus, while linear PEI acquires its positive charge density through the protonation of secondary groups, branched PEI possesses additional primary amine groups for protonation. Based on the existing protonation profile, only every 5 or 6 amino groups are protonated a physiological pH (Suh et al, 1994). In addition to being most basic and also most reactive, the primary amine groups are amenable to chemical modification and have been used to covalently attach different types of molecules with the aim of conferring additional properties to the vector. Nucleic acid-PEI binding slightly changes the PEI protonation profile, one-half to one-third of the amine groups being protonated at physiological pH. Therefore, in contrast to other polymers such as polylysine (PLL), PEI possesses a great buffering capacity over a very wide pH range (Tang and Szoka 1997).

B. PEI-polyplex physico-chemical properties of importance for transfection

As has been commented, polyplex formation occurs as a result of ionic interaction between negative DNA charges provided by phosphate groups, and the positive charge of the cationic polymer (Kabanov and Kabanov 1995) - provided in the case of PEI by protonated amine groups. The size and shape of the resulting polyplex particles depends on the conditions under which they are prepared.

An important part of polyplex transfection activity depends on the polyplex physico-chemical characteristics. Therefore, characterization of the physico-chemical properties and knowledge of the parameters that can modify them could be very useful for predicting and defining the conditions of preparation capable of ensuring optimal transfection performance. The physico-chemical characteristics of polyplexes (structure, size, charge, capability of interaction with biomolecules) are largely dependent on factors inherent to the nature of the polycation (structure, molecular weight, charge density, etc.), but also on properties common to all polymers, such as the charge or mass ratio between polymer and DNA, and also on the characteristics of the solvent used for the electrostatic reaction – such as the ionic force (De Smedt et al, 2000). Of all physico-chemical parameters, the size of the complexes seems to be directly associated to transfection activity (Ogris et al, 1998), while the rest of parameters are relevant to transfection in the degree in which they affect polyplex size. The latter can vary from a few nanometers to several micrometers (Tang and Szoka 1997) - complexes of larger size being aggregates of particles of smaller size. Polyplex size depends on several parameters, such as the cation/anion ratio, DNA and polycation concentration, solution volume, and mixing speed. Moreover, size is greatly influenced by the presence of other electrolytes in the dissolution. Each of these factors will be analyzed separately below.

1. Influence of charge ratio

On examining the variation of size with respect to charge ratio, it is seen that under conditions of non-aggregation (preparation in water), low +/- charge ratios yield small particles. Size progressively increases until the neutralization charge is reached, and decreases again as the net positive charge increases – this being thought to favor solubility of the polyplex particles (Kabanov and Kabanov 1995; Tang and Szoka 1997; Pouton et al, 1998). In the case of PEI-polyplexes, complete condensation takes place from a N/P ratio of 2 or 3 (where N is the number of polymer nitrogen atoms and P the number of DNA phosphorus atoms), with the formation of neutral charge particles (Erbacher et al, 1999a). At these ratios, a tendency towards particle aggregation is observed. The compact particles of smaller size are generally obtained at higher N/P ratios, yielding polyplexes of positive net charge (Erbacher et al, 1999a). At N/P ratios generally used to obtain complete condensation (N/P >4), PEI/DNA complexes present a zeta potential of around + 30-35 mV (Kircheis et al, 1999; Ogris et al, 1999). With respect to shape, small polyplexes have revealed toroidal structures measuring between 40-80 nm, according to electron microscopic estimations (Tang and Szoka 1997) and dynamic light scattering studies (Ogris et al, 1998), as well as globular structures of up to 20-40 nm according to estimations of atomic force microscopy (Dunlap et al, 1997). In comparison, large-size polyplexes are generally spherical or aggregates of micrometric size.

2. Influence of preparation conditions

The preparation conditions greatly influence polyplex size and structure, mostly at aggregation level. The most relevant factors are salt concentration and the concentration of DNA and PEI before and after preparation. In general, polyplexes formed in saline solutions are larger than those formed in water (low ionic force) (Tang and Szoka 1997; Ogris et al, 1998; Kwoh et al, 1999), and size can moreover change over time (Ogris et al, 1998). In addition, even when polyplexes are formed under conditions of low ionic force, and despite the presence of the strong positive polyplex charge, many polyplexes (such as those composed of PLL) effectively aggregate when added to saline solutions of physiological concentration (Pouton et al, 1998). This aggregation tendency is probably related to a decrease in the real zeta potential due to the presence of saline electrolytes (Tang and Szoka 1997). According to these authors, this behavior is partially dependent on the type of cationic

polymer involved. For example, PLL polyplexes and polyamidoamine dendrimers (PAMAM dendrimers) tend to form aggregates, whereas PEI polyplexes and fractured dendrimers (starburst dendrimers) are more resistant to aggregation (Tang and Szoka 1997).

Other authors have demonstrated the importance of DNA and polymer concentration. For example, PLL polyplexes aggregate when the DNA solution is highly concentrated (400 μg/ml), and do not aggregate when the DNA concentration is lower (Duguid et al, 1998). This tendency to aggregate at certain concentrations is frequent in almost all polymers. When equal volumes of prediluted polymer and DNA are used, differences in transfection effectiveness associated to the sequence of the addition of the reagents are scantly relevant (Kircheis et al, 2001c; Wightman et al, 2001), though when the concentrations are high, the mixing order becomes relevant. Thus, transfection activity *in vitro* was found to be 10-fold greater when the polymer (PEI) was added to the plasmid DNA (drop by drop) than when the inverse approach was adopted, i.e., adding the DNA to the polymer (Boussif et al, 1995, 1996). Such differences were in fact associated to differences in the size of the polyplexes prepared in one or other way (larger polyplexes being the most efficacious) (Ogris et al, 1998).

3. Influence of PEI type

While there do not seem to be important differences in zeta potential between polyplexes formed with the different types from PEI (i.e., linear versus branched and high versus low molecular weight)(Kircheis et al, 2001b), the influence of PEI type upon particle size is remarkable under certain preparation conditions. For example, while at low ionic force the sizes of polyplexes prepared with different types of PEI (linear and branched, with different molecular weights) seem to be quite invariable, the behavior of branched and linear PEI polyplexes clearly diverges when the complexes are formed at physiological ionic force. While complexes formed with branched PEI (25 or 800 kDa, indistinctly) are small (50-80 nm) or medium-sized (100 to some hundreds of nm), depending on the DNA concentration, complexes formed with linear PEI of molecular weight 22 kDa conform large aggregates – the size increasing with incubation time (Kircheis et al, 2001b). The same behavior is observed when linear 22-kDa polyplexes initially prepared in a medium without salts are later added to a saline medium (Goula et al, 1998b; Kircheis et al, 2001b; Wightman et al, 2001). As can be expected, these differences in size between linear and branched PEI polyplexes exert a great influence upon transfection activity. In some cell types, the transfection activity of linear PEI of molecular weight 22 kDa is similar to that of branched PEI (Demeneix et al, 1998) (Poulain et al, 2000), whereas in others it is remarkably greater (Poulain et al, 2000; Wightman et al, 2001) – this phenomenon being attributed to the greater size of linear PEI polyplexes when prepared in saline medium. This advantage disappears when the complexes are prepared in nonsaline medium that avoids aggregation (HBG, 5, glucose). In this medium, both linear and branched PEI-polyplexes are small and of similar size (Poulain et al, 2000; Wightman et al, 2001).

