Production and pharmaceutical formulation of plasmid DNA vaccines

Iris van der Heijden

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Production and pharmaceutical formulation of plasmid DNA vaccines

Productie en farmaceutische formulering van plasmide DNA vaccins
(met een samenvatting in het Nederlands)

Proefschrift

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"After all, what nobler thought can one cherish than that the universe lives within us all?"
Neil deGrasse Tyson

CONTENTS

PREFACE

DNA vaccination is an appealing method to obtain an antigen-specific immune response, which could be useful in anticancer therapy [1,2]. When delivered by intradermal tattooing, plasmid DNA (pDNA)vaccination results in a strong and rapid antigen specific cellular response in both mice and non-human primates [3]. In spite of the high immunogenicity of pDNA tattoo, the *in vivo* transfection efficiency of naked pDNA with this technique is extremely low, with approximately 1 out of $5x10^6$ to $5x10^9$ plasmid copies applied being taken up, transcribed and translated [4]. Non-viral carrier systems are extensively used as a transfection agent to deliver nucleic acids for both *in vitro* and *in vivo* applications. In these systems, negatively charged pDNA is bound by electrostatic interaction to an excess of a positively charged carrier resulting in the formation of nanosized particles which protects the pDNA from nuclease degradation, resulting in substantially higher transfection efficiencies compared to naked nucleic acids in *in vitro* assays [5–7]. Cationic polymers are frequently used carriers for plasmid DNA and the resulting DNA/polymer nanoparticles are referred to as polyplexes [8]. When these cationic polyplexes encounter the cells after administration, they are able to interact with the anionic cellular membrane and are taken up into the cells via endocytosis. Within the cells, the polyplexes are transported by endosomes that eventually become acidified and fuse with lysosomes. In this situation, the pDNA is likely to be degraded by the lysosomal enzymes. In order to successfully transport the incorporated pDNA to the nucleus, the polyplexes must be able to escape from the endosomes [9]. An endosomolytic process known as the 'proton sponge effect' [10,11] facilitates the endosomal escape.
The aim of this thesis was the development of a Good Manufacturing Practice (GMP) compliant pDNA production process, including the development of a pharmaceutical formulation of pDNA vaccines for the treatment of Human papilloma viruses (HPV) 16 induced malignancies. Furthermore, this thesis focuses on the development of pDNA-polyplex formulations to further improve the transfection efficiency and immunogenicity of intradermally delivered DNA vaccines.

Chapter 1 gives an overview of currently used pDNA production processes, quality control and formulation of naked pDNA vaccines for early oncology clinical trials.

Chapter 2.1 of this thesis describes a generic method for the production and formulation of plasmid DNA vaccines, complying with current GMP guidelines. This method can be readily used in e.g. academic settings for the in-house production of relative small amounts of plasmid DNA necessary for phase I and II trials.

Chapter 2.2 describes the details of an unexpected contamination during clinical manufacture of a pDNA vaccine as a result of the introduction of a host cell transposon in the pDNA vector. During processing, presence of this movable element was not noticed until quality control of the bulk pDNA vaccine. The identity, source and extend of contamination were investigated during this study.

Chapter 2.3 reports on the shelf-life stability of plasmid DNA as lyophilized powder for reconstitution for intradermal administration, used in an in-house, investigator-initiated clinical phase I study. This study shows that lyophilization is an attractive approach to preserve the quality of the pDNA and can prevent the need for costly and time-consuming additional manufacture of drug product in case of study delays, not uncommon at the early stage of drug development.

To determine if pDNA transfection can be further improved by formulating pDNA into polyplexes, **chapter 3.1** describes the screening of a broad panel of polymers with distinct differences in molecular structure and characteristics. We measured *ex vivo* human skin transfection efficiency and polymer characteristics (size, PDI, charge) for this panel of polyplex formulations and conducted a follow-up experiment in which the best performing polymer was further investigated and tested.

With the aim of developing a pharmaceutical formulation, **chapter 3.2** focuses on the screening for the optimal polymer/pDNA ratio of poly(amido amine)s-polyplexes. Next, in order to also obtain a clinically feasible, stable pharmaceutical formulation we investigated the use of an improved buffer system which also would be applicable in a lyophilization polyplex formulation. An L-histidine buffer instead of the standard HEPES buffer was investigated and appeared not to be inert with respect to transfection efficiency.

Chapter 3.3 describes whether 10 mM L-histidine buffer pH 6.0 in combination with various cryoprotectants (trehalose, sucrose and HPßCD) results in a stable lyophilized product. Physical-chemical characteristics, degradation, transfection efficiency as well as microscopic appearance before and after freeze-drying of several formulations was assessed.

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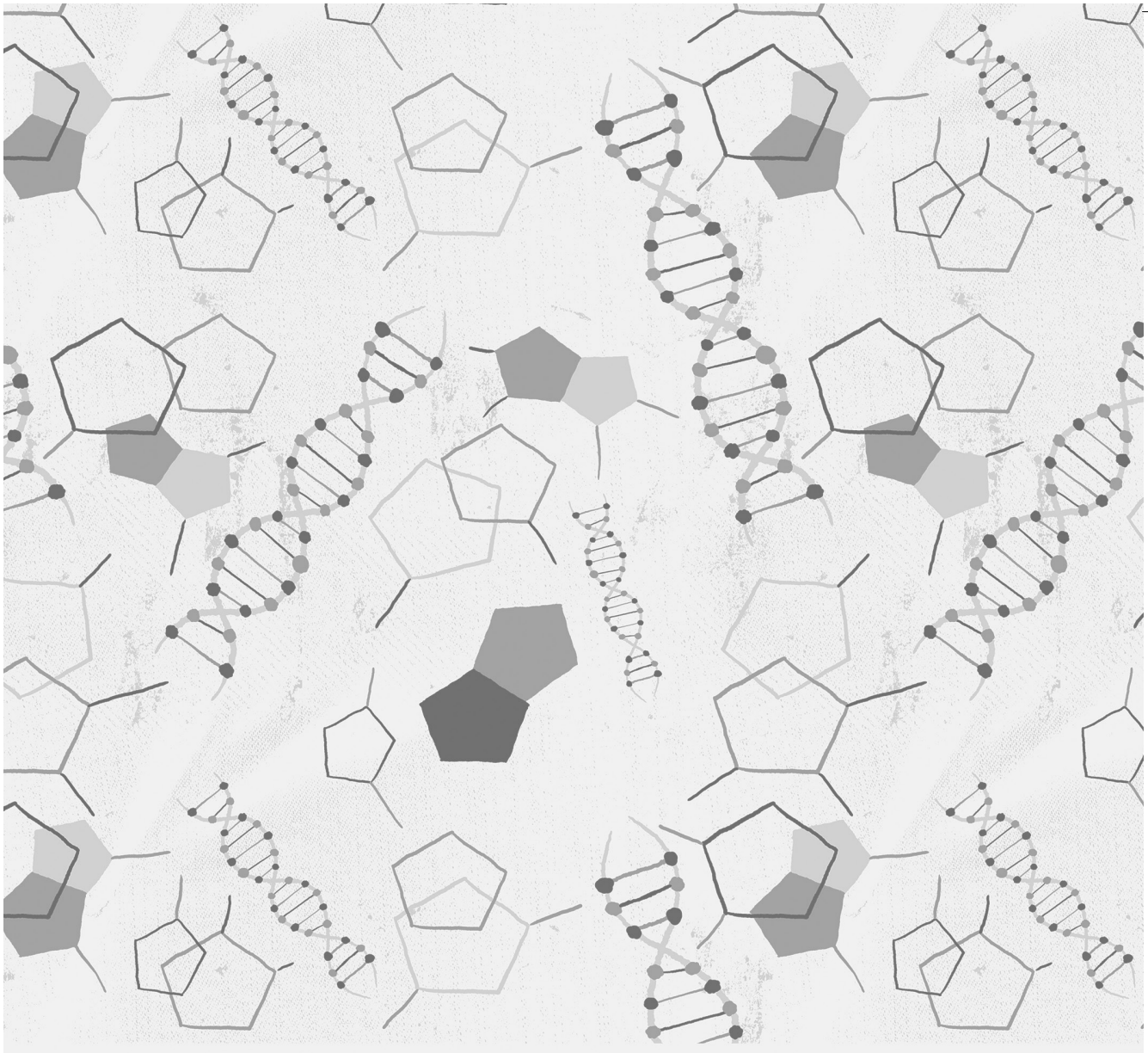

Chapter 1

Introduction

Chapter 1.1

Production and quality control of plasmid DNA vaccines for early oncology clinical trials — a review

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Submitted for publication

ABSTRACT

Gene therapy is based on the concept that human disease may be treated by the transfer of genetic material into specific cells of a patient in order to enhance gene expression, inhibit production of a target protein or increase circulating antigens. For this purpose plasmid DNA (pDNA) or messenger RNA (mRNA) encoding for the desired gene/protein can be administered. Both nucleic acid derivatives (pDNA and mRNA) are simple gene therapy vehicles that can be easily designed and produced *in vitro* from bacteria or bacteria-derived products. Non-viral gene therapy may require considerable amounts (milligram scale) of pharmaceutical grade pDNA per patient, since the efficacy and duration of gene expression is presently relatively low. Most production processes are developed for use at laboratory scale, therefore scaling up can be difficult. We provide an overview of the most important steps during the production process. To increase the shelf life and to obtain highly concentrated solutions for administration, the pDNA can be lyophilized using disaccharides (in combination with polysaccharides) to protect the DNA during lyophilization and storage.

INTRODUCTION

Gene therapy is based on the concept that human disease may be treated by the transfer of genetic material into specific cells of a patient in order to enhance gene expression, inhibit production of a target protein or increase levels of circulating antigens. Amongst the various forms of somatic gene therapy there is a growing interest in DNA vaccination. By means of administering plasmid DNA (pDNA) encoding for a specific protein, in theory several therapeutic goals can be achieved. For example introduction of missing proteins (like insulin for diabetics), replacement of defective gene copies (in cystic fibrosis and Duchenne muscular dystrophy) and introducing immunogenicity against cancer cells, by increasing the circulating target protein (antigen) level [1]. This latter application has gained a lot of interest over the last years as potential anticancer therapy. This review focuses on the small-scale production and quality control of oncology pDNA gene therapy products. Also, pharmaceutical formulation of final dosage forms and administration of pDNA will be discussed. The conduct of early oncology clinical trials with pDNA products requires relatively low amounts of product as only a limited number of patients are treated. The small-scale production of pDNA for anticancer immune therapy concerns investigational medicinal products (IMPs) and therefore Good Manufacturing Practices (GMP) compliance is needed [2,3]. pDNA gene therapy also concerns advanced therapy medicinal products (ATMP) and regulation on advanced therapies (Regulation (EC) 1394/2007). This review is based on extensive literature research and is own experience and illustrates the status of the manufacturing of pDNA vaccination products in oncology.

Plasmid DNA vaccination in oncology

DNA vaccination is a conceptually safe and technically simple way of non-live vaccine immunization. Most important, pDNA vaccination is able to induce not only a humoral immune response, but also a strong cellular response [4,5] that was usually allocated for live infection. The induction of killer cytotoxic T lymphocytes (CTLs) by pDNA vaccination is a result that has not been established by other non-live vaccine platforms [6]. Other advantages of pDNA vaccination above direct injection of the antigen are the ease and possibilities for adjusting the DNA sequence, the relatively low production costs [7], high pharmaceutical stability and improved safety profile when compared to the safety concerns of live vaccines. Because of induction of the killer CTLs DNA vaccination is a very attractive strategy for vaccination against viral infection or tumors. Upon administration, the plasmid enters the cell nucleus using the cellular machinery of the host. In the cell nucleus, the pDNA is transcripted, followed by the production of protein (antigen) in the cytoplasma. Both myocytes and antigen presenting cells (APCs) can be transfected directly. Epitopes of intracellular produced antigens can be presented in the presence of MHC I molecules.

Table 1: Selection of published clinical trials with pDNA vaccines against cancer. Abbreviations: in, intra nodal; im, intramuscular; id, intradermal; it, intra tumoral transfer; sc, subcutaneous; SD, stable disease; PD, progressive disease; CR, complete remission; PR, partial response; na, not applicable.

Target gene(s)	Delivery	Adjuvants	Cancer type	No of subjects	Specific cellular immune response
Two tyrosinase peptide epitopes	in	no	melanoma	26	11/26
gp100	12 id, 10 im	no	melanoma	22	0/13
Melan-A	im	no	melanoma	12	0/11
gp100	id	cDNA of GM-CSF in 6 patients	melanoma	12	1/3
IL-12	id	no	melanoma	24	24/24
CyB1 B1	im	no	Ovarian, breast, colorectal, prostate, renal cell carcinoma	17	6/17
PSMA with CD86	id	GM-CSF in 13 patients	Prostate cancer	26	Partial signs of immunization
PSA	im and id	IL-2 sc, GM-CSF sc	Prostate cancer	9	2/9
PSA	im and id	GM-CSF sc, IL-2 sc	Prostate cancer	9	5/6
IL-2	it	no	Prostate cancer	24	Elevated $CD3^+$, $CD4^+$ and $CD8^+$ levels
CEA/HBsAG	im	no	Colorectal cancer	17	4/17
HLA-B7	it	no	Renal metastatic carcinoma	15	8/14
IL-2	it	no	Renal cell carcinoma	31	6/8
L5238	im	no	Lung cancer	13	0/12
Id linked to MsIg	im, id	no	Lymphoma	13	9/13
HPV16 E7	im, sc	no	Cervical cancer	15	11/15

	Clinical responses	Safety	Phase, location	References
	6/26 SD, 20/26 PD	Local irritation	Phase I, America	(Tagawa et al., 2003)
	1/22 PR, 21/22 PD	Local irritation	Phase I, America	(Rosenberg et al., 2003)
	na	Local irritation	Phase I, America	(Triozzi et al., 2005)
	2/8 SD, 6/8 PD	Local irritation	Phase I, America	(Cassaday et al., 2007)
	2/24 CR, 7/24 SD, 14/24 PD	Local irritation	Phase I, America	(Daud et al., 2008)
	2/17 SD, 15/17 PD	Few mild reactions	Phase I, America	(Gribben et al., 2005)
	Cannot conclude	Local irritation	Phase I/II, Bulgary	(Mincheff et al., 2000)
	4/6 SD, 2/6 PD	Local irritation	Phase I, Sweden	(Pavlenko et al., 2004)
	4/6 SD, 2/6 PD	Not reported	Phase I, Sweden	(Miller et al., 2005)
	16/24 PR, 8/24 PD	Few mild reactions	Phase I, America	(Belldegrun et al., 2001)
	5/17 SD, 12/17 PD	Local irritation	Pilot phase I, America	(Conry et al., 2002)
	7/15 SD, 8/15 PD	Few mild reactions	Phase I, America	(Rini et al., 1999)
	1/31 CR, 2/31 PR, 7/31 SD	Few mild reactions	Phase I/II, America	(Galanis et al., 2004)
	na	Local irritation	Phase I, America	(Nemunaitis et al., 2006)
	7/13 CR, 4/13 SD, 2/13 PD	Local irritation	Phase I/II, America	(Timmerman et al., 2002)
	5/15 CR 10/15 PD	Few mild reactions	Phase I, America	(Sheets et al., 2003)

To efficiently activate naïve T lymphocytes in the lymphatic system, professional APCs present intracellular-produced antigen. Antigen produced by non-APCs can reach MHC I molecules on APCs by cross-presentation. The MHC Class I route is responsible for activating strong killer CTL responses. High titers of killer CTLs are very important because they are able to eliminate cells that are infected by viruses. They are also essential for the elimination of tumors. Some tumors express 'tumor-associated antigens'. By recognizing those antigens, the killer CTLs are able to attack the tumor. Table 1 gives an overview of the clinical oncology trials performed thus far or are ongoing.

Plasmid DNA design

Construction and design of plasmid

Based on the target of vaccination a plasmid DNA active pharmaceutical ingredient (pDNA API) can be constructed. Plasmid DNA is a circular double stranded DNA molecule and is 2 to approximately 15 kbp in size (Fig. 1) [8]. The antigen gene insert needs to be designed and cloned into a backbone and manufactured under GMP/GLP conditions by a dedicated manufacturer with all used materials of known origin. A vector for DNA vaccines should, first of all, be safe in humans. The possible risk of integrating into the human chromosome (the most obvious safety issue for a DNA vaccine vector) should be minimalized. Therefore it should be impossible for the vector to replicate in mammalian cells, which can be obtained by using a non-mammalian origin of replication (ORI) that is suited for efficient propagation in the bacterial host. Secondly, a high yield of plasmid molecules needs to be obtained. As production system a single appropriate bacterial host strain for all manufacturing work does not exist. An appropriate strain should be a clone derived from a host strain stock that is completely characterized and free of contaminations. The bacterial host strain should be safe for the environment, exposed patients, manufacturing employees and health care personnel [9]. There are many different Gram-negative *Escherichia coli* K-12 strains and many of them are suitable host for plasmid propagation [10]. *E.coli* cells should be obtained from dedicated suppliers. Also, the plasmid should contain an antibiotic resistance gene for growth selection. The antibiotic used in the manufacture of plasmid DNA should preferably not belong to the ampicillin family to avoid potential problems with penicillin allergies [8] and more important, such antibiotics are not permitted by the FDA in the manufacture of products for humans. The antibiotic Kanamycin should be used since there is no clinical application for this antibiotic and spontaneous resistance to this antibiotic is rarely seen [11]. It is also possible to perform growth selection without antibiotic resistance gene. A specific origin of replication (ori)-encoded RNAI located on the plasmid is linked to a repressor protein encoded on the host genome that further controls the expression of an essential gene [12]. The amount of plasmid DNA that is internalized *in vivo* is very small (picogram range). It is also impossible for the plasmid to replicate in the cells, so the amount of plasmid available for expression stays small.

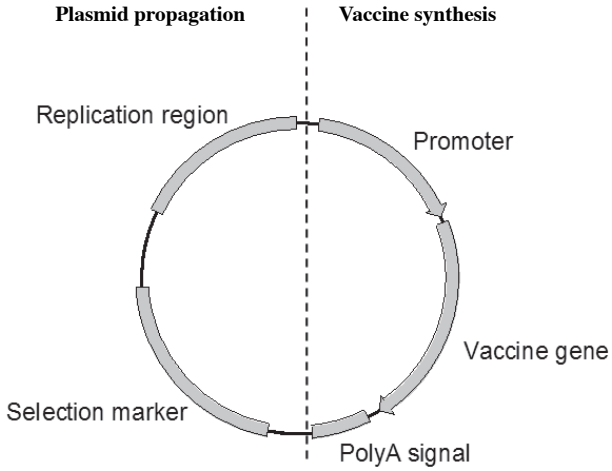

Figure 1: *Schematic overview of plasmid DNA.*

Therefore, a strong mammalian promoter and a polyadenylation termination sequence should be chosen to drive expression of the antigen gene[13]. Finally, different vectors are available for construction of plasmid DNA. Both pUC and pVAX1 vectors designed to stimulate cellular as well as humoral immune resposes [13]. The pVAC1-mcs and pVAC2-mcs vectors from Invitrogen are designed to focus the response on humoral immunity [13]. Based on the idea that more antigen is better, most DNA vaccines use strong viral promoters and are geared towards maximum expression. Other sequences that can be optimized in a plasmid include introns, enhancers and poly-adenylation signals (reviewed by Leitner et al. [14]). To trigger the immunesystem several immune-stimulating sequences can be added to the plasmid DNA backbone. An example is the addition of tetanus toxin fragment-c, which contains the well described "universal" helper epitope p30 [15,16]. This epitope binds to a range of mouse and human MHC class II alleles [17], and the resulting CD4+ T cell stimulation [18] is necessary for the induction of robust CD8+ T cell responses by DNA vaccines. To avoid potential side effects of the origin of replication and the antibiotic resistance gene supercoiled minicircles have been developed [19,20]. They only contain the transcription unit of choice and the essential elements for immobilization [21,22]. A minicircle vector should reduce cytokine induction by the unmethylated CpG motifs in the bacterial sequences [23–25]. Minicircles are about half the size of standard plasmids and therefore have a higher transfection efficiency in vitro [19,20,24,26] and in vivo [19,20,24]. Furthermore due to the absence of bacterial covalent linkages to the expression cassette a more robust and persistent gene expression is seen [23].

Production of Bulk Drug

Master cell bank/working cell bank

Bulk drug production is started by the generation of the master cell bank (MCB). To assure consistent supply of the plasmid usually a working cell bank (WCB) is grown from one vial of the MCB. However, for small scale clinical trials the generation of a WCB is not necessary [7]. Since MCB and WCB need to be tested extensively before further use [27] no large quantities are necessary during phase I clinical evaluation. If more plasmid DNA is needed for future trials a WCB can readily be made from the existing MCB. The MCB is made by introducing the desired plasmid into the production cell. As mentioned before, an appropriate bacterial host strain should be a clone derived from a host strain stock that is completely characterized and free of contaminations[9]. Many different Gram-negative *E.coli* K-12 strains are suitable hosts for plasmid propagation and are commercially available [10]. Candidate strains should be tested with the final vaccine clone to determine which will grow at reasonable rates (2h or less doubling time) achieving a high cell density and give the highest plasmid DNA yields in the fermentation medium in large scale production [14]. The use of DH5 cells can be preferred, for they have given good yields of plasmid DNA and grow at reasonable rates and to high densities [7,13]. Before plasmid DNA uptake, the empty DH5 cells need to be made 'competent' to be able to take up the plasmid. The hydrophilic pDNA molecules won't travel through the bacterial cell membrane by itself and therefore the cells are incubated with $CaCl_2$ to make the cell membranes porous, followed by the transformation of the cells. The plasmid DNA is forced into the cells by incubating the porous bacterial cells with the DNA and placing them briefly at 42°C and putting them back on ice after which the bacterial cells take up the plasmid DNA. The cells are subsequently plated on plates containing kanamycin to make sure only plasmid transformated cells can grow. One single colony is isolated and is again grown in kanamycin containing medium. Afterwards the bacterial cells are aliquoted. Before further use, the MCB needs to be verified to contain kanamycin resistant *E.coli*, no adventitious viruses and no mycoplasma according to Ph. Eur. and FDA guidelines [28,29]. Plasmid identity needs to be confirmed by restriction analysis and sequencing.

Upstream processing

The bulk drug is produced either from the MCB (small scale clinical trials) or the WCB (large scale clinical trials, or registered drug). Fig. 3 represents a process flow chart for the production of pDNA bulk drug substance. All pDNA vaccines are made from identical compounds, but they have different sizes, charge and topology. Hence methods for their production and purification are essentially generic [30]. Safety and potency are directly related to purity, because impurities and contaminants can induce immunological and biological responses [31,32] Table 2 lists currently used production methods with their advantages and disadvantages, where applicable.

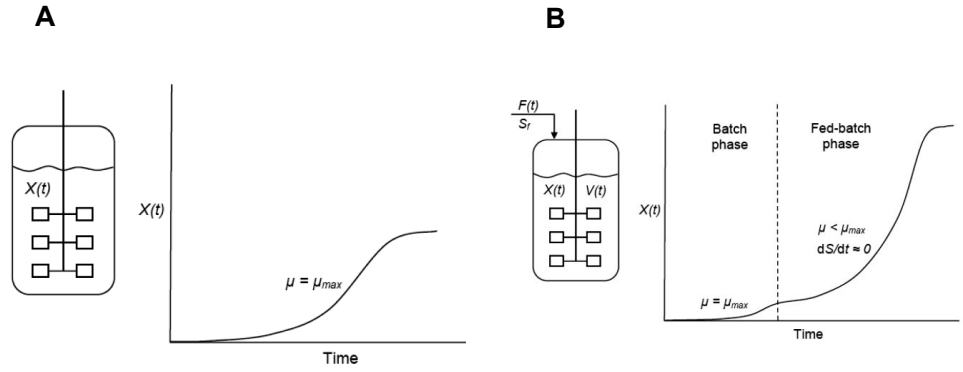

Figure 2: ***(A)*** *Typical batch fermentation. X is biomass concentration, t is time, μ is the specific growth rate.* ***(B)*** *Typical fed-batch fermentation. F is the feed rate, S is the limiting substrate concentration, Sf is the limiting substrate concentration in the feed, V is culture volume. Adapted from [144].*

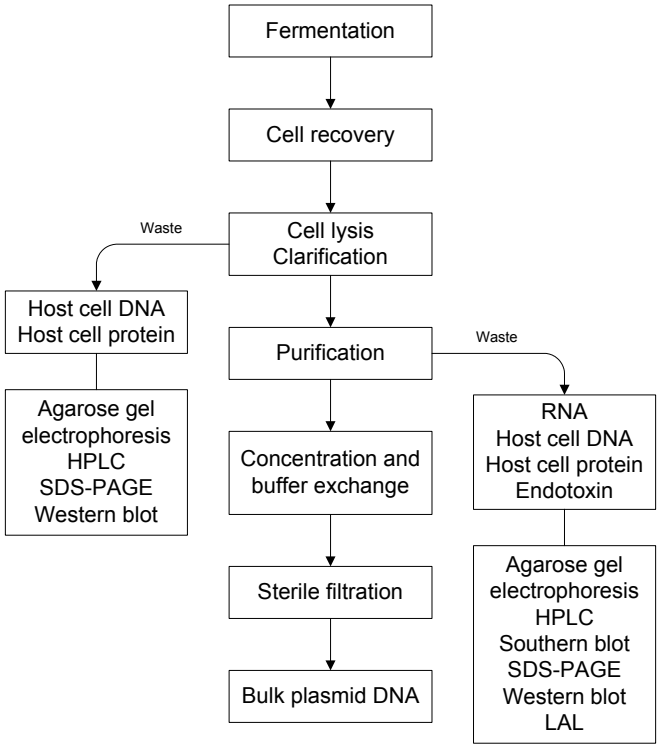

Figure 3: *Process flow sheets for the production of pDNA, with partition of impurities across the manufacturing process. Abbreviations: HPLC, high pressure liquid chromatography; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; LAL, limulus amoebe lysate*

Table 2: Overview of the used methods for each step in the production process in production of pDNA for clinical applications. Abbreviations: TFF, tangential flow filtration; SDS, sodium dodecyl sulphate; ER, endotoxin removal; AEX, anion exchange chromatography; SEC, size exclusion chromatography; TIC, thiophilic interaction chromatography; IPA, isopropyl alcohol; UF, ultra filtration; NH4Ac, ammonium acetate; HIC, hydrophilic interaction chromatography.

Production step	Method	Advantages	Disadvantages	References
Fermentation	Batch	Flexibility in controlling the production	Yield dependent on nutrients	(Horn et al., 1995; Prazeres et al., 1998; Ferreira et al., 1999; Varley et al., 1999; O'Kennedy et al., 2000; Diogo et al., 2000; Eon-Duval, 2003b; Eon-Duval and Burke, 2004; Urthaler et al., 2005; Przybylowski et al., 2007; Quaak et al., 2008; Zhang et al., 2008; Sousa et al., 2009; Cai et al., 2010; Ongkundon et al., 2011)
	Fed batch	High yield No additional special piece equipment	Controlled provision of substrates Extension of working time.	(Lahijani et al., 1996; Chen et al., 1997; O'Kennedy et al., 2003; Urthaler et al., 2005; Listner et al., 2006; Rozkov et al., 2006; Rozkov et al., 2008; Carnes et al., 2011; Bohle et al., 2011; Williams et al., 2009; Cheng et al., 2011; Yang et al., 2009; Ongkundon et al., 2011))
Cell recovery	Centrifugation	Fast	Resuspension of cell paste	(Varley et al., 1999; Przybylowski et al., 2007)
	TFF	Volume reduction and buffer exchange/ cells stay in suspension	Pore size and size of membrane determine process time	(Eon-Duval, 2003b; Quaak et al., 2008)
Lysis	Alkaline/SDS	Complete lysis	Difficult at large scale	(Caplen et al., 1994; Horn et al., 1995; Chen et al., 1997; Prazeres et al., 1998; Schleef and Schorr, 1998; Ferreira et al., 1999; Varley et al., 1999; Diogo et al., 2000; Eon-Duval, 2003b; Eon-Duval and Burke, 2004; Urthaler et al., 2005; Przybylowski et al., 2007; Quaak et al., 2008; Rozkov et al., 2008; Zhang et al., 2008)
Pre-clarification	Centrifugation	Almost complete recovery	Large centrifuges needed (bottles of several liters)	(Schleef and Schorr, 1998; Diogo et al., 2000; Eon-Duval and Burke, 2004; Zhang et al., 2008)
	Filtration	Almost complete recovery	Filter trains necessary	(Schleef and Schorr, 1998; Varley et al., 1999; Eon-Duval, 2003b; Rozkov et al., 2008)
	Vacuum followed by filtration	Fast		(Przybylowski et al., 2007; Quaak et al., 2008)
	TFF followed by dialysis			(Eon-Duval and Burke, 2004)
	NH_4Ac precipitation of proteins			(Horn et al., 1995; Ferreira et al., 1999; Diogo et al., 2000; Urthaler et al., 2005)
Endotoxin removal	ER buffer			(Caplen et al., 1994; Schleef and Schorr, 1998; Przybylowski et al., 2007; Quaak et al., 2008; Rozkov et al., 2008)
Concentration before purification	IPA precipitation of DNA		Introduction of a resuspension step	(Horn et al., 1995; Ferreira et al., 1999; Diogo et al., 2000)
	PEG 8000 precipitation of plasmid			(Horn et al., 1995; Ferreira et al., 1999)
	UF concentration	pDNA stays in solution, buffer exchange possible		(Urthaler et al., 2005; Cai et al., 2010)

Production step	Method	Advantages	Disadvantages	References
Purification	Ultrapure resin	Only one column needed High selectivity	Single use resin Time-consuming	(Caplen et al., 1994; Schleef and Schorr, 1998; Eon-Duval, 2003b; Eon-Duval and Burke, 2004; Przybylowski et al., 2007; Quaak et al., 2008; Rozkov et al., 2008)
	SEC	Fast Buffer exchange possible	Lower SC purity ratio	(Horn et al., 1995; Yang et al., 2009)
	HIC			(Diogo et al., 2000; Urthaler et al., 2005; Cai et al., 2010))
	1. SEC 2. TIC 3. AEX	Highly pure bulk product/ resins can be reused	Three different columns	(Stadler et al., 2004)
	1. AEX 2. SEC		Two different columns	(Ferreira et al., 1999)
	1. Expanded bed adsorption chromatography 2. SEC		Two different columns	(Varley et al., 1999)
	1. AEX 2. SEC 3. AEX		Three different columns	(Zhang et al., 2008)
	Anion Exchange Membrane chromatography	Fast/ smaller volume than columns, therefore less buffer necessary/ RNA reduction Multiple use capsule	Single use Capsule	(Teeters et al., 2003; Zhang et al., 2003; Cai et al., 2010)
	Hydrophobic Interaction Membrane Chromatography			(Pereira et al., 2010)
Concentration and buffer exchange	IPA precipitation followed by resuspension	Fast	Use of organic solvents/ resuspension of pDNA	(Caplen et al., 1994; Schleef and Schorr, 1998; Przybylowski et al., 2007)
	Ethanol precipitation followed by resuspension	Fast	Use of organic solvents/ resuspension of pDNA	(Horn et al., 1995; Zhang et al., 2008)
	TFF	Volume reduction and buffer exchange/ plasmid stays in solution	Takes several hours	(Quaak et al., 2008)

R39 R38 R37 R36 R35 R34 R33 R32 R31 R30 R29 R28 R27 R26 R25 R24 R23 R22 R21 R20 R19 R18 R17 R16 R15 R14 R13 R12 R11 R10 R9 R8 R7 R6 R5 R4 R3 R2 R1

Fermentation

The quality of plasmid DNA is finally determined by fermentation strategy. The primary goal when conducting a fermentation process for plasmid DNA is to maximize both the volumetric (mg/l) and specific (mg/g) yields of supercoiled pDNA. Optimizing volumetric yield allows for smaller and more economical fermentations whereas optimizing specific yield improves plasmid purity in downstream processing [33]. Fermentations can be characterized into two distinct phases. First, a phase of rapid growth occurring from inoculation, followed by a phase characterized by a reduced growth rate to the end of the cultivation cycle. Most of the plasmid content is produced during the second phase [34,35]. When the growth rate is slowed down, cellular metabolic activity is generally low, leaving more nutrients and energy available for plasmid replication [36]. Both Batch and fed-batch cultivation are employed by generating an inoculum. Usually one vial of the MCB is inoculated in a baffled shake flask containing medium and the selection medium (kanamycin). After overnight growth, the seed is inoculated in the fermentor [7,37].

Medium

Plasmid quality and yield are dramatically affected by media composition. A balanced medium is required for energy, biomass and cell maintenance. Also high nucleotide pools in the cells should be supported and energy should be available, although other cell activity should be minimized [33].A balanced medium is based on bacterial composition and energy requirements and contains a carbon source, a nitrogen source, salts and minerals and trace elements [38]. The carbon source can be glucose or glycerol (when acetate production related to high glucose concentration is undesirable) and provides energy and biomass. The nitrogen source is provided by ammonia and ammonium salts. Also complex components can be a source of nitrogen, for example yeast extracts, peptones and casamio acids. The salts and minerals are usually separately added to the base medium and are needed for bacterial metabolism and growth and enzymatic reactions [33]. For shake flask batch cultures, a minimal medium is commonly used. LB broth (Miller's formulation, [39]) is a standard pre-sterilized medium which contains tryptone, yeast extract and NaCl (Sigma Aldrich). A minimal medium can both satisfy the nutritional needs and result in a highly reproducible fermentation process. The production medium for fed-batch cultures is chemically defined and balanced so that the fermentation of the bacteria is carbon-limited once the fed-batch portion of the cultivation is started[34]. Listner et al. formulated a fed-batch medium that could result in a yield of 1.2 g plasmid DNA/L medium. The pre-sterilized base medium contains KH_2PO_4; K_2HPO_4, $(NH_4)2SO_4$, glycerol and is adjusted to pH 7.2 with NaOH. The medium supplement solution (thiamine.HCl and $MgSO_4.7H_2O$) and a trace element solution ($FeCl_3.6H_2O$, $ZnCl_2$, $CoCl_2,6H_2O$, $Na_2MoO_4,2H_2O$, $CaCl_2,2H_2O$, $CuCl_2,2H_2O$, and H_3BO_3) are freshly prepared and filtered before use [38]. Sterilized kanamycin, MSS and TES are added to

the production medium prior to inoculation. During fermentation, both pre-sterilized 15% phosphorus acid and 50% ammonium hydroxide are used for pH adjustment. The latter is also available as a nitrogen source. Feed supplement (85% glycerol) is the (limited) nutrition source during fermentation and acetic acid production should be minimalized. Anti-foam is added during the fermentation responding to foam formation.

Batch fermentation

For shake flask cultures and batch cultures at the time of inoculation, all nutrients for cell growth and plasmid production are available. Examples of batch fermentations can be found in [7,32,40–50]. During the exponential phase of growth all nutrients are in excess so a maximum growth rate can be obtained [33]. Batch temperature is set at 37°C. Eventually, due to limited nutrient supply, it could result in a lower SC content if fermentation is continued too long [51]. For plasmid production in the bacteria, slower growth rates are preferred. In batch fermentation, the growth rate can only be reduced by reducing the maximum growth rate. Lowering the temperature or using glycerol as carbon source can help reducing the maximum growth rate (Fig. 2A).

Fed-batch fermentation

During fed-batch fermentation the addition of limiting nutrients is controlled. Examples of fed-batch fermentations are described in [12,34–36,50–55]. The fed-batch fermentation starts with a 'batch' phase. The bacteria are cultured in an initial amount of medium and all non-limiting nutrients and an initial concentration of limiting nutrients are available from the beginning. When the initial amount of nutrients are consumed by the bacteria, controlled feeding is initiated. A feedback controlled strategy with DO-stat, pH stat, metabolic activity, biomass concentration and nutrient concentration can be very useful[33]. Most fermentor systems are able to monitor DO and pH. Movements in DO or pH can be indicative on the amount of nutrients available for the cells. For example, oxygen uptake is decreased when nutrients are depleted which can be noticed in a rise in DO concentration. pH increase is seen because of an increase in cellular excretion of ammonium ions. Feeding is triggered by DO or pH increase above threshold. In fed-batch fermentation, the feeding 'second' phase of growth is discussed extensively (Fig. 2B). In this phase, the growth rate is smaller than the maximum growth rate. The incidence of low growth rates with high plasmid content of bacteria often leads to the hypothesis that metabolic burden causes decrease of growth rate due to high plasmid amount and/or formation of plasmid encoded proteins [56]. Plasmid copy number (specific yield) within a strain is largely set by process and vector intrinsic factors and the steps of reducing metabolic burden gave rise to improved yield, quality and stability [55]. Although it is generally accepted that plasmid encoding protein production results in metabolic burden, reducing the maximum growth rate and other metabolic parameters, Bohle et al.,

demonstrated that metabolic burden is not the reason for elevated plasmid copy numbers at low growth rates. Instead, the plasmid content of the bacteria is the result of specific plasmid formation rate and dilution by growth. Specific plasmid production rate is therefore identified as a key target for process optimization [56]. In general, plasmid quality from fed-batch is higher rather than batch fermentation[33,51].

