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Design of methacrylate-based polyplexes for tumor targeting

Review Article

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Abbreviations: fetal calf serum, (FCS); gene-directed enzyme prodrug therapy, (GDEPT); immuno-LPP, (ILPP); lipopolyplexes, (LPP); mononuclear phagocyte system, (MPS); poly(aspartic acid), (p(Asp); polyethylene glycol, (PEG); polymer poly(2-(dimethylamino)ethyl methacrylate), (p(DMAEMA))

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Summary

A non-viral gene delivery vector has been developed in our laboratory based on the cationic polymer poly(2-(dimethylamino)ethyl methacrylate) (p(DMAEMA)). This contribution deals with the design of pDMAEMA-based polyplexes for tumor targeting. The first part is concerned with their use for the intraperitoneal therapy of ovarian cancer, the second part with their use for intravenous targeting of solid tumors. It is demonstrated that cell-specific gene delivery to *in vitro* cultured ovarian carcinoma cells can be obtained by coating p(DMAEMA)-based polyplexes with an anionic lipid layer bearing conjugated antibody fragments. As the lipid coat around the so-called lipopolyplexes (LPP) efficiently shields the positive charge of polyplexes, the predominant electrostatic interaction with cell membranes could be avoided. As LPP without antibody did not show transfection, it can be concluded that the presence of a targeting ligand is essential. In addition, the lipid coat around the LPP provided protection of the polyplexes against destabilization by polyanions such as poly(aspartic acid) and hyaluronic acid. This is expected to be essential for *in vivo* application of antibody-targeted LPP as naturally occurring polyanions have been shown to have detrimental effects on plain polyplexes after intraperitoneal administration. After intravenous administration in mice, p(DMAEMA)/[^{32}P]-pLuc complexes distributed primarily to the lungs. The gene expression profile matched the biodistribution profile. *In vitro* evidence was collected pointing to aggregate formation and trapping of the formed aggregates in the lung capillary bed as a primary mechanism explaining the dominant lung uptake and transfection. Therefore, it was investigated whether shielding of the surface positive charge of the polyplexes can increase colloidal stability and prevent dominant lung uptake. Recent mice experiments yielded successful results with surface modification of the p(DMAEMA)-based polyplexes with polyethylene glycol (PEG). Prolonged circulation and avoidance of dominant lung localization were observed after intravenous administration of the PEGylated polyplexes. Most importantly, a significant degree of tumor targeting was observed in the subcutaneous C26 colon carcinoma mouse model.

I. Introduction

The future of cancer gene therapy is dependent on the development of efficient gene delivery systems. Within the realm of non-viral gene delivery systems are complexes of plasmid DNA with cationic lipids or polymers, called lipoplexes or polyplexes, respectively. Within our laboratory, the application of the cationic polymer poly(2-(dimethylamino)ethyl methacrylate (p(DMAEMA)) as polymeric transfectant is currently under investigation.

p(DMAEMA) (**Figure 1**) is able to bind and condense DNA (Cherng et al, 1996; Van de Wetering et al, 1997, 1998). *In vitro* p(DMAEMA) was shown to be an efficient transfection agent for a variety of cell types (Van

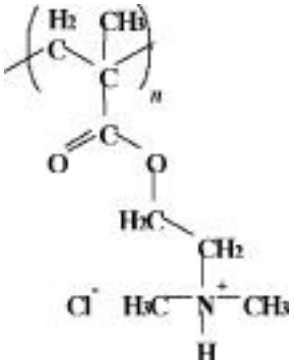

Figure 1. Poly(2-(dimethylamino)ethyl methacrylate (p(DMAEMA))

de Wetering et al, 1997). The size and zeta potential of the polyplexes were shown to be dependent on the polymer/plasmid ratio and important parameters determining the *in vitro* transfection activity and cytotoxicity. Polyplexes with a positive zeta potential (around 30 mV) and a mean size around 0.2 μm possessed the highest transfection activity. p(DMAEMA) polymers with a high molecular weight (> 300 kD) are better transfection agents than low molecular weight polymers (Van de Wetering et al, 1998). Recently, we initiated studies to investigate the application of p(DMAEMA) as a gene carrier in gene-directed enzyme prodrug therapy (GDEPT) (Fonseca et al, 1999).