4. Influence of PEI molecular weight

The first studies of the influence of molecular weight in transfection, involving both linear and branched polyplexes, pointed to the existence of an optimum molecular weight (around 20-25 kDa) at which PEI polyplexes show improved transfection performance (Demeneix et al, 1998; Fischer et al, 1999; Godbey et al, 1999b; Jeong et al, 2001). At higher and lower molecular weights transfection efficacy decreases. Some authors have tried to explain this molecular weight dependency. It has been postulated that low molecular weight constructs show poorer transfection either because they are more unstable and more easily dissociable in saline medium (Papisov and Litmanovich 1988) than high weight constructs, or because their endosomal release capacity is less (Boussif et al, 1996; Kircheis et al, 2001c). The slight decreasing tendency in transfection efficacy for molecular weights larger than 20 kDa is attributed to increased polyplex toxicity (Bieber and Elsasser 2001). Nevertheless, the optimum molecular weight range seems to differ from one cell line to another. Such differences are attributed to an increase in toxicity with growing molecular weight, and to variable cell sensitivity to PEI.

C. Protection against DNase degradation

One of the consequences derived from polyplex formation is nucleic acid protection from degradation by nucleases. Practically all cationic polymers are able to afford variable DNA protection against DNase degradation once the polyplex has been formed (De Smedt et al, 2000) - PEI being one of the most protective polymers (Kircheis et al, 2001c; Moret et al, 2001; Guillem et al, 2002b). This property is of vital importance for transfection activity *in vitro* and *in vivo*, since it allows protection of the nucleic acid from intracellular (endolysosomal digestion) as well as extracellular degradation (through serum nuclease action).

D. *In vitro* transfection properties of PEI-polyplexes

The *in vitro* transfection activity of polyplexes is influenced not only by the intrinsic properties of the latter (as described above), but also by other inherent factors associated to the transfection process, such as polyplex concentration, incubation time, polyplex interaction with the culture medium, and the type of cells used (Boussif et al, 1996). It is difficult to establish systematic comparisons between the transfection activities of different polyplexes, since there is a great variety of cationic polymers, and the optimum transfection conditions vary from one polyplex system to another, as well as from one cell line to another. Perhaps two of the most exhaustive studies comparing the transfection activity of nonspecific polyplexes are those carried out in the 3T3 (Demeneix et al, 1998) and Cos-7 cell lines (Gebhart and Kabanov 2001), employing several

polyplexes - including PEI. According to these studies, PEI and PAMAM polyplexes present the best transfection activities, compared with other polymers, at least in these cell lines. In reference to PEI, the transfection activity *in vitro* has been established in a broad variety of cells. The first form of PEI used for gene transfer was the branched form with a molecular weight of 800 kDa, applied to different cell lines and tissues, as well as in local administration to the brain (Boussif et al, 1995). Posteriorly, these authors described PEI (branched 800 kDa type) mediated transfection in 25 different cell types, including 18 human cell lines as well as primary rat and pig cells (Boussif et al, 1996; Demeneix et al, 1998). Branched PEI of low molecular weight (25 kDa) was introduced soon afterwards (Abdallah et al, 1996), affording superior transfection efficacy and toxicity versus the high molecular weight form. In fact, this form of branched PEI has allowed the transfer and expression of genes incorporated to large gene constructs, as is the case of the artificial 2300-kb chromosomes (Marschall et al, 1999). Such results had not been obtained up until that time with any other type of vector. These two branched forms of PEI have been used with significant efficacy in terms of cell transfection, and have been the standard forms of PEI employed for nucleic acid transference (Godbey et al, 1999a). The linear PEI form was developed soon afterwards (Ferrari et al, 1997). As has been mentioned above, it displays some significant differences in transfection profile (not only *in vitro* but also *in vivo*) that can be interesting for certain applications. However, despite the well demonstrated transfection activity of PEI polyplexes and their widespread use as a regular tool for transfection in different laboratories, our understanding of the PEI transfection process remains incomplete. In the following section we review the mechanistic findings of transfection with PEI polyplexes.

III. Mechanisms of the *in vitro* transfection process with PEI polyplexes

This section describes the pathway of PEI polyplexes in the transfection process, from polyplex addition to the cell culture to arrival of the nucleic acid in the nucleus. To make understanding easier, the section has been divided into different sections referring to the most relevant stages of the polyplex pathway, including interaction with the cell culture medium and the subsequent cellular barriers (cell membrane, endosome-lysosome, cytoplasm and nuclear envelope), and other important issues (influence of particle size, targeting, etc.) in the context of each phase.

A. Interaction with cell culture medium

Once the polyplex has been prepared, the next step consists of polyplex incubation with cells. Polyplex interaction with elements of the cell culture medium (ions, anionic proteins from serum) can originate structural changes in size and surface charge that in turn can affect transfection activity. Although polyplexes generally seem to be less sensitive to serum than lipoplexes (Gebhart and Kabanov, 2001), the presence of serum can reduce or even increase the transfection activity of some polyplexes- concretely when serum absence or presence produces changes in polyplex size. Some authors (Guo and Lee, 2001) have suggested that the inhibiting role of serum on transfection is associated to the stabilization of small PEI polyplexes (of smaller transfection efficacy), in a way similar to what happens with lipoplexes (Turek et al, 2000). According to this hypothesis, initially large complexes or initially small complexes that increase in size on coming into contact with the culture medium, would be resistant to serum inhibition. The influence of polyplex size upon transfection activity is discussed in greater detail in the following sections.

B. Interactions between polyplexes and the cell surface

It can be considered that *in vitro* transfection begins with polyplex interaction with the cell membrane. Different forms of membrane interaction can be defined: nonspecific interactions with receptors or other components of the cell membrane (such as proteoglycans), and specific interactions with membrane receptors (Godbey and Mikos, 2001). The type of interaction depends on whether the polyplex is targeted or not, and on the cell type involved in transfection.

1. Nonspecific cell interaction of untargeted polyplexes

It is generally accepted that the interaction of an untargeted polyplex with the cell essentially consists of an ionic interaction between the positive polyplex charges and the negative charges of the cell membrane (Kabanov and Kabanov, 1995). Specifically, it is thought that polyplex interaction with the cell surface takes place fundamentally with sulfated proteoglycans, which are negatively charged proteins present in the membrane (Kjellen and Lindahl 1991). Evidence to this effect is provided by the fact that cell treatment with heparinase and chondroitinase (enzymes that degrade proteoglycans) or the use of mutant cell lines deficient in proteoglycan production dramatically inhibits transfection with PLL polyplexes (Mislick and Baldeschwieler). A similar mechanism is postulated for other polymers including as PEI polyplexes. Recent studies indicate that such interactions with the membrane proteoglycans are decisive not only in the interaction process, but also in subsequent polyplex internalization (Kircheis et al, 2001a). These studies suggest that the transfection differences observed between different cell types are associated to the levels of proteoglycan expression (Mislick and Baldeschwieler 1996; Labat-Moleur et al, 1996; Godbey and Mikos 2001; Wiethoff et al, 2001). If this were the case, and since several cell types are characterized by low or nil proteoglycan expression (e.g., hematopoietic cells), the latter can be considered difficult to transfect with nonspecific polyplexes (Ogris et al, 2000), and transfection in these cell types would thus require the incorporation of additional elements to the polyplex construct in order to promote cell interaction.