Downstream processing

The design of a plasmid DNA purification process must address removal of contaminants, scalability, and robustness, and should minimize the number of purification steps required [26,40,57]. Most plasmid purification processes described in literature have three steps: cell lysis to release the plasmid DNA, primary purification and concentration using either precipitation centrifugation, or filtration (table 2). The design will for a fast volume reduction to avoid unnecessary costs in equipment and buffers [58].

Lysis

After harvesting the cells from the fermentor, the volume can either be reduced by tangential flow filtration (TFF), precipitation or centrifugation. When using alkaline lysis, TFF can be useful because it concentrates plasmid DNA and provides buffer exchange for further processing, avoiding a resuspension step [7,40]. In TFF the fluid is pumped through a membrane, the cut-off value of this membrane determines which molecules are retained. For this step a cutoff value of $3x10^2$ kDa is sufficient for retaining the cells and exchanging the buffer [59]. The alkaline lysis is challenging since gentle and defined mixing is probably difficult during production up scaling when volumes are increased [57]. After concentration, the cells can be resuspended. RNase can be added with the suspension buffer. The lysis starts with adding an equal amount of sterilized lysis buffer that contains 200mM NaOH and 1% SDS to the cell suspension [7]. The cells need to be exposed for about 5 minutes which will result in an acceptable yield of plasmid DNA without causing undesirable plasmid degradation. The total amount of lysis buffer is added as fast and careful as possible and gentle agitation is applied to the bottle. Vigorous mixing needs to be avoided because high shear stress results in pDNA degradation and genomic DNA contamination [10]. After cell lysis, cellular debris, gDNA and proteins are precipitated by quickly adding an amount of chilled sterilized neutralization buffer (3 M KAc pH 5.5) equal to the amount of cell suspension. The mixture should be thorough but not violent, because the density of the lysed cell suspension differs from the density of the neutralization buffer. Floccules are formed in the solution and they appear to be slightly loose and dry [7,10].

Pre-clarification

To remove debris from the lysate the content needs to be semi-clarified. After transferring the lysate into a vacuum bottle, a small vacuum of 500 mbar is applied to this bottle [7]. During the vacuum, the flocculated debris is lifted to the surface by air bubbles in the solution. After vacuum, the cleared lysate is pumped from the vacuum bottle through a filter. Many filters are suitable for this technique and are commercially available.

Purification

RNA removal

In plasmid DNA production, cellular RNA from the host is a major contaminant. Due to their similar structure, RNA and DNA will compete for binding sites in all chromatography steps and RNA will block the available places for plasmid DNA [60]. RNA is usually removed by enzymatic digestion using ribonuclease A (RNase A) [40,44,60,61]. This enzyme efficiently degrades RNA impurities but is of bovine origin and should be avoided in the production of biopharmaceuticals intended for human application [42,44,62,63]. As an alternative recombinant RNase A may be used, however this will significantly affect the costs [58]. Only a few plasmid purification processes completely avoid the use of RNase A [7,40]. In most cases the RNA is removed by specific precipitation techniques. However, they are applicable on a laboratory scale only [45,57,60,62,64–66]. Combination of two simple techniques provides almost complete removal of RNA. The addition of calcium chloride salt precipitates high molecular weight RNA, and TFF clears low molecular weight RNA [40,58]. Chromosomal DNA, endotoxin and proteins are also precipitated by the addition of calcium chloride [40]. For the removal of RNA by TFF the ionic strength of the diafiltration buffer is important. A low ionic strength results in a reduced RNA content in the retentate. Additionally total nucleic acid load on the membrane has an effect on RNA clearance, a higher plasmid load results in a lower RNA clearance [40]. Also size exclusion [58] and anion-exchange chromatography [31,32,45] can be used for RNA removal. Freitas et al discovered that RNA content can be reduced by simply extending the fermentation time [67]. Streitner et al describe a reverse micellar extraction system in which RNA is removed from a diafiltered cleared lysate [68]. This is a simple, scalable and inexpensive first purification step.

Endotoxin removal

Since *E.coli*, a gram-negative bacterium, is frequently used for amplification in the manufacturing process of plasmids, endotoxins or lipopolysaccharides (LPS) are a common impurity. Lysis of the *E.coli* cells leads to high levels of LPS derived from the outer cell membrane. Residual LPS in pDNA drug products can be potentially toxic, as parenteral injection of LPS can result in a septic shock like syndrome [69,70]. LPS can be efficiently removed by different endotoxin removal steps and current protocols are capable of bringing

LPS levels in pDNA preparations down to 0.01 - 100 International Units (IU)/mg, depending on the purification strategy applied [7,27,41,71–73] Chromatographic methods including hydrophobic interaction [47], gel filtration [42] and anion exchange (e.g. Qiagen). Triton based buffers can successfully be used to encapsulate endotoxins prior to chromatographic analysis [7,52]. HIC issued for endotoxin removal, because it is based on the difference in surface hydrophobicity of pDNA and endotoxins [74]. Foam fractionation is also investigated as a method to separate endotoxin from plasmid DNA and recombinant proteins. This method is ineffective in the purification of plasmid DNA solutions, which are not surface-active and remain in the bulk solution with endotoxin [75].

Topology

Plasmid DNA exists in different topoisoforms, with the only intact and undamaged form being the supercoiled, covalently closed circular (ccc-supercoiled, SC) DNA. Linear forms as well as open circular (OC) forms have been damaged at different gene locations randomly [76] which make these forms inefficient, if promoter or gene coding regions have been destroyed [60]. It is generally believed that supercoiled plasmids are the most active of these forms, based on transcription in vivo [57]. Clearly, separation of the supercoiled form from the other plasmid forms represents one of the most difficult purification challenges, because of the similarity in size and identical nature of nucleotide sequences [77]. However, if expression of the nicked and linear forms of a gene-therapy plasmid was demonstrated to be equivalent to the expression of the supercoiled form, it is conceivable that these plasmid forms could also be considered as active pharmaceutical ingredient (API) – and the cost and difficulty of the purification could be greatly reduced [77]. SC pDNA gives a significantly higher antigen expression than OC and linear pDNA when formulated as PEGylated polyplexes and tattooed in ex vivo human skin, but pDNA topology does not influence antigen expression [78]. While the presence of SC topology guarantees transcriptionally active pDNA molecules, the OC topology does not necessarily imply that the pDNA remained transcriptionally inactive. Successive single-stranded breaks in the OC pDNA sequence could indeed affect the transcription efficiency without affecting the plasmid topology. Unfortunately, at the moment it is unclear how many single-stranded cuts the OC pDNA can bear before it becomes transcriptionally inactive [79,80]. Stability studies in vitro have shown that SC plasmids are sequentially converted to relaxed circles, linear plasmids, and oligomers [81]. Consequently GMP batches of pDNA should contain predominantly the SC form [8,30]. The FDA states that the minimum specification for SC pDNA content should preferably be >80% [82].

Purification techniques

The one step anion exchange process utilizing a silicone based resin, developed for laboratory scale, is available for GMP productions [7,31]. The major disadvantage for usage at larger

scale is the single use application. The membranes used for anion-exchange membrane chromatography developed by Pall (Mustang Q) and Sartorius Stedim (Sartobind Q) are designed for single use. Recently, Pall also developed a membrane capsule for multiple use (Mustang XT). Since pDNA retention capacity of a membrane is much larger than of the anion-exchange resin, the membrane has a smaller volume. This results in smaller volumes of necessary buffers, eventually resulting in a more cost-effective process. Since membrane chromatography has only recently been introduced, the technique is not yet widely implemented in larger scale production processes. Other purification techniques include size exclusion chromatography (SEC) [41,42] and different types of affinity chromatography [83,84]. With SEC molecules are separated solely based on size. The application of SEC is limited, because impurities with the same size as SC pDNA can elute with the plasmid DNA [84]. SEC should be followed by an additional purification step to ensure a low impurity level, or employed as the last purification step to achieve both buffer exchange and further removal of impurities [74]. Affinity chromatography uses a specific binding agent to purify biomolecules on the basis of their biological function or individual chemical structure [84]. For pDNA amino acid- DNA affinity chromatography (with histidine or arginine ligands) is successfully used in a one step process [43]. The underlying interaction mechanism for histidine-agarose chromatography (HIC) is thought to involve hydrophobic interactions and non-specific bio recognition of the nucleic acid bases by the histidine ligand [85,86]. An alternative membrane HIC process is developed, using a membrane support that is derivatized with hydrophobic ligands to yield an HIC matrix [87]. A method for large-scale production of negatively and positively SC plasmid DNA is based upon the known effect of ionic strength on the direction of binding of DNA to an archeael histone, rHMfB, with low and high salt concentrations, leading to positive and negative DNA supercoiling [88]. All different purification methods result in highly pure material, however, the major advantage of a one step process is that it is time saving, compared to a multi-step process.

Quality control

The World Health Organization states that a summary of the characterization of the drug substance should be provided including its identity, strength, biological activity and purity [89]. Rigorous characterization by chemical, physical and biological methods will be essential paying particular attention to the use of a wide range of analytical techniques which are based on different principles. It is recommended that the specification includes an assessment of the identity, nature and quantity of the plasmid, purity, biological activity, endotoxin content and sterility or bioburden. Early in development the specification may be limited and have wide acceptance criteria (World Health Organization, 2007). Various techniques to ensure quality and safety of pDNA have been described (Table 3). UV-vis spectroscopy is widely used to assess the concentration of nucleic acids [90], additionally it gives information on

the purity in terms of protein content [57]. The most frequently used techniques for pDNA analysis are agarose gel electrophoresis (AGE) and HPLC-UV analysis (table 3). AGE analysis includes restriction analysis, intact plasmid and residual genomic DNA/RNA analysis. In AGE analysis traditionally ethidium bromide (EtBr) is used for visualization of the DNA, also more sensitive intercalating dyes like SYBR Green I are being used [7,91]. Also capillary gel electrophoresis (CGE) analysis using different background electrolytes and methods of detection has been used [92–96]. An advantage of CGE over AGE is that it gives better resolution, higher sensitivity and better reproducibility than AGE and also separation time is reduced [57,97–99]. CGE is one of the methods with which the different amount of supercoils can be separated analytically [100], however this has also been shown in AGE analysis [101] and HPLC analysis [102]. Plasmids of different sizes always show the same migration order [103]. CGE requires only 10-50 ng DNA, and quantification is much less prone to errors than scanning of flat agarose gels [98]. When using laser induced fluorescence (LIF) detection, intercalating dyes have to be used. Different intercalating dyes produce different migration orders of supercoiled, linear and nicked plasmid DNA [104]. Another technique for plasmid DNA analysis is the electrophoretic separation on microchips [105]. The advantage of this technique is that several samples can be analyzed simultaneously, and analysis time is considerably shorter than AGE or CGE analysis (1 min per sample). Recently, an arginine-affinity chromatography analytical technique is developed that previously has been used as a purification technique [106]. Plasmid DNA on gold particles, used for particle mediated epidermal delivery, can be quantified by inductively coupled plasma mass spectrometry (ICP MS). When plasmid DNA is desorbed onto the gold particle UV-vis spectroscopic methods no longer yield accurate results. ICP MS is able to detect the phosphorus atoms on any single nucleotide base in the plasmid DNA [107]. For residual RNA analysis also a RiboGreen assay has been developed [108]. This assay allows detection of as little as 1.0 ng/ml RNA, surpassing the sensitivity achieved with EtBr by 200-fold. Determination of nucleic acids in solution with fluorescent nucleic acid stains results in less contaminant interference than 260-nm absorbance measurements. For precise measurements the sample needs to be treated with RNase-free DNase I to remove DNA. DNA interferes due to strong fluorescence enhancement upon binding to the RiboGreen reagent.

Table 3: Overview of important quality control methods with their respective methods. Abbreviations: AGE, agarose gel electrophoresis; LAL, limulus amoebocyte lysate; EU, European units; HPLC, high performance liquid chromatography; PCR, polymerace chain reaction; BCA, bicinchoninic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; CGE, capillary gel electrophoresis; SC, supercoiled

Test	Method	References
Identity	AGE restriction digests and plasmid size	(Horn et al., 1995; Schleef and Schorr, 1998; Varley et al., 1999; Ferreira et al., 2000; Diogo et al., 2000; Schalk et al., 2001; Przybylowski et al., 2007; Quaak et al., 2008)
	Sequencing	(Schleef and Schorr, 1998; Schalk et al., 2001; Quaak et al., 2008)
Concentration	Measuring absorbance at 260 nm, calculation	(Schleef and Schorr, 1998; Varley et al., 1999; Schalk et al., 2001; Przybylowski et al., 2007; Quaak et al., 2008; Zhang et al., 2008)
	ICP MS	(Medley et al., 2010)
Sterility	Conform current pharmacopeia	(Horn et al., 1995; Schleef and Schorr, 1998; Schalk et al., 2001; Przybylowski et al., 2007; Quaak et al., 2008)
Endotoxin	Kinetic LAL test	(Horn et al., 1995; Schleef and Schorr, 1998; Varley et al., 1999; Ferreira et al., 2000; Diogo et al., 2000; Schalk et al., 2001; Eon-Duval, 2003b; Urthaler et al., 2005; Przybylowski et al., 2007; U.S.department of health and human services, 2007; Quaak et al., 2008; Zhang et al., 2008)
RNA	HPLC	(Horn et al., 1995; Schleef and Schorr, 1998; Varley et al., 1999; Diogo et al., 2000; Eon-Duval, 2003b; Przybylowski et al., 2007)
	AGE	(Horn et al., 1995; Schleef and Schorr, 1998; QIAGEN, 1998; Varley et al., 1999; Ferreira et al., 2000; Diogo et al., 2000; Schalk et al., 2001; Urthaler et al., 2005; U.S.department of health and human services, 2007; Quaak et al., 2008; Zhang et al., 2008)
	Ribogreen assay	(Jones et al., 1998)
E.coli DNA	Southern blot	(Horn et al., 1995; Schleef and Schorr, 1998; Ferreira et al., 2000; Diogo et al., 2000; Schalk et al., 2001; Eon-Duval, 2003b; Urthaler et al., 2005; U.S.department of health and human services, 2007; Zhang et al., 2008)
	AGE	(Ferreira et al., 2000; Schalk et al., 2001; Przybylowski et al., 2007; Quaak et al., 2008)
	PCR analysis	(Varley et al., 1999; Schalk et al., 2001; Urthaler et al., 2005; U.S.department of health and human services, 2007)
Residual Protein	BCA test	(Horn et al., 1995; Schleef and Schorr, 1998; Ferreira et al., 2000; Diogo et al., 2000; Schalk et al., 2001; Eon-Duval, 2003b; Urthaler et al., 2005; Quaak et al., 2008)
	SDS-PAGE	(Diogo et al., 2000; Schalk et al., 2001; Przybylowski et al., 2007)
	ELISA	(U.S.department of health and human services, 2007; Zhang et al., 2008)
Purity	Absorbance ratio 260:280 nm	(Schleef and Schorr, 1998; Schalk et al., 2001; Quaak et al., 2008; Zhang et al., 2008)
	Isoform characterisation by agarose gel electrophoresis	(Horn et al., 1995; Schleef and Schorr, 1998; Varley et al., 1999; Ferreira et al., 2000)
	HPLC	(Schmidt et al., 1999; Eon-Duval, 2003b; Urthaler et al., 2005; Przybylowski et al., 2007; Zhang et al., 2008)
	CGE	(Schmidt et al., 1999; Holovics et al., 2010)
	Arginine-affinity chromatography	(Sousa et al., 2011)
Potency	Transfection/ immunostaining	(Horn et al., 1995; Schalk et al., 2001; Przybylowski et al., 2007; Quaak et al., 2008)
	Transformation efficiency	(Ferreira et al., 2000; Diogo et al., 2000; Schalk et al., 2001)
	ELISA	(Zhang et al., 2008)

R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34 R35 R36 R37 R38 R39

Formulation

Pharmaceutical formulation concerns the development of a dosage form suitable for its intended use with respect to e.g. administration route, shelf life (stability). Plasmid DNA may be administered either in a simple salt solution ("naked DNA") or complexed with a carrier or adjuvant [109–115]. To date most naked pDNA vaccines have been formulated in either water for injections (WFI) [103,116–118] or in (phosphate/citrate buffered) saline [44,81,118–122]. The physical and chemical stability of pDNA is a requirement for DNA-based pharmaceuticals, in order to be stored, shipped and applied even under extreme environmental conditions. Storage conditions can influence pDNA topology. Storage of solutions at 4°C promote plasmid instability, which could have an impact on transfection efficiency [60]. Formulation of naked pDNA in 0.9% sodium chloride irrigation USP results in a stable product for 12 months when stored at 4°C and for at least three years when stored at -20°C [44]. This is in contrast to the stability data presented by Walther et al., who demonstrated degradation of naked pDNA in water after storage for 6 months at 4°C, and rapidly increasing thereafter [103]. However, pDNA size also plays a major part in susceptibility to degradation[123]. Condensation of the pDNA prevents degradation usually multivalent cations are used for condensation [124–127], however possibly sodium can partly condense the pDNA resulting in better stability when stored in solution. To improve plasmid DNA stability lyophilization can be used [7,128,129]. Lyophilization may increase the stability of DNA under long-term storage, but may also cause damage during the freezing step of the lyophilization process [129,130].

In general lyophilization is used to ensure a low moisture content for drugs that are susceptible to hydrolysis [128]. Lyophilization causes the removal of the hydration sphere around a molecule. Only a few water molecules most tightly bound to DNA do not form an ice-like structure upon low-temperature cooling [129]. It has been reported with X-ray diffraction, infrared and ultraviolet spectroscopy that hydration water is necessary for maintaining the structural integrity of the DNA molecule. In the absence of water, the ordered conformation is lost and base stacking is destroyed [131]. Lyophilization without protecting agent results in loss of plasmid DNA activity (up to 75%), due to loss of hydration water [129,132]. Agents that can substitute for non freezable water, such as sugars, have demonstrated cryoprotective properties for DNA and other molecules during lyophilization [129,133]. Sugars have hydroxyl groups and are therefore able to form hydrogen bonds with proteins or DNA preventing aggregation [134,135]. Often crystalline bulking agents (like mannitol or glycine) are preferred for lyophilization because they form stronger dried cakes, with better dissolution properties than amorphous agents do [136]. Nevertheless, the use of such excipients generally requires an annealing step in the lyophilization process, before sublimation to promote the complete crystallization of the bulking agent [137]. Fakes et al have described the moisture sorption behavior of mannitol, sucrose, trehalose, dextran and polyvinylpovidone (PVP)

[128], agents frequently used for protection during lyophilization [128,129]. In general cakes are more stable in the presence of amorphous bulking agents and the Tg of lyophilized cakes must be maintained above planned storage conditions to assure long-term stability. Naked pDNA lyophilized with sucrose as bulking agent has been shown to have a shelf life stability for more than 5 years [138].

Crystalline bulking agents are essentially anhydrous and any residual moisture will localize in the amorphous drug phase only, resulting in degradation [139]. Additionally only a small rise in residual moisture content decreases the T_g value of freeze-dried sugars significantly [140]. Residual moisture contents of lyophilized cakes can be controlled by the use of appropriate bulking agents, or by changing the secondary drying cycle during lyophilization [128]. Moisture level after lyophilization should be as low as possible since amorphous disaccharides will convert to the crystalline state at moisture levels above 10% and the sorbed moisture will be liberated [128]. The amorphous solid systems involving polymers (like dextran, maltodextrin or PVP) may retain relatively high amounts of moisture and exhibit high glass transition temperature values, thus representing an interesting option [128,136]. These polymeric materials may also serve as good "sinks" for moisture liberated from stoppers, thus preventing the conversion of the drug or other excipients to crystalline states [141]. In formulations, they may be used either alone or in combination with other bulking agents, e.g. low-molecular-weight sugars [128]. Dextran/sucrose mixtures give the opportunity to concentrate solutions upon reconstitution, transfection is maintained even when rehydrated to only 10% of the original volume [132]. An advantage of using PVP is that it has been shown to increase the expression of pDNA. This polymer is thought to interact with pDNA through hydrogen bonding and may enhance the expression of pDNA *in vivo* at higher concentrations of pDNA by improving its tissue dispersion or cellular uptake [113]. Not only the residual water content of lyophilized formulations should be controlled, but also the formation of reactive oxygen species (ROS) [142]. Molina et al show that chemical degradation by ROS during storage contributes to a consistent loss of supercoiled content observed at all storage temperatures [143].

CONCLUSIONS

In this review aspects of the pharmaceutical development of plasmid DNA vaccines for early oncology clinical trials have been discussed. It was shown that to date there is no standardized pDNA production process available. Since most methods used during production are developed for laboratory scale, difficulties can arise when the process needs to be scaled up for larger trials. Fermentation can be scaled-up using fed-batch instead of batch fermentation, however care must be taken to select the proper medium, feed solution and feed rate. In

the down stream process the lysis step is a critical step when using alkaline lysis, since pDNA is only able to withstand the high pH for a defined period of time. Gentle mixing with large volumes is challenging, therefore in-line lysis methods are being developed. For pDNA purification ideally a one step process with a high pDNA capacity should be used. Membrane chromatography is very promising, since relatively small membranes can capture large amounts of pDNA. To avoid the use of organic solvents, TFF can be used to exchange the elution buffer for the desired solution and concentrate the solution for further processing. If a lyophilized product is required, disaccharides are sufficiently able to protect the pDNA from stresses during lyophilization. In combination with polymers, the cake will remain its structure even when exposed to high humidity. The polymers like dextran, maltodextrin and PVP serve as good sinks for moisture, preventing collapse of the cake.

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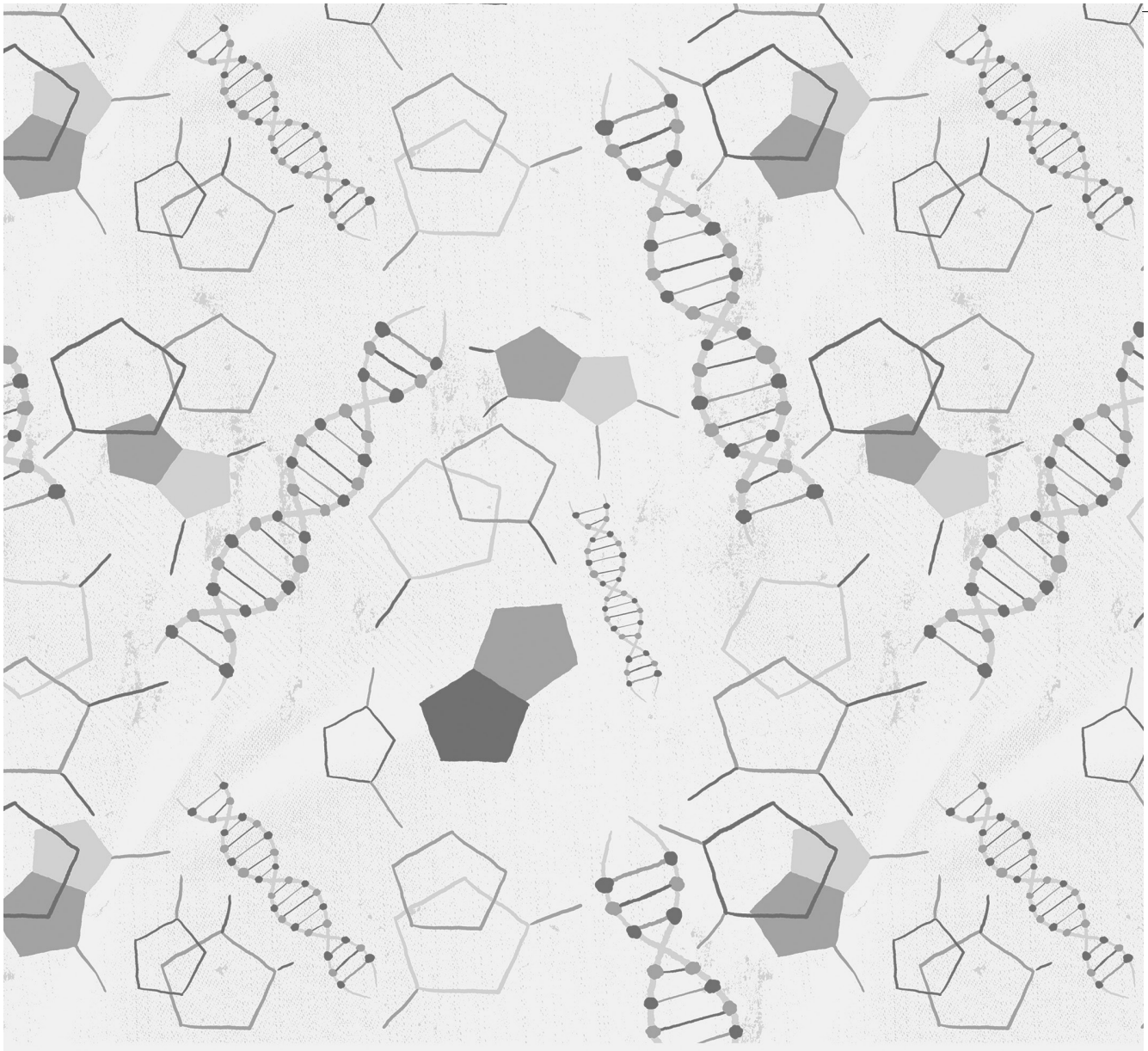

Chapter 2

Pharmaceutical production of plasmid DNA vaccines

Chapter 2.1

GMP compliant production of a plasmid DNA vaccine for the treatment of HPV16 induced malignancies

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ABSTRACT

To induce a specific cytotoxic T-cell response targeting Human Papilomavirus (HPV) 16 induced malignancies, we developed an HPV16 E7 based DNA-vaccine. The vaccine is composed of a pVAX backbone with an insert encoding a gene-shuffled version of HPV16 E7 that is genetically fused to an immunostimulatory sequence (tetanus toxin fragment-c), and is termed pVAX TTFC-E7SH. In a phase I study the plasmid will be administered via intradermal tattoo vaccination to assess both the toxicity and potency of inducing oncogene-specific T-cell immunity. To facilitate this study a Good Manufacturing Practice (GMP)-compliant plasmid manufacturing process was set up with fed-batch upstream processing, anion exchange membrane downstream processing and a pharmaceutical dosage form was produced. We manufactured approximately 825 mg plasmid DNA of a high purity >90% supercoiled DNA, an $A_{260/280}$ ratio 1.80-1.95, undetectable or low residual endotoxins, Escherichia coli host cell protein, RNA, and DNA. After sterile filtration, the concentration of the plasmid solution was approximately 1.0 mg/mL. For the scheduled phase I study a concentration of 5 mg/mL is desired, and further concentration of the solution was achieved by lyophilization. Upon reconstitution of the lyophilized material with an isotonic sucrose solution, a solution of 5mg/mL TTFC-E7SH could be made. Lyophilized TTFC-E7SH was sterile with >90% supercoiled DNA, an $A_{260-280}$ ratio 1.80-1.95, content 90-110% of labeled, and residual water content <2% (w/w) and stable for at least three months at 25°C. Upon restriction-enzyme digestion, the product yielded the predicted profile. Immunogenicity was confirmed in an *in vivo* mouse model. We have thus developed not only a reproducible process to manufacture pharmaceutical grade plasmid DNA but also a stable dosage form for use in clinical trials.

INTRODUCTION

Persistent infection with high-risk Human Papilomavirus (HPV) subtypes is strongly associated with the development of cancers of the cervix, vulva, vagina, penis, anus and oropharynx. One of the prerequisites for carcinogenesis is the continuous expression of the oncogenic early proteins E6 and E7 [1]. As E6 and E7 are strictly intracellular proteins, T-cell mediated immunity is needed to recognize the (pre)malignant cells. Therefore, DNA vaccination with a plasmid encoding these oncogenes is an attractive immunotherapeutic approach for the induction of a HPV 16 specific cytotoxic T-cell response against HPV-induced lesions [2]. We developed an HPV16 E7 based DNA-vaccine composed of a pVAX backbone with an insert encoding a gene-shuffled version of HPV16 E7 (E7SH) that was genetically fused to an immunostimulatory sequence, Tetanus toxin fragment-c (TTFC), termed pVAX TTFC-E7SH [3]. In preclinical studies this HPV16 E7 directed vaccine proved to be highly effective, and importantly a detailed safety study demonstrated lack of oncogenic potential warranting clinical evaluation [4]. In an upcoming phase I clinical study, pVAX TTFC-E7SH will be administered using our in-house developed tattoo strategy [5]. It was shown previously that this short-interval intradermal DNA vaccination leads to the rapid and sustained development of both T-cell and B-cell responses [6].

To generate pVAX TTFC-E7SH for clinical use, we set up a manufacturing process according to Good Manufacturing Practices (GMP) guidelines [7]. In summary, this process consists of the production of a Master Cell Bank (MCB), production of the bulk drug from this MCB and subsequently production of the final product from E7 bulk drug [8]. The MCB, expanded from a single host cell transformed with the plasmid of interest, is established in order to enable the repeated manufacture of the pDNA bulk drug of consistent quality in time. The manufacture of bulk drug pDNA consists of the expansion by fed-batch fermentation (upstream processing) of an MCB aliquot followed by purification of the pDNA product (downstream processing). In this process, contaminants like genomic DNA, RNA, proteins, and endotoxins are reduced to predefined, acceptable levels. Production of pDNA final product involves an aseptic fill & finish process resulting in the desired pharmaceutical dosage form containing the pDNA of interest in the required dose unit. All abovementioned production steps are monitored by various in-process controls, focused on pDNA concentration and purity during processing [8]. MCB, pDNA bulk drug and final product are subject to extensive quality control testing (also in-process) before release for (clinical) use. Several papers have been published on the (small-scale) manufacture of clinical grade plasmid DNA [8–13].

In this article we describe the GMP compliant production of pVAX TTFC-E7SH via fed-batch fermentation in *E.coli* followed by a one-step purification, and organic solvent free, protocol. Tangential Flow Filtration (TFF) in combination with lyophilization was used in order to achieve a highly concentrated and stable dosage form.

MATERIALS AND METHODS

Production facility

The plasmid DNA production facility for manufacturing of the Master Cell Bank (MCB) and bulk drug consists of two GMP EU class A with class B background cleanrooms [14](Interflow, Wieringerwerf, The Netherlands). The first cleanroom is dedicated to handling bacteria and the second cleanroom is dedicated to purification. Both cleanrooms contain a class 100 (A) biosafety cabinet. The fermentor is placed in a class 100 (A) down-flow booth. Manufacturing of pVAX TTFC-E7SH final product is performed in a third class 100 (B) cleanroom, containing a class 100 (A) down-flow cabinet, as described earlier [15]. All cleanrooms are subjected to a monitoring program for viable and non-viable particles at operating and resting state [16]. During manufacture only product dedicated glassware and sterile disposables are used. Buffer components, pharmaceutical excipients and primary packaging materials used in the manufacture of pVAX TTFC-E7SH were of European Pharmacopeia (Ph. Eur.) grade (if possible) and provided with a Certificate of Analysis (CoA) by the supplier. All materials and excipients were approved on the basis of in-house quality controls carried out according to monographs in the mentioned pharmacopoeia (if applicable).

Source plasmid DNA (active pharmaceutical ingredient (API))

TTFC-E7SH consists of a pVAX1 plasmid backbone with CMV promotor sequence, a T7 bacteriophage promotor/priming sequence, a multiple cloning site, a bovine growth hormone (BGH) reverse priming site, a BGH polyadenylation site, a kanamycin resistance-gene (S.aureus), and a pUC origin of replication site for propagation of the plasmid in *E.coli*. The genetic sequence for the fusion-protein is cloned in the HindIII/XbaI cloning site. It consists of the non-toxic domain 1 of tetanus toxin fragment C and a shuffled version of the HPV type 16 E7 protein. Tetanus toxin fragment C (from Clostridium tetani) is used as stimulator of T-helper cells which increase the cytotoxic T cell reaction against the shuffled E7 protein. See also Fig. 1 [4]. Before further use, the obtained pVAX TTFC-E7SH source pDNA was analyzed for purity and identity by agarose gel electrophoresis (AGE), restriction analysis and sequencing.

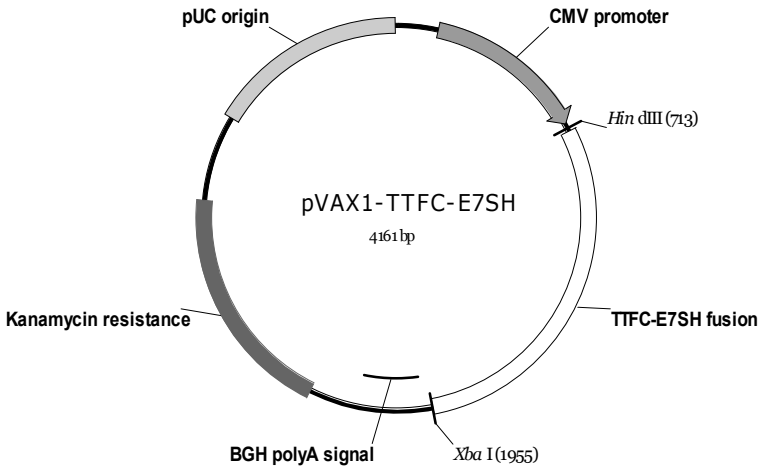

Figure 1: *Plasmid DNA map of pVAX TTFC-E7SH.*

Master Cell Bank (MCB)

E.coli DH5 bacteria were used for transformation (ATCC #53868, Teddington, Middlesex, UK). Bacteria were made competent with $CaCl_2$ and subsequently transformed with TTFC-E7SH using a standard heat shock method [17]. These bacteria were plated on Luria Bertani (LB) plates containing 100 µg/mL kanamycin (Biotrading Benelux, Mijdrecht, The Netherlands) and grown in an Incubator Shaker Innova 4230 (New Brunswick Scientific BV. Nijmegen, The Netherlands). A single colony was isolated and grown in 175 mL LB-Miller broth (Sigma Aldrich Chemicals BV. Zwijndrecht, The Netherlands) containing 100 µg/mL kanamycin (Roche diagnostics Nederland BV. Almere, The Netherlands) at 37°C in a 1L sterile baffled shake flask (Nalgene, Rochester, NY, USA) at 200 rpm. When an OD600 ≥ 0.7 was reached, glycerol (BUFA, Uitgeest, The Netherlands) was added to the culture (30% v/v). Aliquots of 1 mL were frozen at -80°C. One MCB lot was produced. Quality control of the MCB was performed according to Ph. Eur. [16] and FDA guidelines [18,19]. Plasmid identity was confirmed by sequencing and restriction analysis.

Fermentation

Two vials of the pVAX TTFC-E7SH MCB were inoculated into 15 mL LB broth containing 0.1 mg/mL kanamycin. After growing for 7 hours at 37°C and 200 rpm each solution was transferred to 235 mL LB broth containing 0.1 mg/mL kanamycin for overnight growth. After calibration of the pH and Dissolved Oxygen (DO)-electrode, this culture was inoculated into a BioFlow 3000 benchtop fermentor with a working volume of 10L (New Brunswick Scientific BV, The Netherlands) containing 7L growth medium (7 g/L KH_2PO_4, 7 g/L K_2HPO_4, 6 g/L $(NH_4)2SO_4$ and 14 ml/L glycerol 85%) 58.1 ml medium supplement solution (240

g/L thiamine.HCl and 72 g/L $MgSO_4.7H_2O$ in WFI), 7 ml trace element solution (2.7 g/L $FeCl_3.6H_2O$, 2 g/L $ZnCl_2$, 2 g/L $CoCl_2.6H_2O$, 2 g/L $Na_2MoO_4.2H_2O$, 1 g/L $CaCl_2$, 1.3 g/L $CuCl_2.2H_2O$, and 0,5 g/L H_3BO_3 in 1.2N HCl)[20], 14 mL kanamycin 50 mg/mL and 700 µL Antifoam 204 (100% active components, mixture of organic non-silicone polypropylene basted polyether dispersions, Sigma-Aldrich Chemicals BV. Zwijndrecht, The Netherlands) preheated to 37°C under fed-batch conditions. During fermentation pH was controlled at 7.1 with 35% ammonia solution and 15% phosphoric acid solution. Addition of Antifoam 204 was controlled with a solution level probe and agitation speed (max. 800 rpm) and air inlet (max 15) were automatically feed-back controlled based on DO at a set point of 30% and monitored using the BioCommand software (New Brunswick Scientific BV. Nijmegen, the Netherlands).

24 hours after inoculation, addition of feed supplement is started at 4 g/L/h. During fermentation, addition of feed supplement is increased, due to increase in fermentor content. After 70 hours fermentation is stopped. During fermentation approximately 1400 mL feed supplement, 300 mL ammonia, 150 mL 15% Phosphoric Acid and 20 mL Antifoam 204 is added.