This contribution deals with the design of pDMAEMA-based polyplexes for tumor targeting. The first part is concerned with their use for the intraperitoneal therapy of ovarian cancer, the second part with their use for intravenous targeting of solid tumors.

II. Intraperitoneal administration

A. p(DMAEMA)-based polyplexes

Ovarian cancer is one of the most common fatal gynecological malignancies. The OVCAR-3 human ovarian carcinoma cell line growing i.p. in nude mice provides a model system suitable for studying ovarian cancer (Hamilton et al, 1984). Since ovarian cancer remains confined to the peritoneal cavity throughout most of its lifetime, it has been considered that ready access to the peritoneal cavity and containment of the disease progress within the peritoneal cavity favor development of anticancer gene therapy strategies. Therefore, we have defined intraperitoneally localized OVCAR-3 cells as transfection targets which should be accessible to DNA delivery systems injected directly into the peritoneal cavity (Van de Wetering et al, 1999b).

The approach taken to investigate whether OVCAR-3 cells can be transfected in vivo was a comparative in vitro - ex vivo - in vivo study utilizing similiar exposure conditions of the cells to the p(DMAEMA) transfection complexes in vitro and in vivo. The transfection results can be summarized as follows: p(DMAEMA)/plasmid (pCMVLacZ) complexes can transfect OVCAR-3 cells in vitro with an overall transfection efficiency of 10%. Cells grown in vivo can be transfected ex vivo with p(DMAEMA)/plasmid complexes with an overall transfection efficiency of 1-2%. However, cells grown in vivo were very difficult to transfect in vivo: transfection of intraperitoneally localized OVCAR-3 cells was negligible after i.p. injection of the transfection complexes into nude mice bearing OVCAR-3 cells in the peritoneal cavity (Van de Wetering et al, 1999a).

The following reasons might explain this discrepancy:

(i) The polyplexes may have formed aggregates induced by one or more components of the ascites fluid. We have previously observed that large-sized polyplexes are less efficient in transfection (Van de Wetering et al, 1997).

(ii) A potential reason for the differences found in vitro and in vivo transfection experiments may be sought in the clustering of cells growing in the peritoneal cavity. OVCAR-3 cells cultured in vitro grow adherently while in vivo cells grow in suspension in the peritoneal cavity. Clusters of cells are formed with consequent reduced accessibility of a major fraction of the cells. In order to investigate whether declustering of the cells would result in improved accessibility and consequently higher transfection efficiency ex vivo, cells isolated from mice were treated with trypsin before incubation with the transfection complexes. Trypsin-mediated declustering did not improve transfection.

(iii) Another difference between the in vitro and the in vivo situation is the presence of body fluids, peritoneal ascites fluid in case of the particular tumor used here. The influence of ascites fluid on the transfection activity of the polyplexes was investigated in vitro. In parallel, the influence of fetal calf serum (FCS) was studied in the same experiment. When ascites and FCS are absent during the experiment, the transfection optimum was observed at a polymer/plasmid ratio of 1.6/1 (w/w). With an increasing ascites or FCS concentration, the optimum polymer/plasmid ratio shifted to higher values. This is in good agreement with the results obtained by Yang and Huang (Yang and Huang, 1997) who showed that the inhibitory effect of serum on lipofection could be overcome by increasing the cationic lipid/DNA ratio. The transfection activity was increased 2-fold in the presence of FCS at the optimum ratio which is possibly caused by a stimulating effect of certain FCS components on the interaction of the polyplexes with the cells. However, the in vitro transfection activity was strongly reduced in the presence of ascites fluid. To elucidate which component(s) of ascites had such a detrimental effect on the in vitro transfection activity, the influence of hyaluronic acid, which has been reported to be present in relatively high concentrations in ascites (Veatch et al 1995; Catterall et al, 1997), on the transfection activity was studied. Hyaluronic acid, a polymer consisting of a regular repeating sequence of disaccharide units (glucuronic acid and N-