2. Specific cell interaction of targeted polyplexes. Influence of targeting upon vector properties: specificity, efficacy, cell internalization

Considering the need to improve polyplex specificity and efficacy, effort has centered on combining and even exchanging nonspecific interaction between polyplexes and the cell surface via a specific cellular internalization mechanism, by incorporating ligands attached to the vectors. The development of targeted polyplexes has as main aim their application to *in vivo* therapy, where selectivity in gene delivery is particularly important. Nevertheless, *in vitro* targeting, in addition to testing the selectivity of a possible ligand for subsequent *in vivo* use, is especially interesting when transference through nonspecific interaction is very low. This is the case of cells that grow in suspension, such as lymphocyte derived cell lines, whose proteoglycan expression is very low and nonspecific polyplex transfection fails (Ogris et al, 2000). In the case of PEI-polyplexes, it has been demonstrated that the incorporation of targeting elements not only contributes to improve the specificity of delivery but also increases the activity of transfection in different cell lines (Erbacher et al, 1999b).

In general, targeted polyplexes have been based on the covalent attachment of a targeting element to the polymer, PLL and PEI being the most commonly used elements. This strategy began with the experiments of Wu et al, (1987), which targeted complexes of asialoorosomucoid-PLL/DNA to the asialoglycoprotein receptors of hepatic cells. Other ligands frequently used for selective nucleic acid delivery are: a) transferrin (Wagner and al.), whose receptor is abundant in tumor cells (Wagner et al, 1990; Cotten et al, 1993); b) galactosylated ligands (Plank et al, 1992) or asialofetuin (Dasi et al,) for hepatocyte targeting; c) epithelial growth factor (EGF) (Chen et al, 1994, Cristiano and Roth 1996) and basic fibroblast growth factor (bFGF) (Sosnowski et al, 1996) for targeting lung cancer cells; and d) antibodies that recognize specific membrane elements, such as anti-PECAM (platelet endothelial cell adhesion molecule), for selective transference to endothelial cells (Li et al, 2000). In this last group, one of the best developed models is based on specific gene transfer to T cells using antibodies against membrane antigens that are expressed fundamentally in these cells, such as JL1 (Suh et al, 2001), CD3 (Erbacher et al, 1999a; O'Neill et al, 2001) and CD4 (Puls and Minchin, 1999).

Although in some models these targeted polyplexes have produced interesting results, the need for specific synthesis of the vector for each target cell greatly limits their use and increases the economic cost - especially when a monoclonal antibody is used as targeting element. A more versatile targeting method is based on the use of the streptavidin-biotin system, which had been previously used to prepare targeted immunoliposomes (Alino et al, 1999). In this case, targeted gene delivery was based on the attachment of biotinylated antibodies (against membrane antigens) on the cell surface, with the subsequent addition of polyethylenimine-avidin-DNA complexes to interact with cell-attached antibodies (Wojda and Miller, 2000) through the specific avidin-biotin interaction. The *in vitro* transfection results in terms of effectiveness obtained with this procedure are limited, though the main disadvantage is that for further *in vivo* development, complete vector assembly must be made before administration.

Taking these previous studies as reference, we attempted to construct a targeted polyplex (which we have called immunopolyplex), the salient characteristic of which is the possibility of easily replacing the targeting element, leaving the polyplex backbone intact. Streptavidin protein was thought to be attached covalently to PEI, acting as a bridge molecule for direct binding of biotinylated proteins (targeting elements) to the vector. The streptavidin-biotin system is considered to allow targeting element replacement without complicated protocol modifications, avoiding the need for specific synthesis of the vector for each case, and moreover allowing considerable savings in time and money. Since a great amount of biotin-labeled antibodies against membrane antigens are commercially available, they can easily be used to determine the most suitable targeting element for many targeted nucleic acid strategies. Due to the therapeutic interest and difficulty of hematopoietic cell gene transfer, our work with immunopolyplexes has focused on the transference of genes and oligonucleotides to cell lines of hematological origin, which proved hard to transfect through nonspecific pathways. Thus, we selected as targeting elements several biotinylated antibodies that specifically recognize some membrane antigens of hematopoietic cells. Initially we started with a set of commercial biotin labeled antibodies against the following antigens: CD4 and CD3 for T lymphocyte targeting, CD19, CD20, CD21, CD22 for B lymphocyte targeting and CD45 and CD71 for panlymphocytic targeting. The best results were obtained with immunopolyplexes carrying CD3 antibody for T cell transfection (Guillem et al, 2002a, 2002b) and CD19 antibody for B cell transfection (Guillem et al, 2002b) (**Figure 1**). We found that immunopolyplex activity was fundamentally specific and mediated mainly through specific antigen-antibody interaction, and that anti-CD3 immunopolyplex is more efficacious in T cells than anti-CD19 in B cells (4- or 5-fold in terms of the percentage of positive cells, and 6- to 12-fold in terms of fluorescence intensity per cell). In this case, abundance of antigen could be a parameter for partly explaining observed differences in transfection activities: we found CD3 in T cell line (Jurkat) to be about 3-fold more abundant than CD19 in B cell line (Granta 519). However, this is not the only parameter to be taken into account for explaining or predicting transfection activities in general. As some authors have suggested (O'Neill et al, 2001), the efficiency of transgene expression could be affected by signaling events following antibody-antigen interaction. For example, we observed that although CD45 is 4-fold more abundant than CD3 in Jurkat cells, transfection with anti-CD45 immunopolyplexes displayed poor results (data not shown). The lack of transfection is probably related to the notion that CD45 does not internalize upon antibody binding, as previously reported (van der Jagt et al, 1992). In this case, although anti-CD45 immunopolyplex does bind to CD45 membrane antigen,