Downstream Processing

Recovery

After 70 hours of fermentation the bacteria were harvested from the fermentor. The culture was mechanically pumped into the Flexstand Tangential Flow Filtration (TFF) system equipped with a Hollow Fibre Cartridge model UFP-300-E-6A with a membrane area of 0.28m^2 (GE healthcare, Diegem, Belgium). The bacteria were concentrated to a volume of approximately 1.5 L Subsequently the fermentation media were exchanged, in eight steps, for TE buffer (50 mM tris, 10 mM EDTA), the first buffer in the lysing protocol.

Lysis and pre-clarification

The harvested bacteria were lysed using a standard alkaline lysis procedure. In brief, the biomass was resuspended in TE-buffer supplemented with 600 µL 100 mg/mL certified BSE free RNase A solution (Sigma Aldrich Chemicals BV. Zwijndrecht, The Netherlands) [21] and lysed by the addition of lysis buffer (200 mM NaOH; 1% SDS) and inverted mixing by hand. After 5-10 min. after lysis, cellular debris, gDNA and proteins were precipitated by gently adding pre-cooled (4-10°C) neutralization buffer (3M potassium acetate, KAc pH 5.5). The lysate was clarified by applying a vacuum of 500 mbar followed by dead end filtration with both a Sartopure PP2 0.2m^2/5µm filter and a Sartopore 2 XLG 0.8µm/0.2µm filter (Sartorius AG, Goettingen, Germany). Subsequently, an endotoxin removal buffer was added to the pre-cleared lysate.

Purification

One-step plasmid purification was performed using the 140 mL Pilot Scale Mustang Q XT capsule (Pall Netherlands, Mijdrecht, The Netherlands) connected to the Äkta Pilot (GE Healthcare, Diegem, Belgium) [22]. The capsule membrane is preconditioned with subsequently 1400 mL 1M NaOH and 1L 1M NaCl in 25 mM phosphoric acid (280 mL/min). The capsule membrane is equilibrated with 1L equilibration buffer (0.5M NaCl, 10mM tris, 0.1M EDTA, 280mL/min). After equilibration the cleared lysate is loaded onto the membrane (280 mL/min). Once the loading is finished, the column is washed to remove unwanted E. coli host DNA and RNA. The first wash step consists of 3.5 L wash buffer (0.6 M NaCl, 10mM tris, 0,1M EDTA, 280 mL/min) pumped over the membrane until the UV signal is close to zero and then 2 membrane volumes wash buffer are used, ca. 280 mL. The plasmid DNA is eluted from the membrane with elution buffer (1,2M NaCl, 10mM tris, 0,1M EDTA, 200 mL/min). As soon as the UV signal starts to rise the elution fraction is collected in a 1 L volumetric cylinder. Collection is paused once the UV signal is <5% of the maximum peak height. The pump is stopped for 20 minutes. Then elution is proceeded: as soon as the UV signal starts to rise, the elution fraction is collected in another 1 L volumetric cylinder and collection is stopped again once the UV signal is <5% of the maximum peak height. After elution, the capsule is cleaned with 1.5 L 1 M NaOH and flushed with 1.5 L 1 M NaCL in 0.1M NaOH for storage of the capsule (280 mL/min). During the run UV260, UV280, pH, conductivity and pressure over the column are monitored.

Concentration and sterile filtration

After purification, the plasmid was concentrated by TFF using a Quixstand TFF system equipped with a Hollow Fibre Cartridge model UFP-300E-4MA with a membrane area of 420 cm^2 (GE Healthcare, Diegem, Belgium) to concentrate the solution. The concentration of the elution fraction was determined with an Eppendorf BioPhotometer 6131 (Hamburg, Germany). The solution was concentrated to concentration of approximately 1.1 mg/ml. The elution buffer was exchanged for sterile water for injection (WFI) in eight steps, followed by sterile filtration with a 0.2 µm Mini Kleenpak filter (Pall, Hitma BV Uithoorn the Netherlands). The final plasmid solution was stored at -80°C in sterile United States Pharmacopeia (USP) grade poly propylene (PP)-bottles (Nalgene, VWR, Amsterdam, The Netherlands) until further processing.

Formulation

Formulation Development

A lyophilized product was developed in order to be able to reach high concentrations of TTFC-E7SH after reconstitution for intradermal injection, since this administration route limits the volume which can be administrated. Furthermore, lyophilization increases storage

stability at more convenient storage conditions. We developed our final formulation using a previously developed pDNA vaccine, pDERMATT (plasmid DNA encoding recombinant MART-1 and tetanus toxin fragment-c), as active ingredient. pDERMATT contains the same backbone (pVAX) and helper protein (TTFC) as TTFC-E7SH, but encodes for a MART-1 epitope instead of the gene shuffled version of E7. Several excipients were tested as lyoprotectant, cryoprotectant and bulking agent amongst which polyvinylpyrrolidone (PVP), sucrose, trehalose and sucrose/mannitol, trehalose/mannitol mixtures. Accelerated stability studies (at 25˚C/60% RH and 40˚C/75% RH conditions) with content and purity (% ccc) as primary outcome parameters showed that sucrose offers the best protection for pDNA during manufacture and storage as lyophilized product [8,23].

Manufacturing process

To manufacture 370 vials TTFC-E7SH 2 mg/vial lyophilized product, 14.8 g sucrose (Merck, Darmstadt, Germany) is weighed in a tarred volumetric flask. The TTFC-E7SH drug substance is subsequently quantitatively transferred to the volumetric flask. The sucrose is subsequently dissolved under continuous magnetic stirring. The solution is then filtered through a sterile 0.2 µm Mini Kleenpak filter (Pall, Hitma BV Uithoorn, The Netherlands) unit using a peristaltic pump and 4.8mm x 1.6mm platinum catalyzed silicone tubing (Watson Marlow, Cheltenham, UK). The peristaltic pump is calibrated for a filling volume of 2.0 mL and 2.0 mL aliquots are filled into 8 mL colorless glass injection vials (hydrolytic class I Type Fiolax-clear, Aluglas, Uithoorn, The Netherlands) using 1.6mm x 1.6mm platinum catalyzed silicone tubing. Grey butyl rubber lyophilization stoppers (Type FM157/1, Helvoet Pharma N.V., Alken, Belgium) are partially inserted into each injection vial and the injection vials are loaded into a Model Lyovac GT 4 freeze-dryer (GEA lyophil GmBH, Hürth, Germany) at ambient temperature and pressure.

Lyophilization Protocol

During the freeze-drying process, the product temperature is continuously monitored with at least 1 Model Pt-100 resistance thermometer. The product is frozen to –35°C in 2 hours. This temperature is maintained for 3 hours. After this period, the primary drying phase is started by establishing a vacuum of 0.1 mbar in 1 minute and holding the temperature at –35°C. This temperature and pressure are maintained for 1.0 hour, after which the temperature is linearly increased to – 25°C in 6 hours. This condition is maintained for 38 hours, after which the temperature is linearly increased to –12°C in 5 hours and subsequently to +25°C in 5 hours. The secondary drying phase is carried out for 5 hours at a shelf temperature of +25°C and a chamber pressure of 0.02 mbar. The total length of the freeze-drying cycle is approximately 65.0 hours. Finalization of the lyophilization process is checked with a pressure increase test. The closures are subsequently put into the injection vials pneumatically under vacuum,

vials are removed from the freeze-dryer, sealed with aluminum caps (West Pharmaceutical, Germany), labeled and packed into boxes.

Quality Control

All chemicals used for quality control were of Ph. Eur. or analytical grade and used without further purification.

Agarose gel electrophoresis including endonuclease digestion

IPCs and final product were analyzed by electrophoresis using 25 cm, 1% agarose (ABGene, Epsom, Surrey, UK) self-cast gels. Agarose gels were run in a horizon 20·25 horizontal gel electrophoresis unit coupled to a Whatmann Biometra power supply (Westburg B.V., Leusden, The Netherlands). Running buffer was a 40 mM Tris-Acetate 1mM EDTA, pH 8.3 solution and electrophoresis was carried out at 30V for 21 hours. After electrophoresis, gels were stained for 1.5 hours with a 1x Sybr green I solution (Sigma Aldrich, Zwijndrecht, The Netherlands) and then visualized and photographed under UV light (Gene Genius, The Netherlands). Restriction enzyme analysis was performed using the indicated enzyme combinations and resulting DNA fragments were analyzed by agarose gel electrophoresis. Digestions were performed according to instructions of the manufacturer.

For AGE the samples were diluted, if necessary, to approximately 20 ng TTFC-E7SH /µL; for determination of residual *E.coli* host RNA/DNA in the bulk drug and lyophilized products the sample was diluted to 50 ng TTFC-E7SH /µL. Subsequently 2 µL of 6x loading dye was added to 10 µL of the solutions and loaded onto the gel.

Capillary gel electrophoresis

Capillary gel electrophoresis (CGE) of TTFC-E7SH MCB, bulk drug and final product was performed at PlasmidFactory (Bielefeld, Germany) as previously reported [24].

UV analysis

UV analysis was used to determine the concentration and purity of the plasmid DNA. Samples were diluted to 30 µg/mL and measured with a Biophotometer (Eppendorf, Hamburg, Germany). Absorbance was measured at 230, 260, 280 and 320 nm. The A260/A280 ratio was calculated.

Protein analysis

A bicinchoninic acid (BCA) assay from Pierce (Rockford, Il, USA) was used to measure residual protein content of the bulk drug product and TFF samples.

Sequencing

The sequence of the whole plasmid construct is completely sequenced (both strands) using custom primers by Verification Sequencing. The test is performed at Qiagen (Hilden, Germany).

Bioburden

The bioburden of the solution was determined using the total viable aerobic count according to Ph. Eur. 2.6.12 using 10 mL of the bulk drug product [16].

Sterility and bacterial endotoxins

Sterility of the final lyophilized product was determined by the filtration method and the presence of bacterial endotoxins with the Pyrochrome ® limulus amoebocyte lysate assay (Cape Cod Associates, Cape Cod, MA, USA). Both carried out according to Ph. Eur. 2.6.1 and 2.6.14 [16].

Mice

Female C57BL/6J mice (6–8 weeks) were obtained from Charles River (Wilmington, MA, USA). They were allowed to acclimate for approximately 1 week after arrival. All mice were housed n=5/cage, in a climate-controlled room. Food and water were provided *ad libitum*. All testing occurred between 10.00 and 18.00 hours. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals at our institute.

DNA immunization

Mice were vaccinated with the reconstituted pVAX TTFC-E7SH final product, or with empty pVAX vector as a control. For intradermal DNA vaccination, the hair of the left hind leg of the mice was re- moved with depilatory cream (Veet sensitive, Reckitt Benckiser, Berkshire, UK). Next, 15 ml of the pVAX TTFC-E7SH solution was applied to the skin and a sterile disposable 9-needle cartridge (MediUm-Tech, Berlin, Germany) mounted on an Aella® tattoo machine for medical use (MT.DERM, Berlin, Germany) was used to apply the vaccine. Needle depth was adjusted to 1.0 mm, and the needle bar oscillated at 100 Hz. DNA vaccines were punched into the skin by a 45 s tattoo. Mice were vaccinated with a standard vaccination scheme on day 0, 3 and 6 [5]. All mice were anesthetized with isoflurane (Abbott Laboratories, Illinois, USA), during treatment.

Cytotoxic T cell (CTL) assay

To measure specific CTL responses against the HPV16 E7 epitope, peripheral blood lymphocytes were stained at the indicated time points with Phyco Erythrin (PE)-conjugated H-2Db $E7_{49-57}$-tetramers and Allo Phyco Cyanin (APC)-conjugated CD8 (BD Pharmingen, San Jose, USA) at 20 °C for 15 min in FACS buffer (1x Phosphate Buffered Saline (PBS), 0.5% Bovine Serum Albumin (BSA) and 0.02% sodium azide) as described before [25]. $CD8^+$ cells expressing the HPV16 E7 epitope-specific T-cell receptor will bind to the tetramer and can be detected by flow cytometry. After incubation cells were washed three times in FACS buffer and analyzed. Living cells were selected based on propidium iodide (PI) exclusion. Data acquisition and analysis was done with a FACSCalibur (Becton Dickinson, Franklin Lakes, USA) using Flowjo software.

RESULTS

Upstream processing

Previously, we reported the small-scale manufacture of pDERMATT 2 mg lyophilized powder for reconstitution for intradermal administration. In this process, for the upstream processing, we made use of a batch-fermentation method, resulting in a yield of approximately 20 mg/L after fermentation at a 10L scale resulting in ca. 200 mg total plasmid. In order to increase the yield per fermentation run, it was decided to switch to a fed-batch fermentation method, using the continuous administration of nutrition to the bacteria culture which is necessary for growth beyond a certain OD. Table 1 gives the combined results of the upstream processing of pVAX TTFC-E7SH and the closely related pDNA vaccine pVAX sig-HELP E6SH-KDEL, using the same host cell and fermentation process [23]. As can be seen the applied fermentation process is highly reproducible during n=4 fermentation runs.

10L Fed-batch fermentation runs with a duration of approximately 70 hours resulted in a final OD_{600} > 110, all with a plasmid content of 200 mg/L (ca. 2000 mg total yield) as determined on small samples that were purified using an Endofree Qiagen Plasmid Mini kit (QIAGEN GmbH, Hilden, Germany). This fermentation strategy improved yield extensively when compared to previous protocols reaching an OD600 of approximately 7 with a total yield of about 200mg [8]. Before further processing, harvested material was stored double-concentrated at -80°C. Stability tests on frozen culture showed comparable recovery results (> 90%) and same topology of the recovered pDNA product for at least 6 weeks after harvesting (recovery t=0 of 93.5% vs. 94.5% after 6 weeks.

Table 1: IPCs Quality Control results and reproducibility of TTFC-E7SH upstream processing including set specifications and test methods. *Third and fourth fed-batch fermentations for reproducibility performed for production pVAX sig-HELP-E6SH-KDEL. Abbreviations: IPC, in-process control; OD_{600}, optical density at 600nm; UV, ultra violet; NA, non applicable.

Test	Test Method	Specification	Fermentation run			
			1	2	3*	4*
IPC						
OD_{600} inoculum	UV	≥ 0.7	8.15	0.822	4.46	3.95
Fermentation time (hh:mm)	NA	For information	68:38	70:00	71:00	72:00
OD_{600} fermentation	UV	For information	121	110.8	123.2	135.5
$A_{260/280}$ after fermentation	UV on miniprep material	1.80-1.95	1.9	1.9	1.9	1.8
Estimated amount DNA after fermentation (mg)	UV on miniprep material	For information	2000	2000	3000	3000

Downstream processing

For downstream processing, i.e. purification of TTFC-E7SH, we made again use of alternative methods as we previously reported [8]. In order to minimize RNA contamination originating from the host cell, RNase was added to the cell culture during the lysis procedure. Indeed, the addition of RNAse, obtained from certified BSE free origin, resulted in a significant decrease in residual RNA as compared to results obtained during the development phase (data not shown). Moreover, we made a switch in purification system making use of a Mustang XT filter unit instead of the QIAGEN purification resin [8]. We have shown that the QIAGEN resin reproducibly resulted in pDNA of excellent quality without the necessity of additional polishing steps (e.g hydrophobic interaction chromatography (HIC)[26]). The Mustang XT membrane (pore size 0.8 μm) was selected, as it does not require laborious column preparation and very large amounts of buffer. Also, the membrane can be used multiple times. Moreover, since pDNA binding to the QIAGEN resin (stationary phase) is based on diffusion, the binding is flow-dependent and is therefore a slow process. In the Mustang XT membrane binding involves a direct interaction of the pDNA and the stationary phase. The binding is flow independent and is therefore much faster when compared to binding to the QIAGEN resin (280 ml/min to 14 ml/min respectively. Recovery of Mustang XT was relatively low, approximately 50% when compared to the recovery from the QIAGEN resin (> 80%).

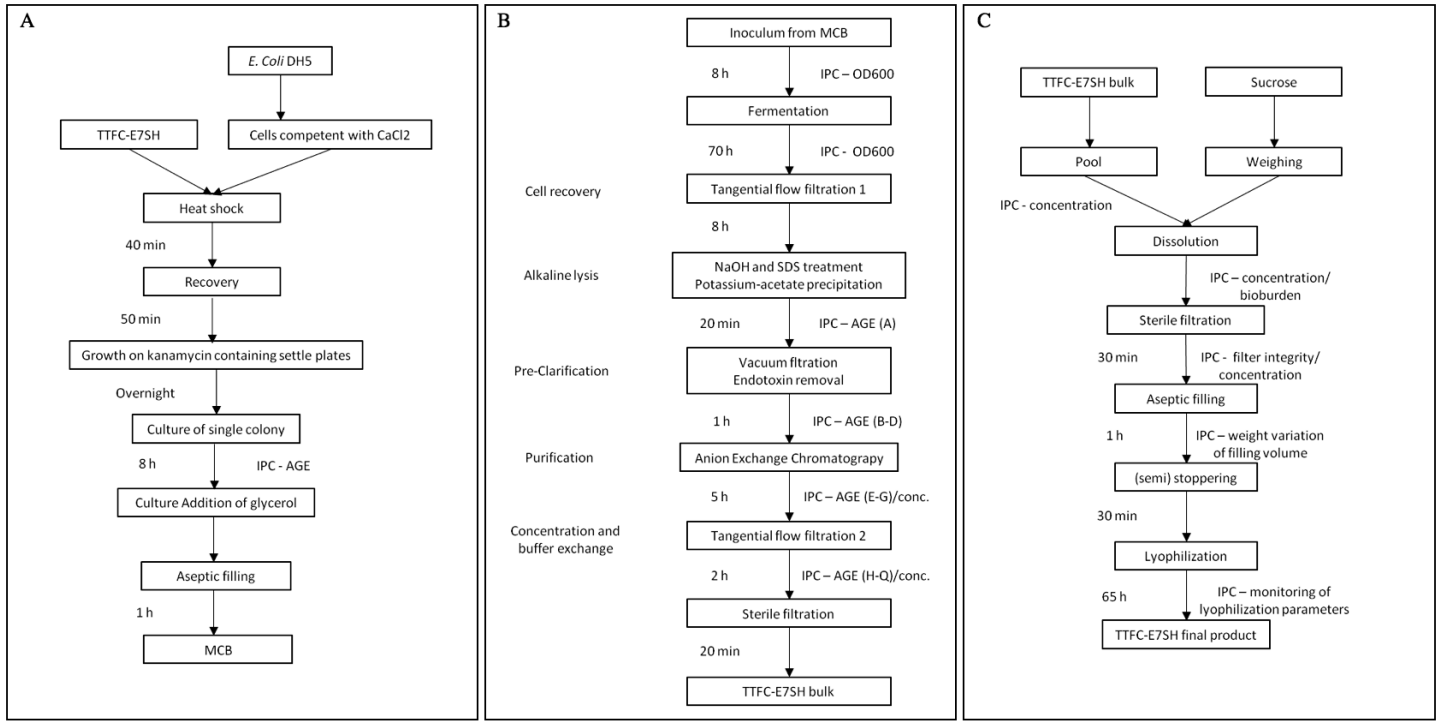

Figure 2: *Process flow sheets for the production of pVAX TTFC-E7SH at our facility, production of Master Cell Bank (MCB) (A), TTFC-E7SH bulk (B) and TTFC-E7SH final product (C).*

R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34 R35 R36 R37 R38 R39

Table 2 gives the result of three upstream processing runs for pVAX TTFC-E7SH, using material from two separate upstream fermentation runs. Fig. 3 shows that *E.coli* host RNA is efficiently removed during purification. As can be seen the *E.coli* host RNA that is not removed by the RNAse solution. is effectively removed from the Mustang XT capsule during the salt wash. In the elution phase, pDNA is released from the membrane by a further increase in salt concentration in a multiple-step elution process, illustrated in Fig. 4. AGE data show no changes in topology during the downstream processing (Table 2 and Fig. 3). Table 2 gives the in-process and quality control test results of the three TTFC-E7SH bulk drug batches. The specifications for the bulk drug are based on specifications found in literature and adapted from our previous production process. All batches contained a high supercoiled (SC) pDNA content (>90%). Linear plasmid DNA was not seen in any of the measured samples. In AGE results, a band is visible just above the open circular (OC) band. When compared to the supercoiled ladder the size of this band is approximately twice the size of the supercoiled plasmid. CGE measurements confirm the presence of dimer pDNA. Two fermentation runs allowed for the production of 825 mg bulk drug, a sufficient amount of plasmid DNA for the clinical trial. The TTFC-E7SH bulk drug products were all conform specifications and are released for formulation. From this, it can be concluded that the down-stream process selected is capable of the reproducible purification of TTFC-E7SH pDNA.

Formulation

Formulation development

For the upcoming clinical study, a dose unit content of 5 mg/ml was required. Since the TTFC-E7SH bulk drug could only be concentrated to approximately 1 mg/ml due to high viscosity of the pDNA solution, lyophilization was chosen for further concentration [27]. Vials were filled with 2 mg TTFC-E7SH and upon reconstitution with 400 µl an isotonic solution containing 5 mg/ml TTFC-E7SH was achieved. Sucrose was selected as bulking agent based on previous data with a similar sized and formulated pDNA showing stability of the lyophilized product of over 66 months [23].

Manufacturing process

An overview of our lyophilized pDNA production can be found in Fig 1C. First, the three TTFC-E7SH bulk drug products are pooled and gently mixed, to ensure a uniform end product. The TTFC-E7SH concentration of the formulation solution before and after filtration and filling was shown to be equal, indicating no retention of TTFC-E7SH in the filter or tubing. IPCs and QC results for TTFC-E7SH 2 mg/vial final product are given in Table 3. All tested items were conform specifications.

Table 2: Downstream processing IPCs and Quality Control results of TTFC-E7SH bulk drug including set specifications and test methods. Abbreviations: IPC, in-process control; QC, quality control; AGE, agarose gel electrophoresis; AEX-HPLC anion exchange high performance liquid chromatography; UV, ultra violet; BCA bicinchoninic acid; RP-HPLC, reversed phase high performance liquid chromatography; cfu, colony forming units; Ph. Eur., European Pharmacopoeia; EU, Endotoxin Unit

Test		Test Method	Specification	Bulk product (batch)		
IPC				**1**	**2**	**3**
	$A_{260/280}$ eluate	UV	1.80-1.95	1.8	1.9	1.9
	Purity SC band	AGE	Approximate size predicted	Conforms	Conforms	Conforms
	Amount DNA in eluate (mg)	UV	For information	330.0	451.95	298.0
	Recovery (%)	UV	For information	75.7	80.88	70.47
	$A_{260/280}$ bulk drug	UV	1.80-1.95	1.8	1.9	1.8
	Amount of DNA bulk drug (mg)	UV	For information	249.85	365.55	210.8
QC						
Appearance		Visual inspection	Clear, colorless solution	Conforms	Conforms	Conforms
Identity	Sequence	Sequencing	Conform reference	Conforms	Conforms	Conforms
	Restriction map	AGE	Compares to theoretical	Conforms	Conforms	Conforms
	Plasmid size	AGE	Approximate size predicted	Conforms	Conforms	Conforms
	Retention time	AEX-HPLC	Identical to standard	Conforms	Conforms	Conforms
Concentration (mg/mL)		UV	Conform declaration	1.1	0.95	1.454
Purity	$A_{260/280}$ ratio	UV	1.7-2.0	1.8	1.9	1.8
	Circular plasmid DNA (%)	CGE	≥ 90% supercoiled	91.4	94.2	91.2
	Genomic DNA	AGE	< 5% (<0.05 mg/mg plasmid)	Conforms	Conforms	Conforms
	RNA	AGE	< 4% (<0.04 mg/mg plasmid)	Conforms	Conforms	Conforms
	Protein	BCA assay	< 5 µg/mL	Conforms	Conforms	Conforms
Bioburden (cfu)		RP-HPLC	None detected	0	0	0
Endotoxin (EU/mg)		Ph. Eur.	< 100 EU/mg	Conforms	Conforms	Conforms

R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34 R35 R36 R37 R38 R39

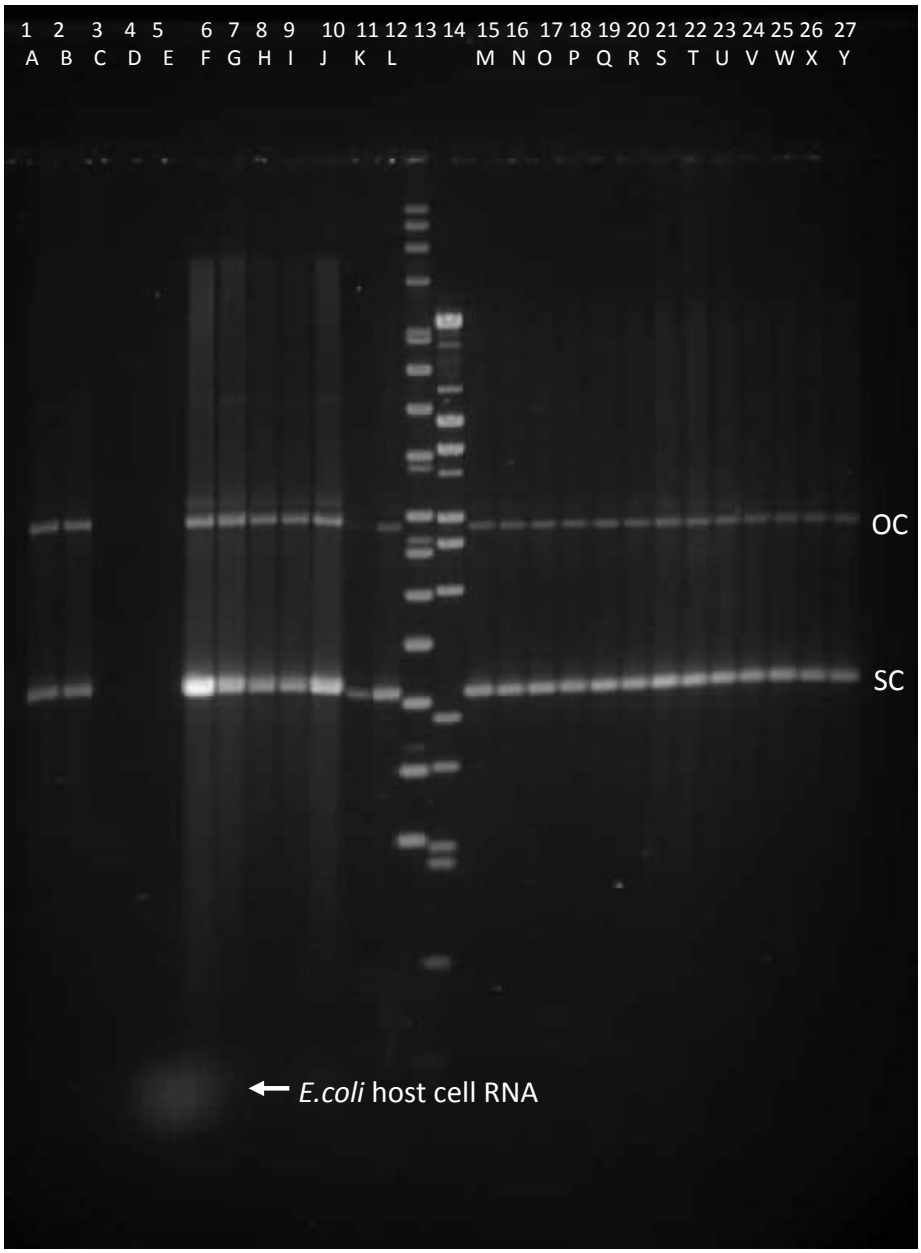

Figure 3: *Analysis of IPCs and bulk product by 1% agarose gel electrophoresis: lane 1-12 and 15-26 contain IPCs; lane 13 supercoiled DNA ladder; lane 14 lambda DNA BstEII digest; lane 27 TTFC-E7SH bulk product. Letters corresponding to the process step as indicated in figure 2B.*

Table 3: Overview of in-process controls (IPC) and quality control (QC) results of TTFC-E7SH 2mg/vial final product. Mean values are given with standard deviations within parentheses. Abbreviations: CGE, capillary gel electrophoresis; UV, ultra violet; AGE, agarose gel electrophoresis; cfu, colony forming units; Ph. Eur., European Pharmacopoeia; EU, Endotoxin Unit.

Test		Specification	Result
IPC			
	Concentration of pool (mg/ml)	1.0-1.1	0.918
	Concentration formulation solution (mg/ml)	0.9-1.1	1.005
	Concentration after filtration (mg/ml)	0.95-1.05	0.909
	Bioburden before filtration (cfu)	< 2.7 X 10^6 cfu/ml (retention capacity filter)	Conforms
	Filter integrity test, bubble point (psi)	≥ 1200	3150
	Weight variation of filling volume (%)	≤ 3.0	0.3698
QC			
Appearance		White, freeze-dried cake	Conforms
Reconstitution		Complete, leaving no visible residue as undissolved matter and resulting in a clear colorless solution	Conforms
CGE analysis	Purity	≥ 90% CCC	96.9%
UV analysis	Content	90-110% of labeled content	107.7%
	Purity	$A_{260/280}$ = 1.80-1.95	1.86
AGE analysis	Identification	Size of CCC band is 3745-4577 bp	4254 bp
	Purity	Report size of other bands (compared to supercoiled of linear DNA ladder)	1. 7850bp (SC) 2. 4498bp (lin)
	Residual *E.coli* host DNA	< 5% (<0.05 mg/mg plasmid)	< 5%
	Residual *E.coli* host RNA	< 4% (<0.04 mg/mg plasmid)	< 4 %
Uniformity of Dosage units		Conforms Ph. Eur. <2.9.40> Acceptance value < 15%	Conforms 8.84%
Residual water content (%w/w)		≤ 2%	0.86%
Endotoxin level (EU/vial)		< 10	Conforms
Sterility		Sterile	Conforms

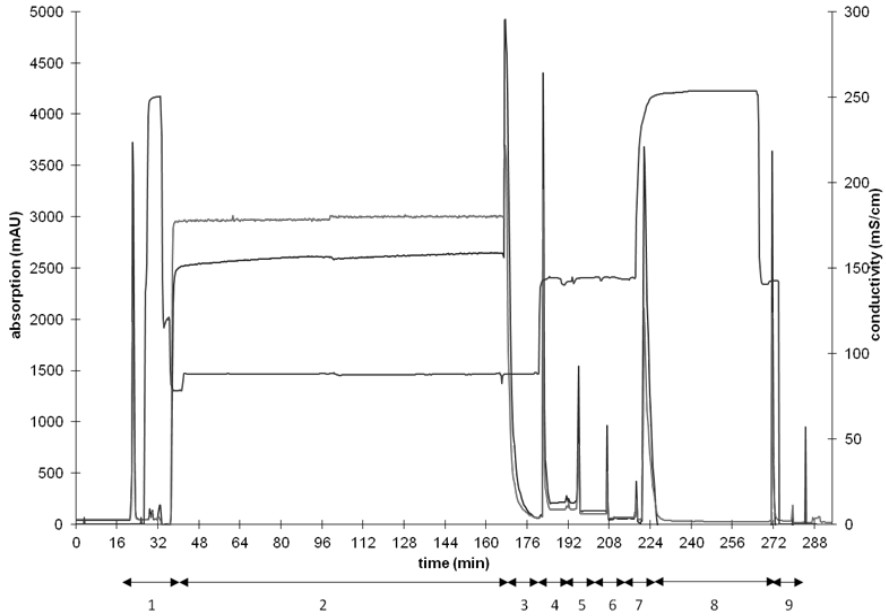

Figure 4. *Mustang XT purification profile. Blue = UV260, red = UV280, orange = conductivity. Numbers correspond with purification steps. 1= equilibration 2= loading cleared lysate 3= washing step 4= first elution step 5= second elution step 6= third elution step 7= fourth elution step 8= NaOH wash 9= storage flush.*

Immunogenicity

To test the immunogenicity of the produced TTFC-E7 pDNA vaccine, mice were vaccinated with TTFC-E7SH final product by intradermal DNA tattooing. Figure 5 shows the CTL response upon dermal vaccination. All vaccinated mice developed a strong E7-specific CTL response, with a peak level at day 21. The control group did not develop a vaccine specific immune response.

Stability upon storage

Stability of TTFC-E7SH 2mg/vial lyophilized product was evaluated at -20°C, +5±3°C and the accelerated storage condition of +25±2°C/60±5% RH (climate chamber HEKK 0057, Weiss Technik Ltd.) in the dark. After 3 months of storage, SC pDNA content as determined by CGE analysis was found to be 92.2% (-20°C), 91.6 % (+5±3°C) and 92.5% (+25±2°C/60±5% RH), respectively.

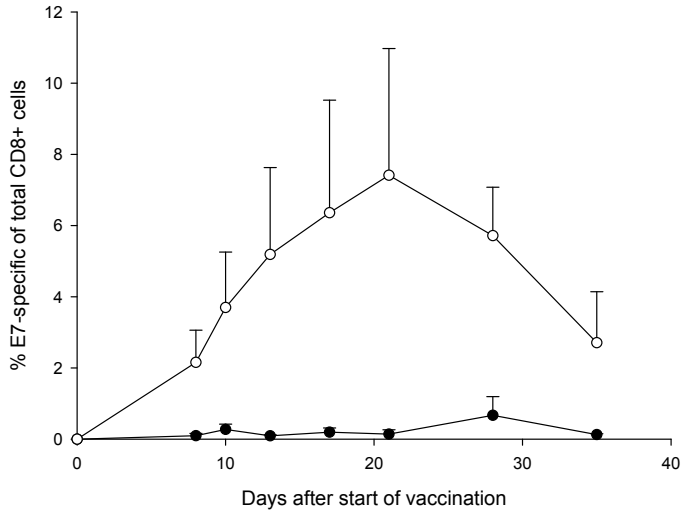

Figure 5: *E7-specific CD8⁺ T-cell responses in peripheral blood lymphocytes in mice upon vaccination with TTFC-E7SH (n=5, open circles) by DNA tattooing. Control mice were vaccinated with empty pVAX vector (n=5, filled circles). Values represent the Mean + Standard Deviation (error bars).*

DISCUSSION

We chose to further develop our previous small-scale batch fermentation strategy [8] to a small-scale fed-batch strategy followed by membrane based ion-exchange chromatography in order to improve plasmid DNA yield and process efficiency. Indeed we succeeded in a yield-increase of OD = 7 (20 mg/L) for the batch fermentation to OD = 120 (200 mg/L) for the fed-batch fermentation method. This fed-batch fermentation strategy initiates cell growth in the first 24 hours (batch phase), 24 hours after inoculation the fed-batch phase is started by the addition of feed supplement solution at an initial speed of about 4.6g/l/h, starting as a carbon-limited process. This results in a steep increase in the growth rate of the culture as can be deducted from the increased oxygen consumption (agitation/air inlet/%O2 enrichment) and a strong increase in the OD. The feed rate was manually adjusted once the growth is arrested as can be judged from a decrease in agitation/air-inlet or $\%O_2$ enrichment. Our 'semi defined' fermentation medium clearly supports higher cell densities in comparison to our strategy used in the batch fermentation but is still highly reproducible due to the reliable sources of our medium components (Table 1). Glycerol was selected as carbon source as it does not cause as much acetate excretion as compared to use of high glucose concentration [28]. Therefore, it can be used at higher concentrations without becoming inhibitory to the fermentation process. Trace elements are added to the medium to benefit the growth and

production of homogeneous supercoiled plasmid monomers [28]. Although exponential feeding profiles further increase higher biomass and plasmid yields in contrast to constant feeding, this fermentation strategy resulted in lower plasmid stability and percentage of viable cells, therefore we chose to apply feedback controlled constant feeding, i.e. feeding of glycerol based on a decrease in agitation/air-inlet or $\%O_2$ enrichment [29–31]. However, recent large-scale fed-batch fermentation strategies with optimized strains and vectors with exponential feeding reported to be able to produce very high pDNA amounts of 2220 mg/L and 2600 mg/L respectively [29,32,33]. Therefore, for further optimization of plasmid yields, this leaves room for improvement. We made use of a 'standard' *E.coli* vector instead of an engineered vector combination reported in these articles. Apart from product yield, we previously showed that in terms of final product quality the selection of an appropriate host cell is important as well [34]. After fermentation, the culture was harvested with TFF, which enabled to concentrate the bacteria and subsequently exchange the culture medium for TE buffer. The bacteria are immediately ready for further processing, and do not require an additional resuspending step which would be necessary after concentration by means of centrifugation. The culture is collected and stored at -80°C upon further processing which has been shown to have no negative effect on recovery or plasmid DNA topology. Previously, using the batch fermentation mode, all material was subsequently purified. Applying the fed-batch strategy, the amount of harvested material requires multiple times downstream processing. In the case of TTFC-E7SH presented, from one fermentation run two batches of bulk drugs could be produced. To ensure complete RNA removal, RNase A was added during the first lysis step. However, the use of animal derived enzymes is not preferred for the production of clinical grade plasmid DNA [35–37]. During pilot runs, very low recovery percentages were reached during the purification phase. A limited, sub-optimal amount of pDNA was able to bind to the membrane, because genomic RNA, still in solution, competed for the membrane binding sites. When RNase A was used in the lysis, the recovery was restored to approximately 50%. Therefore, we decided to use BSE-free certified RNase A. For the lysis, the frozen culture is allowed to thaw and 250 ml of 2x concentrated culture is added to four 5L lysis bottles each to ensure efficient lysis of the bacterial cells. After lysis, large, flock-like particles of gDNA, host cell protein and cell debris are formed. After applying a small vacuum (~500 mbar) the flocks start to float on the solution. With this separation technique air escapes from the solution under vacuum to enable the formation of a compact bed of flocculent material. The lysate is pre-cleared sufficiently after 15 minutes, to allow filtration using two capsule filters placed in line. We improved the pre-clarification step by replacing the Aervent membrane Opticap filter (1.2/0.5/0.22 µm 9/16 in. Millipore, Amsterdam, the Netherlands) with the Sartopure PP2 0.2m²/5µm filter and Sartopore 2 XLG 0.8µm/0.2µm filter (Sartorius AG, Goettingen, Germany), both with polyethersulfone (PES) filter membrane placed in line. Because of the increased cell density, after lysis the amount of cell debris was also increased. The Opticap

filter was not able to clear the lysate, but the new in line placed filter system was able to filter large amounts of lysate without being clogged instantly. Apparently, the different pore sizes used in the filter train allowed us to more efficiently clear the lysate during the filtration step. Also, the filter-train concept made it possible to replace the first Sartopure PP2 0.2m^2/5µm filter when it had reached capacity, and only replacing the Sartopore 2 XLG 0.8µm/0.2µm filter when necessary. pDNA purification can be performed by a lot of techniques [38,39]. Since an one-step purification is important for us (as done in the previous batch-fermentation), we selected the re-usable Mustang XT 140 mL membrane capsule for pDNA purification as it is a single-step method and approved for production of clinical grade pDNA [12,40]. After loading approximately 60L of the pre-cleared lysate, the pDNA was eluted from the membrane. During the development phase, we found that after elution still a significant fraction of plasmid DNA was bound to the membrane. We chose to apply a multiple-step elution to increase yield of the Mustang XT purification. Using this elution technique, we were able to elute the pDNA more efficiently from the membrane, from 64% from a single elution step to 79% with multiple elution steps. In about four elution phases, all pDNA is eluted from the membrane. The elution fractions are then combined, mixed and stored for further processing. As compared to QIAGEN resin, we found that this multiple-step elution was a (technical) disadvantage of Mustang XT when compared to the one-step elution from the QIAGEN resin.