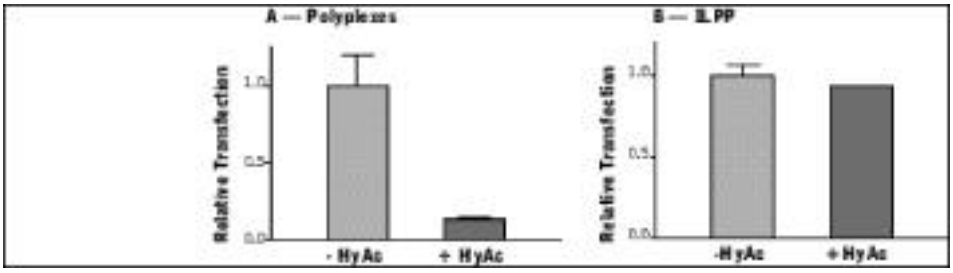

Figure 2. Influence of hyaluronic acid (HyAc) on the transfection efficiency of polyplexes vs ILPP. OVCAR-3 cells (1.1×10^4 cells/well) were exposed for 1 hour at 37°C to (**A**) polyplexes or (**B**) ILPP in the absence (-HyAc) or presence (+HyAc) of 2.5 mg/ml hyaluronic acid. Gene carriers were removed by washing and cell culture was continued for another 47 h prior to evaluation for β-galactosidase expression.

acetylglucosamine), interacts withcells and is studied for its potential role in metastases proliferation. Due to its polyanionic character, hyaluronic acid might have interacted with the positively charged polyplexes resulting in a reduction of the transfection activity, as has been reported for the effect of heparin on lipoplexes (Xu and Szoka, 1996; Mounkes et al, 1998). As shown in **Figure 2**, indeed, the in vitro transfection activity was strongly reduced in the presence of hyaluronic acid in concentrations which are in the range (up to 11 mg/ml) observed to be present in peritoneal effusions from cancer patients (Roboz et al, 1985; Catterall et al, 1997). No negative effect of hyaluronic acid on cell viability was observed. This outcome suggests that one of the components of ascites fluid, hyaluronic acid, may have induced a negative effect on the transfection capability of p(DMAEMA)-based polyplexes.

B. Protection and targeting of p(DMAEMA)-based polyplexes

Clearly, in order to obtain effective gene transfer to OVCAR-3 cells under in vivo conditions, the p(DMAEMA)-based polyplexes need to be protected from the inactivating effects of tumor ascitic components. For achieving protection we developed a detergent removal method to coat the cationic polyplexes with anionic lipids (**Figure 3**) (Mastrobattista et al, 2001). Lipid-coated polyplexes (further referred to as lipopolyplexes (LPP)) were formed by adding p(DMAEMA)-based polyplexes (3:1 w/w ratio of polymer:DNA) to a mixture of lipids (with egg-phosphatidylglycerol as the anionic component) solubilized in 150 mM octylglucoside and subsequent slow removal of the detergent by adsorption to hydrophobic polystyrene BioBeads. With this method spherical particles of about 120 nm and bearing a negative charge were obtained.