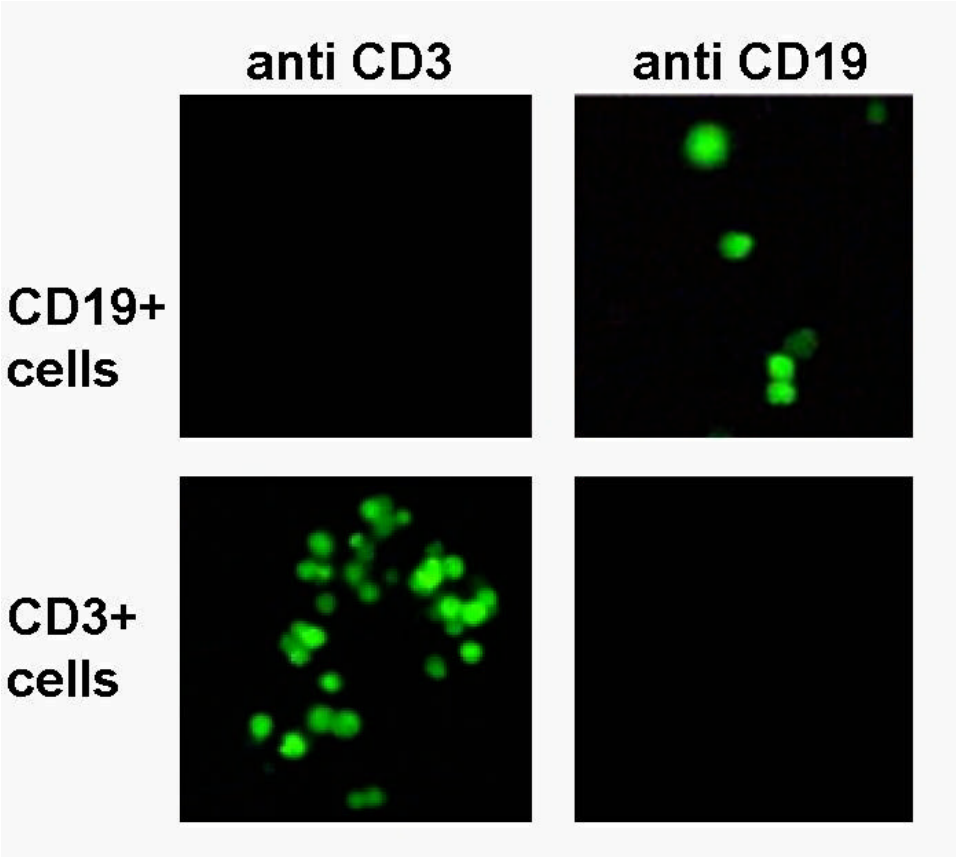

Figure 1: Fluorescence imaging of EGFP transfection with immunopolyplexes. Granta 519 B cell line (CD3-/CD19+, up) and Jurkat T cell line (CD3+/CD19-, down) were transfected with p3CEGFP (5 mg/ml), employing anti-CD3(left,up and down) and anti-CD19 (right, up and down) immunopolyplexes as vehicles . The imaging shows cells seen under fluorescence microscopy 24 h after transfection.

its internalization might not be promoted. In the case of CD3, the fact that CD3 antibody binding stimulates cell proliferation can be taken to constitute a collateral effect favoring transfection efficacy, since it eliminates the nuclear membrane in the transfection period. Conversely, antibodies that after antigen binding stimulate cell apoptosis, such as CD20 (Cardarelli et al, 2002), would dramatically impair the transfection process by eliminating targeted cells. All these facts should be taken into account when designing a targeting model, though when the antigen-antibody profile is not known, antibody screening could easily be performed with immunopolyplex until the most suitable targeting option is identified.

C. Polyplex internalization: size does matter

Although endocytosis is accepted to be the general mechanism responsible for cellular internalization of polyplexes (Kircheis et al, 1997; Godbey et al, 1999c), the term comprises very different forms of internalization, including fluid phase endocytosis (Remy-Kristensen et al, 2001), nonspecific absorptive endocytosis (Labat-Moleur et al, 1996; Mislick and Baldeschwieler 1996), phagocytosis, macropinocytosis (Remy-Kristensen et al, 2001), and receptor mediated endocytosis (Boussif et al, 1996; Ogris et al, 1998). The first studies of polyplex internalization mechanisms were performed with transferrin-polylysine polyplexes (Tf-pLL)(Cotten et al, 1990; Zenke et al, 1990; Wagner et al, 1991). These authors reported important *in vitro* transfection with small particles (diameter ≤100 nm), and suggested that clathrin-coated pits were implicated in receptor mediated endocytosis (Wagner et al, 1990). Without further evidence, it was quickly assumed that this mechanism could be the preferential internalization route for other polyplexes and, at the same time, that it should restrict the internalization of complexes greater than 100 nm. This correspondence seemed to be satisfied by PLL (Wagner et al, 1991) and pDMAEMA (2-(dimethylamino)ethyl methacrylate) polyplexes (van de Wetering et al, 1998), since complexes of a few hundreds of nanometers transfected better than those of micrometric size. Subsequent research with PEI-polyplexes (Ogris et al, 1998) demonstrated that polyplexes of great size can also benefit from specific internalization mediated by receptor, resulting in even greater transfection levels than with small constructs (Ogris et al, 1998; Wightman et al, 2001).

In an attempt to account for these apparently contradictory findings, some authors have suggested hypotheses to explain the relation between transfection efficacy and construct size. One hypothesis suggests that larger (and therefore heavier) polyplexes settle upon the cells, creating a greater local polyplex concentration which would force interaction with the cells. In contrast, small

polyplexes remain in suspension and their contact with the cells would be more limited (Boussif et al, 1996; Ogris et al, 1998). This hypothesis is sustained by the fact that on promoting sedimentation of small polyplexes over cells by centrifugation, transfection efficacy increases (Boussif et al, 1996). This hypothesis assumes that there are no significant internalization differences between large and small PEI-polyplexes, since if the internalization of large polyplexes were greatly impaired, the effect of the higher local concentration could be neutralized. This explanation by itself, which could help account for the differences with PEI-polyplexes, fails to explain the behaviour of polyplexes in general - since it does not account for PLL polyplexes behaving in exactly the opposite way, i.e., large polyplexes transfect worse than small constructs. It could be argued that the assumption that PEI and PLL polyplexes follow the same internalization pathway has not been demonstrated, since some authors have proposed different internalization pathways for PEI and PLL polyplexes (Godbey et al, 2000), and these could be influenced differently by polyplex size. Moreover, the influence of size upon transfection seems to be strongly dependent on the type of cell involved, though the PEI and PLL polyplex experiments mentioned above were performed in the same cell line model (K562 cells).

Another proposed explanation suggests that the reduced transfection efficacy of small PEI-polyplexes is due to their lesser capacity to destabilize the endosomes compared with larger PEI-polyplexes. Since PEI is though to behave as a proton sponge that destabilizes the endosome (Behr 1996)(see the following section), these authors assume that a critical minimum amount of PEI must reach the endosome to cause its rupture, and suggest that small PEI-polyplexes do not contain sufficient polymer to promote endosome disruption as effectively as the larger constructs. This hypothesis is supported by the observation that the transfection efficacy of small particles increases in the presence of lysosomotropic agents (chloroquine or endosomolytic peptides), whereas the efficacy of large particles is not substantially modified (Ogris et al, 1998).

In the case of polylysine, and since the latter does not exert an intrinsic destabilizing effect upon the endosome, large particles would not have an advantage over small ones in relation to endosomal release, and transfection efficacy would fundamentally depend on internalization effectiveness - where small polyplexes supposedly would be favored by the possibility of using the clathrin coated pit internalization route. In support of this explanation, some studies of the kinetics of internalization of fluorescent labeled transferrin PEI-polyplexes show that while small polyplexes are rapidly and fully internalized, those of great size remain attached to the membrane and are internalized more slowly (Ogris et al, 2001b). Still, total fluorescence and membrane binding fluorescence are greater in the case of the large polyplexes that for the small particles–thus supporting the hypothesis postulating a greater local concentration of large polyplexes. On the other hand, although relative internalization is less efficient in the case of large polyplexes, the associated transgene expression is eleven times greater than in the case of the smaller constructs. This supports the hypothesis of an increased endosomal release for large PEI polyplexes.