After purification, a second TFF step was applied to concentrate the pDNA solution to approximately 1.1 mg/ml TTFC-E7SH in approximately 2000 ml and to exchange the elution buffer for WFI. During the development phase, we found that circulation of the eluate during 30 minutes before start of the buffer exchange improved recovery from the TFF membrane (from 43 to 75%).

For the final E7 drug product manufacture, all bulk drug batches were combined. Indeed, final product complied to all specifications with high purity (>96% SC) (Table 2). Recovery after purification is only 33% of the theoretical amount in the fed-batch culture, however, this method is capable of fast processing the large volumes that comes with fed-batch fermentation strategies. Though, further optimization of the purification method to increase purification recovery is necessary. In addition, the final product showed effective immunogenicity as reflected in the induced in an E7-specific CTL response in a murine model (Figure 5). Stability results obtained thus far are in line with previously reported long term stability data with similar formulation[23].

CONCLUSION

In conclusion, we developed a reproducible, GMP-compliant manufacturing process for the manufacture of clinical grade TTFC-E7SH plasmid DNA for early clinical trial use. By the introduction of fed-batch fermentation and an alternative purification procedure, we developed a relatively fast, high yield, and straightforward production process. This manufacturing method can be generically applied for the small scale manufacture of plasmid DNA for early clinical studies.

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R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34 R35 R36 R37 R38 R39

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Chapter 2.2

Transposon leads to contamination of clinical pDNA vaccine

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ABSTRACT

We report an unexpected contamination during clinical manufacture of a Human Papilomavirus (HPV) 16 E6 encoding plasmid DNA (pDNA) vaccine, with a transposon originating from the E. coli DH5 host cell genome. During processing, presence of this transposable element, insertion sequence 2 (IS2) in the plasmid vector was not noticed until quality control of the bulk pDNA vaccine when results of restriction digestion, sequencing, and CGE analysis were clearly indicative for the presence of a contaminant. Due to the very low level of contamination, only an insert-specific PCR method was capable of tracing back the presence of the transposon in the source pDNA and master cell bank (MCB). Based on the presence of an uncontrolled contamination with unknown clinical relevance, the product was rejected for clinical use. In order to prevent costly rejection of clinical material, both in-process controls and quality control methods must be sensitive enough to detect such a contamination as early as possible, i.e. preferably during plasmid DNA source generation, MCB production and ultimately during upstream processing. However, as we have shown that contamination early in the process development pipeline (source pDNA, MCB) can be present below limits of detection of generally applied analytical methods, the introduction of "engineered" or transposon-free host cells seems the only 100% effective solution to avoid contamination with movable elements and should be considered when searching for a suitable host cell-vector combination.

INTRODUCTION

Persistent infection with high-risk Human Papilomavirus (HPV) subtypes is strongly associated with the development of cancers of the cervix, vulva, vagina, penis, anus and oropharynx. One of the prerequisites for carcinogenesis is the continuous expression of the oncogenic early proteins E6 and E7 [1]. As E6 and E7 are strictly intracellular proteins, T-cell mediated immunity is needed to recognize the premalignant and malignant cells. Therefore, DNA vaccination with a plasmid encoding these oncogenes is an attractive immunotherapeutic approach for the induction of a HPV 16 specific cytotoxic T-cell response against HPV-induced lesions [2]. We developed an HPV16 E6 based DNA-vaccine composed of a pVAX backbone with an insert encoding a fusion protein composed of a signal peptide, a series of minimal CD4 helper epitopes, a gene-shuffled version of HPV16 and a C-terminal ER retention signal KDEL: pVAX sig-HELP-E6SH-KDEL further referred to as the HPV16 E6 vaccine [3]. In preclinical studies this HPV16 E6 vaccine proved to be highly effective and safe, and warranted clinical evaluation [4]. In an upcoming clinical study, HPV 16 E6 vaccine will be administered using the in-house developed tattoo strategy [5]. It was shown previously that this short-interval intradermal DNA vaccination leads to the rapid and sustained development of both T-cell and B-cell responses [6].

To generate the HPV16 E6 vaccine for clinical use, we set up a manufacturing process according to Good Manufacturing Practices (GMP) guidelines [7]. In summary, this process consists of the production of an E6 Master Cell Bank (MCB), production of E6 bulk drug from this MCB and subsequently production of E6 final product from E6 bulk drug[8]. The MCB, expanded from a single host cell transformed with the plasmid of interest, was established in order to enable the manufacture of sufficient pDNA bulk drug of consistent quality in time. The manufacture of bulk drug pDNA consists of the expansion by fermentation (upstream processing) of an MCB aliquot followed by purification of the pDNA product (downstream processing). In this process, contaminants like genomic DNA, RNA, proteins, and endotoxins are reduced to predefined, acceptable levels. Production of pDNA final product involves an aseptic fill & finish process resulting in the desired pharmaceutical dosage form containing the pDNA of interest in the required dose unit. All abovementioned production steps are monitored by various in-process controls, focused on pDNA concentration and purity during processing [8]. MCB, pDNA bulk drug and final product are subject to extensive quality control testing (also in-process) before release for (clinical) use. Several papers have been published on the (small-scale, ≤ 10L) manufacture of both non-clinical and clinical grade plasmid DNA [8–13].

Saedler et al. already reported in 1973 multiple copies of the IS2 element in *E.coli* [14]. Fifteen years later, the spontaneous insertion of an IS2 element was described by de Togni et al., when spontaneous insertion of an IS2 element into the plasmid pUC 18 had occurred. IS2

insertions also resulted in incorrect annotation of genes and proteins from many different species. A detailed search of GenBank showed that the IS2 is present within many eukaryotic nucleotide sequences and it is likely to be incorporated into the insert during the cloning process [15]. IS elements can act as genomic parasites, and play a role in promoting the evolutionary adaptation of their hosts and they are able to generate some of the beneficial mutations that increase organism fitness [16].

Multiple sites of IS2 integration have been identified, in example Oliveira et al. have found IS2 transposition upstream of the kanamycin resistance gene in a pDNA vaccine vector (pCIneo::IS2). pCIneo spontaneously recombines due to the presence of two 28 bp direct repeats[17]. Certain regions of the target genome serve as so-called "hot spots" (*e.g.,* repeated sequences) for integration [18]. For example, regions of homology with an internal sequence of IS2 and a pentanucleotide GGTAT sequence might be involved. Also the site of insertion is identified as pentanucleotide ATACC. Insertion likely occurs during growth of a single colony and segregation of the progeny plasmids [18].

We report an unexpected contamination of the HPV16 E6 bulk drug, which appears to originate from the introduction of a host cell transposon in the pDNA vector.

MATERIALS AND METHODS

E6 source pDNA

The sig-HELP-E6SH-KDEL insert was constructed by standard molecular cloning techniques and cloned into the pVAX1 backbone (No. V260-20, Invitrogen, Grand Island, NY, USA) using HindIII and XbaI at The Netherlands Cancer Institute (NKI-AVL, Amsterdam, The Netherlands). Subsequently, the pVAX sigHELP-E6SHKDEL plasmid of 4.1 kb with a kanamycin resistant marker (E6 vaccine) was produced using *Escherichia coli* (*E.coli)* DH5α as host cell and purified with the Qiagen 'mega' endotoxin-free plasmid purification kit (Qiagen, Venlo, the Netherlands) to obtain HPV16 E6 source pDNA. Before further use, the obtained HPV16 E6 source pDNA was analyzed for purity and identity by agarose gel electrophoresis (AGE), restriction analysis and sequencing.

E6 MCB and bulk drug production

All production steps were performed under cleanroom conditions (GMP EU class A with class B background)[19] *E.coli* DH5 cells were used for transformation (ATCC #53868, Teddington, Middlesex, UK). Cells were made competent with $CaCl_2$ and subsequently transformed with E6 using a standard heat shock method[20]. These cells were plated on Luria Bertani (LB) plates containing 100 µg/mL kanamycin (Biotrading Benelux, Mijdrecht, The Netherlands) and grown. One single colony was isolated and grown in 175 mL LB-Miller

broth (Sigma Aldrich Chemicals BV. Zwijndrecht, The Netherlands) containing 100 µg/mL kanamycin (Roche diagnostics Nederland BV. Almere, The Netherlands) at 37°C in a 1L sterile baffled shake flask (Nalgene, Rochester, NY, USA) at 200 rpm. When an $OD_{600} \geq 0.7$ was reached, glycerol (BUFA, Uitgeest, The Netherlands) was added to the culture (30% v/v). Aliquots of 1mL were frozen at -80°C. One MCB lot was produced. Quality control of the MCB was performed according to European Pharmacopoeia (Ph. Eur.) [21] and FDA guidelines [22,23]. Plasmid identity was confirmed by sequencing and restriction analysis.

The production process of E6 bulk drug is summarized in Fig. 1. The culture grown from one MCB vial was inoculated in a BioFlo 3000 benchtop fermentor with a working volume of 10L (New Brunswick Scientific BV, Nijmegen, The Netherlands) containing 7 L growth medium (KH_2PO_4, K_2HPO_4 $(NH_4)_2SO_4$ and glycerol (14 g/L)) feed supplement (4 g/L/h), 58.1ml medium supplement solution (thiamine.HCl and $MgSO_4.7H_2O$ in WFI), 7ml trace element solution ($FeCl_3.6H_2O$, $ZnCl_2$, $CoCl_2.6H_2O$, $Na_2MoO_4.2H_2O$ $CaCl_2$, $CuCl_2.2H_2O$ and H_3BO_3 in 1.2N HCl) and 50 mg/mL kanamycin at 37 °C under fed-batch conditions [24]. pH, dissolved oxygen (DO) and agitation were monitored using the BioCommand software (New Brunswick Scientific BV. Nijmegen, The Netherlands). After fermentation, the cell culture was harvested by tangential flow filtration (TFF) using a Flexstand TTF system equipped with a Hollow Fibre Cartridge model UFP-300-E-9A with a membrane area of 0.84 m^2 (GE Healthcare, Diegem, Belgium) and stored in TE-buffer (50 mM trometamol, 10 mM EDTA) at -80°C until further use. In total, two HPV16 E6 bulk drug batches were manufactured.
The harvested cells were lysed using a standard alkaline lysis procedure. In brief, the biomass was resuspended in TE-buffer supplemented with 600 µL of a 100 mg/ml RNase A solution [25] and lysed by the addition of lysis buffer (200 mM NaOH; 1% SDS) for 5-10 min. Cellular debris, gDNA and proteins were precipitated by gently adding neutralization buffer (3M potassium acetate (KAc)). The lysate was clarified by applying a vacuum of 500 mbar followed by dead end filtration with both a Sartopure PP2 0.2m^2/5µm filter and a Sartopore 2 XLG 0.8µm/0.2µm filter (Sartorius AG, Goettingen, Germany). Subsequently, an endotoxin removal buffer was added. One-step plasmid purification was performed using the 140 mL Pilot Scale Mustang Q XT capsule (Pall Netherlands, Mijdrecht, The Netherlands) connected to the Äkta Pilot (GE Healthcare, Diegem, Belgium) [12]. After purification, the plasmid was concentrated by tangential flow filtration (TFF) using a Quickstand TTF system equipped with a Hollow Fibre Cartridge model UFP-300E-4MA with a membrane area of 420 cm^2 (GE Healthcare, Diegem, Belgium) in sterile water for injection (WFI), followed by sterile filtration (0.2 µm).

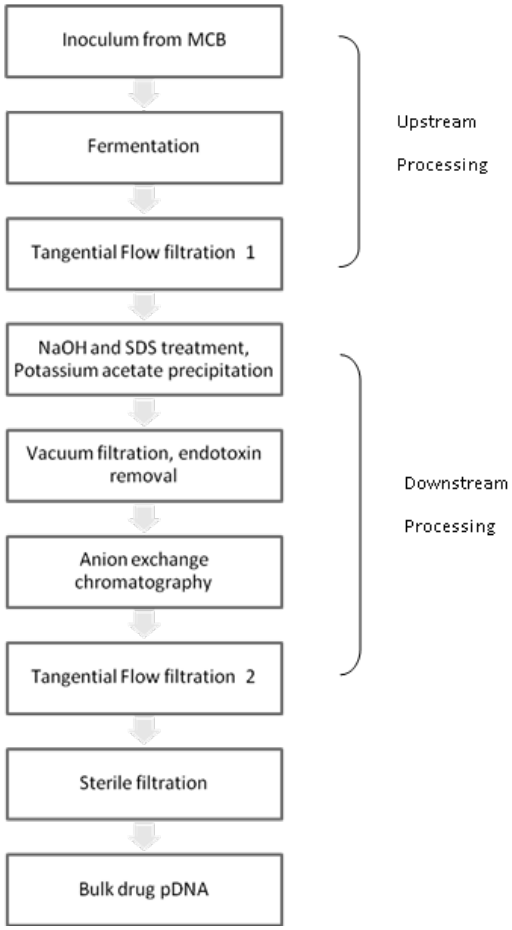


Figure 1: *Process flow sheet for the production of E6 bulk drug.*

CGE analysis

Capillary gel electrophoresis (CGE) of E6 MCB and bulk drug was performed at PlasmidFactory (Bielefeld, Germany) as previously reported [26].

Isolation, ligation and transformation

The contamination product (+/- 5.5 kb band) was isolated from gel and re-ligated using T4 ligase. Ligation product was transformed in DH5α and bacteria were plated on LB-plates containing kanamycin (100 µg/mL). Colonies were selected for an overnight culture and pDNA was isolated using a mini-prep kit and subsequently sequenced.

Sequencing and insert analysis

Primers spanning the pVAX backbone and insert were used. Samples were sequenced on a 3730 DNA analyzer (Applied Biosystems, Carlsbad, CA, USA). Sequence alignment was performed using Vector NTI software (Invitrogen Grand Island, NY, USA) and Seqman software (DNAStar, Madison, WI, USA). Resulting sequences were aligned and matched to the known pVAX sequence. The non-matching region was analyzed using the Basical Local aligment search tool from NCBI-BLAST [27] within the nucleotide collection.

Deep sequencing

Deep sequencing was performed at the Central Genomics Facility of The Netherlands Cancer Institute (NKI-AVL, Amsterdam, The Netherlands). In brief, samples were sheared using a Covaris DNA shearing device (Woburn, MA, USA), and analyzed on an Illumina HiSeq2000 sequencing machine (LGC Genomics, Berlin, Germany). As an internal control for the sensitivity of the analysis, pVAX containing either green fluorescent protein (GFP) or far-red fluorescent protein (TurboFP635) were spiked at ratios 1:1,000 and 1:10,000 respectively.

Batch fermentation and specific transposon PCR

Additional HPV16 E6 batch fermentation was performed as described previously[8]. In contrast to the fed-batch fermentation, all nutrients and growth components are already present from the start. Batch fermentation culture is started with inoculation with the same HPV 16 E6 MCB product as in the fed-batch fermentation. Cell culture samples were purified with the Qiagen 'maxi' endotoxin-free plasmid purification kit (Qiagen, Venlo, The Netherlands). Plasmid DNA samples were analyzed by restriction analysis and transposon specific PCR. PCR mixtures were prepared for each sample using both forward primer GATAACGGAGAGAGACTGCG for the insert and reversed primer GAGAACCTGCGTCAATC for the pVAX backbone.

RESULTS

Characterization of E6 source pDNA, MCB and bulk drug

Quality control analysis of the HPV16 E6 source pDNA and the MCB, including AGE, restriction and sequence analysis was conform specifications (data not shown), and E6 was subsequently released for manufacture of HPV16 E6 bulk drug. Upstream and downstream processing of both batches of HPV16 E6 bulk drug were performed without deviations and purity of HPV16 E6 in-process control samples, as determined by AGE and UV analysis, was within specifications (Table 1).

Table 1. In-process controls during fed-batch fermentation and downstream processing and quality control results of E6 bulk drug.
Abbreviations: IPC, in-process control; QC, quality control; AGE, agarose gel electrophoresis; AEX-HPLC anion exchange high performance liquid chromatography; UV, ultra violet; BCA bicinchoninic acid; ND, not detected; RP-HPLC, reversed phase high performance liquid chromatography; cfu, colony forming units; Ph. Eur., European Pharmacopoeia; EU, Endotoxin Unit

Test		Test Method	Specification	Bulk product (batch)	
				1	2
IPC					
	OD_{600} inoculum	UV	≥ 0.7	4.46	3.95
	Fermentation time (hh:mm)	Visual inspection	For information	71:00	72:00
	OD_{600} fermentation	UV	For information	123.2	135.5
	$A_{260/280}$ eluate	UV	1.80-1.95	1.9	1.9
	Purity SC band	AGE	Approximate size predicted	Conforms	Conforms
	Amount DNA in eluate (mg)	UV	For information	410	768.6
	Recovery (%)	UV	For information	52.26	66.40
	A260/280 final product	UV	1.80-1.95	1.9	1.8
	Amount of DNA final product (mg)	UV	For information	214.28	510.35
QC					
Appearance		Visual inspection	Clear, colorless solution	Conforms	Conforms
Identity	Sequence	Sequencing	Conform reference	*Extra 1340 bp insert*	*Extra 1340 bp insert*
	Restriction map	AGE	Compares to theoretical	*Extra band*	*Extra band*
	Plasmid size	AGE	Approximate size predicted	Conforms	Conforms
	Retention time	AEX-HPLC	Identical to standard	Conforms	Conforms
Concentration (mg/mL)		UV	Conform declaration	0.974	0.865
Purity	$A_{260/280}$ ratio	UV	1.7-2.0	1.9	1.8
	Circular plasmid DNA (%)	AEX-HPLC	≥ 90% supercoiled	94	92
	Genomic DNA	AGE	< 5% (<0.05 mg/mg plasmid)	ND	ND
	RNA	AGE	< 4% (<0.04 mg/mg plasmid)	ND	ND
	Protein	BCA assay	< 5 µg/mL	ND	ND
Bioburden (cfu)		Ph. Eur.	None detected	0	0
Endotoxin (EU/mg)		Ph. Eur.	< 100 EU/mg	Conforms	Conforms

Quality controls of the HPV16 E6 bulk drug conformed to specifications except for restriction analysis, size, and sequencing data (Table 1). A clear, additional band appeared in restriction digestion analysis, indicating the presence of a contaminant with a size between the 5-6 kb (Fig. 2a). This finding was confirmed by CGE analysis (Fig. 2b). In contrast to the MCB, an additional peak appeared in the chromatogram of the bulk drug eluting separately from the supercoiled (SC) pDNA, SC dimer and open circular (OC) form of E6 pDNA, indicative for a contaminant.

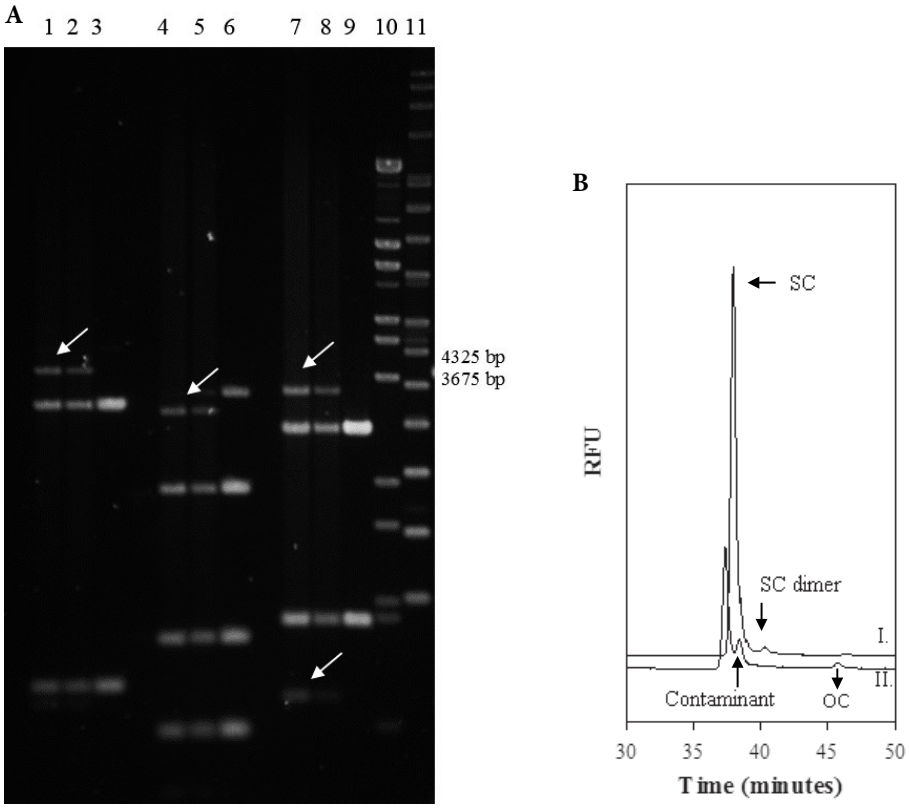


Figure 2: (A) *Restriction analysis of E6 MCB and bulk drug: lane 1-3 E6 bulk drug batch 1,2 and MCB AvaI and XbaI digest; lane 4-6 E6 bulk drug batch 1,2 and MCB SpeI and XbaI digest; lane 7-9 E6 bulk drug batch 1,2 and MCB HindIII and XbaI digest; lane 10 linear ladder (Lambda DNA BstEII digest); lane 11 supercoiled DNA ladder.*

Expected fragment sizes: AvaI and XbaI, 898 and 3259bp; SpeI and XbaI, 81, 684, 1140 and 2256bp; HindIII and XbaI, 1242 and 2915bp. Actual fragment sizes: AvaI and XbaI, 868, 3922 and 4395bp; SpeI and XbaI, 680, 1130, 2249 and 3821bp; HindIII and XbaI, 825, 1264, 3520 and 4104bp.

White arrows point at unexpected fragments after restriction analysis.

(B) *Representative CGE chromatogram of E6 MCB (I.) and bulk drug (II.).*

Identification of contaminant

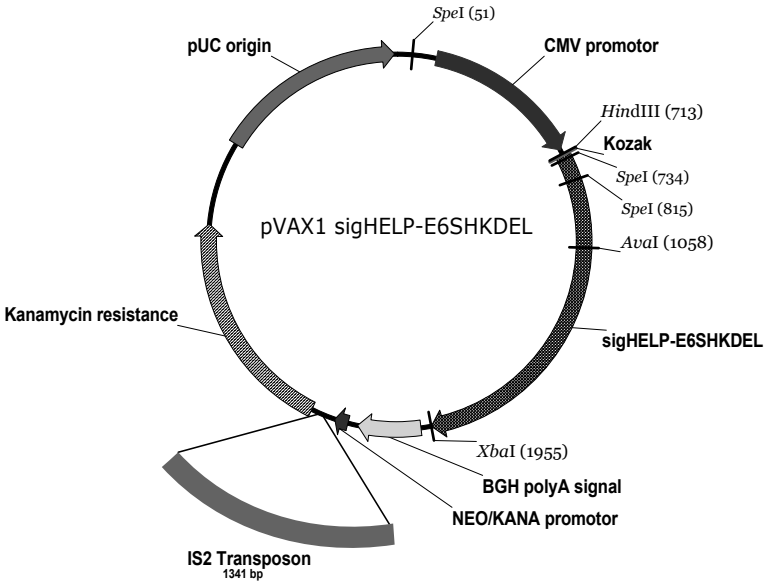
In order to identify the 5.5 kb contamination, this extra band was isolated from the restriction analysis gel, transformed in DH5α cells and plated on LB-plates containing kanamycin. As it was possible to grow colonies that solely contained the 5.5 kB product it was clear that the contamination product harbored a kanamycin resistance gene and was therefore likely to be a plasmid, possibly pVAX related. Therefore, pVAX-based primers were used for sequencing analysis.

Sequencing could identify the contaminant as a 1340 bp insert in the HPV16 E6 vaccine, with a sequence that showed a match with a sequence originating from the *E.coli* DH1 (ME8569) host cell genome (GenBank: AP012030.1, 100% hit after BLAST with insertion element 2 (IS2) insertion element repressor InsA). The *E.coli* DH5 cell line originates from the DH1 cell line after being mutated to *deoR*, and the resulting *E.coli* DH5 cell line is more transformation efficient and frequently employed as the host cell in pDNA manufacture [8,28–34]. As they are both K-12 strain derivates, they share the same host cell genome (except for the *deoR* mutation). For K-12 strains 6-7 IS1 elements, 6 IS2 elements and 5 IS3 elements have been reported [35]. The IS2 element that is transposed from the DH5 genome, is the same as in DH1 [36]. The contaminating IS2 insert is located in the pVAX-backbone of the HPV16 E6 vaccine, between the BGH polyadenylation signal and the kanamycin resistance gene (Fig. 3) and resulted in a deletion of 5 bp from the original backbone.

Extent of contamination

In order to determine the extent of the contamination, HPV16 E6 source pDNA and MCB (purified from single cells from the MCB stock without multiplication) and HPV16 E6 bulk drug from both fed-batch fermentations were re-analyzed (see supplementary Fig. 1). Restriction analysis of E6 source pDNA and MCB showed no additional 6 kb band, which would be indicative for the presence of the contaminant. However, as restriction analysis is not sensitive enough to detect very low concentrations of contamination, starting at 10 ng plasmid DNA per sample, we interpreted this result with some caution and decided to perform additionally a deep-sequence analysis on the HPV16 E6 MCB and bulk drug.

Results of this analysis clearly confirmed contamination of the E6 bulk drug, with 12.85% of the E6 plasmids containing the IS2 insert. Deep sequence analysis of the MCB, however, in line with the results of the restriction analysis, was negative for the presence of IS2 transposase. Spiked low frequency contaminations as controls (GFP and pTurbo at concentrations of 1 plasmid per 1,000 and 10,000, respectively) were detected during analysis, suggesting that if the contaminant would be present in the MCB, it would have to be at a frequency below 1:10,000.



Primer	5' TO 3'
pvax1 forw	GACTCTTCGCGATGTACG
pvax1 rev	CCCTATAGTGAGTCGTATTA
pvax2 forw	CCTCGACTGTGCCTTCTA
pvax2 rev	GCCACAGTCGATGAATC
pvax3 forw	GCATACGCTTGATCCG
pvax3 rev	GCCTTATCCGGTAACTATCG
pvax4 forw	GCGTAATCTGCTGCTTG
pvax4 rev	CAAGTGGGCAGTTTACCG
Transposase Insert Forw	GCAATTGTTCAGCAGAGC
Pvax5 rev	AGAACCTGCGTGCAATC

Figure 3: (A) *E6 vaccine (pVAX sigHELP-E6SHKDEL) with identified site of IS2 transposon insertion.****(B)*** *Primers used for sequencing.*

In parallel with these experiments, it was investigated whether the upstream processing method was responsible for triggering transposase-activity. Therefore, E6 bulk drug was manufactured using a batch-fermentation method as described previously[8]. Both E6 bulk drug product obtained by fed-batch (FB) and batch (B) fermentation were subsequently analyzed by restriction digestion analysis. As the contamination did not appear in the batch fermentation in contrast to the fed-batch E6 bulk drug samples (Fig.4), growth conditions might provide selective pressure for the outgrowth of the IS2 contaminated material.

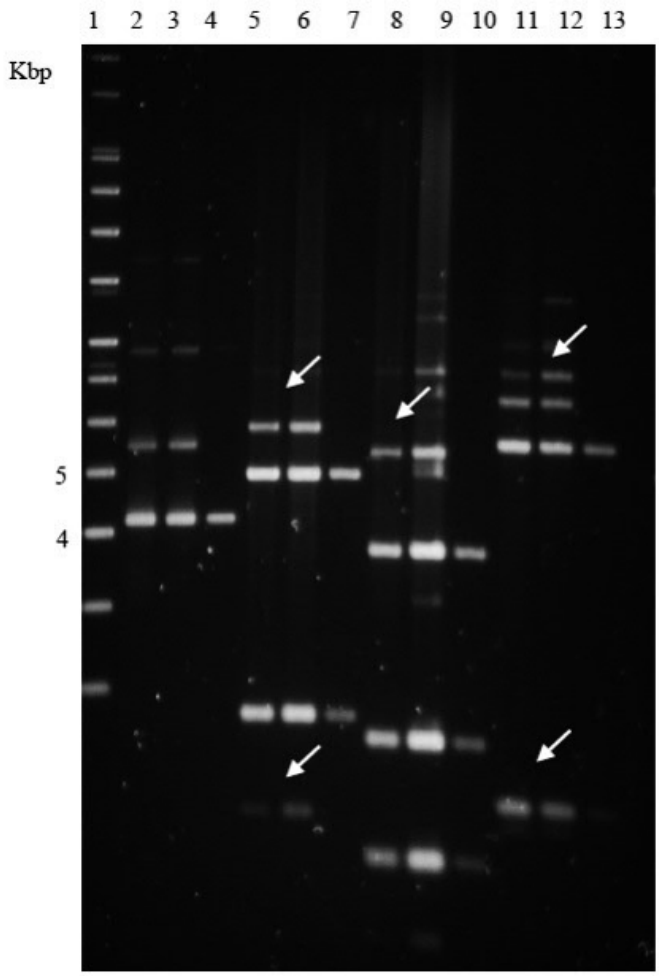


Figure 4: *Restriction analysis of E6 bulk drug obtained by fed-batch (FB) and and batch (B) fermentation: lane 1 linear ladder (Lambda DNA BstEII digest); lane 2 supercoiled DNA ladder; lane 3-5 FB batch 1,2, B uncut; lane 6-8 FB batch 1, 2, B, HindIII and XbaI digest; lane 9-11 FB batch 1, 2, B SpeI and XbaI digest; lane 12-14 FB batch 1, 2, B AvaI and XbaI digest.*

Expected fragment sizes: HindIII and XbaI, 1242 and 2915bp; SpeI and XbaI, 81, 684, 1140 and 2256bp; AvaI and XbaI, 898 and 3259bp. Actual fragment sizes: HindIII and XbaI, 821, 1246, 2964 and 4249bp; SpeI and XbaI, 672, 1118, 2269, 3196 and 4249bp; AvaI and XbaI, 833, 3272, 3805, 4200, 4692, and 5488bp.

White arrows point at unexpected fragments after restriction analysis.

As conclusive analytical method, a highly specific PCR analysis was performed. Primers consisted of one specific to the IS2 sequence and the other specific to the pVAX1 backbone. The primers were selected to amplify the transposon-specific sequence in the contaminated HPV16 E6 plasmid, resulting in a 600 bp PCR product. Analysis was performed on all material generated thus far (HPV16 E6 source pDNA, MCB, and bulk drug obtained by fed-batch and batch fermentation, respectively). In order to obtain a semi-quantitative measurement of the level of transposase-contamination samples were diluted from 1 up to 1,000,000 fold. PCR results show that, in contrast to the results of the restriction digestion and deep sequence analysis, all products tested were contaminated with the IS2 transposon (Fig. 5). The bulk drug obtained by fed-batch fermentation contains the highest percentage of pDNA contamination.

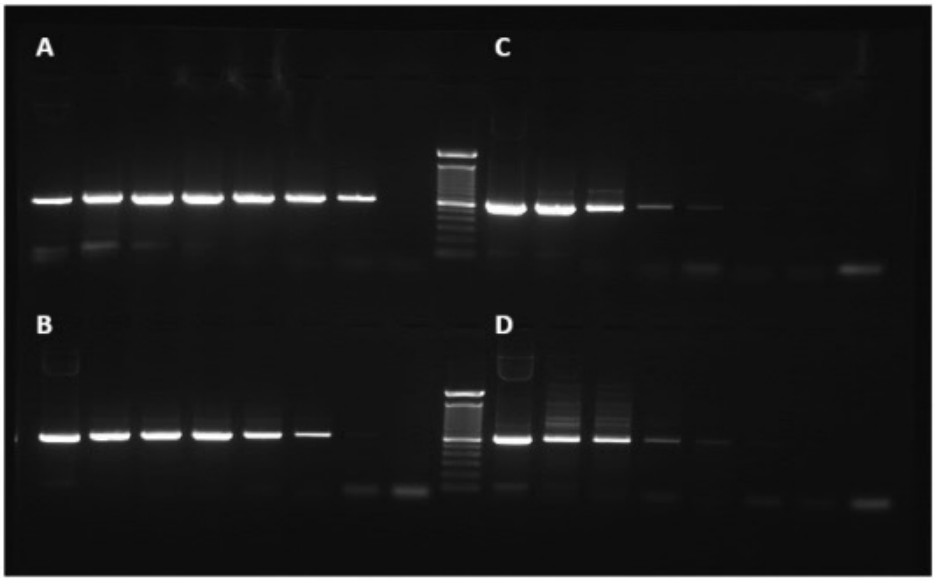


Figure 5: *Contamination specific PCR using 100 ng of template diluted 1x, 10x, 100x, 1,000x 10,000x 100,000x, 1,000,000x and water controls of* ***(A)*** *E6 bulk drug obtained by fed-batch fermentation* ***(B)*** *E6 bulk drug obtained by batch fermentation,* ***(C)*** *E6 MCB and* ***(D)*** *E6 source pDNA.*

DISCUSSION

We report the contamination of an HPV16 E6-based plasmid DNA vaccine with an IS2 insertion element originating from the *E.coli* DH5 host cell. The introduction of a transposon from a host cell into the plasmid of interest has been reported before in relation to non-clinical pDNA production [17,37], but not to clinical pDNA production. Using a broad panel of analytical techniques, we were able to show transposon contamination in the HPV16 E6

source pDNA. During processing, however, it was not noticed until quality control of E6 bulk drug when results of restriction digestion, sequencing, and CGE analysis were clearly indicative for the presence of a contaminant. Apparently, both the in-process controls during manufacture of the bulk drug as well as the quality control methods used for HPV16 E6 source pDNA and the MCB were not sensitive enough to identify contamination at an earlier stage. For instance, the contamination level of source pDNA is 10,000-100,000 fold less compared to the E6 bulk drug obtained by fed-batch fermentation (Fig. 5), which was quantified at approximately 13% by deep-sequence analysis. This very low contamination level and thus the possibility of sampling only "clean" material likely explains why the IS2 insertion was not identified e.g. during sequence analysis of HPV16 E6 source pDNA.
We found insertion of IS2 in the pVAX-backbone between the BGH polyadenylation signal and the kanamycin resistance gene. Also, Prather et al., described IS1-insertion in or near the antibacterial resistance gene of an HIV DNA vaccine vector [37]. In addition, the specific combination of host cell and pDNA vector of interest may trigger transposon activity.