Previous work from our group has demonstrated that p(DMAEMA)-based polyplexes are destabilized when exposed to poly(aspartic acid) (p(Asp)), which liberates the DNA from the polyplexes and making it susceptible to DNase I-induced degradation (Arigita et al, 1999). We investigated whether polyplexes present in LPP are protected against destabilization with p(Asp). LPP and polyplexes admixed with empty anionic liposomes were incubated with DNase I in the presence of a large excess of p(Asp). Before and after the addition of DNase I samples were analyzed by gel electrophoresis for the presence of non-degraded plasmid DNA (**Figure 4**). It was demonstrated that there is no difference in DNA staining intensity between LPP before (lane 2) and after (lane 1) treatment with DNase I, indicating that a large amount (if not all) of the polyplexes within LPP is protected from destabilization with p(Asp). In contrast, polyplexes that were admixed with empty preformed liposomes with the same lipid composition as in the LPP coat appeared very sensitive to destabilization with p(Asp), as DNase I completely degraded complexed DNA (lanes 3 and 4). These results indicate that coating of polyplexes with lipids protects the polyplexes from destabilization by p(Asp).

Unfortunately, besides the positive effect of conferring protection, the presence of a negatively charged lipid coat had a strong negative impact on the transfection capability of the LPP. This negative effect is likely due to loss of cationic charge-mediated electrostatic interaction with the cells. This problem could be overcome by coupling specific antibody fragments to the LPP surface (**Figure 3**). Targeting of LPP to OVCAR-3 cells was realized by coupling Fab'-fragments of the mAb 323/A3 (anti-EGP-2 receptor) to the surface of LPP (immuno-LPP (ILPP)). It was demonstrated that the presence of the targeting ligand mediates cellular binding and uptake of the coated particles and compensates for the loss of electrostatic interaction with the cell membrane by the introduction of the lipid coating on the polyplex surface. It is also noteworthy that – in sharp contrast with the plain polyplexes – ILPP did not induce any cytotoxicity to the cells (as monitored with the XTT assay). An important observation from the ovarian carcinoma application point of view is the stability of the ILPP system in the

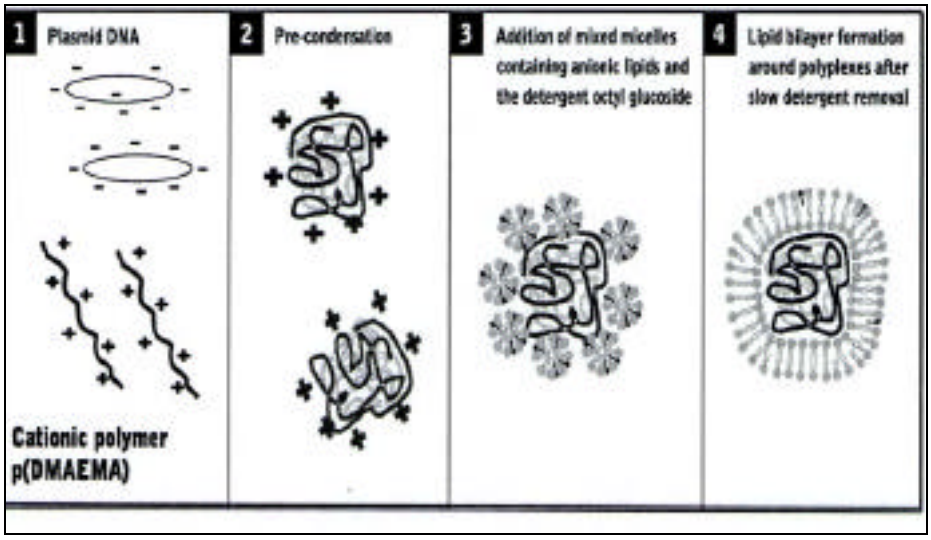

Figure 3. Schematic representation of lipopolyplex formation. (**1**) Plasmid DNA is condensed by adding the cationic polymer p(DMAEMA) to the DNA at a weight/weight ratio of 3:1, respectively. The formed polyplexes (**2**) are added to mixed micelles containing the detergent OG and a total amount of 3 μmol detergent-solubilized lipids (**3**). Upon slow removal of detergent by adsorption to hydrophobic BioBeads, lipid coats are preferentially formed around positively charged polyplexes due to electrostatic interactions (**4**).