In our studies of PEI polyplex characterization, we have observed that when PEI-polyplexes of different sizes are treated with DNase I, the large complexes (N/P ratios close to charge neutrality) totally protect plasmid DNA from degradation, while the smaller ones (high N/P ratios) experience discrete cuts in the DNA sequence (Guillem et al, 2002b). This would occur because a small particle would have more DNA exposed at the polyplex surface per unit mass than a larger particle – thereby increasing the probability of exposure of some DNA regions at the polyplex surface, with increased accessibility to nucleases. We hypothesize that this same process may occur at intralysosomal level, and can partly explain the transfection advantage of large polyplexes versus small constructs.

Probably the influence upon transfection efficacy of all these processes would be the sum of the contribution of each individual effect, favoring transfection in one of the stages (internalization, endosomal release, access to the nucleus), while impairing it in others.

Regarding the upper polyplex size limit for penetrating the cell, there are at least two alternative possibilities. One option is to accept that penetration occurs via the internalization of polyplex particles in large vesicles. This hypothesis receives growing support from many studies that show that polyplexes (targeted or not) with a size of hundreds of nanometers and of micrometric size (Pouton et al, 1998), or even aggregates or precipitates (such as DNA complexes with calcium phosphate or DEAE-dextran), are able to transfect cultured cells (De Smedt et al, 2000). Some authors have even detected the endocytosis of large polyplex particles using electron microscopy (Bieber et al, 2002). Retaining the hypothesis of small particle endocytosis as preferential internalization mechanism, the other possibility would be to admit that large polyplexes might not be internalized entirely, but could remain attached to the external cell membrane surface - as suggested for transfection with large fluorescent labeled transferring PEI polyplexes (Ogris et al, 2001b) - and would then be internalized as smaller fragments detached from the large ones. Both processes could coexist, and the variable predominance of either could depend not only on particle size, but also on polyplex type, and the cell type involved (Kircheis et al, 2001c). In fact, some authors (Remy-Kristensen et al, 2001) have observed that in certain cells (EAhy 926 cells), small PEI-polyplexes, initially homogeneously attached to cell membrane, migrate to particular areas of the cell surface, yielding large aggregates that are further taken up in vesicles several micrometers in size (macropinocytosis). In contrast, in other cells (L929 fibroblasts), the same polyplexes are quickly and homogeneously internalized by submicrometric endosomes (fluid phase endocytosis).

D. Endosome trafficking. The proton-sponge effect: influence on the transfection efficacy of PEI-polyplexes

It is believed that after internalization, the particles

are directed towards the lysosomal route to be degraded (Klemm et al, 1998; Lecocq et al, 2000). For most polycations such as polylysine, accumulation and degradation in the endosomal compartment is an important obstacle in the transfection process (Mislick et al, 1995), and explains the relatively low levels of transfection obtained. Different strategies have been developed to overcome this obstacle, such as the addition of lysosomotropic agents (e.g., chloroquine) (Erbacher et al, 1996) to the culture medium, or the use of membrane destabilizing peptides (Plank et al, 1994) or inactivated viral particles possessing endosomolytic activity (Curiel et al, 1991) and which can be added to the medium or bound to the vector.

Nevertheless, some polycations such as PEI and PAMAM fractured dendrimers (starburst dendrimers) do not require lysosomotropic agents to exhibit substantial transfection *in vitro* (Haensler and Szoka 1993; Kukowska-Latallo et al, 1996; Tang et al, 1996; Tang and Szoka 1997). In these cases, the addition of chloroquine has little or no effect. Attempts have been made to explain this behavior through the proton sponge hypothesis, which assumes that PEI and fractured dendrimers are able to buffer the endolysosomal pH and cause endosome disruption via osmotic swelling (Berh 1996). The key to the proton sponge effect would be the degree of protonation of the polycation amine groups. Whereas at physiological pH the amine groups of PLL are fully protonated (pKa between 9 and 10), the amine groups of PEI and the starburst dendrimers are only partially protonated. Consequently, after endocytosis of such polyplexes (PEI or PAMAM), the amine groups are able to uptake protons from the acidic endosomal interior, which is thought to buffer endosomal pH and induce proton accumulation within the endosome–this in turn being coupled to a simultaneous flow of chloride anions towards the interior. The above authors on one hand hypothesize that the net increase in ion concentration would lead to a massive water input, with swelling and ultimately rupture of the endosome, while on the other hand it is postulated that increasing PEI protonation could contribute to its separation from DNA via the repulsion of internal positive charges - thereby contributing to polyplex dissociation (Berh). However, the authors did not take into consideration that the presence of negative DNA charges can compensate the increase in the PEI protonation, and therefore the internal cationic repulsion effect. Besides, other investigators report that the differences in transfection efficacy between PEI and PLL cannot be sustained on the buffering effect of PEI upon lysosomal pH, because according to their measurements the intralysosomal pH of cells transfected with PEI-polyplexes remains unaltered (Godbey et al, 2000; Forrest and Pack 2002). In any case, the different authors interpret their respective findings in different ways. Thus, according to Godbey el al., the increased effectiveness of PEI with respect to PLL is explained by the capacity of PEI to avoid the lysosomal degradation route followed by PLL polyplexes. These authors accordingly proposed different intracellular processing mechanisms for each type of polyplex (Godbey et al, 1999a; Godbey et al, 2000). On the other hand, Forest et al, maintain that it is necessary for PEI-polyplexes to be exposed to an acidic environment (endosome-lysosome fusion) in order to achieve endosome DNA release. Moreover, they observe no trafficking of PLL-polyplexes towards lysosomes in some cell lines. Again, different routes for PEI and PLL polyplexes are postulated, though in this case the situation is opposite that proposed above. Uncertainty therefore remains about the intracellular fate of polyplexes and their endolysosomal processing.

Apart from such discrepancies regarding the particle processing mechanisms, there seems to be general agreement that knowledge of the relation between PEI-polyplexes and intralysosomal pH is critical for understanding PEI polyplex transfection activity. We therefore decided to further investigate the influence of pH upon the interaction between DNA and PEI. To this effect, we added PEI polyplexes to solutions at different pH (from 3.5 to 12) and studied the intensity of the resulting interaction between DNA and PEI based on a fluorescence decay assay (Guillem et al, 2002b). Our results indicate that the intensity of interaction between DNA and PEI decreases at basic pH and is enforced at acid pH values. Considering the physico-chemical properties of PEI, this seems logical, since at acid pH values PEI positive charge increases and its capacity to interact with negatively charged DNA should also increase. In contrast, as pH becomes less acidic, the PEI positive charge decreases, and DNA-PEI ionic interaction can be expected to decrease gradually, releasing DNA. These data suggest that, at intracellular level, an acidic environment, far from stimulating the dissociation of PEI-DNA complexes (which, if lysosomal pH is not modified by PEI polyplexes, would threaten DNA integrity in the lysosome), seems to actually strength PEI-DNA interaction - and this could contribute to protect DNA from lysosomal degradation.