In literature it is reported that transposon activity can be induced by starvation or other stressful conditions but also under conditions favorable to growth, including abundant resources and moderate temperature [15,38]. We found an approximately < 10,000-fold higher contamination levels of HPV16 E6 bulk drug produced using fed-batch compared to classical batch fermentation. Apparently, fed-batch fermentation, in which the cells are in the continue state of starvation, is more supportive for transposon activity, and provides a growth advantage for host cells carrying the plasmid with the IS2 insert.
Recently, to avoid the undesirable transposon insertion the application of engineered reduced genome (transposon free) *E.coli* strains has been reported [29,39]. This involves the introduction of subtle alterations in the host genome or vector backbone or the use of non-traditional host strains, devoid of IS elements, for plasmid DNA production [40]. Multiple-deletion series (MDS) strains of *E.coli* can be used to overcome the insertion of IS elements [41,42]. Future work could include growing our current HPV16 source material on such a transposon free E. coli strain in order to test whether this could prevent the outgrowth of the contaminant.

CONCLUSION

We have shown that the introduction of a transposon from a well-defined host cell in a pDNA vector can occur. In order to prevent costly rejection of clinical material, both the quality control of the source pDNA, the MCB and in-process controls during manufacture of pDNA bulk drug must be sensitive enough to detect a contamination as early as possible, i.e.

preferably during source pDNA generation, MCB production and ultimately during upstream processing. However, as we have shown that contamination early in the process flow (source pDNA, MCB) can be present below limits of detection, the introduction of "engineered" or transposon-free host cells seems the only 100%-solution to avoid contamination with movable elements and should be considered when searching for a suitable host cell-vector combination.

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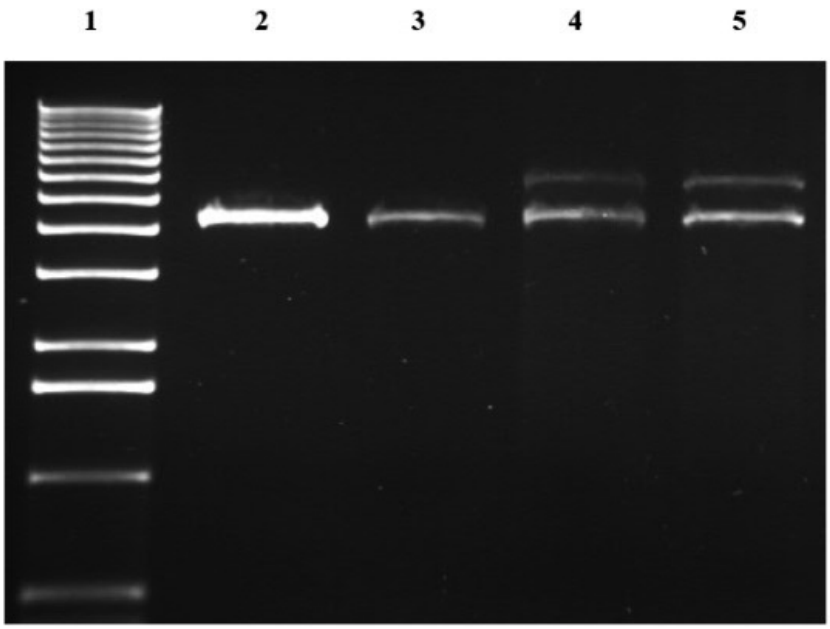

Supplementary figure 1: *Re-analysis single XbaI cut lane 1 100 bp ladder; lane 2 E6 source pDNA; lane 3 MCB; lane 4 E6 bulk drug batch 1; lane 5 E6 bulk drug batch 2*
Expected fragment size: XbaI digest 4161bp. Actual fragment size: XbaI digest 4161 and 5500bp.

Chapter 2.3

Long term stability of lyophilized plasmid DNA pDERMATT

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ABSTRACT

In this short note we report on the shelf-life stability of pDERMATT (plasmid DNA encoding recombinant MART-1 and tetanus toxin fragment-c) 2 mg lyophilized powder for reconstitution for intradermal administration, used in an in-house, investigator-initiated clinical phase I study. pDERMATT was stored at 25°C/60% relative humidity (6 months), 2-8°C (24 months), and -20°C (66 months) in the dark and analyzed at several time points during the conduct of the clinical study for appearance, identity, purity (plasmid topology), content and residual water content. pDERMATT appeared stable at all storage conditions for the periods tested which, although patient inclusion in the study was significantly delayed, ensured the clinical supply needs. This study shows that lyophilization is an useful tool to preserve the quality of the pDNA and can prevent the need for costly and time-consuming additional manufacture of drug product in case of study delays, not uncommon at the early stage of drug development. To our knowledge, this is the first study reporting shelf life stability of a pDNA formulation for more than 5 years.

An important aspect of Investigational Medicinal Products (IMPs) used in clinical studies is their shelf-life. Often, at an early stage of development (phase I-II), costs of (biological) IMP-manufacture are high and patient inclusion rates are prognosted but not guaranteed. In particular investigator-initiated studies, often limited-funded, may run into problems as a result of extended inclusion periods and limited stability of the IMP of interest. Therefore, the development of an IMP drug product with a substantial shelf-life already at this early stage of development can be crucial. In this short note we report on the shelf-life stability of pDERMATT (plasmid DNA Encoding Recombinant MART-1 and Tetanus toxin fragment-c), an in-house developed anti-cancer vaccine encoding for the MART-1 melanoma associated antigen (Fig. 1) [1] and used in a phase I clinical trial investigating the toxicity and efficacy inducing tumor-specific T cell immunity in advanced-stage melanoma patients using intradermal tattooing as route of administration [2]. Previously, we reported on the development of a GMP-manufacturing process of pDERMATT and the development of a lyophilized dosage form, using sucrose as lyoprotectant [3]. We stored pDERMATT 2mg lyophilized powder for reconstitution for intradermal use at 25±2°C/ 60±5% relative humidity (RH) (6 months), 2-8°C (24 months), and -20°C (66 months) in the dark, and analyzed the drug product with our stability indicating assay method at several timepoints during the clinical study for appearance, identity, purity, content and residual water content as described earlier [3,4]. Moreover, plasmid topology (supercoiled (SC), open circular (OC), and linear isoforms) was assessed in time using anion exchange high performance liquid chromatography (AEX) and capillary gel electrophoresis (CGE) analysis [4]. To our knowledge, this is the first study reporting shelf life stability of a pDNA formulation for more than 5 years.

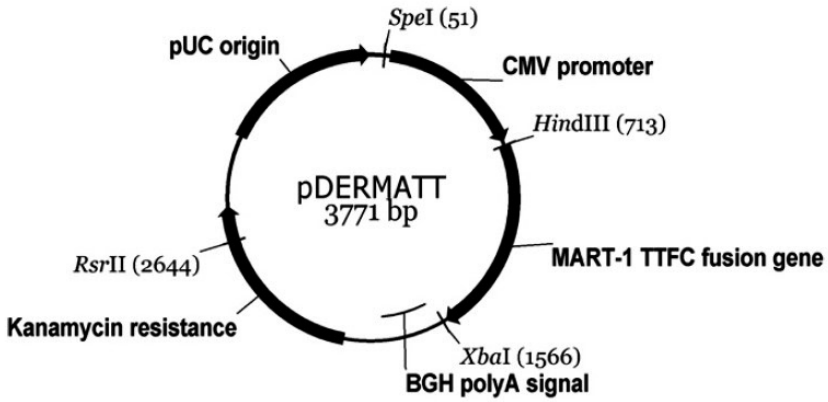

Figure 1: *Plasmid DNA map of pDERMATT (plasmid DNA encoding recombinant MART-1 and Tetanus Toxin Fragment-c) including selected restriction sites.*

Table 1 presents the stability endpoints of the different storage conditions with test items and release specifications. Apart from the residual water content, for all three storage conditions no significant changes in appearance, identity, purity, and content were observed during the respective test periods and still conformed to the initial release specifications. Dependent on the storage condition, the residual water content increases in time, with the highest content reached at the highest temperature condition (+25±2°C/60±5% RH). This limited increase in water content is common for lyophilized products and is caused by water sorption from the rubber closures of the vials, followed by moisture uptake of the hygroscopic freeze-dried cake [5,6]. More important, however, is that this increase in water content does not affect product quality during the periods tested at the respective storage conditions (Table 1). No aggregation as a result of the residual moisture increase was seen as earlier reported by Sharma *et al.* [7].
As for the plasmid topology, AEX-analysis showed only minor, non-significant variation in supercoiled and open circular (SC/OC) contents during the period and storage conditions tested, with no appearance of linear isoforms (Fig. 2A). Additional CGE measurements gave comparable results with identical topology-profiles for pDERMATT at time zero compared to the stability study endpoints (Fig. 2B). Also AGE measurements indicate presence of both SC and OC species at all timepoints (See Table 1 and Fig. 3). This is an important stability aspect, as degradation of pDNA can result in a shift in topoisoform distribution by disruption of the tertiary structure of the molecule, resulting in a conversion of SC pDNA into the OC or linear topoisoform. As SC pDNA is considered to be most efficient and results in the highest transfection efficiency [8,9], degradation to other isoforms can potentially influence clinical outcome.

Table 1. pDERMATT quality control in time at different storage conditions. Abbreviations: AEX-HPLC, anion exchange high performance liquid chromatography; R_t, Retention time; UV, ultra violet; AGE, agarose gel electrophoresis.

	Storage condition		**25°C/ 60%RH**	**2-8°C**	**-20°C**
Test Item	**Release specification**	**T = 0 months**	**T = 6 months**	**T = 24 months**	**T = 66 months**
Visual inspection	White, freeze-dried cake	Conforms	Conforms	Conforms	Conforms
Reconstitution	Complete, leaving no visible residue as undissolved matter and resulting in a clear colorless solution	Conforms	Conforms	Conforms	Conforms
AEX-HPLC analysis					
1. *Identification*	R_t reference standard = R_t powder for ID injection	Conforms	Conforms	Conforms	Conforms
2. *Purity*	≥ 90% SC	95.7 %	95.1 %	99.0 %	98.8 %
UV analysis					
1. *Content*	90.0-110.0% of labeled content	99.6 %	97.5%	97.4 %	98.8 %
2. *Purity*	$A_{260/280}$ = 1.80-1.95	1.80	1.88	1.85	1.9
AGE analysis					
1. *Identification*	3395-4149 bp	3774 bp	3680 bp	3809 bp	3762 bp
2. *Purity*	Report size of other visible bands	1. 7595 bp 2. 3959 bp	1. 7430 bp 2. 3607 bp	1. 7729 bp 2. 4105 bp	1. 7538 bp 2. 3810 bp
Residual water content (%w/w)	≤ 2.0%	0.7 %	2.41 %	2.39 %	1.49 %

R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34 R35 R36 R37 R38 R39

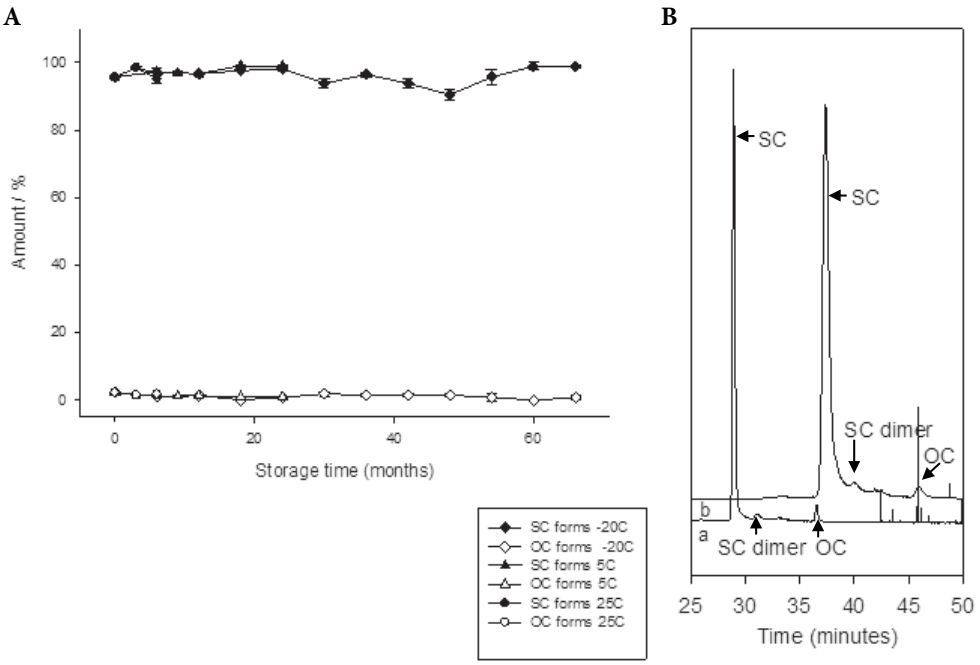

Figure 2: *pDERMATT topology in time at different storage conditions:* ***(A)*** *AEX was used for quantitative analysis of plasmid DNA isoforms. Formulations were stored at 25±2°C/60% RH, 2-8°C, and -20°C, all in the dark over a period of 6, 24, and 66 months respectively.* ***(B)*** *Electropherigram from CGE analyzing different plasmid topologies of pDERMATT plasmid DNA at t= 0 months (a) and t = 66 months (stored at -20°C) (b).*

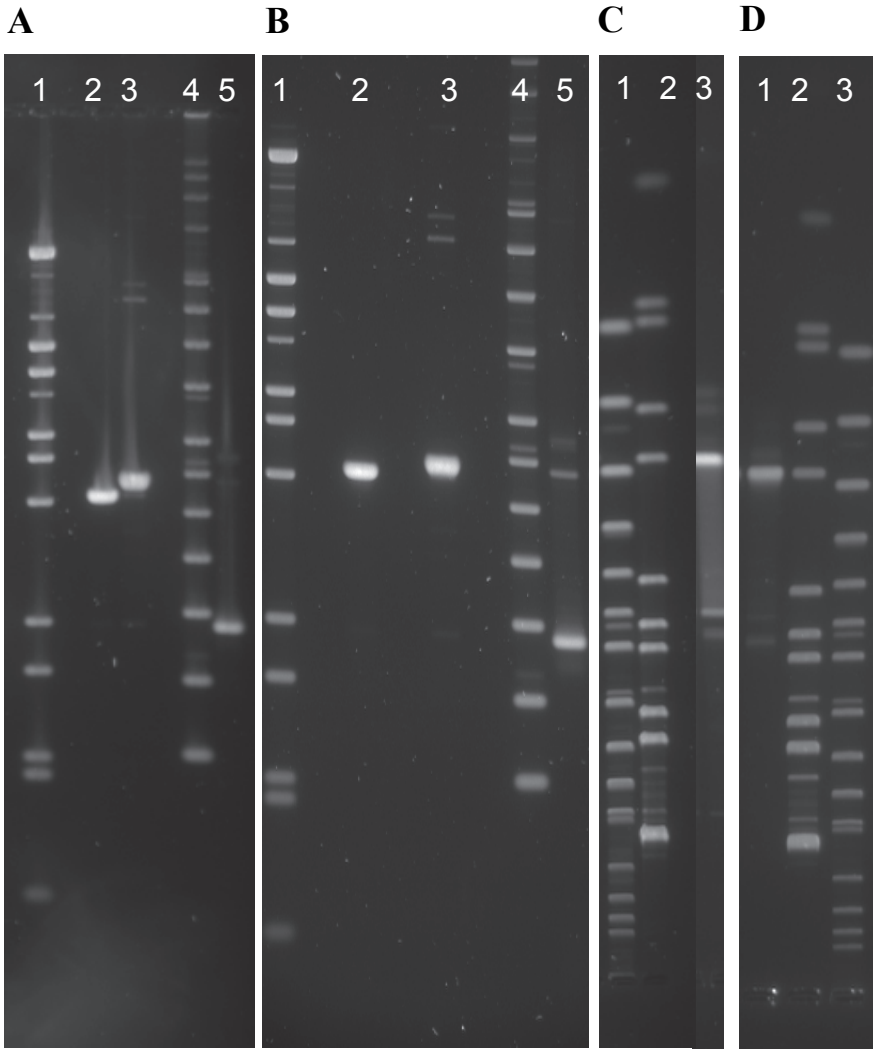

Figure 3: *Analysis of pDERMATT in time at different storage conditions by 1% agarose gel electrophoresis: (**A) t=0 months**, lane 1 Lambda DNA BstEII digest; lane 2 pDERMATT linear standard; lane 3 pDERMATT open circular standard; lane 4 supercoiled DNA ladder ; lane 5 pDERMATT final product. **(B) t=6 months,** lane 1 lambda DNA BstEII digest; lane 2 pDERMATT linear standard; lane 3 pDERMATT open circular standard; lane 4 supercoiled DNA ladder; lane 5 pDERMATT final product 25°C, lane 6 pDERMATT final product 2-8°C; lane 7 pDERMATT final product -20°C, lane 8 pDERMATT reference standard. **(C) t=24 months,** lane 1 lambda DNA BstEII digest; lane 2 supercoiled DNA ladder; lane 3 pDERMATT reference standard; lane 4 pDERMATT final product 2-8°C; lane 5 pDERMATT final product 2-8°C. **(D) t=66 months,** lane 1 supercoiled DNA ladder; lane 2 lambda DNA BstEII digest; lane 3 pDERMATT final product -20°C; lane 4 pDERMATT final product -20°C; lane 5 pDERMATT reference standard; lane 6 pDERMATT reference standard.*

Indeed, several authors have reported on pDNA stability and although quite stable during production, storage and administration [10], it undergoes various types of stress which can lead to degradation [11]. Naked plasmid DNA in solution stored at 2-8°C undergoes a conversion from SC topology into OC topology within a week. Additionally, after prolonged storage the OC pDNA was converted into linear pDNA. This was prevented by storage at -20°C, however repeated freeze thaw cycles did also affect the topology [3]. Similar findings have also been described by Walther *et al.* [12] who showed a rapid conversion of SC forms into OC forms after 6 months of storage of pDNA in solution at 4°C and the appearance of the linear form after 9 months, which is a strong indicator of pDNA instability. These conversions did not occur when stored at -80°C. No extensive studies on the long term stability of lyophilized naked DNA are reported other than for pDNA formulated into nanomedicines (*e.g.*, DNA-polycation/lipid complexes, gold particles) and during limited periods of time, mostly as a part of accelerated stability studies [13–17]. Reports on freeze-dried pDNA mostly refer to continued degradation in the dried state and damage that increasingly accumulates even during storage at lower temperatures, albeit al slower rates. Stability data of storage studies of naked DNA and DNA-based therapeutics range from 3 weeks to about a year at several storage conditions varying from -20°C to 75°C [18]. Therefore, the results of our study are of added value and, given the similar molecular structure of DNA plasmids, might be extrapolated to other pDNA drug products.

During the conduct of the study, pDERMATT lyophilized product was stored at -20°C. Indeed, also in this trial patient inclusion was delayed and the strategy of stabilization of pDNA by dehydration through lyophilization in combination with storage at low temperature ensured a stable pharmaceutical pDNA product of sufficient quality throughout the clinical study and prevented costly and time-consuming additional manufacture of drug product. Also, the stability results generated are indicative for significant stability of lyophilized pDNA at a higher storage temperature (i.e. > 24 months at +2-8°C). Therefore, for an upcoming clinical trial at our institute examining the toxicity and efficacy of a plasmid DNA directed towards human papilloma virus (HPV) induced cancers, the same formulation composition [3] and storage condition (-20°C) will be used. In parallel, stability testing will be performed at higher storage temperature conditions.

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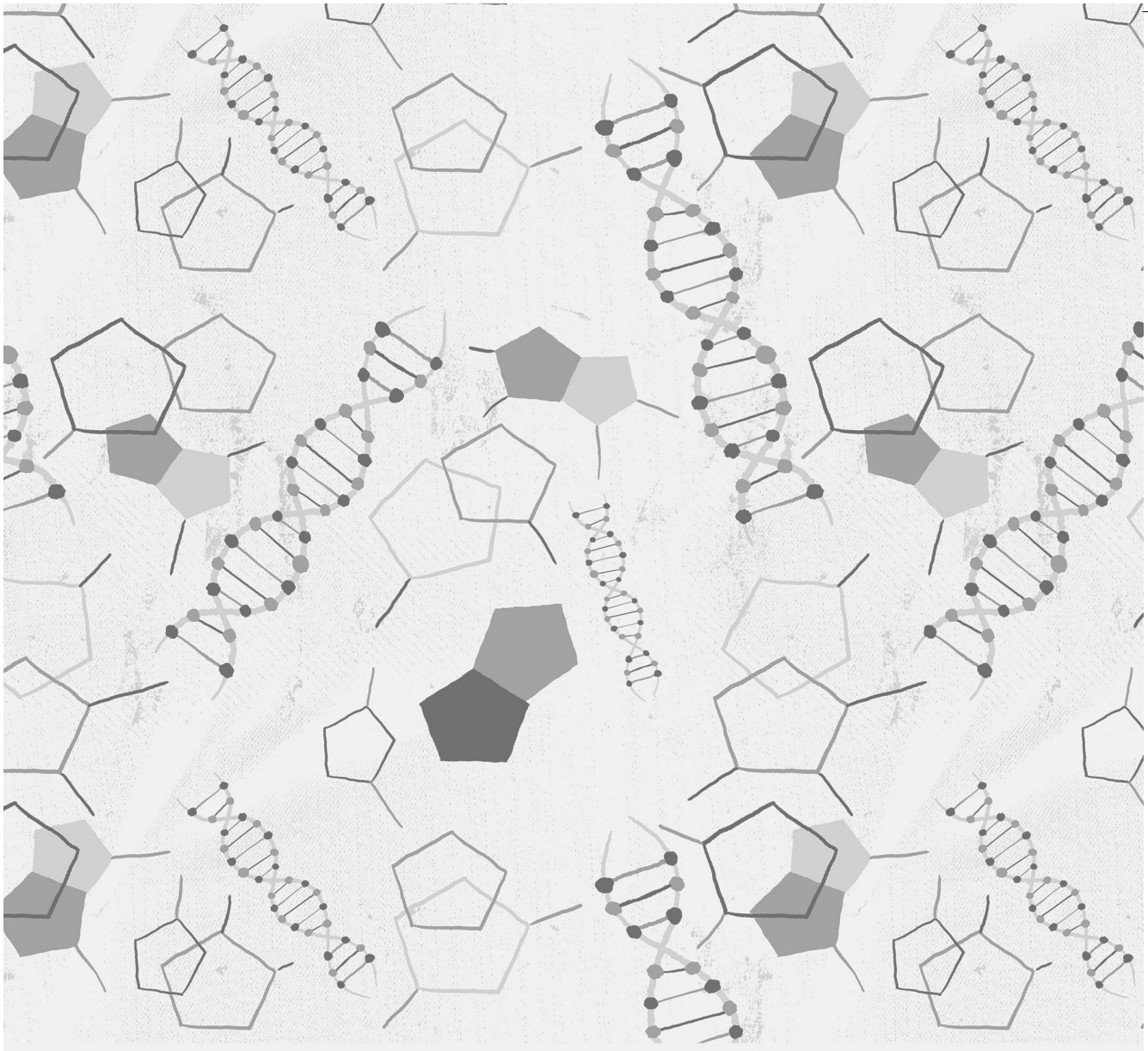

Chapter 3

Pharmaceutical development of nanoparticle-formulated dermal DNA vaccines

Chapter 3.1

Screening of polymer candidates for polyplex vaccination by intradermal tattooing

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Submitted for publication

ABSTRACT

To determine if pDNA transfection can be further improved by formulating pDNA into polyplexes, a broad panel of polymers with distinct differences in molecular structure was characterized and investigated using an ex vivo human skin model as read-out.

We measured ex vivo human skin transfection efficiency and polymer characteristics (size, PDI, charge) for all polyplex formulations and conducted a follow-up experiment in which the best performing polymer was further investigated and tested.

We found that only poly(amino amide)s polyplexes were successful in enhancing transfection efficiency of plasmid DNA when compared to the performance of the other polymers in the panel. Variations in PEG-chain MW and degree of PEGylation resulted in PAA 7.5% $mPEG_{1100}$ polyplexes with highest size and with lowest transfection efficiency, therefore the least favorable polymer of the series. Also, for most polyplexes higher N/P ratios are resulting in increased transfection efficiencies. Next, PAA 15% $mPEG_{1100}$, $mPEG_{2000}$ and $mPEG_{5000}$ polymers resulted in the highest transfection efficiencies of al sets (N/P 50).

We conclude that PAA-PEG based polyplexes are the most promising candidates for improving pDNA transfection efficiency. Further optimization of these polyplexes indicates that PAA 11% $MPEG_{2250}$ results in optimal transfection efficiency for ex vivo human skin DNA tattooing.

INTRODUCTION

Non-viral carrier delivery systems are widely used as transfection reagents to deliver nucleic acids for both *in vitro* and *in vivo* applications. In these systems, negatively charged DNA is bound by electrostatic interaction to an excess of a positively charged carrier. In this complexation process, DNA is condensed into positively charged, nanosized particles and protected from nuclease degradation, resulting in substantially higher transfection efficiencies compared to naked nucleic acids in *in vitro* assays[1]. Cationic polymers are frequently used carriers to enhance transfection, and the resulting DNA nanoparticles are referred to as polyplexes [1–4]. DNA vaccination is an appealing method to obtain an antigen-specific immune response, e.g. to be applied in anticancer therapy [5]. When delivered by intradermal tattooing, plasmid DNA (pDNA) vaccination results in a strong and rapid antigen specific cellular response in both mice and non-human primates [6]. In spite of the high immunogenicity of pDNA tattoo, the in vivo transfection efficiency of naked pDNA with this technique is extremely low, with approximately 1 out of $5x10^6$ to $5x10^9$ plasmid copies applied being taken up, transcribed and translated[7]. The *ex vivo* human skin model that has been previously developed in our group is an adequate model for the characterization and optimization of intradermal DNA vaccines [7]. In this study, the *ex vivo* human skin model was used to determine the ability of various non-viral DNA polymeric carriers to improve intradermal antigen delivery and transfection. Therefore, we screened a broad panel of polymers with distinct differences in molecular structure. Polymers were selected on the basis of promising results or reported use in *in vitro* and *in vivo* non-viral pDNA and siRNA delivery [8–12]. Merkel et al. investigated formulation of a 25/27mer 2′-OMe siRNA with polyethylene imine (PEI) and polyethylene glycol (PEG) grafted PEI (PEG-PEI) into nanosized complexes with 1 μM siRNA. While all of the polymers used this study were able to effectively condense siRNA, stability of the complexes depended on the PEGgrafting degree. *In vivo* experiments suggested that PEG-PEI/siRNA complexes are promising nanomedicines for pulmonary siRNA delivery [8]. pHPMA-DMAE cationic nanoparticles have been investigated by de Wolf and Novo et al. [10,13]. Polyplexes based on pHPMA-DMAE with a pDNA concentration of 50 μg/mL were devoid of any cytotoxicity and mediated highest transfection activity at N/P ratio of 50 *in vitro* and *in vivo*. Particle size was 130 nm ± 10 and charge + 35 ± 6. Verheul et al. investigated the use of positively charged nanoparticles loaded with fluorescently labeled ovalbumin that were made from thiolated TMCs and thiolated hyaluronic acid. The stability of these particles was confirmed in 0.8 M NaCl, in contrast to particles made from non-thiolated polymers that dissociated under these conditions, demonstrating that the particles were held together by intermolecular disulfide bonds. Size of these particles was around 200 nm and charge varied from 13 ± 0.8 to 21 ± 0.5 [11]. Vader et al. investigated several bioreducible poly(amido amine) copolymers for their ability to deliver siRNA to a carcinoma cell line and induce gene silencing.

Transfection resulted in effective gene silencing at higher w/w ratios with only minor toxicity and particle size of approximately 50 nm and charge of approximately 30 mV. Efficient uptake was only found at low w/w ratios, indicating that an excess of polymer hindered uptake at higher w/w ratios. However, uptake of polyplexes with low w/w ratios did not result in effective gene silencing, probably due to lack of sufficient polymer to induce endosomal escape. Only siRNA that was effectively complexed was taken up by cells and excess of polymer seemed to hinder that process. At the same time, sufficient amount of polymer was necessary for endosomal escape[12].

All polymers were PEGylated in order to shield the cationic charge of the polyplexes which was previously shown to be essential for antigen expression [14]. In our experiments, we applied a significant higher concentration of pDNA compared to studies previously reported [0.5 mg/mL vs. 0.05 and 0.02 mg/mL [9,10] because this relatively high concentration is necessary for *ex vivo* testing. On the basis of the results of this screening phase, a polymer candidate was selected for further formulation and optimization by varying the PEG-chain MW and degree of PEGylation of the polymer as it has been shown that both factors were found to strongly influence DNA condensation of the polymer and therefore also affect the biological activity [9].

MATERIALS AND METHODS

Materials

The pVAX:LucNP plasmid [6] encodes the influenza A $NP_{366-374}$ epitope as a genetic fusion with firefly luciferase gene, inserted in the EcoRI/NotI site of minimal pVAX1 plasmid backbone (Invitrogen, Carlsbad, USA). The plasmid was expressed and amplified in *E.coli* DH5 and was purified with the Endofree™ QIAGEN® Mega-kit (QIAGEN®, Hilden, Germany). Non-degradable linear poly(ethylene imine) (PEI) was PEGylated with PEG chains of different molar masses (2 and 20 kDa), resulting in block copolymers poly(ethylene glycol)-poly(ethylene imine) PEI25k-PEG(20k)1 (46 mol % $PEG_{20,000}$) and PEI(25)-PEG(2)10 (50 mol % PEG_{2000}) [8,9]. Biodegradable methacrylamide block polymer pHPMA-DMAE-b-PEG was PEGylated with PEG_{5000}-ABCPA, resulting in 20 mol% PEG [10]. Partially thiolated trimethylated chitosan was cross-linked with thiolated hyaluronic acid and PEGylated with 13 mol % $MPEG_{2000}$ to obtain TMC-PEG [11]. PEGylated poly(amido amine) (PAA) copolymers possessing protonable amino groups and bioreducible linkages in the main chain and hydroxybutyl groups in the side chains (CBA-ABOL), were synthesized by Michael addition polymerization of N,N'-cystaminebisacrylamide with the appropriate amine according to the procedure described previously [15]. PEGylated analogues (PAA-PEG and DM-PAA-PEG) were prepared using 11 mol % or 8 mol % of MeO-PEG-NH_2 respectively, in the total

amino monomer feed during the PAA synthesis using $mPEG_{2250}$. Additional PEGylated PAA analogues were prepared with 7.5; 15 and 25 mol % of MeO-PEG-NH_2 in the total amino monomer feed during the PAA synthesis, using three different PEG moieties: $mPEG_{1100}$, $mPEG_{2000}$ and $mPEG_{500}$ (Table 2). L-Histidine was purchased from VWR (Amsterdam, the Netherlands). HEPES was purchased from Sigma-Adrich, Zwijndrecht, the Netherlands. All other chemicals were of analytical grade.

Polyplex preparation

All different polyplexes were prepared by mixing an equal volume of dissolved polymer and the pVAX:LucNP plasmid. The ratios of polymer to plasmid DNA are expressed as the molar ratio of nitrogens within the polymers to phosphates in the plasmid DNA (N/P). N/P ratios in the range of 1 to 50 were tested. All polyplexes were formulated with a final DNA concentration of 0.5 mg/mL in 20 mM HEPES pH 7.4, 10% sucrose buffer with a high viscosity and a low ionic strength, conditions previously shown to be favorable for obtaining small and stable DNA complexes [14,16]. Control naked DNA was diluted to the same concentration in the same buffer. 10mM L-histidine pH 6.0 buffer was used for follow-up experiments with PEGylated PAA-pDNA (pVAX:LucNP) polyplexes as it has shown to benefit PAA-polyplex expression.

Polyplex characterization

The size of obtained particles was measured in 20 mM HEPES pH 7.4, 10% sucrose, or 10mM L-histidine pH 6.0 buffer with dynamic laser scattering using an ALV/GCS-3 (Malvern Instruments, Malvern, UK). Particle size distribution is described using the polydisperity index (PDI), ranging from 0 for a monodisperse to 1 for a heterodisperse preparation. The zeta potential of obtained particles was determined in 20 mM HEPES pH 7.4 or 10mM L-histidine pH=6,0 buffer, using a Zetasizer Nano Z (Malvern Instruments, Malvern, UK). Both instruments were calibrated using polystyrene latex beads of defined size and electrophoretic mobility. The presence of unbound pDNA was visualized by electrophoresis at 85V using a 1% agarose gel containing 0.5µg/mL etidium bromide.

DNA tattooing of ex vivo human skin

Formulations and naked DNA controls encoding firefly luciferase were administered to intact skin by DNA tattooing [6] to allow luciferase expression measurements. The skin model used in these experiments has been described previously for the optimization of tattooing of naked DNA in skin [7]. In brief, 10 µL of the indicated formulation at a final DNA concentration of 0.5 mg/mL was applied to the skin into a custom fabricated mould to keep the area of tattooing constant (diameter 8 mm, surface 50 mm^2). The formulation was subsequently administered into the skin using a Permanent Make Up (PMU)® tattoo machine (kindly provided by MT.DERM GmbH, Berlin, Germany) (Fig 1A). For all tattoos, 9-needle cartridges

at an oscillating frequency of 100Hz were used. The needle depth was adjusted to 1.5 mm and tattoo duration was 20s. After tattooing, skin samples were kept at 5% CO_2, 37°C in complete keratinocyte serum free medium (SFM) containing 1% penicillin/streptomycin and 0.25µg/mL amphotericin B (all Invitrogen, Carlsbad, USA) to allow expression measurements of luciferase. During this incubation, skin was cultured at the air–medium interface with the epidermis exposed to the air to mimic the natural situation. N=5 tattoos/Formulation, transfection efficiency is expressed as relative transfection efficiency (percentage) of the transfection efficiency of naked pDNA in the same piece of skin.

Measurement of antigen expression using intravital imaging of ex vivo human skin

The expression of luciferase was measured in intact skin samples approximately 17 h after tattooing to obtain maximal antigen levels[7]. The substrate luciferin (Xenogen, Hopkinton, USA) was added to the medium in a final concentration of 45 µg/ml. During this procedure extra medium was added to the box in which skin was incubated, to cover the complete epidermis of skin samples with fluid to guarantee full accessibility of luciferin to the tattooed areas. 30 min after the addition of the substrate, luminescence produced by active luciferase was acquired during 30 s with an IVIS Lumina II CCD camera (Xenogen, Hopkinton, USA). Signal intensity was quantified as the sum of all detected light within the tattoo area of interest. In all measurements, background luminescence was determined for non-treated skin to allow correction during data analysis (Fig 1B).

Transmission electron microscopy (TEM)

The size and shape of the polyplexes were visualized using TEM. To this end, samples were applied on Agar® formvar/carbon coated copper grids (van Loenen instruments, Zaandam, The Netherlands). The samples were negatively stained by uranyl acetate and dried on air. The samples were visualized under a Tecnai12 transmission electron microscope (Philips, Eindhoven, The Netherlands) using a GATAN 626 cryoholder (Gatan GmbH, München, Germany). Samples were observed at 120 kV. Images were recorded on TemCam-0124 camera (TVIPS GmBH, Gauting, Germany) and processed with AnalySIS software. The magnification ranged from 30,000 to 265,000 times.

Statistical analysis

A Welch Two Sample t-test (one-way anova) was used for statistical analysis with the aid of 'R' software. Data are represented as mean ± standard deviation. P values < 0.05 were considered statistically significant.

RESULTS

Screening phase – characterization

Polymers were selected on the basis of distinct differences in molecular structures and their potential as polyplex-polymer. In Table 1, the groups and their basic molecular structures tested are depicted. In total, more than 25 polyplex-formulations were characterized and based on the results of acceptable size, PDI, charge and pDNA condensation, approximately 15 were tested in the ex vivo human skin model. Characteristics (N/P, size, PDI, and charge) of the best performing candidate in the ex vivo human skin model within each group and N/P range tested are given. Optimal N/P ratios were typical for the polymer that was used with lower N/P ratios for PEI(25)-PEG(20)1, PEI(25)-PEG(2)10, TMC-PEG (5:1), intermediate N/P ratios for pHPMA-DMAE-b-PEG (12:1) and higher N/P ratios for PAA-PEG and DM-PAA-PEG (25:1). All polyplexes were < 200 nm and of comparable size with the exception of DM-PAA-PEG (around 100 nm) and in particular TMC-PEG (around 50 nm) which resulted in smaller particles. The PDI values of the different polyplexes varied between 0.3 for PEI(25)-PEG(20)1 to 0.66 for DM-PAA-PEG. Charge is directly influenced by the number and configuration of PEG chains available for shielding and is very diverse for the polymer groups. All polymers have a positive charge, ranging from almost neutral (+0.6 mV) for DM-PAA-PEG to 14.5 mV for PEI(25)-PEG(2)10 polyplexes.