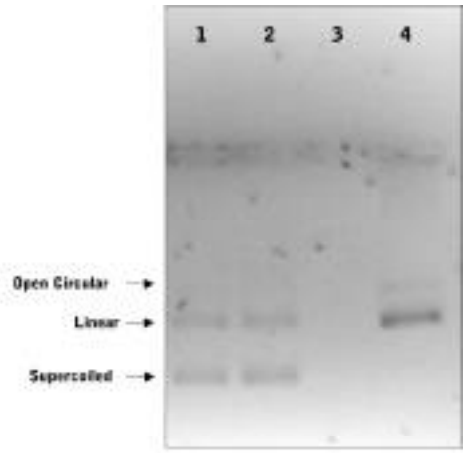

Figure 4. Nuclease resistance assay of DNA in polyplexes and in LPP in the presence of p(Asp). LPP (lanes 1 and 2) and polyplexes admixed with empty liposomes (lanes 3 and 4) were incubated with DNase I in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of p(Asp) (1 mg/ml) for 30 min at 37°C and subsequently analysed by gel electrophoresis to visualize the presence of intact plasmid DNA. Image colors have been inverted for clarity.

presence of hyaluronic acid (**Figure 2B**). Whereas the transfection efficiency of polyplexes is drastically reduced in the presence of hyaluronic acid, this reduction is not observed with ILPP, indicating that hyaluronic acid does not negatively affect the transfection efficiency of ILPP.

In conclusion, the ILPP system features colloidal stability and transfection capability under conditions mimicking the in vivo situation and therefore show promise for use in plasmid-based approaches to gene therapy of ovarian cancer. At present, ILPP are being investigated for their efficiency to deliver genes to ovarian carcinoma cells for GDEPT purposes.

III. Intravenous administration

It is obvious that direct injection of gene medicines in the cavity containing the tumor burden, like in the case of intraperitoneally localized ovarian carcinoma metastases, represents a simpler task than targeted delivery to tumor tissue from the systemic circulation. For systemic gene delivery mediated by polyplexes additional biological barriers have to be considered: non-specific interactions with blood components, colloidal instability, rapid uptake by the cells of the mononuclear phagocyte system (MPS), and limited extravasation into tumor tissue. The intravenous fate of the cationic p(DMAEMA)-based polyplexes is in line with other reports describing the location and extent of gene expression after intravenous administration of DNA complexes and can be summarized as follows. Plasmid DNA (encoding for the firefly luciferase enzyme) was labelled with [α-32P]-dCTP by nick translation. Approximately six-week-old, female Balb/c mice received positively charged polyplexes (polymer/DNA ratio 3:1 w/w) labelled with trace amounts of radioactivity, in an injection volume of 200 μl by tail vein injection. At various time points, blood was collected from the vena cava under ether anaesthesia and subsequently the mice were killed. Radioactivity levels in each organ were determined. It was observed that the positively charged pDMAEMA/[32P]-DNA polyplexes