Another point still far from being clarified is how polyplexes leave the endosomes. While some authors have used electron microscopy to detect endolysosomal microrupture (Bieber et al, 2002), other investigators have failed to detect any endolysosome vesicle alterations (Remy-Kristensen et al, 2001) - even when transgene expression is subsequently achieved. Again, the results obtained seem to depend on the cell type involved. In any case, if endosome disruption effectively occurs, it appears to be on a non-massive scale, since the phenomenon has been only scarcely detected. Nevertheless, the fact that peptides such as melittin, which has endosomolytic properties (thus contributing to nucleic acid release into the cytoplasm), increase the transfection efficacy of PEI polyplexes (Ogris et al, 2001a) speaks in favor of the convenience of promoting endosomal release.

E. Cytoplasm transport and nuclear accession

In reference to cytoplasmic transport, some authors who have studied the dependence of inert particle cytoplasmic diffusion upon size, concluded that particle mobility is effectively dependent upon particle size – those measuring more than 54 nm presenting impaired diffusion

(Luby-Phelps et al, 1987). Nevertheless, it has been found that large particles can migrate through the cytoplasm not only by diffusion, but also via other mechanisms in which cytoskeletal components such as the microtubuli or actin filaments are involved, thereby facilitating polyplex transport (Meyer et al, 1997). Accordingly, if finally PEI polyplexes are released into the cytoplasm following endosomal disruption, they theoretically could be transported to the nucleus – especially those particles measuring less than 54 nm in size. With respect to the nuclear envelope, one aspect that suggests the latter to be an important barrier for nucleic acid transference to the nucleus is the fact that when cells are allowed to undergo mitosis after adding polylysine (Brunner et al, 2000) or PEI polyplexes (Brunner et al, 2000; Remy-Kristensen et al, 2001), these are transfected much more efficiently than when the cell cycle has been arrested. Thus, it can be concluded that mitosis (and consequently nuclear dismantling) facilitates transfection – this being the reason why superior transfection efficacy is generally obtained with rapidly proliferating cells than in cells that either do not divide or do so only slowly. Nevertheless, since some cells that do not divide can be transfected, there must be mechanisms for penetrating the nucleus in the presence of the nuclear envelope.

Some authors have proposed that polyplex entry to the nucleus could involve polyplex fusion with the nuclear membranes, mediated by polyplex interaction with the negatively charged membrane phospholipids (Godbey et al, 1999c). According to these authors, at a certain moment during polyplex trafficking, the particles could establish contact with phospholipids - those synthesized continuously for membrane regeneration or those from the endosomal membrane. In any case, polyplexes could become coated with a lipid envelope and perhaps on interacting with the phospholipids of the nuclear envelope, the coated polyplexes could finally fuse with the nuclear membranes and thus access the interior of the nucleus.

Another potential route for polyplex access to the nucleus that would not imply nuclear envelope modification or rupture is based on the existence of the nuclear pores. In this context, while pore diameter is 80 nm, pore structure leaves free only a central water channel of 9 nm - though particles up to 28 nm in diameter can be transported to the nucleus via the activation of transport mechanisms that imply energy consumption (Nigg, 1997). It is therefore theoretically possible for small polyplex particles (less than 28 nm in size) to access the nucleus through the nuclear pores. The nuclear importation of molecules larger than 40,000 Da (generally proteins) is known to be highly selective and depends on the presence of a short amino acid sequence called a nuclear location signal (NLS)(Newmeyer 1993). For this reason, with the aim of facilitating nuclear delivery, and this improving transfection efficacy, many polyplex formulations also incorporate nuclear location sequences (Branden et al, 1999; Zanta et al, 1999) or peptides such as melittin (Ogris et al, 2001a) which in addition to possessing endosomolytic activity also has nuclear targeting properties. Improvements in transfection efficacy associated to the use of NLS suggest that polyplexes can at least partially benefit from transport mechanisms through nuclear pores.

F. PEI polyplex dissociation within the nucleus: nuclear availability

In order for vehiculized nucleic acid to modify gene expression (by means of a transgene or oligonucleotides), it is assumed that the non-nucleic component in general, or the cationic polymer in the case of polyplexes, must separate from the nucleic acid at some point. In the case of lipoplexes, the use of fluorescent labeled DNA and lipids has shown that whereas labeled DNA appears in the nucleus, the cationic lipids do not. This suggests that lipoplex disassembly takes place before the DNA reaches the nucleus (Marcusson et al, 1998). However, in the case of polyplexes, the evidence suggests that the polymer (fundamentally PEI) not only accompanies the nucleic acid to the nucleus but moreover targets it to the latter (Boussif et al, 1995; Pollard et al, 1998; Godbey et al, 1999c; Wightman et al, 2001). Thus, in experiments involving cytoplasmic injection, the DNA vehiculized in polyplexes produced an increase in the portion of DNA released into the nucleus (up to 10-fold in the case of PEI-polyplexes) with respect to naked DNA (Pollard et al, 1998).

Internalization experiments in certain cell models involving fluorescent PEI administered either alone or forming part of polyplexes have revealed preferential fluorescence location in the nucleus (Godbey et al, 1999c). With respect to disassembly, the destination seems to depend on the nucleic acid size. Thus, in the case of oligonucleotides, some evidence indicates that the latter separate from the polymer (PEI) once within the nucleus (Dheur et al, 1999; Guillem et al, 2002a). In our transfection experiments with immunopolyplexes or untargeted PEI-polyplexes carrying FITC labeled oligonucleotides in Jurkat (non-adherent cells) and B16 (adherent) cells, respectively, we observed that the initially quenched fluorescence of oligo-FITC in the polyplexes (at 95% to N/P 10) is progressively recovered once polyplex or immunopolyplex has been incorporated into the cell and disassembled - a process which can be seen with fluorescence microscopy (**Figure 2**). Fluorescence is located mainly in the nucleus in both models (**Figure 3**), thus indicating that targeting does not alter the intracellular processing of polyplexes - though the kinetics are different (immunopolyplex trafficking being faster).

In the case of PEI polyplexes carrying plasmid DNA, it seems that although the former reach the nucleus, most polyplexes remain undissociated. This at least is the interpretation of the experiment conducted by Godbey and coworkers (Godbey et al, 1999c). In effect, when PEI and nucleic acid are labeled with green and red fluorescent probes, respectively, and polyplex is subsequently formed, the fluorescence observed is yellow–thus indicating that the green and red probes are located sufficiently close to allow fluorescence overlapping. When the intracellular route of these labeled PEI-polyplexes is followed, fluorescence labeling in the nucleus is seen to be mainly of a yellow color (undissociated polyplexes) - though some green and red dots (corresponding to dissociated