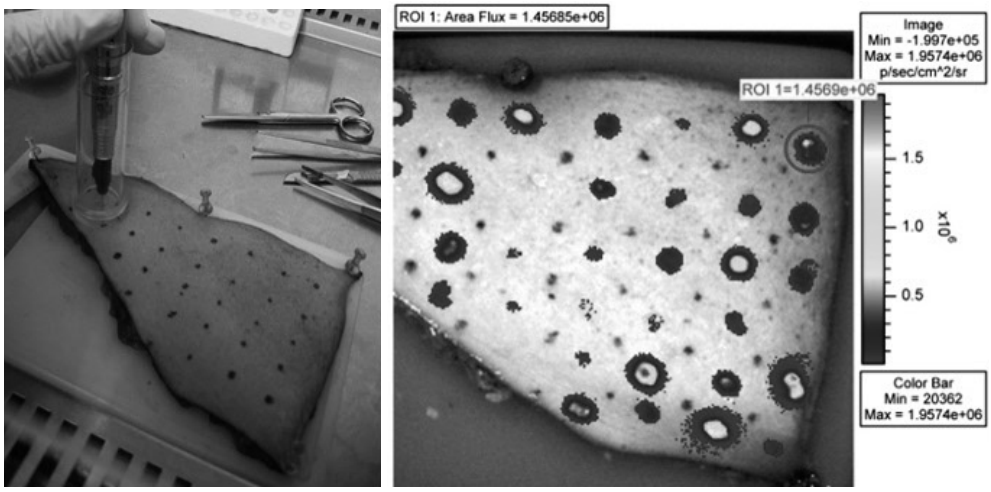
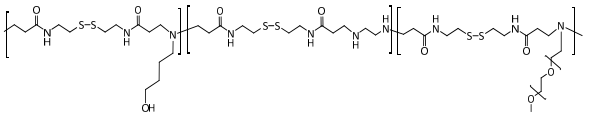
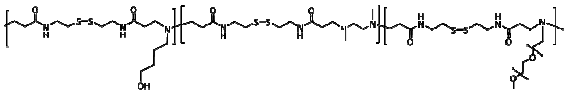
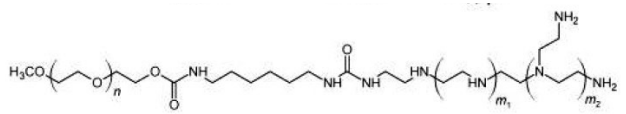
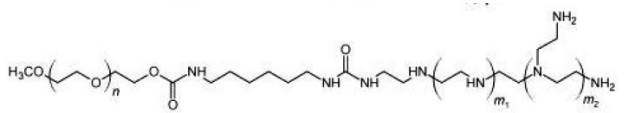
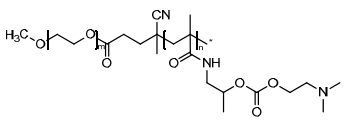
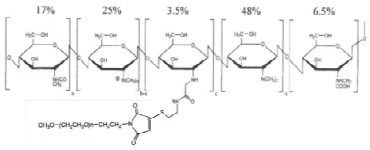


Figure 1: *Tattooing procedure of the human skin (A) and typical expression of luciferase (B), visualized with a light sensitive camera, 18 hours after tattooing [7].*

Table 1: Polyplex characteristics. PAA-PEG, DM-PAA-PEG: PEGylated poly(amido amine); PEI(25)-PEG(20)1, PEI(25)-PEG(2)10 PEGylated polyethylene imine; pHPMA-DMAE-b-PEG: PEGylated methacrylamide block polymer; TMC-PEG: PEGylated partially thiolated trimethylated chitosan. N/P:charge ratio cationic nitrogens/ anionic phosphate groups ; PDI: Poly Dispersity Index.

Polymer	PEG chain	N/P	Size (nm)	PDI	Charge (mV)	Polymer stuctural formula
PAA-PEG	PEG_{2250} 11 mol %	25:1 Range: 2,5-50	117 ± 25	0.46 ± 0.03	+ 5 ± 2	
DM-PAA-PEG	PEG_{2250} 8 mol %	25:1 Range: 2,5-50	98 ± 13.6	0.66 ± 0.09	+ 0.6 ± 8.6	
PEI(25)-PEG(20)1	$PEG_{20,000}$ 46 mol %	5:1 Range: 3-15	146 ± 32.0	0.3 ± 0.04	+ 5.84 ± 3.22	
PEI(25)-PEG(2)10	PEG_{2000} 46 mol %	5:1 Range: 3-15	151 ± 37.2	0.45 ± 0.02	+ 14.5 ± 4.36	
pHPMA-DMAE-b-PEG	PEG_{5000} 20 mol %	12:1 Range: 3-50	134 ± 62.7	0.520 ± 0.21	+ 8.45 ± 10.9	
TMC-PEG	PEG_{2000} 13 mol %	5:1 Range: 2,5-30	52 ± 29.3	0.45 ± 0.08	+5.87 ± 5.30	

Screening phase – ex vivo transfection efficiency

Previously, we showed that PEGylation of polyplexes is necessary to restore transfection efficiency of polyplexes in *ex vivo* human skin. To prevent electrostatic interactions with the anionic extracellular matrix (ECM) components of the skin and consequent immobilization of the cationic polyplexes, shielding the cationic surface charge is required [14]. Fig. 2 gives the antigen expression of the selected polyplex-formulations per group (Table 1) relative to the naked pDNA expression. Compared to naked DNA, the luciferase expression of PAA-PEG and DM-PAA-PEG polyplexes was highly increased up to 1750%. This increase in expression was absent for all other polyplexes, with only a minor increase with pHPMA-DMAE-b-PEG (Figure 2). Moreover, luciferase expression was almost blocked by PEI(25)-PEG(20)1 and even reduced by PEI(25)-PEG(2)10 and TMC-PEG.

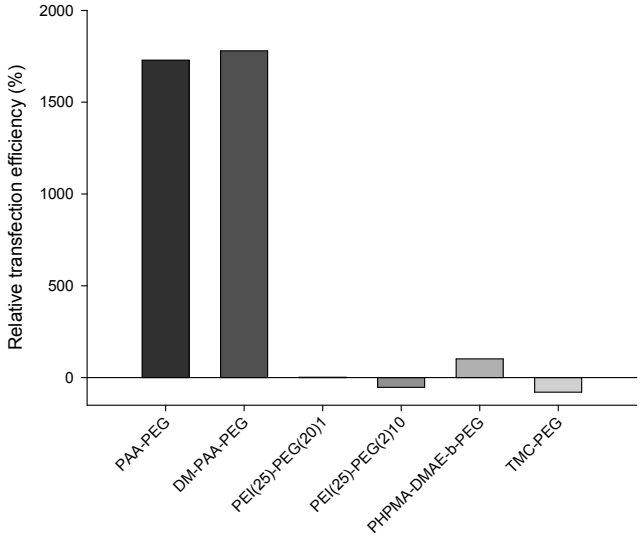


Figure 2: *Luciferase expression of the best performing polyplexes per group as percentage of naked pDNA expression. N = 5 tattoos/polyplex formulation*

PEGylation effect

Given the results above, PAA-PEG was selected for further testing and formulation development. In order to investigate the relationship between PEG chain length and content and transfection efficiency, a series of PAA-PEG polymers with variable PEG content (7.5, 15 and 25%) and chain length ($mPEG_{1100}$; $mPEG_{2000}$ and $mPEG_{5000}$), using the same backbone as the initial PAA-PEG polymer was developed. In addition, we varied the N/P ratios in the range of 3-50 in the polyplex-formulations prepared from these polymers. A total number of 45 polyplex-formulations was thus obtained, which were fully characterized for size, PDI and charge, and transfection efficiency. All polyplexes appeared of comparable size of around

100 nm (range 73 to 123 nm) and PDI (range 0.231 to 0.441), with the exception of the polyplexes with 7.5 % $mPEG_{1100}$, which showed larger particle sizes as well as PDIs at all N/P ratios (N/P 25 and N/P 50 > 400 nm and PDI 0.660), indicative of aggregation. PDI values increase with increasing N/P values, thus increasing polymer content in the polyplex solution results in a more heterodisperse mixture. A similar pattern for charges is also visible. At low N/P values (mostly 3) the anionic charge indicates the presence of naked pDNA, so not all pDNA is condensed in these polyplexes. A logic result of the increased polymer content of the polyplexes is increase of cationic charge, which is reflected in increasing polyplex charge of e.g. -13 for N /P 3 and +6 for N/P for PAA 25 % $mPEG_{5000}$, respectively. Figure 3 summarizes the relative transfection efficiencies for the corresponding polymers. Antigen production is expressed as percentage of the naked pDNA expression. In general, polymers with a high N/P ratio of 25-50 perform best, except for 7.5 % $mPEG_{1100}$ and 7.5% $mPEG_{5000}$. When it comes to PEG content, for all polymer sets the 15 % mPEG content results in highest transfection efficiencies. In addition, increasing PEG chain length results in an higher transfection efficiency with an optimum between $mPEG_{2000}$ and $mPEG_{5000}$. Of all polymers tested, the 11% $mPEG_{2250}$ formulation at N/P 25 performs best.

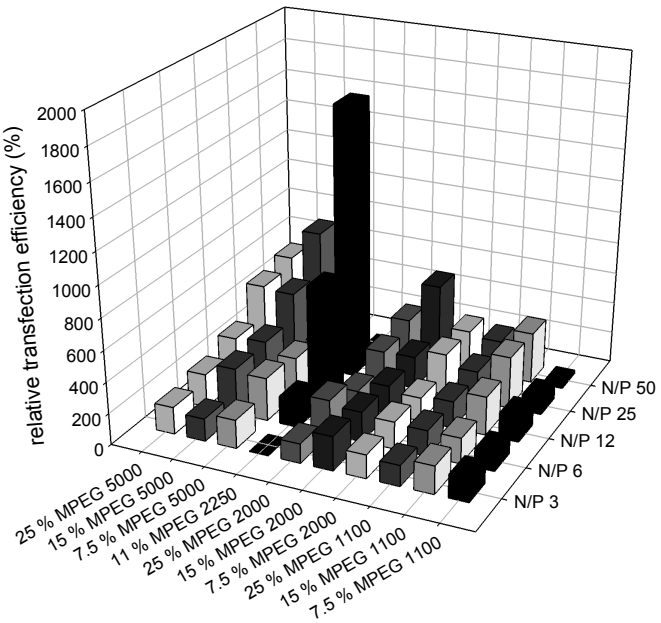


Figure 3: *Luciferase expression PEGylated poly(amido amine)s PAA-PEG analogues as percentage of naked pDNA expression. N = 5 tattoos/polyplex formulation*

DISCUSSION

In this study a broad panel of polymers with distinct differences in molecular structure was characterized and tested in order to investigate if pDNA transfection could be further improved by formulating pDNA into polyplexes, using an *ex vivo* human skin model as read-out. Polymers were selected on the basis of promising results or reported use in *in vitro* and *in vivo* non-viral pDNA and siRNA delivery [8–12]. All polymers were PEGylated in order to shield the cationic charge of the polyplexes which was previously shown to be essential for antigen expression [14]. However, PEGylation of polymers other than the PAA-PEG polymers did not result in improved transfection efficiency when compared to naked pDNA expression. Only poly(amino amide)s polyplexes were successful in enhancing transfection efficiency of plasmid DNA when compared to the performance of the other polymers in the panel. An explanation could be found in the nature of the polymer. As PAA-PEG polymers are biodegradable polymers because of the cleavable disulfide linkages in the backbone, they are very successful in intracellular pDNA cargo release. However, other polymers tested were also able to form stable polyplexes by electrostatic interaction, and the fact that all polyplexes have a cationic charge highlights effective condensation of the pDNA. It is questionable whether the plasmid DNA cargo cannot escape the polyplexes, or the plasmid DNA is not protected well enough by the polymers, leaving its efficiency to the same level of naked (unprotected) plasmid DNA.

After the screening phase, PAA-PEG was selected for further formulation and optimization. Fig. 4 shows typical TEM-images of polyplex formulations of PAA-polyplexes without PEG (A,B) and with 11 mol % $mPEG_{2250}$ (C,D). These images expose the differences of appearance of the PEGylated and non-PEGylated PAA-polyplexes. PAA-polyplexes without PEG form sphere-like nanoparticles < 200 nm. These sphere-like nanoparticles are not formed when PEGylated PAA-polymers are used for polyplex formation. The formulation seems highly disorganized with free pDNA noticeable in the matrix-like substance with absence of sphere-like particles.

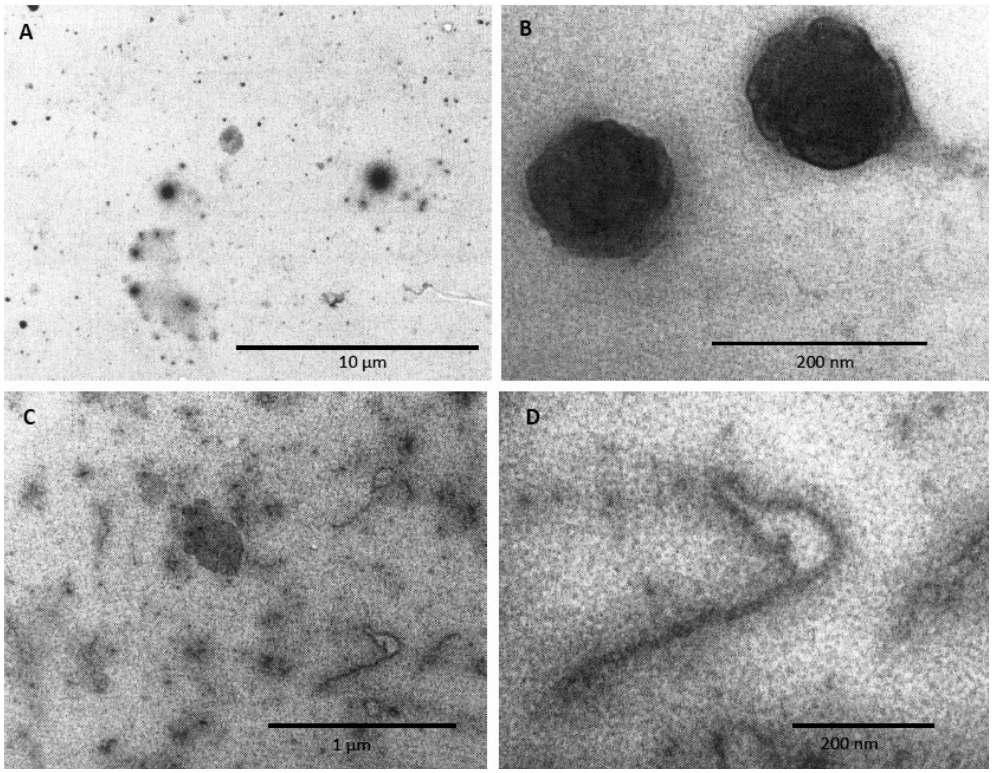


Figure 4: *TEM measurements of Poly (amido amine)s polyplexes without* ***(A,B)*** *and with* ***(C,D)*** *11 mol % $mPEG_{2250}$*

As we found in parallel experiments with the polymer that 10 mM L-Histidine pH 6.0 was able to significantly increase transfection efficiency when compared to the 20 mM HEPES pH 7.4, 10% sucrose buffer, we choose to replace the HEPES buffer with the L-Histidine buffer in our follow-up experiments as they were going to be conducted with PAA-PEG analogues. The purpose of these follow-up experiments was to further investigate the relationship between physical and molecular properties of the PAA-PEG polymer by examining the transfection efficiency in relationship to the PEGylation rate. A series of PAA-PEG polymers was developed with alternating PEG content (7.5, 15 and 25%) and PEG chain length ($mPEG_{1100}$; $mPEG_{2000}$ and $mPEG_{5000}$) with the similar backbone as the initial PAA-PEG polymer. We investigated different mPEG MW and degree of PEGylation, as it has been shown that both factors were found to strongly influence DNA condensation of PEI and therefore also affect the biological activity of the PEI-g-PEG/ DNA complexes [9]. Petersen et al. found that copolymers with many short PEG blocks reduced the diameter of the spherical complexes from 142 ±59 to 61 ± 28 nm. (charge ~5 mV). Co-polymers with only a few but long PEG blocks self- assembled to small and compact condensates of low surface charge (size: 51 ± 23 nm, charge ~3 mV).

Copolymers with many long PEG blocks generated complexes of ill-defined shape and of almost no shielding of the PEI charge (size 130 ± 60 nm, charge ~16 mV) [17]. Best *in vitro* performing system was the copolymer that formed large complexes with a high positive surface charge and a low toxicity profile, as it was found for the copolymer with many short PEG 550 Da blocks[9].

After full characterization and DNA tattooing in our *ex vivo* human skin model, we were able to find relationships in physical and molecular properties. First, the PAA 7.5% $mPEG_{1100}$ polyplexes were the largest and with lowest transfection efficiency, therefore the least favorable polymer of the series because aggregation results in unstable polyplex formation. Second, for all polyplexes (except from PAA 7.5% $mPEG_{1100}$ and PAA 7.5% $mPEG_{5000}$) higher N/P ratios are resulting in increased transfection efficiencies as the availability of free polycations is beneficial for transfection efficiency, an effect Boeckle et al. found for *in vitro* and *in vivo* PEI transfection [18] and that was confirmed by Thibault et al. in chitosan-based polyplexes [19]. Third, PAA 15% $mPEG_{1100}$, $mPEG_{2000}$ and $mPEG_{5000}$ polymers resulted in the highest transfection efficiencies of al sets (N/P 50). Interestingly, the charge of these polymers did not correlate to transfection efficiency levels but were the highest charge for any polymer (from N/P 3 to N/P 50). All together, the best transfection efficiency was achieved by the PAA 11 mol % $mPEG_{2250}$ polymer.

CONCLUSION

Based on the finding in this study, PAA-PEG based polyplexes are the most promising candidates for improving pDNA transfection efficiency by intradermal tattoo vaccination. Variations in PEG chain length and degree showed that 15% mPEG for all PEG MWs was best for transfection efficiency, probably as a result of the size and charge of these polyplexes. From the panel tested, the polyplex formulation containing 11 mol % $mPEG_{2250}$ resulted in the best transfection efficiency in the *ex vivo* human skin DNA tattoo-model.

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Chapter 3.2

Histidine enhances transfection efficiency of poly(amido amine)s polyplexes for intradermal DNA tattoo vaccination

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ABSTRACT

We have previously shown that poly(amido amine)s PAA-polyplexes can significantly increase the transfection efficiency of DNA vaccines upon intradermal tattooing. In order to translate this promising PAA-polyplex concept to the clinic, an optimal pharmaceutical formulation is warranted. In this study we screened for the optimal polymer/pDNA ratio of the PAA-polyplexes, making use of an *in vitro* High Content Screening (HCS) platform. In addition, we investigated the use of an alternative buffer system for the PAA-polyplex formulation. We examined the applicability of an L-histidine buffer, an approved and commonly used excipient in parenteral formulations. PAA-polyplexes in different polymer/pDNA ratios in the different buffer systems were tested in the HCS platform. It was found that PAA-polyplexes at a N/P ratio of 25 formulated in L-histidine significantly improved the percentage of transfected cells in comparison to the HEPES formulation (from 9.9 ± 4.7 % to 17.0 ± 1.6 %, $p = 0.007$) and the LPEI control (9.2 ± 2.7 % to 17.0 ± 1,9 % $p = 0.03$). Subsequently, promising polyplex formulations were PEGylated and tested in an ex *vivo* human skin model and *in vivo* by vaccination of mice with these formulations which allowed simultaneous measurement of antigen expression and T cell responses. When formulated in 10 mM L-histidine buffer pH 6.0, the transfection efficiency of PAA-polyplexes *ex vivo* is 2-fold enhanced ($p = 0.02$). Additionally, as *in vivo* antigen expression of the polyplexes in L-histidine buffer is enhanced, *in vivo* immunogenicity is not improved.

The better buffering capacity of L-histidine in the lower pH region of endosomal acidification, together with a higher concentration of cationic charge carriers (polymer and protonated histidine) is hypothesized to result in a more pronounced proton sponge effect and higher polymer-endosomal membrane interactions, and/or different particle conformation giving rise to increased efficiency of endosomal escape and thus transfection. In conclusion, the results presented in this study show that a 10 mM L-histidine buffer pH 6.0 has an additive effect in the transfection activity and therefore is a promising excipient in the pharmaceutical formulation of PAA-PEG polyplexes for intradermal tattoo vaccination.

INTRODUCTION

DNA vaccination is an appealing method to obtain an antigen-specific immune response, which could be useful in anticancer therapy [1,2]. When delivered by intradermal tattooing, plasmid DNA (pDNA) vaccination results in a strong and rapid antigen specific cellular immune response in both mice and non-human primates [3]. In spite of the high immunogenicity of pDNA tattoo, the *in vivo* transfection efficiency of naked pDNA with this technique is extremely low, with approximately 1 out of $5x10^6$ to $5x10^9$ plasmid copies applied being taken up, transcribed and translated [4]. Non-viral carrier systems are extensively used as a transfection vehicle to deliver nucleic acids for both *in vitro* and *in vivo* applications. In these systems, negatively charged pDNA is bound by electrostatic interaction to an excess of a positively charged carrier resulting in the formation of nanosized particles which protects the pDNA from nuclease degradation, giving substantially higher transfection efficiencies compared to naked nucleic acids in *in vitro* assays [5,6]. Cationic polymers are frequently used carriers for plasmid DNA and the resulting DNA/polymer nanoparticles are referred to as polyplexes [7]. When these cationic polyplexes encounter the cells after administration, they are able to interact with the anionic cellular membrane and are taken up into the cells via endocytosis. Within the cells, the polyplexes are transported by endosomes that eventually become acidified and fuse with lysosomes (Fig. 1). In this situation, the pDNA is likely to be degraded by the lysosomal enzymes. In order to successfully transport the incorporated pDNA into the nucleus, the polyplexes must be able to escape from the endosomes [8]. An endosomolytic process known as the 'proton sponge effect' [9,10] facilitates the endosomal escape. Endosomal escape can also be assisted by use of cell-penetrating peptides (CPPs) that either form complexes with the pDNA or can be incorporated into polymers [11]. It has been suggested that the opening of transient pores in the lipid bilayer of endosomes is involved [12], but also conformational changes in response to the acidification inside the endosomes leads to destabilization of the endosomal membrane bilayer [13]. After endosomal release, the polyplexes have to unpack and transfer the pDNA load close to the nucleus to facilitate successful uptake and processing. In addition to the beneficial effect on *in vitro* transfection efficiency, formulation of pDNA into cationic particles has also been shown to result in a higher transfection efficiency than naked pDNA upon intramuscular injection [14,15].

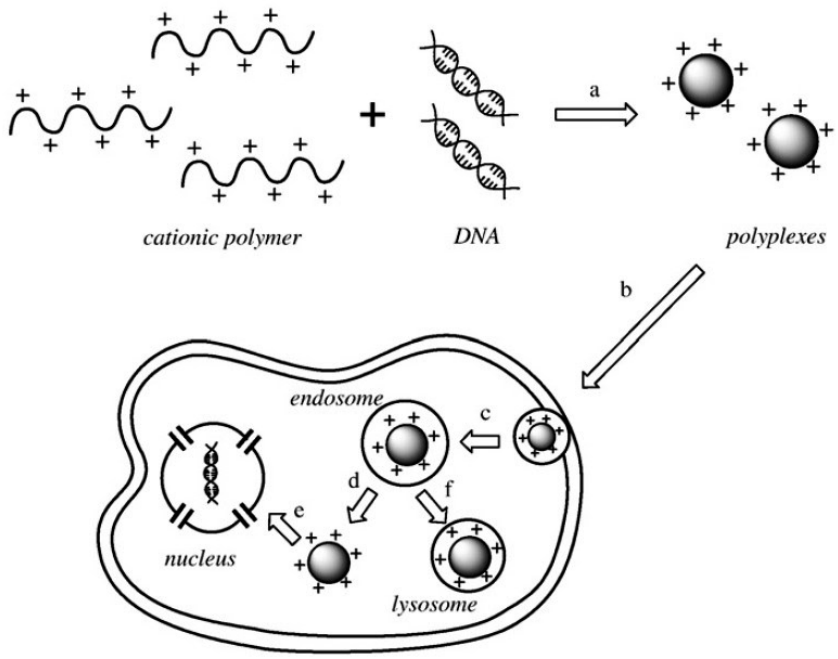


Figure 1: *Schematic illustration of cationic polymer-mediated gene delivery: a) formation of cationic polymer/DNA complexes (polyplexes); b) cellular uptake of polyplexes by endocytosis; c) endosomal pathway of polyplexes; d) endosomal escape of polyplexes; e) polyplex unpacking and nuclear translocation of DNA; f) degradation of polyplexes in lysosome, adapted from [39].*

Previously, we have shown that intradermal tattooing of polyplexes making use of (PEGylated)-poly(amido-amine)s polymers results in a significantly enhanced transfection efficiency both *in vitro* as well as *in vivo* models [2]. Poly(amido-amine)s (PAAs) are a unique family of synthetic functional polymers that have been widely developed for use both as biomedical materials and polymer therapeutics (see Fig. 2) [16] and can be easily synthesized by Michael-type polyaddition of primary or bis-secondary amines to bis(acrylamide)s [17,18]. These cationic polymers are water-soluble, biodegradable and have lower cytotoxicity than other usual polycationic vectors [9,19]. The tertiary amines in the main chain of the PAAs give these polymers high buffer capacity in the pH range 5.1-7.4 and this property enables PAA polyplexes once taken up by cells to escape from the endosomes by increasing polymer-membrane interaction and the assumed 'proton sponge effect' [9,20].

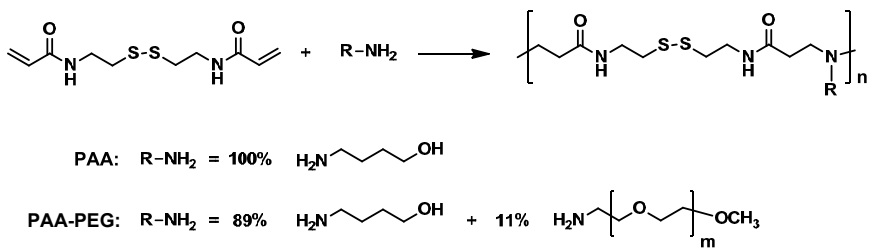


Figure 2: *Bioreducible poly(amido amine)s without (PAA) and with $mPEG_{2250}$ (PAA-PEG) in the side chains.*

In order to translate this promising PAA-polyplex concept to the clinic, a suitable pharmaceutical formulation is warranted. In this study, we screened for the optimal polymer/pDNA ratio of the PAA-polyplexes making use of the *in vitro* High Content Screening (HCS) platform [21]. In addition, we investigated the use of an alternative buffer system for the PAA-polyplex formulation. Commonly, in the experimental setting low molarity buffers such as 20 mM HEPES (pH 7.4) are used and tonicity is adjusted using sugars (glucose, sucrose) [2,22,23]. However, HEPES is not listed as an approved inactive ingredient by the US Food and Drug Administration, which precludes the use of this buffer in a clinical formulation [24]. Therefore, we examined the applicability of a histidine buffer, an approved and commonly used excipient in parenteral formulations [25]. In addition, the L-histidine buffer has been applied in the development of a lyophilized LPEI polyplex-formulation [23].

The low aqueous stability of polyplexes is a major hurdle for clinical translation, and freeze-drying is an attractive option to increase shelf-life [22,23,26,27]. In the present study, PAA-polyplexes in different polymer/pDNA ratios in the different buffer systems were tested in the *in vitro* HCS platform [23]. Subsequently, promising polyplex formulations were tested in an *ex vivo* human skin model [4] and *in vivo* by vaccination of mice which allowed simultaneously measurement of antigen expression and T cell responses [3].

MATERIALS AND METHODS

Materials

The pVAX:LucNP plasmid [3] encodes the influenza A $NP_{366-374}$ epitope as a genetic fusion with firefly luciferase gene, inserted in the EcoRI/NotI site of minimal pVAX1 plasmid backbone (Invitrogen, Carlsbad, USA). pVAX:EGFP was constructed as previously prescribed [28]. Plasmids were expressed and amplified in *E.Coli* DH5 and were purified by EndofreeTM QIAGEN® Mega-kit (QIAGEN®, Hilden, Germany). Non-PEGylated and PEGylated poly(amido amine) (PAA) copolymers possessing protonable amino groups and bioreducible

linkages in the main chain and hydroxybutyl groups in the side chains (CBA-ABOL), were synthesized by Michael addition polymerization of N,N'-cystaminebisacrylamide with the appropriate amine according to the procedure described previously [18]. The PEGylated analog was prepared using 11 mol% of MeO-PEG-NH_2 (MW *ca.* 2250 after dialysis purification) in the total amino monomer feed during the PAA synthesis. ExGen500 (22 kDA linear polyethylenimine (LPEI)) was purchased from Fermentas, St. Leon-Rot, Germany. DAPI was purchased from Invitrogen, Carlsbad, USA. L-Histidine was purchased from VWR (Amsterdam, the Netherlands), DMEM (Dulbecco's modified Eagle's Medium, with 1 g/l glucose, 584 mg/ml L-glutamine), Fetal Bovine Serum (FBS), and Phosphate Buffered Saline (PBS) were purchased from PAA Laboratories GmbH, Pasching, Austria. Chloroquine, HEPES, paraformaldehyde, amphidicolin and Triton® X-100 were purchased from Sigma-Adrich, Zwijndrecht, The Netherlands. All other chemicals were of analytical grade.

Cell culture

COS-7 African Green Monkey kidney cells were grown in DMEM supplement with 5% heat inactivated FBS and antibiotics/antimycotics. Cells were maintained at 37°C in a 5% CO_2 humidified air atmosphere and split twice weekly.

Polyplex preparation and characterization

Polyplexes were prepared by mixing an equal volume of plasmid and dissolved polymer. The ratios of polymer to plasmid DNA are expressed as the molar ratio of nitrogens within the PAAs to phosphates in the plasmid DNA (N/P). PAA-pDNA (pVAX:EGFP) polyplexes in the N/P range of 1-50 were formulated in either 20 mM HEPES pH 7.4 or 10 mM L-histidine pH 6.0 buffer (pDNA concentration of 50 μg/ml). LPEI controls (N/P 6) were prepared by adding 4 volumes of polymer solution to 1 volume of plasmid solution (50 μg/ml) and mixing. PEGylated PAA-pDNA (pVAX:LucNP) polyplexes in the N/P range of 1-50 were formulated in either 20 mM HEPES pH 7.4 or 10 mM L-histidine pH 6.0 buffer (pDNA concentration of 0.5 mg/ml). All polyplexes were incubated for 30 min at room temperature. Formulation characterizations were performed with the PEGylated PAA pVAX:LucNP construct. N/P Ratios were calculated assuming that 302 and 532 g/mol correspond with each (protonable) nitrogen containing-repeating unit of PAA and PEG–PAA, respectively. For plasmid DNA 330 g/mol corresponds with the average mass of a repeating unit bearing one negative phosphate group. Control naked DNA was diluted to the same concentration in the same buffer. The size of obtained particles was measured in 20 mM HEPES pH 7.4, or 10 mM L-histidine pH 6.0 buffer with dynamic laser scattering using an ALV/GCS-3 (Malvern Instruments, Malvern, UK). Particle size distribution is described using the polydisperity index (PDI), ranging from 0 for a monodisperse to 1 for a heterodisperse preparation. The zeta potential of obtained particles was determined in 20 mM HEPES pH 7.4 or 10 mM L-histidine pH 6.0 buffer, using

a Zetasizer Nano Z (Malvern Instruments, Malvern, UK). Both instruments were calibrated using polystyrene latex beads of defined size and electrophoretic mobility.

HCS transfections

COS-7 cells (n=10,000) were seeded into black, clear bottom, 96-well tissue culture plates (Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands) 24 h prior to transfection. Immediately prior to transfection the culture medium was refreshed with 100 µl complete medium. PAA–pDNA (pVAX:EGFP) polyplexes in the N/P range of 1-50 formulated in either 20 mM HEPES pH=7.4 or 10 mM L-histidine pH 6.0 buffer were tested (n=3 wells/formulation), with 22 kDa LPEI (ExGen 500) as a positive control. 25 µl of the polyplex samples (corresponding to 0.25 µg plasmid DNA/well) was added per well and after 4 h incubation, medium was replaced with 100 µl complete medium. Cells were incubated for indicated times at 37 °C in a 5% CO_2 humidified air atmosphere.

HCS Fixation and nuclear staining

48 h post transfection, cells were washed once with 100 µl PBS and fixed for 30 min with 100 µl 4% paraformaldehyde in PBS. After fixation, cells were washed once with 100 µl PBS and nuclei were stained with 21.8 µM DAPI in PBS. After DAPI staining, cells were washed twice with PBS. To each well, 100 µl PBS was added and cells were analyzed using a Cellomics Arrayscan V HCS Reader.

High capacity automated fluorescence imaging and image analysis

A Cellomics Arrayscan V HCS Reader (Thermo Fisher Scientific, Waltham, MA, USA) was used to analyze the cells. For the analysis of the HCS data, the Cellomics algorithm Target Activation BioApplication (Thermo Fisher Scientific, Waltham, MA, USA) was used. The Target Activation BioApplication allows for measurements of up to 4 different fluorescent channels. One fluorescent channel is reserved for the identification of individual cells using major cellular organ- elles/compartments (e.g. nuclei or cytoplasm) and provides the basis for the intracellular region where intensity measurements are made. In this study, single cells were identified according to the morphology of their nuclei using DAPI staining. After identification of single cells, the fluorescence intensity of the used fluorescent probes was measured in a predefined region. If the fluorescence intensity is higher than the defined threshold, the single cell is 'positive' or a 'responder' for this fluorescent signal. From these values, the percentage of responders can be calculated (Fig 3).

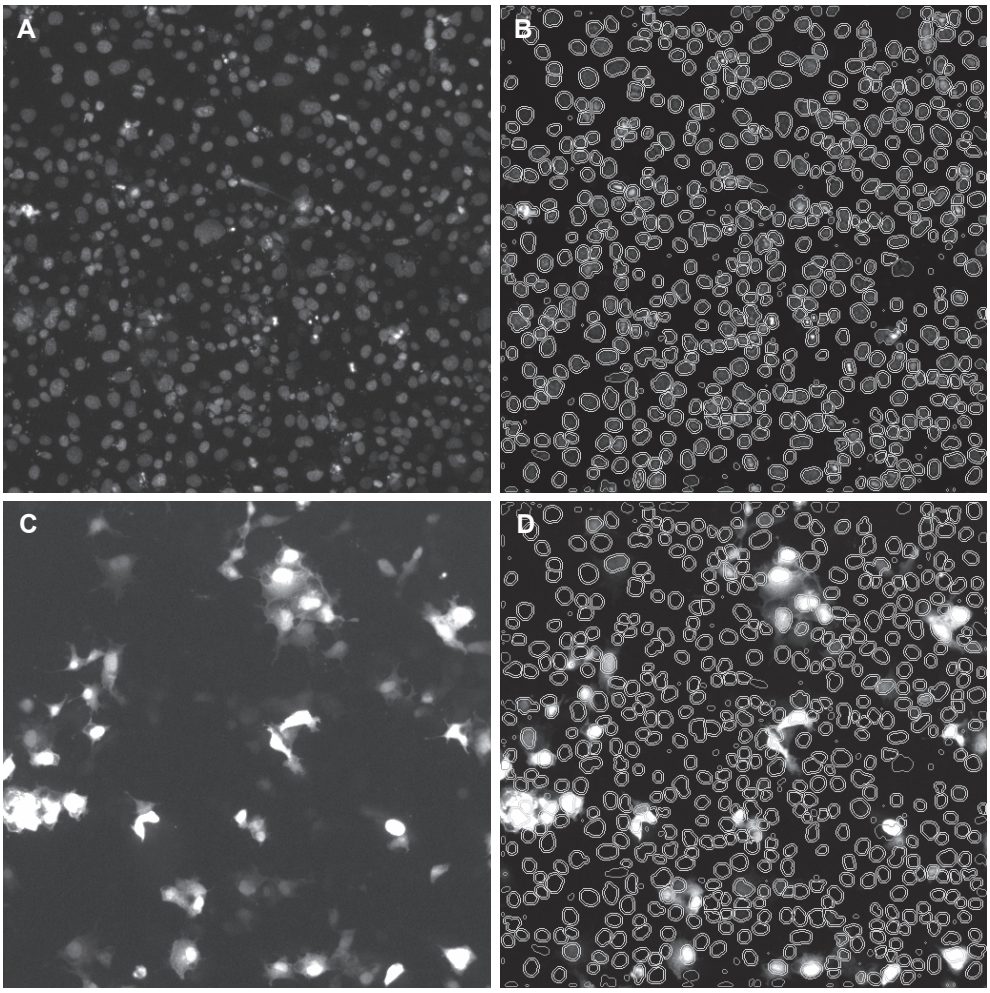


Figure 3: *Cell number and transfection efficiency measurements using the Target Activation BioApplication (Thermo Fisher Scientific, Waltham, MA USA). COS-7 cells were incubated for 4 hours with 22 kDa linear PEI and pDMV-EGFP complexes at N/P ratio of 6. Forty eight hours after transfection, cells were imagined on a Cellomics Arrayscan V HCS Reader and analyzed by the Target Activation BioApplication. Nuclei were stained with DAPI* ***(A)*** *and cells were identified using their nuclear morphology* ***(B)****. Included cells are shown outlined in green, rejected cells in red. The % EGFP expressing cells* ***(C)*** *was determined using the identified cells* ***(D)****.*

DNA tattooing of ex vivo human skin

Formulations and naked DNA controls encoding firefly luciferase were administered to intact skin by DNA tattooing [3] to allow luciferase expression measurements. The skin model used in these experiments has been described previously for the optimization of tattooing of naked DNA in skin [4]. In brief, 10 µL of the indicated formulation at a final DNA concentration of 0.5 mg/mL was applied to the skin into a custom fabricated mould to keep the area of tattooing

constant (diameter 8 mm, surface 50 mm^2). The formulation was subsequently administered into the skin using an Aella® tattoo machine (MT.DERM GmbH, Berlin, Germany) (Fig. 4A). For all tattoos, 9-needle cartridges at an oscillating frequency of 100 Hz were used. The needle depth was adjusted to 1.5 mm and tattoo duration was 20 s. After tattooing, skin samples were kept at 5 % CO_2, 37 °C in complete keratinocyte serum free medium (SFM) containing 1 % penicillin/streptomycin and 0.25 µg/mL amphotericin B (all Invitrogen, Carlsbad, USA) to allow expression measurements of luciferase. During this incubation, skin was cultured at the air–medium interface with the epidermis exposed to the air to mimic the natural situation.