Table 1. Overview of circulation time properties of shielded polyplex systems

Author	Polymer	Dose	Ratio	Lungs	Liver	Blood	Time	Misc
Howard *et al.*	TMAEMA-HPMA 30 kD copolymer	20 µg	N/P=2	<5%	± 50%	<10%	30 min	
Collard *et al.*	pLL peptide-PEG$_{5000\ D}$	50 µg	N/P=2		>60%		30 min	crosslinked
Oupický *et al.*	PLL$_{25\ kD}$ post PEG$_{30000}$	80-100 µg	N/P=2		45%	40%	30 min	crosslinked
Ogris *et al.*	PEI$_{56\ kD}$ post PEG$_{5000\ D}$-transferrin	50 µg	N/P=6			30%	30 min	Neuro2A sc tumors
Oupický *et al.*	PLL$_{25\ kD}$ post pHPMA$_{\sim 50\ D}$	24 µg	N/P=2		60%	<10%	30 min	
Oupický *et al.*	pTMAEMA$_{\sim 50\ D}$-co-pHPMA$_{\sim 50\ D}$	2 µg	N/P=1	<10%	50%	<10%	30 min	
Kircheis *et al.*	PEI$_{25\ kD}$-transferrin	30 µg	N/P=5		75%		4h	Neuro2A sc tumors
Mullen *et al.*	PLL$_{\sim 15\ kD}$-graft-PEG$_{5000\ D}$	20 µg	+/-3	<10%	20%	70%	5 min	
Nguyen *et al.*	PEI$_{25\ kD}$-PEO$_{10\ kD}$	50 µg	N/P=16		0.3 ng Luc/mg			
Mao *et al.*	Chitosan$_{\sim 50\ kD}$ – post PEG $_{5000\ D}$	5 µg	N/P=6	<5%	12 %	< 5%	15 min	

distributed primarily to the lungs. Within minutes 80 percent of the injected dose was recovered from the lungs. In a second set of experiments, distribution of transfection activity was studied. Twenty-four hours after i.v. administration of pDMAEMA-based polyplexes (polymer/DNA ratio 3:1 w/w), luciferase levels were determined in lungs, liver, spleen, kidneys and heart. The results showed that the gene expression profile matched the biodistribution profile of the administered positively charged polyplexes. Most of the expression was seen primarily in the lungs. A third set of experiments was designed to shed more light on the mechanism involved in the dominant lung uptake of polyplexes. In vitro turbidity experiments in serum were performed providing evidence for severe aggregation occurring upon addition of the polyplexes to the serum. Hemaglutination experiments provided evidence that positively charged complexes induce the formation of extremely large structures upon addition to erythrocytes. If formed in vivo, such large aggregates are likely to block the blood flow in the lungs. Another potential in vivo factor may be electrostatic interaction between the cationic polyplexes and the negatively charged lung cell membranes. However, incubation of polyplexes with serum albumin showed that the zeta potential of the complexes drops to negative values, making the possibility of electrostatic interactions less likely.

The 'first pass' distribution of polyplexes to the lungs severely impedes the utility of cationic polymers for gene delivery. Therefore, we and other groups (**Table 1**) are currently investigating whether shielding of the surface positive charge of the polyplexes can prevent dominant lung uptake and increase colloidal stability. Recent experiments yielded some success with surface modification of the p(DMAEMA)-based polyplexes with PEG. Aggregation in serum as demonstrated for non-PEGylated polyplexes in turbidity experiments in vitro could be prevented by coupling covalently PEG to the surface of the polyplexes. Also, PEGylation yielded a drop in the zetapotential of the complexes to almost neutral. Severe hemagglutination was not observed when washed erythrocytes were incubated with the PEGylated complexes. Most importantly, in vivo experiments showed prolonged circulation and avoidance of dominant lung localization in case of intravenous administration of PEGylated polyplexes. So far, the best results were obtained when PEG with a high molecular weight (20,000) was used: at 30 min after intravenous administration into Balb/c mice about 50% of the injected dose was still circulating in the bloodstream which is substantially higher when compared to the 2% of injected dose still circulating in case of the uncoated polyplexes. Localization and gene expression in the lungs is almost absent, which is likely related to the improved colloidal stability of the complexes. For evaluating tumor targeting, we have utilized the subcutaneous C26 colon carcinoma mouse model. In this tumor model, the degree of tumor accumulation amounted to about 4% of injected dose per gram tumor tissue.

A comparison of our best results with those reported in the literature (**Table 1**) tells us that we are well underway towards our goal to develop a nonviral carrier system for systemic gene delivery to a distant tumor. As the approach taken appears realistic, our research is continued with the ultimate aim to adopt this delivery.

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