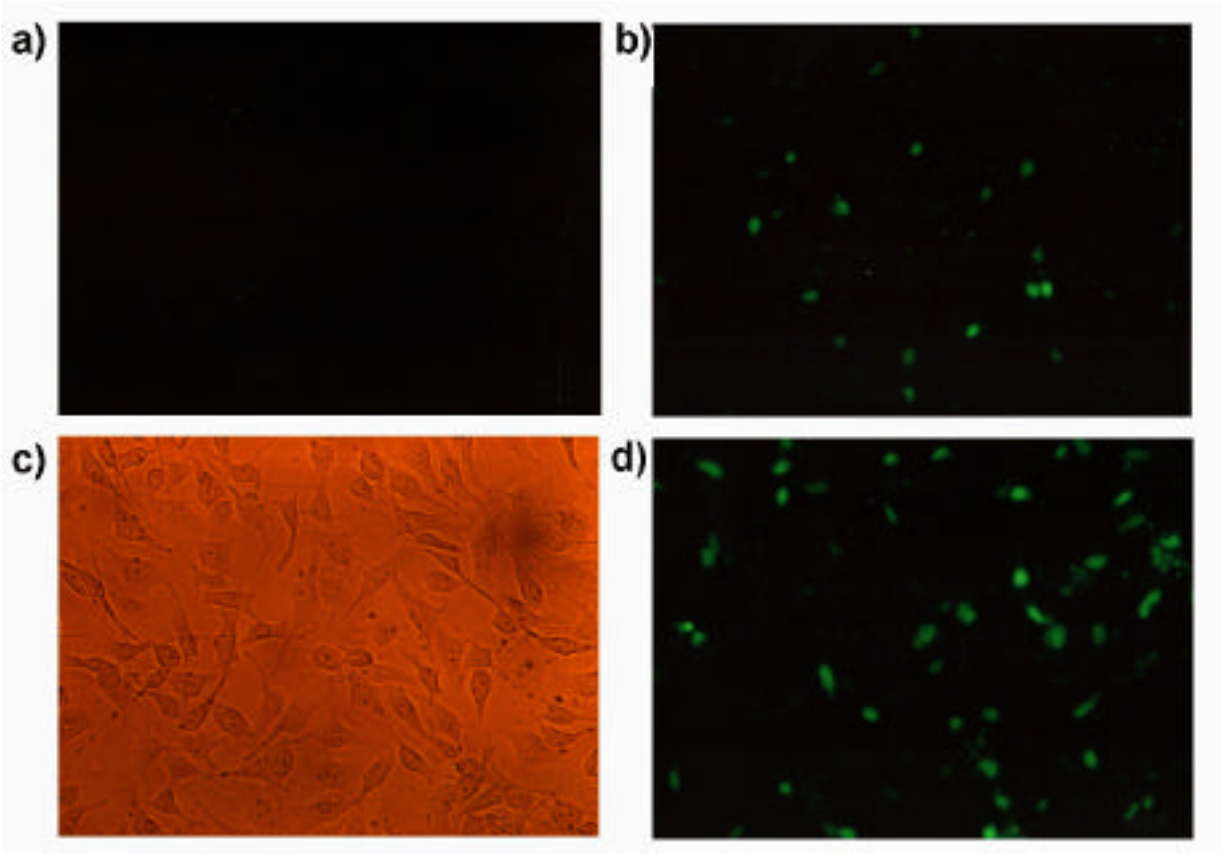

Figure 2. Imaging of B16 cells treated with PEI-polyplexes bearing FITC labeled oligonucleotides. Cells were visualized under fluorescence microscopy at 0 (a) 6(b) and 24 hours(d) after PEI polyplex addition (c ,cells seen under transmitted light).

complexes) appear extranuclearly. However, as mentioned in previous sections, there is evidence to suggest that polyplex destination is strongly dependent upon the cell type involved (Remy-Kristensen et al, 2001; Bieber et al, 2002). In many studies it has not been possible to detect the presence of exogenous DNA in the nucleus (either along or accompanied by PEI), though transgene expression has been detected (Remy-Kristensen et al, 2001; Bieber et al, 2002). This on one hand indicates that it is not possible to know whether in these cases the expressed DNA has reached the nucleus in free form or accompanied by the polymer. On the other hand, it suggests that the presence of many DNA copies is not needed to ensure transgene expression (this being the reason why transgene expression observed in the Godbey nuclear location experiment could be due to the small proportion of DNA dissociated from the polymer). This hypothesis is reinforced by the observations of direct DNA injection experiments: only 10 copies per nucleus sufficed to achieve transgene expression (Pollard et al, 1998). Nevertheless, the fact that direct polyplex injection (PEI or PLL polyplexes with a small number of transgene copies in different cell types) into the nucleus affords transgene expression, and that this does not happen with lipoplexes (Pollard et al, 1998), indicates that polyplex can be disassembled, at least partially, within the nucleus. This in turn generates new questions, however: Is it possible for exogenous gene expression to occur without polyplex disassembly? Without discarding that exogenous gene expression could originate from a small part of plasmid molecules that can be released, the possibility exists that the transcription machinery (as if PEI were the cationic nuclear proteins associated to genomic DNA), could temporarily separate DNA from polymer – this in turn being sufficient to allow transgene transcription. To date, the limited experimental evidence in this direction is provided by the work of Bieber and coworkers (Bieber et al, 2002). In order to verify whether PEI-DNA interaction could be a critical stage for transfection, these investigators conducted tests of *in vitro* transcription with PEI polyplexes, observing that transcription is not altered by the presence of the PEI. This speaks in favor of the hypothesis of transcriptional disassembly.

IV. *In vivo* transfection of polyplexes

Although it was not our aim to conduct an in-depth review of the mechanisms of polyplex *in vivo* transfection, a summarized account will be provided of some critical aspects of PEI that could be important for understanding the transfection profile of PEI-polyplexes *in vivo*, compared with other polymers also used *in vivo*.

In vivo gene expression mediated by polyplexes was first reported by Wu and coworkers (Wu et al, 1991) in a murine model of gene transfer to the liver using polyplexes based on galactosylated PLL. Despite the time elapsed since these first results were published, only few subsequent reports have appeared involving the use of