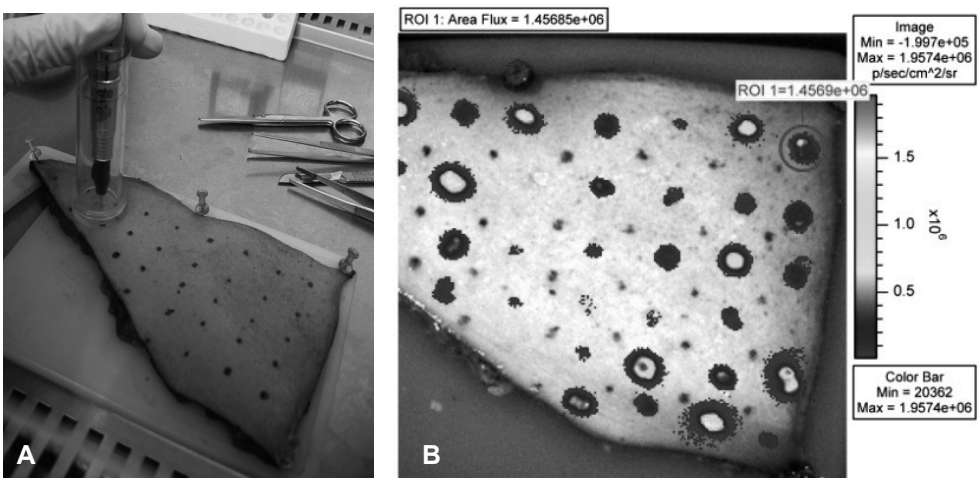


Figure 4: *Tattooing procedure of the human skin (A) and typical expression of luciferase (B), visualized with a light sensitive camera, 18 hours after tattooing.*

Measurement of antigen expression using intravital imaging of ex vivo human skin

The expression of luciferase was measured in intact skin samples approximately 17 h after tattooing to obtain maximal antigen levels [4]. The substrate luciferin (Xenogen, Hopkinton, USA) was added to the medium in a final concentration of 45 µg/ml. During this procedure extra medium was added to the box in which skin was incubated, to cover the complete epidermis of skin samples with fluid to guarantee full accessibility of luciferin to the tattooed areas. 30 min after the addition of the substrate, luminescence produced by active luciferase was acquired during 30 s with an IVIS Lumina II CCD camera (Xenogen, Hopkinton, USA). Signal intensity was quantified as the sum of all detected light within the tattoo area of interest. In all measurements, background luminescence was determined for non-treated skin to allow correction during data analysis (Fig. 4B).

Mice

Female C57BL/6J mice (6–8 weeks) were obtained from Charles River (Wilmington, MA, USA). They were allowed to acclimate for approximately 1 week after arrival. All mice were housed n=5/cage, in a climate-controlled room. Food and water were provided *ad libitum*. All testing occurred between 10.00 and 18.00 hours. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals at our institute.

DNA immunization

To allow simultaneous measurement of antigen expression and T cell responses, mice were immunized by pDNA tattooing with formulations containing the pVAX:LucNP construct. For intradermal DNA vaccination, the hair of the left hind leg of the mice was removed with depilatory cream (Veet sensitive, Reckitt Benckiser, Berkshire, UK). Next, 15 ml of the polyplex formulation or naked pDNA solution was applied to the skin and a sterile disposable 9-needle cartridge (MT.DERM GmbH, Berlin, Germany) mounted on an Aella® tattoo machine for medical use (MT.DERM GmbH, Berlin, Germany) was used to apply the vaccine. Needle depth was adjusted to 1.0 mm, and the needle bar oscillated at 100 Hz. DNA vaccines were punched into the skin by a 45 s tattoo. Mice were vaccinated with a standard vaccination scheme on day 0, 3 and 6 [3]. All mice were anesthetized with isoflurane (Abbott Laboratories, Illinois, USA), during treatment. At indicated time points after immunization, approximately 50 µl of peripheral blood was collected by tail bleeding for the measurement of T cell responses.

Measurement of antigen expression using intravital imaging

Antigen expression upon DNA vaccination was measured by a light-sensitive camera to allow longitudinal *in vivo* expression of firefly luciferase after vaccination on day t=1, t=2 and t=3. Mice were anesthetized with isoflurane. A solution of the substrate luciferin in PBS (150 mg/kg, Xenogen, Hopkinton, USA) was intraperitoneally injected and after 18 min the luminescence produced by active luciferase was acquired during 45 s in an IVIS Lumina II CCD camera (Xenogen, Hopkinton, USA). Signal intensity was quantified as the sum of all detected light within the region of interest, after subtraction of background luminescence.

Cytotoxic T cell assay

To measure antigen-specific CTL responses, peripheral blood lymphocytes were stained at the indicated time points with Phyco Erythrin (PE)-conjugated H-2D^b/NP$_{366–374}$-tetramers and Allo Phyco Cyanin (APC)-conjugated CD8a antibody (BD Pharmingen, San Jose, USA) at 20 °C for 15 min in FACS buffer (1x Phosphate Buffered Saline (PBS), 0.5 % Bovine Serum Albumin (BSA) and 0.02 % sodium azide) as described before [29]. CD8$^+$ cells expressing

the NP epitope-specific T cell receptor will bind to the tetramer and can be detected by flow cytometry. After incubation cells were washed three times in FACS buffer and analyzed. Living cells were selected based on propidium iodide (PI) exclusion. Data acquisition and analysis was done with a FACSCalibur (Becton Dickinson, Franklin Lakes, USA) using Flowjo software (Becton Dickinson, Franklin Lakes, USA).

Statistical analysis

A Welch Two Sample t-test (one-way anova) was used for statistical analysis with the aid of 'R' software. Data are represented as mean ± standard deviation. P values < 0.05 were considered statistically significant.

RESULTS

HCS transfection experiment

In order to optimize the formulation, transfection efficiency of polyplexes was first assessed in an *in vitro* cell culture. HCS was used to simultaneously measure cell number (by way of fluorescent staining of the nuclei) and EGFP expression for each individual cell. COS-7 cells were transfected with PAA-polyplexes with pDNA encoding pVAX:EGFP with varying N/P ratios (N/P 1-50) or the LPEI control (N/P 6). The cells were imaged on a Cellomics Arrayscan V HCS Reader with the criteria that 4 image fields were collected for each well and these were analyzed by the Target Activation BioApplication[21]. To measure cell number, cells were identified according to their nuclear morphology (Fig 3). Viable cells have a nuclear morphology that differs from that of dead or dying cells. As a consequence, only the viable cells will be counted which makes cell number a good measure for potential toxic effects due to exposure to transfectants. To determine the transfection efficiency, EGFP expressing cells were identified among all the detected cells. It was found that PAA-polyplexes at a N/P ratio of 25 formulated in L-histidine significantly improved the percentage of transfected cells in comparison to the HEPES formulation (from 9.9 ± 4.7 % to 17.0 ± 1.6 %, p = 0.007) and the LPEI control (9.2 ± 2.7 % to 17.0 ± 1,9 % p = 0.03) (Fig. 5).

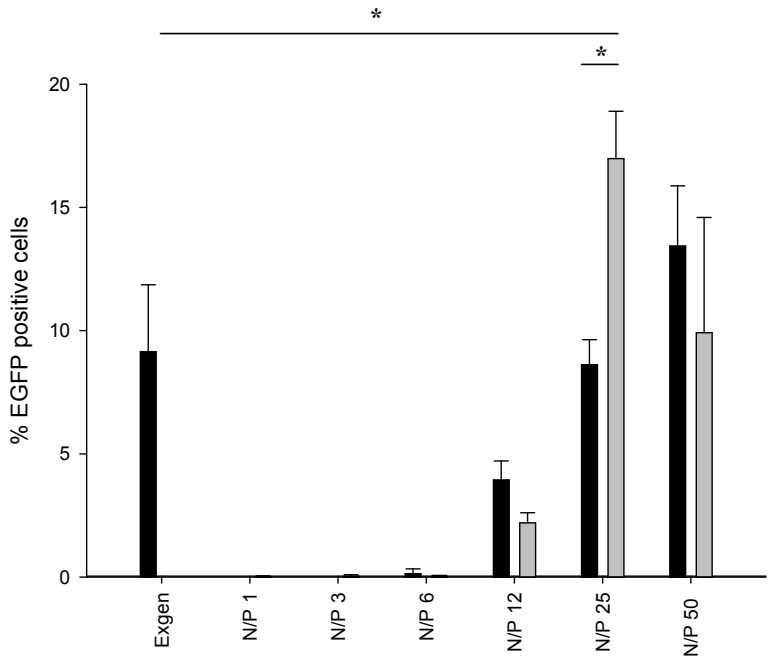


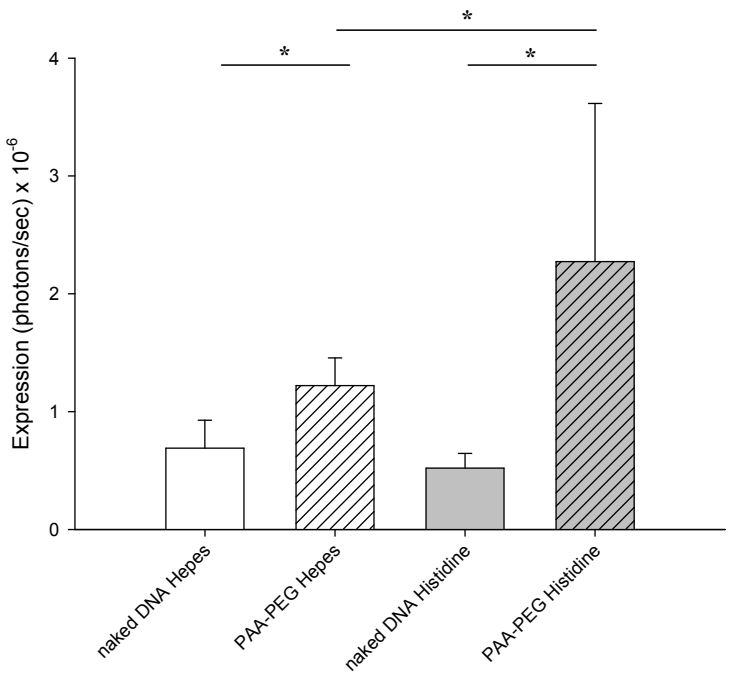
Figure 5: *Transfection efficiency measured using HCS. COS-7 cells were incubated for 4 h with PAA-polyplexes in HEPES (black bars) and L-histidine buffer (grey bars) or LPEI complexes. Forty eight hours after transfection, cells were imaged on the Cellomics Arrayscan V HCS Reader and analyzed by the Target Activation BioApplication. Data are presented as mean + SD. N=3 (Control: 22kDa LPEI ExGen). *Values significantly different from buffer system and Exgen control.*

Ex vivo human skin intradermal tattoo vaccination

Previously, we showed that PEGylation of the PAA-PEG polymers is necessary to restore transfection efficiency of polyplexes in *ex vivo* human skin. Shielding of the cationic surface charge is hypothesized to prevent electrostatic interactions with the anionic extracellular skin matrix components with immobilization of the polyplexes as a consequence [2]. Given the results from the HCS assay with the PAA-polymers without PEG and the previous optimization of PAA-PEG polyplexes in the skin model, the PAA-polyplex at N/P of 25 was selected for testing in the *ex vivo* human skin model. For further testing, PAA-polymers were PEGylated by coupling $mPEG_{2250}$ chains to the PAA backbone (Fig. 2, [30]). For PAA-PEG polyplexes in either HEPES or L-histidine buffer, luciferase expression was significantly increased compared to naked pDNA (1.8 fold $p = 0.007$ and 4.4 fold $p = 0.04$, respectively) in the *ex vivo* human skin model (Fig 6A). Moreover, a significant 2-fold enhanced transfection efficiency of PAA-PEG polyplexes when formulated in L-histidine buffer was found compared to HEPES ($p = 0.02$).

Fig. 6B gives the characteristics of the PAA-PEG polyplex formulated in HEPES and L-histidine buffer. When formulated in L-histidine buffer, size and charge of the polyplexes are increased in comparison to the same polyplexes formulated in HEPES buffer from size 118 ± 10 nm to 197 ± 41 nm and charge 5.5 ± 0.2 mV to 13.8 ± 1.2 mV respectively. PDI was not affected by change in buffer system, indicating similar homogeneousity of the polyplexes in solution (Fig. 6B).

A



B

Buffer	Size (Z-ave nm)	PDI	Charge (mV)
HEPES	118±10	0.49±0.04	5.5±0.2
L-histidine	197±41	0.46±0.04	13.8±1.2

Figure 6: ***(A)*** *Luciferase expression of PAA-PEG polyplexes (N/P 25) in HEPES and L-histidine buffers compared to naked DNA controls in intact ex vivo human skin. Each point represents the mean + SD of 5 data points, randomized tattooed over one biopsy of skin. *Values significantly different from naked DNA control and buffer system.* ***(B)*** *PAA-PEG polyplex characteristics. The results are expressed as mean values of 3 measurements ± SD.*

In vivo transfection efficiency and immunogenicity

The *in vivo* antigen expression upon tattoo vaccination and $NP_{366-374}$ specific T cell responses were studied in C57/B6 mice. Mice were vaccinated with naked DNA or polyplex in HEPES or L-histidine buffer formulations using a standard tattoo vaccination protocol, with administrations on day 0, 3 and 6 [3]. To simultaneously measure vaccination-induced

antigen expression and vaccination-induced antigen-specific T cell responses, a pVAX:LucNP model pDNA vaccine was used. This model pDNA construct allows the monitoring of *in vivo* antigen expression by assessment of luciferase activity, while vaccine immunogenicity can be determined by monitoring of T cell responses against the vaccine-induced influenza $NP_{366\text{-}374}$ epitope. Although the longitudinal *in vivo* antigen expression of the polyplexes was increased (time points 36 and 60 hours after first vaccination) in comparison to the naked pDNA controls, the increase with use of L-histidine that was so apparent in both the *in vitro* and *ex vivo* assays was not present in this assay. However, the polyplexes in both buffers showed an increased transfection efficiency when compared to the naked pDNA controls (Fig. 7A). Nonetheless, the increase of the polyplex formulations did not translate in an enhanced immunogenicity when compared to the naked pDNA controls. In line with previous results [2], the magnitude of the antigen-specific T cell response induced by vaccination with naked DNA or with polyplexes in either L-histidine or HEPES buffer was not significantly different (Fig 7B).

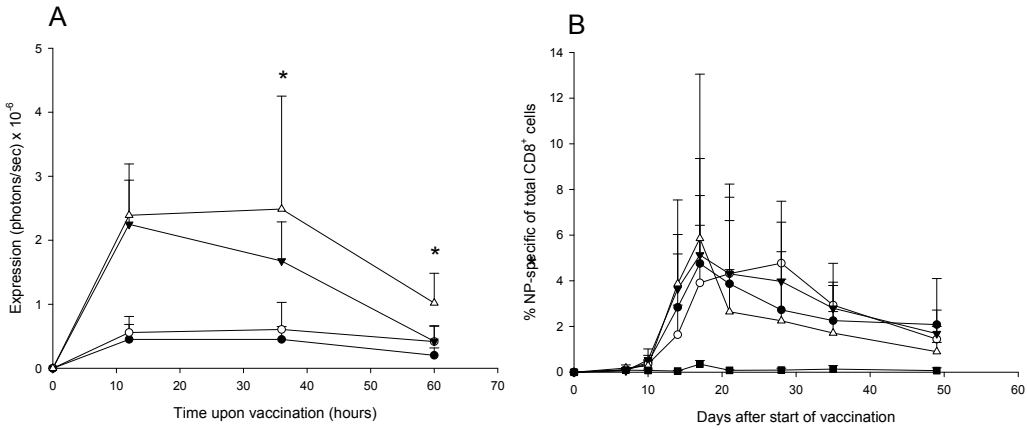


Figure 7: (A) *In vivo antigen expression in mice upon tattoo vaccination of: Naked DNA in HEPES buffer (●) or naked DNA in L-histidine buffer (○), PAA-PEG polyplexes in HEPES buffer (▼) or PAA-PEG polyplexes in L-Histidine buffer (Δ) (both polyplexes at N/P ratio of 25). Expression of the vaccine-encoded antigen (luciferase) was measured at the indicated time points upon tattooing with a light-sensitive camera. *Values significantly different from other buffer system. Each point represents the mean + SD of 8 mice.*
(B) *T cell responses upon tattoo vaccination of DNA polyplexes. $NP_{366\text{-}374}$ specific T cell responses upon tattoo vaccination with the LucNP construct are shown for mice vaccinated with naked DNA in HEPES buffer (●) or naked DNA in L-histidine (○), PAA-PEG polyplexes in HEPES buffer (▼) or PAA-PEG polyplexes in L-histidine buffer (Δ) and non-vaccinated control mice (▪, n = 3). (Both polyplexes at N/P 25). $NP_{366\text{-}374}$ specific T cell responses were measured by direct ex vivo MHC tetramer staining of peripheral blood lymphocytes. Each point represents the mean + SD of 8 mice.*

DISCUSSION

Previously, Kasper et al. [23] reported the use of L-histidine pH 6.0 buffer as a potential excipient in the development of a lyophilized LPEI-polyplex formulation as an alternative for the non-pharmaceutical HEPES buffer. To protect the active compound, the lyophilization medium should be of low ionic strength and have a pH close to the pKa of histidine, i.e. around 6.1. In this case L-histidine provides high buffer capacity at increasing ionic strength during cryoconcentration of the complexes (or other solutes) in solution. [23]. As the size and charge of polyplexes is influenced by the ionic strength of the buffer and the degree of protonation of the polymer depending on the surrounding pH, Kasper et al. reported that due to the lower pKa and thus higher degree of protonation of the LPEI polymer, smaller particles, a lower PDI and an increased zeta-potential were seen in L-histidine buffer. However, the changes in size and charge did not affect *in vitro* transfection efficiency. In contrast, we found an increased zeta-potential and an increased particle size when using L-histidine compared to HEPES. This increase in particle size at lower pH may be an unique PAA-effect as Coué et al. also reported an PAA-nanoparticle size increase, explaining this by the capacity of PAA polymers to undergo a conformational change from a coiled structure at pH 7.4 to a more extended one when exposed to acidic pH [17]. Also, a significantly increased *in vitro* and *ex vivo* transfection efficiency for our PAA polyplexes formulated in L-histidine buffer is shown. We found that the N/P ratio of 25 was most advantageous for the transfection efficiency, indicating the optimal balance in anionic pDNA charge and cationic PAA-polymer charge. Furthermore, the use of L-histidine is only beneficial to the PAA-PEG polyplexes, as the use of L-histidine has hardly an effect on the transfection efficiency of naked pDNA (Fig. 6A). We hypothesize that these findings may be attributed to one or a combination of the following effects. First, the tertiary amines in the PAA-PEG backbone of the polymer in the L-histidine buffer solution (pH 6.0) are protonated to a higher degree than in HEPES buffer (pH 7.4). The buffer capacity of these PAAs, defined as the percentage of amino groups becoming protonated in the pH range from pH 7.4 to 5.1, is supposed to be a relevant parameter for endosomal escape of the polyplexes. The PAAs have hydroxyalkyl side groups and are proposed to bind protons during the endosomal acidification process and subsequently promotes endosomal escape of polyplexes by osmolysis (proton sponge effect). This results in polyplexes with higher surface charge (Table 4B). It is expected that such polyplexes are taken up more efficiently by endocytosis due to increased polyplex-cell membrane interactions. Second, L-histidine (pKa 6.1) that is taken up simultaneously with the endocytosis of the polyplexes has a significantly higher buffer capacity in the endosomal acidification process to pH ca. 5 than HEPES (pKa 7.55) for which acidification below pH 6.5 falls outside its buffering range. The 'proton sponge' hypothesis involves that unprotonated amines of the polymer can absorb protons, resulting in more protons being pumped into the endosomes with a concomitant influx of

chloride-ions and water. A combination of the osmotic swelling and a swelling of the polymer due to repulsion between protonated amine groups causes destabilization of the endosomal membrane with subsequent release of its contents into the cytoplasm [9,10,31]. Also, high pKa cationic polymers including chitosan with histidine (imidazole group) has been reported to bring about dramatic enhancement of transfection efficiency [32–34]. Several histidine-rich polymers have been developed for improved nucleic acid delivery such as biodegradable PEG-co-poly(L-lysine)-g-histidine multiblock copolymers [35] and aminated or alkylated poly(1-vinylimidazole) for non-viral transfection [36,37]. Further experiments with PAA-PEG polyplexes should investigate whether coupling l-histidine-groups to the PAA-backbone also would lead to increased transfection efficiencies. Third, the tertiary amines in the PAA-backbone of the polymer in the L-histidine buffer solution (pH 6.0) are protonated to a higher degree than in HEPES buffer (pH 7.4), resulting in a more extended structure and thereby formation of polyplexes of increased size and charge. The physic-chemical alternations of these polyplexes could be responsible for the increase in transfection efficiency. These combined pH and buffer capacity-effect hypothesis is substantiated by experiments using a 10 mM citrate buffer (pKa 6,4) of pH 6.0, which also resulted in a positive effect on PAA-PEG polyplex *ex vivo* transfection efficiency (increase of 1.4 fold, not significant when compared to HEPES). The *in vivo* antigen expression results are, though less pronounced, in line with the *in vitro* and *ex vivo* results and indicative for a higher transfection efficiency of the PAA-PEG polyplexes (Fig. 6A). The immunogenicity of the PAA-PEG polyplexes in either buffer, however, is not increased when compared to the naked pDNA controls (Fig. 6B). The attenuated increase of transfection efficiency of polyplexes in L-histidine when compared to the polyplexes in HEPES that is noticed *in vivo* could be the result of differences in the murine skin structure as compared to the human skin, as these polyplexes can induce very different antigen expression patterns in the *ex vivo* human skin model. Additionally, polyplexes even formulated in HEPES are not able to enhance immunogenicity. Furthermore, the assumption that a high transfection efficiency automatically will translate in a high immune response likely is too simple and dependent on other factors, such as antigen presentation and co-stimulation. Although it has been shown that the level and duration of vaccine-induced antigen expression is correlated with the magnitude of vaccine-specific CTL response in mice upon tattoo immunization when using naked DNA [3], we show here that a significant increase in transfection efficiency does not consequently result in an enhanced immune response for the tested plasmid formulations used in this study. As the murine skin has different properties (e.g. a higher density of hair follicles and a different thickness) when compared to humans, it is difficult to connect the results of these vaccination models.

CONCLUSION

When formulated in L-histidine buffer, the transfection efficiency of PAA-polyplexes *in vitro* and in an *ex vivo* human skin model is enhanced compared to the same polyplexes dispersed in HEPES buffer. The better buffering capacity of L-histidine in the lower pH region of endosomal acidification, together with a higher concentration of cationic charge carriers (polymer and protonated histidine) is hypothesized to result in a more pronounced proton sponge effect and higher polymer-endosomal membrane interactions, giving rise to increased efficiency of endosomal escape and thus transfection.

Although the *in vivo* antigen expression of the polyplexes in L-histidine buffer is enhanced, *in vivo* immunogenicity is not improved. It may be that the murine model, for instance because of significant differences in skin structure, is not suited as a read-out model for the tattoo-application. Also, the assumption that a high transfection efficiency automatically will translate in a high immune response likely is too simple and dependent on other factors. In conclusion, the results presented in this study show that an L-histidine buffer has a distinct additive effect in the transfection activity and therefore is a promising excipient in the pharmaceutical formulation of PAA-PEG polyplexes for intradermal tattoo vaccination.

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Chapter 3.3

Lyophilization of plasmid DNA-poly(amido amine)s polyplex formulations

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ABSTRACT

In this study, we investigated if lyophilization can preserve our PEGylated poly(amido amine)s PAA-polyplexes. Therefore, polyplexes formulated with potential lyoprotectants (trehalose, sucrose or HPßCD) and with or without L-histidine-buffer were investigated for physico-chemical characteristics (appearance, size, PDI and charge) as well as transfection efficiency before lyophilization and after reconstitution of the freeze-dried products. After initial testing, sucrose was selected as lyoprotectant and the development of this formulation was further pursued.

Characteristics of the PAA-PEG polyplexes before and after lyophilization were different with respect to size, PDI, and charge. Before drying, polyplexes formulated in 10 mM L-histidine buffer pH 6.0 with 10 % sucrose had the largest size and charge. After lyophilization, all formulations increased in size and PDI. Only the polyplexes lyophilized in 10% sucrose showed an increased charge after freeze-drying. TEM measurements indicate that the sucrose formulation reconstituted with 10 mM L-histidine buffer pH 6.0 displays a significantly higher level of organization with the presence of particle-like structures sized < 100 nm.

In this study we showed that lyophilization induces differences in physico-chemical characteristics (appearance, size, PDI and charge) of polyplex formulations before freeze-drying and after reconstitution of the lyophilized product. As the polyplex formulations, analyzed by TEM, all appear highly disorganized before and after freeze-drying these characteristics may be of less importance. PEGylated PAA-polyplexes formulated in sucrose and reconstituted with L-histidine buffer, however, show a more organized, particle-like formulation after reconstitution of the lyophilized product. Indeed, this formulation shows preserved transfection efficiency in an *ex vivo* human skin model, probably related to a charge increase, related to more protonated polymers.

INTRODUCTION

Non-viral carrier delivery systems are widely used as transfection reagents to deliver nucleic acids for both *in vitro* and *in vivo* applications. In these systems, negatively charged DNA is bound by electrostatic interaction to an excess of a positively charged carrier. In this complexation process, DNA is condensed into positively charged, nanosized particles and protected from nuclease degradation, resulting in substantially higher transfection efficiencies compared to naked nucleic acids in *in vitro* assays [1]. Besides cationic lipids, cationic polymers are frequently used carriers to enhance transfection, and the resulting DNA nanoparticles are referred to as polyplexes [1–4]. DNA vaccination is an appealing method to obtain an antigen-specific immune response, e.g. to be applied in anticancer therapy [5]. When delivered by intradermal tattooing, plasmid DNA (pDNA) vaccination results in a strong and rapid antigen specific cellular response in both mice and non-human primates [6]. In spite of the high immunogenicity of pDNA tattoo, the in vivo transfection efficiency of naked pDNA with this technique is extremely low, with approximately 1 out of $5x10^6$ to $5x10^9$ plasmid copies applied being taken up, transcribed and translated [7]. We have shown that this transfection efficiency is significantly increased when formulating pDNA into PEGylated poly(amidoamine)s (PAA)-polyplexes [8]. Moreover, when formulated in an aqueous histidine-buffer instead of the commonly applied HEPES buffer, we found an additional enhanced transfection effect in *in vitro*, *ex vivo* and *in vivo* models [9].

Stability of these polyplexes in aqueous solutions, however, is very low [10]. They need to be freshly prepared prior to administration in order to prevent quality loss due to particle aggregation in liquid formulations resulting in decrease in transfection efficiency [11–14]. As this limited shelf-life is a major limiting factor for clinical applicability, a stable pharmaceutical formulation is warranted. Freeze-drying is a widely used method in order to preserve small molecules as well as biologicals and to extend their shelf-life [12,14–16]. As for these formulations, during the lyophilization process polyplexes are exposed to subsequent freezing and drying steps that can be detrimental to the quality of the product, unless appropriate stabilizers are used [10,17]. Several authors have identified appropriate lyoprotectants like 10% sucrose [12,18], 14% lactosucrose and a mixture of 10% HPßCD and 6.5% sucrose [14], a combination of Dextran 3000 and sucrose [19] and trehalose, mannitol and sucrose [3]. Previously, we reported on the long-term stability of a lyophilized naked pDNA formulation, also making use of 10% sucrose as lyo- and cryoprotectant. The choice of stabilizer is critical but also the mass ratio stabilizer/nanoparticle is important. Our formulation requires a relatively high pDNA concentration of 0,5 mg/mL for *ex vivo* transfection efficiency testing and because of tonicity of the parenteral formulation we want to keep our lyoprotectant concentration around 10%, resulting in a relatively low pDNA/lyoprotectant ratio of 200. Allison et al. also found a lower stabilizing sucrose/pDNA ratio of 500 [16]. Talsma et al

showed that a sucrose/pDNA ration of 10.000 was sufficient to protect transferring-PEI complexes [12]. Kasper et al. found that 20% sucrose was necessary to stabilize pDNA/LPEI polyplexes, corresponding to a sucrose/pDNA weight ratio of 4000 [20]. Successful ratios of 7500 for PEI-based polyplexes [21] and 1250 for DMAEMA-based polyplexes [15] also have been reported. In this study, we investigated if lyophilization can preserve our PEGylated PAA-polyplexes. Therefore, polyplexes formulated with potential lyoprotectants (trehalose, sucrose or HPßCD) and with or without L-histidine-buffer were assessed for physico-chemical characteristics (appearance, size, PDI and charge) as well as transfection efficiency before lyophilization and after reconstitution of the freeze-dried products.

MATERIALS AND METHODS

Materials

The pVAX:LucNP plasmid [6] encodes the influenza A $NP_{366-374}$ epitope as a genetic fusion with firefly luciferase gene, inserted in the EcoRI/NotI site of minimal pVAX1 plasmid backbone (Invitrogen, Carlsbad, USA). The plasmid was expressed and amplified in *E.coli* DH5 and was purified with the Endofree™ QIAGEN® Mega-kit (QIAGEN®, Hilden, Germany). PEGylated poly(amido amine) (PAA) copolymers possessing protonable amino groups and bioreducible linkages in the main chain and hydroxybutyl groups in the side chains (CBA-ABOL), were synthesized by Michael addition polymerization of N,N'-cystaminebisacrylamide with the appropriate amine according to the procedure described previously [22]. The PAA-polymers were PEGylated using 11 mol % of MeO-PEG-NH_2 (PEG_{2250}) in the total amino monomer feed during the PAA synthesis. L-Histidine was purchased from VWR (Amsterdam, the Netherlands). All other chemicals were of analytical grade. Furthermore, the solutions contained either 0% or 10% of trehalose (Merck, Darmstadt, Germany), sucrose (BUFA, Uitgeest, The Netherlands), HPßCD (Roquette Pharma, Lestrem, France) in sterile water for injections (WFI) (B.Braun, Melsungen, Germany) or 10mM L-histidine buffer pH = 6.0.

Polyplex preparation

Polyplexes with a final DNA concentration of 0.5 mg/mL were prepared by pipette-mixing an equal volume of dissolved PEGylated PAA-polymer and the pVAX:LucNP plasmid in a molar ratio of nitrogens within the polymer to phosphates in the plasmid DNA (N/P) of 25, this identified previously as the most optimal formulation in terms of transfection efficiency [9]. Formulation solutions with either 0% or 10% of trehalose, sucrose, HPßCD in sterile water for injections (WFI) or 10mM L-histidine buffer pH 6.0. As a control, naked DNA was diluted to the same concentration in 10mM L-histidine buffer pH 6.0.

Freeze-drying of the polyplexes

200 µL aliquots of the formulation solution were filled into 1.8 mL Type 1, Class A, borosilicate glass HPLC autosampler vials (VWR, Amsterdam, the Netherlands). Vials were loaded into the freeze dryer (Model Lyovac GT 4 freeze-dryer, GEA lyophil GmBH, Hürth, Germany). The lyophilization program was based on literature [23]. Vials were frozen to -35°C at 0.5°C/min in two hours. The shelf temperature of -35°C was maintained for 24 hours during the primary drying phase, while a vacuum of 10 Pa was established. At the end of primary drying the temperature was linearly increased to 0°C in 2 hours while the pressure was reduced to 0.9 Pa, to start secondary drying. These conditions were maintained for another 48 hours after which the vials were removed from the freeze-dryer, manually stoppered with 11 mm aluminum PTFE seal finishes (VWR, Amsterdam, the Netherlands) and stored at -20°C prior to characterization and further testing.

Polyplex characterization

The size of obtained particles was measured in 10mM L-histidine pH 6.0 buffer or WFI (with or without 10% sucrose) with dynamic laser scattering using an ALV/GCS-3 (Malvern Instruments, Malvern, UK). Particle size distribution is described using the polydisperity index (PDI), ranging from 0 for a monodisperse to 1 for a heterodisperse preparation. The zeta potential of obtained particles was determined in 10mM L-histidine pH 6,0 buffer or WFI, using a Zetasizer Nano Z (Malvern Instruments, Malvern, UK). Both instruments were calibrated using polystyrene latex beads of defined size and electrophoretic mobility. The presence of unbound pDNA was visualized by electrophoresis at 70V using a 1% agarose gel containing 0.5 µg/mL etidium bromide. Upon loading into the gel, pDNA was released from the polyplexes by adding 60 µL Heparine 5.000 I.E./ml (LEO Pharma, Ballerup, Denmark) to 10 µL polyplex solution [24].

DNA tattooing of ex vivo human skin

Formulations and naked DNA controls encoding firefly luciferase were administered to intact skin by DNA tattooing [6] to allow luciferase expression measurements. The skin model used in these experiments has been described previously for the optimization of tattooing of naked DNA in skin [7]. In brief, 10 µL of the indicated formulation at a final DNA concentration of 0.5 mg/mL was applied to the skin into a custom fabricated mould to keep the area of tattooing constant (diameter 8 mm, surface 50 mm^2). The formulation was subsequently administered into the skin using a Permanent Make Up (PMU)® tattoo machine (kindly provided by MT.DERM GmbH, Berlin, Germany) (Fig 1A). For all tattoos, 9-needle cartridges at an oscillating frequency of 100Hz were used. The needle depth was adjusted to 1.5 mm and tattoo duration was 20s. After tattooing, skin samples were kept at 5% CO_2, 37°C in complete keratinocyte serum free medium (SFM) containing 1% penicillin/streptomycin and 0.25µg/

mL amphotericin B (all Invitrogen, Carlsbad, USA) to allow expression measurements of luciferase. During this incubation, skin was cultured at the air–medium interface with the epidermis exposed to the air to mimic the natural situation.

Measurement of antigen expression using intravital imaging of ex vivo human skin

The expression of luciferase was measured in intact skin samples approximately 17 h after tattooing to obtain maximal antigen levels[7]. The substrate luciferin (Xenogen, Hopkinton, USA) was added to the medium in a final concentration of 45 µg/ml. During this procedure extra medium was added to the box in which skin was incubated, to cover the complete epidermis of skin samples with fluid to guarantee full accessibility of luciferin to the tattooed areas. 30 min after the addition of the substrate, luminescence produced by active luciferase was acquired during 30 s with an IVIS Lumina II CCD camera (Xenogen, Hopkinton, USA). Signal intensity was quantified as the sum of all detected light within the tattoo area of interest. In all measurements, background luminescence was determined for non-treated skin to allow correction during data analysis (Fig 1B).

Transmission electron microscopy (TEM)

The size and shape of the polyplexes were visualized using TEM. To this end, samples were applied on Agar® formvar/carbon coated copper grids (van Loenen instruments, Zaandam, The Netherlands). The samples were negatively stained by uranyl acetate and dried on air. The samples were visualized under a Tecnai12 transmission electron microscope (Philips, Eindhoven, The Netherlands) using a GATAN 626 cryoholder (Gatan GmbH, München, Germany). Samples were observed at 120 kV. Images were recorded on TemCam-0124 camera (TVIPS GmBH, Gauting, Germany) and processed with AnalySIS software. The magnification ranged from 30,000 to 265,000 times.