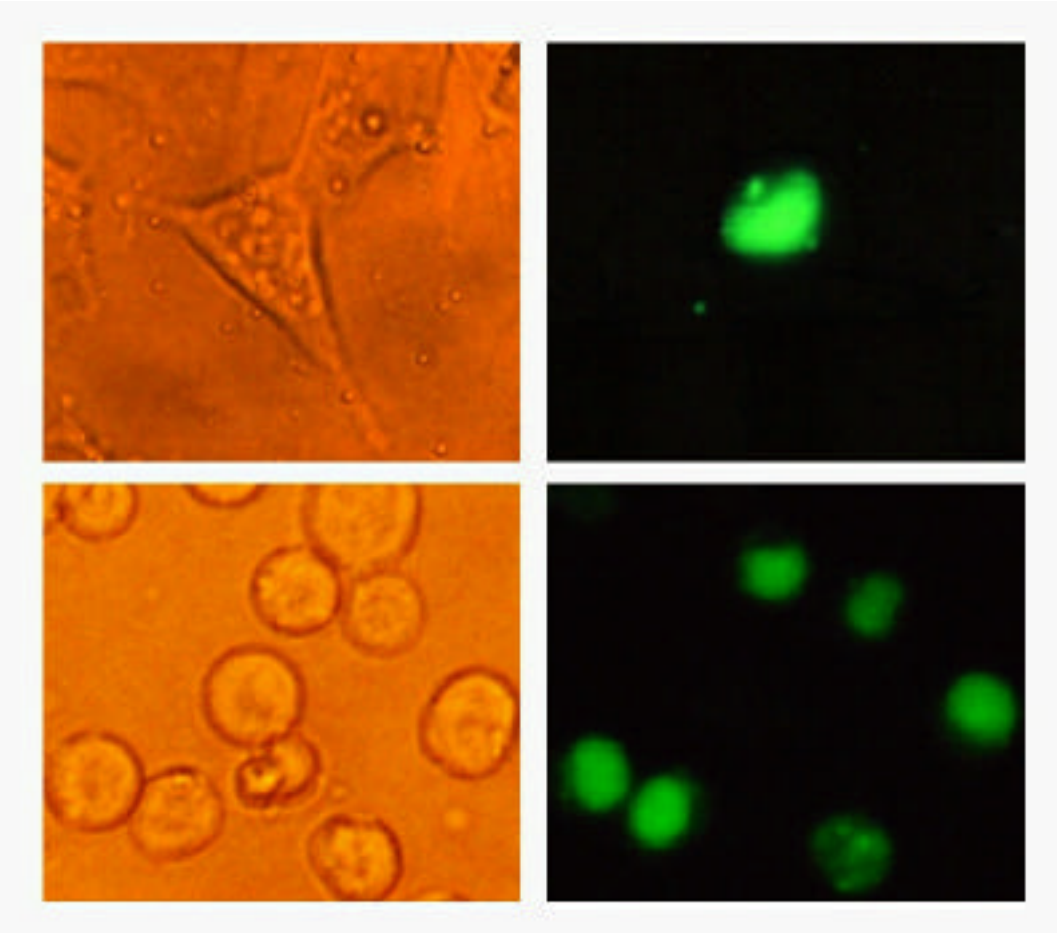

Figure 3: Nuclear localization of oligo-F transferred with PEI based vectors. Imaging of B16 cells (Up) transfected with PEI-oligo polyplexes (24 after trnasfection) and Jurkat cells (down) transfected with anti CD3 immunopolyplexes (6 h after transfection) (left,transmitted light; right , fluorescent light)

polyplexes *in vivo*, and with limited success (De Smedt et al, 2000). Regarding PEI polyplexes, the more successful systemic administration models refer to lung gene transfer (Goula et al, 1998a), though in this case transfection depends on the formation of aggregates that cause pulmonary capillary obstruction secondary to microthrombus formation (Chollet et al, 2002). One of the important and specific obstacles of *in vivo* gene transfer is systemic clearance, i.e., polyplex elimination from blood before the particles are able to cross the vascular endothelial fenestrations and interact with the target tissues. The two main characteristics controlling the systemic stability of nonviral vectors in general, and of polyplexes in particular, are particle size and surface charge. In order to overcome this important problem, several works have been conducted with the aim of obtaining small-size polyplexes. By varying the type of polymer, the preparation conditions, the zeta potential, the nucleic acid-cationic polymer ratio, and via the addition of other molecules, it has been possible to reach sizes of under 200 nm for almost all kinds of polyplexes (Erbacher et al, 1998). Even with such small-size polyplexes, interaction of the latter with serum proteins (Dash et al, 1999) and/or later activation of the complement system (Plank et al, 1996), induces the formation of large particles that are recognized by the macrophage elimination system. In order to avoid charge mediated aggregation, covering of the positively charged surface of the polyplexes has been performed. Some of the more widely used covering molecules are hydrophilic polymers, mainly polyethyleneglycol (Lee et al, 2002; Lim et al, 2000; Ogris et al, 2001b), and to a lesser extent poly-[N-(2-hydroxypropyl)methacrylamide] (pHPMA) (Toncheva et al, 1998), anionic lipids (Mastrobattista et al, 2001), and even the targeting elements themselves - as is the case of galactose, (Hashida et al, 1998), the asialoorosomucoids (Kwoh et al, 1999) or transferrin (Ogris et al, 2001b). In general, the coated polyplexes exhibit a neutral or negative zeta potential (surface charge), in addition to much lesser binding to anionic proteins and scant induction of serum aggregation compared with uncovered polyplexes (Kircheis et al, 1999; Ogris et al, 1999).

On the other hand, with the purpose of increasing *in vivo* transfection efficacy and specificity, several targeting elements have been incorporated to the polyplexes. One of the best worked models is the targeting to the liver of PLL polyplexes, with the use mainly of ligands that are recognized and internalized by the hepatic receptors of asialoglycoproteins, such as the asialoorosomucoids (Wu et al, 1991; Chowdhury et al, 1993), natural glycosidic residues like galactose (Perales et al, 1994; Nishikawa et al, 1998; Wu and Wu 1988) or mannose (Nishikawa et al, 2000), and glycopeptides (Merwin et al, 1994).

Another of the *in vivo* systemic administration

models affording improved results involves gene transfer to tumors with PLL or PEI polyplexes targeted with transferrin and EGF (Frederiksen et al, 2000; Kircheis et al, 2001b). Polyplex targeting with antibodies has been applied for *in vivo* transfer to respiratory epithelium. Different targeting elements, such as anti-PECAM, an antibody against PECAM1 (platelet endothelial cell adhesion molecule 1) (Li et al, 2000) attached to a PEI backbone, or the Fab fragment of polyclonal antibodies with specificity for the polymeric Ig receptor abundantly expressed in cells of the pulmonary epithelium, attached to PLL backbone (Ferkol et al, 1995) have been used. Another approach has been the search of alternative routes to systemic administration, including local administration by direct addition of polyplexes over the targeted tissues or organs. One type of polymer used *in vivo* via local administration is represented by the fractured dendrimers. The latter have been used for the transfer of a gene with immunosuppressor activity, with the purpose of prolonging graft survival in a murine model of heart transplantation (Qin et al, 1998), obtaining good results. Also chitosan has been used in pulmonary local administration with a good toxicity profiles and good transfection efficacy (Koping-Hoggard et al, 2001). However, PEI is the polymer offering the greatest success and efficacy *in vivo* via local administration. PEI-polyplexes have been used for nucleic acid transfer to different organs including the kidneys (Boletta et al, 1997), brain (Boussif et al, 1995; Lemkine et al, 1999), lungs (Ferrari et al, 1997; Ferrari et al, 1999), and tumors in diverse locations (Coll et al, 1999; Aoki et al, 2001). However, few clinical tests have been conducted based on nucleic acid transfer with polyplexes. This shows that the field is still in its beginnings, and development will depend on the improvement of polyplex formulations for *in vivo* use.

V. Conclusions

As we have seen, PEI based vectors have become important nonviral gene transfer vehicles, mostly because of the intrinsic properties of PEI. In effect, the latter is positively charged, thus allowing it to interact spontaneously with polyanionic nucleic acids and form stable polyplex particles that can interact with cell membrane; PEI protects DNA from degradation; and it allows linker molecule binding (through its primary amine groups), which in turn facilitates further covalent coupling of several elements that can improve the transfection profile of the vector in terms of efficacy and specificity, such as targeting proteins, nuclear localization sequences, etc. The transfection pathway of PEI polyplexes has not been fully elucidated, but they seem to follow an endocytic route in which PEI protects DNA from lysosomal degradation and promotes accession of DNA to the nucleus.

Further efforts are needed to achieve better results with *in vivo* use, including improvements in the toxicity profile and stability in blood circulation, as well as other aspects involving *in vivo* nucleic acid transfer efficacy and specificity.

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