Statistical analysis

A Welch Two Sample t-test (one-way anova) was used for statistical analysis with the aid of 'R' software. Data are represented as mean ± standard deviation. P values < 0.05 were considered statistically significant.

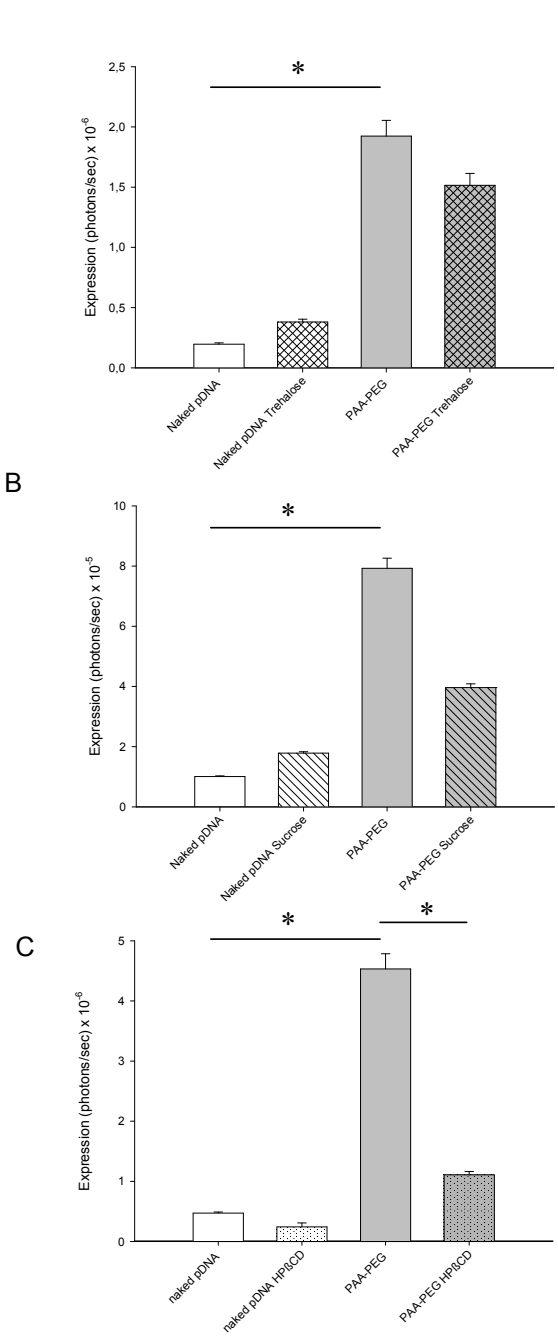

Figure 1: *Luciferase expression of PAA-PEG polyplexes in 10mM L-histidine buffer compared to naked DNA controls with 10% (w/v)* ***(A)*** *trehalose;* ***(B)*** *sucrose;* ***(C)*** *HPβCD. N=5 tattoos/formulation * means significantly different ($p < 0.05$)*

RESULTS

Buffer composition and luciferase expression with addition of lyoprotectants

Previously, we showed that PEGylation of the PAA-polymers is necessary to restore transfection efficiency of polyplexes in *ex vivo* human skin. To overcome the negative influence of the presence of the extracellular matrix (ECM) in skin tissue, shielding the cationic surface charge was required to prevent electrostatic interactions with the anionic ECM components and consequent immobilization of these polyplexes in the ECM [8].

In addition, we reported that luciferase expression was significantly increased when formulating PAA-PEG polyplexes in a 10 mM L-histidine buffer pH 6.0 as compared to naked pDNA (4.4 fold $p = 0.04$) as well as compared to PAA-PEG polyplexes formulated in standard HEPES buffer (1.8 fold, $p = 0.007$) [9]. Therefore, 10 mM L-histidine buffer pH 6.0 was selected as buffering system in the lyophilized product formulation or as reconstitution vehicle of the lyophilized product.

To assess the effect of excipients on antigen expression, solutions of PAA-PEG polyplexes in 10 mM L-histidine buffer pH 6.0 containing 10 % (w/v) of one of the lyoprotectants (trehalose, sucrose and HPßCD) were applied to *ex vivo* human skin. In line with earlier results with a naked DNA solution, absence of lyoprotectants resulted in higher antigen expression (Figure 1). In our experiment, trehalose and sucrose both show decreased transfection efficiency, but this was not found statistically different. HPßCD, however, showed a 4 fold significant decrease ($p = 0.01$) in transfection efficiency. In addition, different amounts (100, 200, 300 and 400 µl) of placebo solutions containing 10mM L-histidine buffer pH 6.0 without lyoprotectant, or with trehalose, sucrose or HPßCD, all 10 % (w/v) were freeze-dried in order to investigate cake formation at low volumes. A uniform, intact cake matrix was obtained after freeze-drying at all volumes for the sucrose solutions, in contrast to the two other excipients which showed less attractive appearance or even collapse. As expected, L-histidine alone was not able to form a cake without support of a lyoprotectant/bulking agent. Given the results above and the experience gained on the stability of freeze-dried pDNA when formulated into a sucrose solution [25], development of this formulation was further pursued.

Characterization of the polyplexes

Table 1 gives the polyplex characteristics before freeze-drying and after reconstitution of the different lyophilized products as determined using DLS and zeta sizing. As can be seen, characteristics of the polyplexes before and after lyophilization were different with respect to size, PDI, and charge. Before drying, polyplexes formulated in 10mM L-histidine buffer pH 6.0 with 10% sucrose were largest in size and had the highest charge. For reference, also the characteristics of PAA-PEG polyplexes formulated in only 10 mM L-histidine buffer pH 6.0 are given, displaying the smallest size and lowest charge but comparable PDI.

Table 1. Effect of lyophilization on the size, PDI and charge of the PAA-polyplexes. A-F corresponds with Fig. 4 and 6. NA= not applicable

Formulation composition		**Reconstitution medium**		**Mean size**		**PDI**		**Charge**	
				Before freeze-drying	After freeze-drying	Before freeze-drying	After freeze-drying	Before freeze-drying	After freeze-drying
10% sucrose + 10mM L-histidine	A	WFI	D	164 ± 34	189,8 ± 12,4	0,491 ± 0,02	0,775 ± 0,182	15,6 ± 0,29	14,5 ± 0,45
10% sucrose	B	WFI	E	126,9 ± 8,9	221,8 ± 42	0,473 ± 0,05	0,700 ± 0,02	8,59 ± 0,33	14,4 ± 0,45
		10 mM L-histidine	F	126,9 ± 8,9	159,0 ± 8,15	0,473 ± 0,05	0,733 ± 0,09	8,59 ± 0,33	17,47 ± 0,55
10 mM L-histidine	C	NA		106 ± 3,48	NA	0,461 ± 0,05	NA	7,73 ± 0,08	NA

Upon rehydration, reconstitution for all freeze-dried formulations was fast, complete, and leaving no visible residue as undissolved matter and resulting in a clear colorless solution. All formulations showed an increased PDI, indicating a more polydisperse solution after freeze-drying. Furthermore, all freeze-dried formulations showed an increase in size upon reconstitution (A vs. D and B vs. E, F). For the freeze-dried product only containing sucrose this increase in size, however, was dependent on the reconstitution medium used, with a significantly smaller size-increase when using 10 mM L-histidine buffer pH 6.0 as compared to WFI. The charge of the freeze-dried product containing 10 mM L-histidine buffer 10% sucrose upon reconstitution (D) was comparable with the solution before freeze-drying (A). This in contrast to the formulation just containing sucrose which showed a significant increase in charge upon reconstitution with the largest increase using 10 mM L-histidine buffer (Table 1).

Upon visual inspection, freeze dried polyplex formulations showed no differences in structures and were all white, stable cakes without collapse. Looking microscopically instead of macroscopically, however, marked differences could be observed for the various formulations before lyophilization and after reconstitution of the freeze-dried products. Fig. 2 shows showed typical TEM-images of all polyplex formulations. Most striking is that all formulations, to a more or lesser extent, show the presence of free pDNA, surrounded by particle-like 'dots'. Apart from the lyophilized product only containing sucrose and reconstituted with 10mM L-histidine buffer pH 6.0, no marked differences can be observed before lyophilization and after reconstitution for the various formulations which all seem highly disorganized (A vs. D, B vs. E). When compared to the TEM analysis of naked pDNA (G), however, this disorganization level is just relative. It appears that PAA-PEG forms a sort of matrix around the pDNA molecules (G vs. A-C), which is preserved upon lyophilization and subsequent reconstitution. Moreover, the sucrose formulation reconstituted with 10mM L-histidine buffer pH 6.0 displays a significantly higher level of organization with the presence of particle-like structures sized < 100 nm. The addition of heparin shows the release of the pDNA cargo of the polyplexes during AGE analysis. In case of degradation, a pDNA 'smear' or multiple bands would be visible. As this is not the case for any of the formulations, AGE analysis shows no structural differences in pDNA before and after freeze drying when released from the particles after heparin treatment (Fig. 3). Indeed, heparin seems to have an effect on the distribution of the pDNA over the gel, as the SC band looks much lighter when compared to the untreated naked DNA, used for the preparation of the polyplexes. As this effect, however, is also visible for naked DNA with heparin, this is not considered an effect of the freeze-drying.

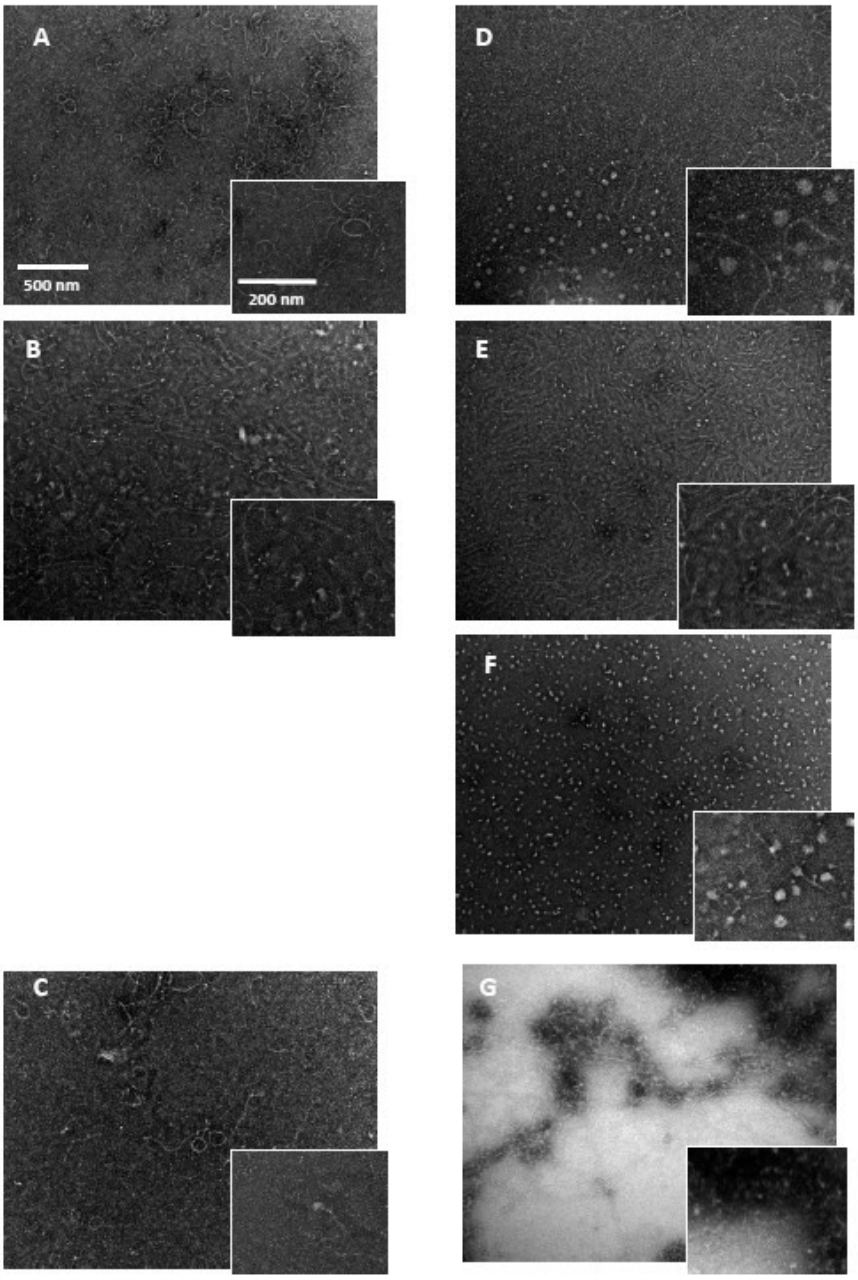

Figure 2: *TEM measurements before (A, B, C) and after lyophilization (D,E, F). (A) PAA-polyplexes 10% sucrose + 10mM L-histidine; (B) PAA-polyplexes in 10% sucrose. (C) PAA-polyplexes 10% sucrose + 10mM L-histidine (D) PAA-polyplexes in 10% sucrose and 10mM L-histidine, reconstituted with WFI (E) PAA-polyplexes in 10% sucrose reconstituted with WFI (F) PAA-polyplexes with 10% sucrose, reconstituted with 10mM L-histidine (G) naked pDNA in 10mM L-histidine*

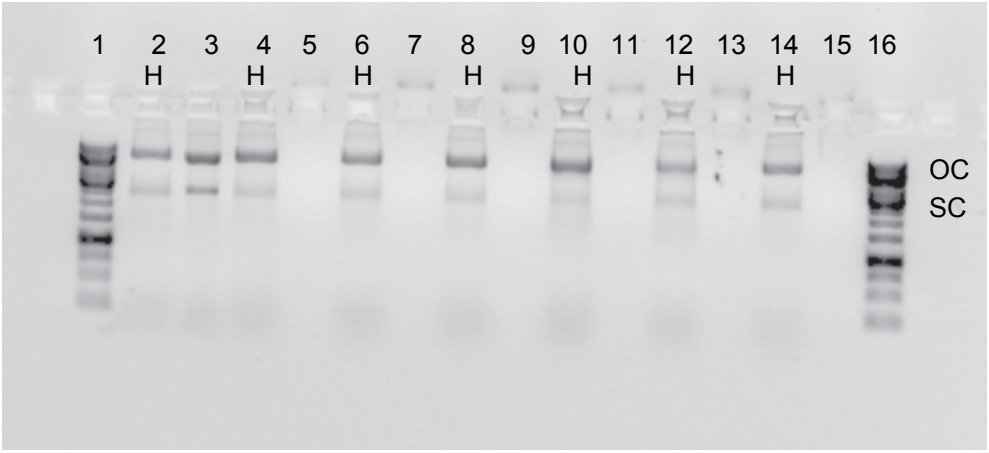

Figure 3: *Agarose gel electrophoresis of naked DNA and PAA-PEG polyplexes in the presence of heparin (H). Lane 1 1Kb ladder, lane 2,3 naked pDNA (G); lane 4,5 PAA-polyplexes in 10mM L-histidine (C); lane 6,7 PAA-polyplexes in 10% sucrose (B); lane 8,9 PAA-polyplexes 10% sucrose + 10mM l L-histidine (A); lane 10,11 PAA-polyplexes 10% sucrose reconstituted with 10mM L-histidine (F); lane 12,13 PAA-polyplexes 10% sucrose reconstituted with WFI (E); lane 14, 15, PAA-polyplexes 10% sucrose + 10mM L-histidine (D); lane 17 1Kb ladder.*

Transfection efficiency in ex vivo human skin

Before freeze-drying all PAA-polyplex formulations showed a significant *ex vivo* transfection efficiency increase when compared to the naked pDNA solution (A-C vs. G; $p < 0.05$; Figure 4). After reconstitution of the freeze-dried samples, however, the *ex vivo* transfection efficiencies were found to be different. All freeze-dried formulations showed a significant *ex vivo* transfection efficiency increase when compared to the naked pDNA solution (D-F vs. G; $p < 0.05$). The PAA-PEG polyplex formulation with 10 % sucrose in WFI showed a similar transfection efficiency after freeze-drying. Interestingly, when reconstituted with L-histidine the formulation freeze-dried with 10% sucrose in WFI transfection efficiency was significantly increased when compared to the other freeze-dried formulations ($p < 0.05$).

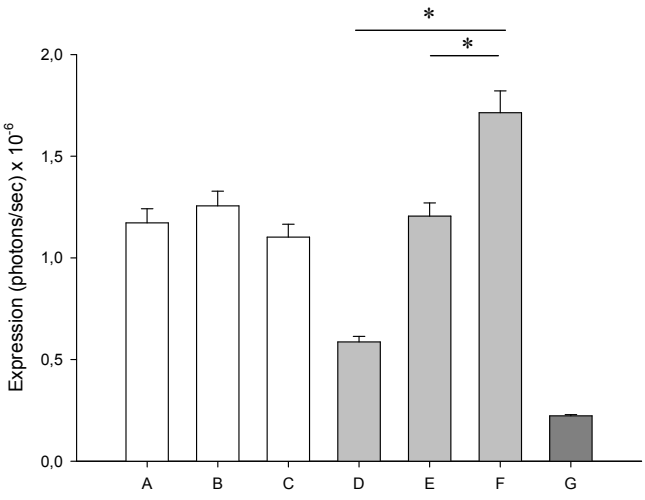

Figure 4: *Luciferase expression of PAA-PEG polyplexes compared to naked DNA controls before (A, B, C) and after (D, E, F) lyophilization and naked pDNA (G). (A) PAA-polyplexes 10% sucrose + 10mM L-histidine; (B) PAA-polyplexes in 10% sucrose in WFI. (C) PAA-polyplexes 10mM L-histidine (D) PAA-polyplexes in 10% sucrose in 10mM L-histidine (E) PAA-polyplexes in 10% sucrose in WFI (F) PAA-polyplexes 10% sucrose + 10mM L-histidine (G) naked pDNA in 10mM L-histidine. N=5 tattoos/ formulation. * means statistically different ($p < 0.05$)*

DISCUSSION

During lyophilization, the stability of polyplexes can be drastically affected. Without presence of stabilizers, the size of the polyplex complex size can be severely increased [14,18,26,28]. This increase demonstrates the necessity of using lyoprotectants to inhibit freezing-induced aggregation e.g. as a result of increased salt concentrations which reduces the hydrate layer around the particles. It has been reported with X-ray diffraction, infrared and ultraviolet spectroscopy that hydration water is necessary for maintaining the structural integrity of the pDNA molecule in the polyplex [26,27]. We chose to use a 10 mM L-histidine buffer pH 6.0 as it is an attractive lyophilization buffer because of its pKa of 6.1, close to the buffers' pH thereby providing a high buffer capacity at low buffer concentrations (10 mM), and its low ionic strength [14]. As we know that lyoprotectants or bulking agents influence the transfection efficiency *ex vivo* [26], we first investigated the effect on transfection efficiency before freeze drying. HPßCD had the most dramatic effect on transfection efficiency, probably as a result of a complexation interaction. Trehalose and sucrose, however, had a non-significant decrease on transfection efficiency. Yet, as we have to freeze dry in very low volumes, we noticed that the freeze dried cake of sucrose appeared most stable and attractive.

As sucrose also has been shown to be very effective as a lyoprotector [25] we chose to continue with this lyoprotectant. Polyplex characterization before and after freeze-drying showed an important change in particle size. Interestingly, we noticed an increased particle size for all formulations after freeze-drying, but the reconstitution medium had a great influence on the particle size. When reconstituted with L-histidine instead of WFI, polyplexes in L-histidine were much smaller. These formulations were both freeze-dried in WFI + 10 % sucrose, so L-histidine reconstitution influences particle size, and this decrease is not an effect of the freeze-drying, but of the L-histidine instead. However, the increased PDIs after reconstitution are indicative for a lyophilization-induced heterogeneity, as the solution is more polydisperse than before freeze drying. Interestingly, these changes in polyplex characteristics were not reflected by the transfection efficiency of the freeze dried polyplexes. Only the transfection efficiency of polyplexes in 10 mM L-histidine buffer pH 6.0 was decreased after freeze drying. The polyplexes freeze-dried in 10 % sucrose and reconstituted with 10 mM L-histidine buffer pH 6.0 even showed an significant increase of transfection efficiency, therefore acting as the best formulation (F). Likely, this can be explained by the physico-chemical properties of the polyplexes which show that these particles are the smallest and of highest charge. In addition, the polyplexes freeze-dried in 10% sucrose and reconstituted with 10 mM L-histidine buffer pH 6.0 appear more like particles as visualized by TEM analysis and compared to polyplexes freeze-dried in 10% sucrose and reconstituted with WFI. Apparently, the addition of an L-histidine buffer to the freeze-dried polyplexes instead of building it into the lyophilized formulation results in increased transfection efficiency, probably because of the better buffering capacity of L-histidine in the lower pH region of endosomal acidification, together with a higher concentration of cationic charge carriers (polymer and protonated histidine) is hypothesized to result in a more pronounced proton sponge effect and higher polymer-endosomal membrane interactions, giving rise to increased efficiency of endosomal escape and thus transfection.

CONCLUSION

In this study we showed that lyophilization induces differences in physico-chemical characteristics (appearance, size, PDI and charge) of polyplex formulations before freeze-drying and after reconstitution of the lyophilized product. As the polyplex formulations, analyzed by TEM, all appear highly disorganized before and after freeze-drying these characteristics may be of less importance. PEGylated PAA-polyplexes formulated in sucrose and reconstituted with L-histidine buffer, however, show a more organized, particle-like formulation after reconstitution of the lyophilized product. Indeed, this formulation shows preserved transfection efficiency in an *ex vivo* human skin model, probably related to a

charge increase, related to more protonated polymers. Long term stability testing of 50 mg/ml PEGylated PAA-polyplexes 11 mol % $mPEG_{2250}$ with 0.5 mg/ml pDNA in 10 % sucrose is initiated.

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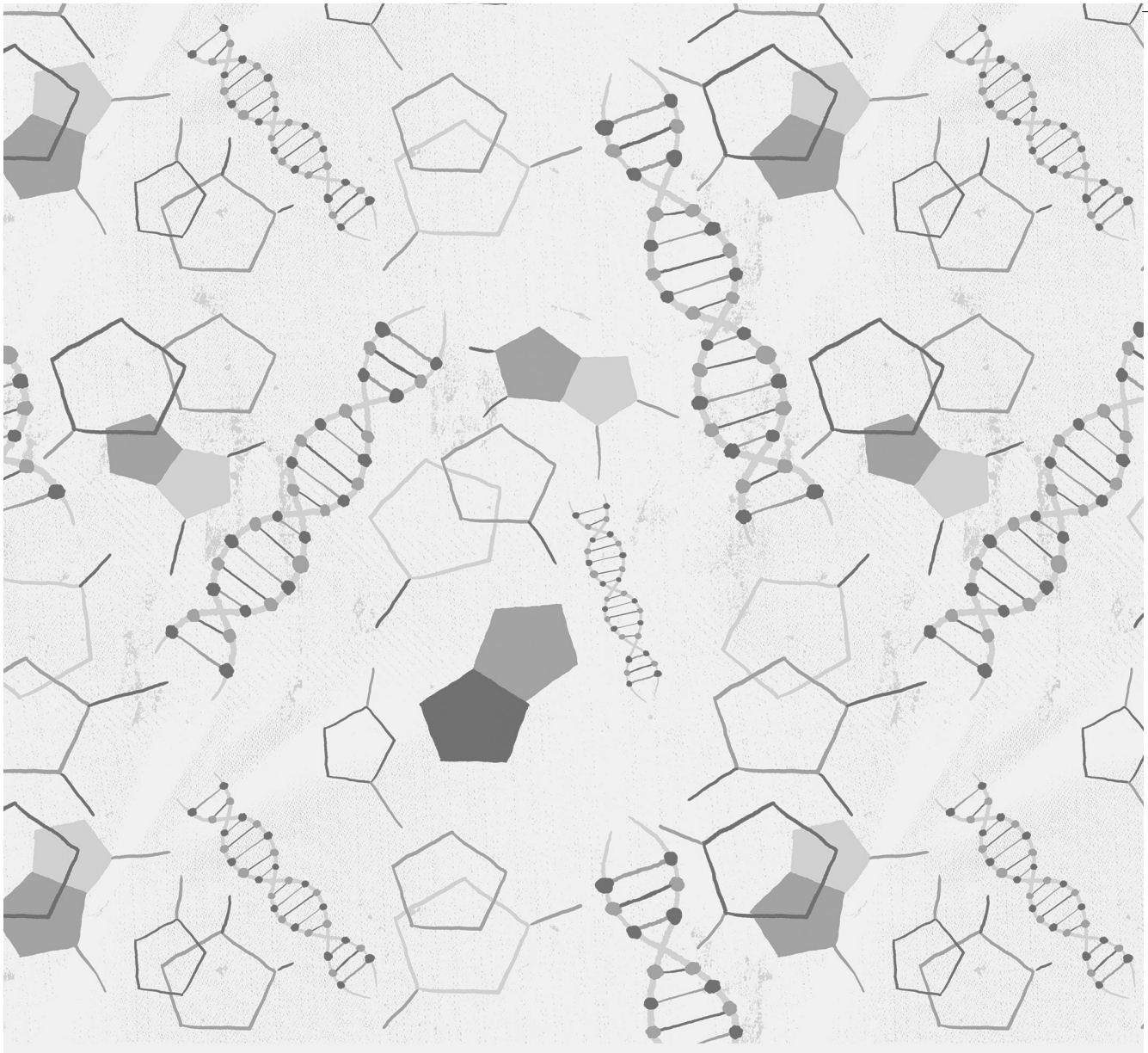


Chapter 4

Summary and perspectives

SUMMARY AND PERSPECTIVES

The aim of this thesis was the development of a Good Manufacturing Practice (GMP) compliant plasmid DNA production process, as well as the development of a pharmaceutical formulation of pDNA vaccines for the treatment of Human papilloma viruses (HPV) 16 induced malignancies. Furthermore, this thesis focuses on the development of pDNA-polyplex formulations to further improve the transfection efficiency and immunogenicity of intradermally delivered DNA vaccines.

Chapter 1 gives an introduction into the work that has been performed on the pharmaceutical development of anticancer plasmid DNA vaccines. Non-viral gene therapy may require considerable amounts (milligram scale) of pharmaceutical grade pDNA per patient, since the efficacy and duration of gene expression is presently relatively low. Most production processes are developed for use at laboratory scale, therefore scaling up can be difficult. We provide an overview of the most important steps during the production process. To increase the shelf life and to obtain highly concentrated solutions for administration, the pDNA can be lyophilized using disaccharides (in combination with polysaccharides) to protect the DNA during lyophilization and storage. Also, clinical pDNA anticancer clinical trials are reviewed.

Chapter 2.1 describes the production process of the pDNA vaccine pVAX TTFC E7SH. A Good Manufacturing Practice (GMP)-compliant plasmid manufacturing process was set up with fed-batch upstream processing, anion exchange membrane downstream processing and a pharmaceutical dosage form was produced. We manufactured approximately 825 mg plasmid DNA of a high purity >90% supercoiled DNA, an $A_{260/280}$ ratio 1.80-1.95, undetectable or low residual endotoxins, *Escherichia coli* host cell protein, RNA, and DNA. After sterile filtration, the concentration of the plasmid solution was approximately 1.0 mg/mL. For the scheduled phase I study a concentration of 5 mg/mL is desired, and further concentration of the solution was achieved by lyophilization. Upon reconstitution of the lyophilized material with an isotonic sucrose solution, a solution of 5 mg/mL TTFC-E7SH could be made. Lyophilized TTFC-E7SH was sterile with > 90% supercoiled DNA, an $A_{260-280}$ ratio 1.80-1.95, content 90-110% of labeled, and residual water content <2% (w/w) and stable for at least three months at 25°C. Upon restriction-enzyme digestion, the product yielded the predicted profile. Immunogenicity was confirmed in an *in vivo* mouse model.
In particular, the downstream processing can be further optimized to more efficiently release the pDNA from the capsule in this step. First, the lysis can be improved by investigating the introduction of an in-line lysis method, thereby decreasing the time the pDNA is exposed to high pH for a shorter time and thus decreasing pDNA degradation during this step. Second, the elution phase of the anion exchange chromatography can be improved by investigating

different elution buffers and flow rates. As we are able to bind most of the pDNA present in the lysate, optimization of elution buffer(s) may further improve the elution yield of the pDNA from the membrane.

In **chapter 2.2** of this thesis we point out the importance of a careful selection of host cell-vector combination in the production of clinical pDNA. We report an unexpected contamination of pDNA during clinical manufacture with a transposon originating from the *E.coli* DH5 host cell genome. During processing, presence of this transposable element, insertion sequence 2 (IS2) in the plasmid vector was not noticed until quality control of the bulk pDNA vaccine when results of restriction digestion, sequencing, and CGE analysis were clearly indicative for the presence of a contaminant. Due to the very low level of contamination, only an insert-specific PCR method was capable of tracing back the presence of the transposon in the source pDNA and master cell bank (MCB). Based on the presence of an uncontrolled contamination with unknown clinical relevance, the product was rejected for clinical use. In order to prevent costly rejection of clinical material, both in-process controls and quality control methods must be sensitive enough to detect such a contamination as early as possible, i.e. preferably during plasmid DNA source generation, MCB production and ultimately during upstream processing. However, as we have shown that contamination early in the process development pipeline (source pDNA, MCB) can be present below limits of detection of generally applied analytical methods, the introduction of 'engineered' or transposon-free host cells seems the only 100% effective solution to avoid contamination with movable elements and should be considered when searching for a suitable host cell-vector combination.
In **chapter 2.3** we report on the shelf-life stability of pDERMATT (plasmid DNA encoding recombinant MART-1 and tetanus toxin fragment-c) 2 mg lyophilized powder for reconstitution for intradermal administration. pDERMATT was stored at 25°C/60% relative humidity (6 months), 2-8°C (24 months), and -20°C (66 months) in the dark and analyzed at several time points during the conduct of the clinical study for appearance, identity, purity (plasmid topology), content and residual water content. pDERMATT appeared stable at all storage conditions for the periods tested which, although patient inclusion in the study was significantly delayed, ensured the clinical supply needs. This study shows that lyophilization is an useful tool to preserve the quality of the pDNA and can prevent the need for costly and time-consuming additional manufacture of drug product in case of study delays, not uncommon at the early stage of drug development.

To determine if pDNA transfection can be further improved by formulating pDNA into polyplexes, we describe in **chapter 3.1** the screening of a broad panel of polymers with distinct differences in molecular structure and characteristics using an *ex vivo* human skin model as read-out.

We measured *ex vivo* human skin transfection efficiency and polymer characteristics (size, PDI, charge) for all polyplex formulations and conducted a follow-up experiment in which the best performing polymer was further investigated and tested. We found that only poly(amino amide)s polyplexes were successful in enhancing transfection efficiency of plasmid DNA when compared to the performance of the other polymers in the panel. Variations in PEG-chain MW and degree of PEGylation resulted in PAA 7.5 % $mPEG_{1100}$ polyplexes with highest size and with lowest transfection efficiency, therefore the least favorable polymer of the series. Also, for most polyplexes higher N/P ratios are resulting in increased transfection efficiencies. Next, PAA 15 % $mPEG_{1100}$, $mPEG_{2000}$ and $mPEG_{5000}$ polymers resulted in the highest transfection efficiencies of al sets (N/P 50). It can be concluded that PAA-PEG based polyplexes are the most promising candidates for improving pDNA transfection efficiency. Further optimization of these polyplexes indicates that PAA 11 % $MPEG_{2250}$ results in optimal transfection efficiency for *ex vivo* human skin DNA tattooing.

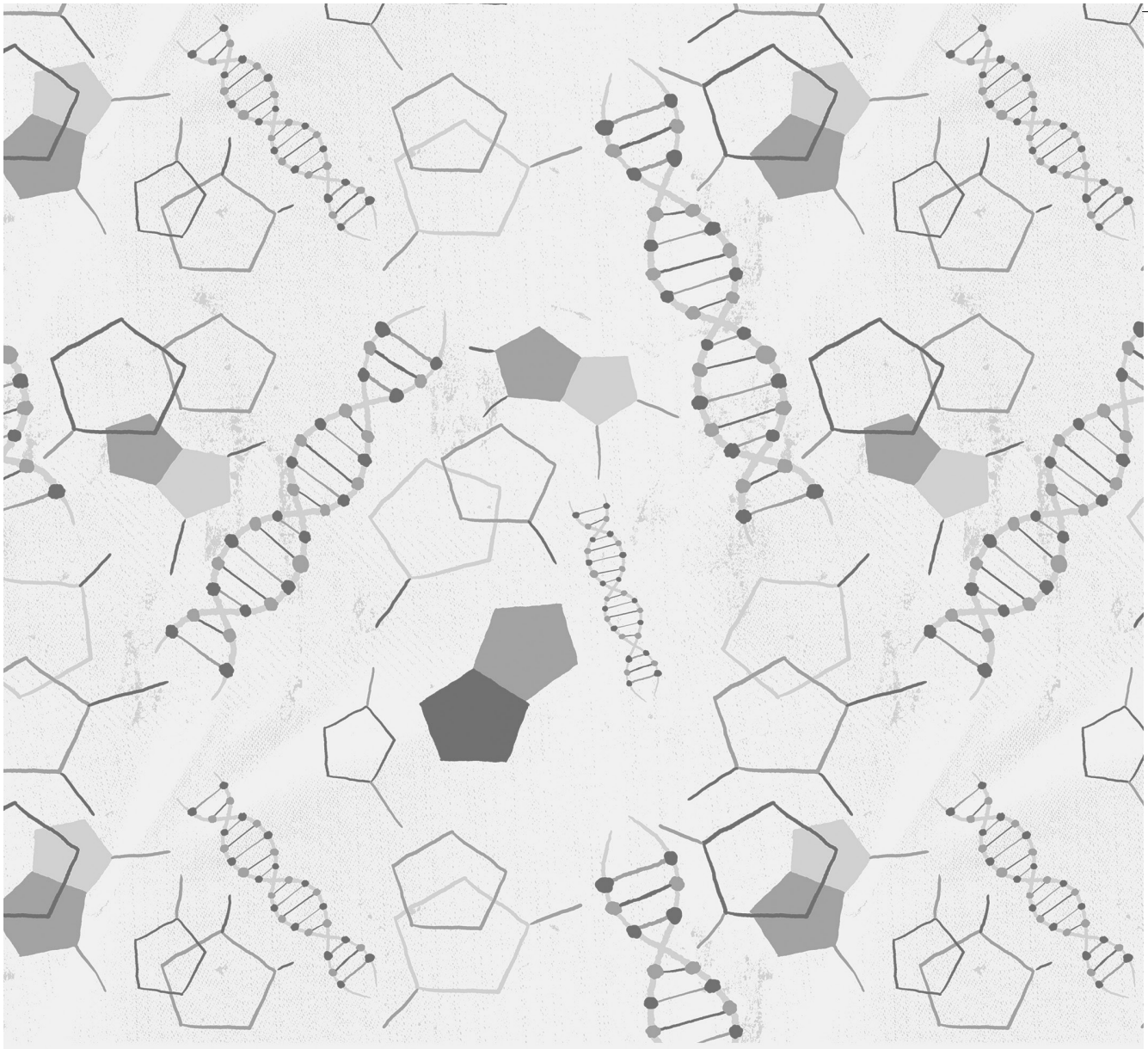
In order to translate this promising PAA-polyplex concept to the clinic, an optimal pharmaceutical formulation is warranted. In **chapter 3.2** we screened for the optimal polymer/pDNA ratio of the PAA-polyplexes, making use of an *in vitro* High Content Screening (HCS) platform. In addition, we investigated the use of an alternative buffer system for the PAA-polyplex formulation. We examined the applicability of an L-histidine buffer, an approved and commonly used excipient in parenteral formulations. PAA-polyplexes in different polymer/pDNA ratios in the different buffer systems were tested in the HCS platform. It was found that PAA-polyplexes at a N/P ratio of 25 formulated in L-histidine significantly improved the percentage of transfected cells in comparison to the HEPES formulation (from 9.9 ± 4.7 % to 17.0 ± 1.6 %, p=0.007) and the LPEI control (9.2 ± 2.7 % to 17.0 ± 1,9 % p = 0.03). Subsequently, promising polyplex formulations were PEGylated and tested in an *ex vivo* human skin model and *in vivo* by vaccination of mice with these formulations which allowed simultaneous measurement of antigen expression and T cell responses. When formulated in 10mM L-histidine buffer pH 6.0, the transfection efficiency of PAA-polyplexes *ex vivo* is 2-fold enhanced (p = 0.02). Additionally, as *in vivo* antigen expression of the polyplexes in L-histidine buffer is enhanced, *in vivo* immunogenicity is not improved.

The better buffering capacity of L-histidine in the lower pH region of endosomal acidification, together with a higher concentration of cationic charge carriers (polymer and protonated histidine) is hypothesized to result in a more pronounced proton sponge effect and higher polymer-endosomal membrane interactions, and/or different particle conformation giving rise to increased efficiency of endosomal escape and thus transfection. In conclusion, the results presented in this chapter show that a 10 mM L-histidine buffer pH 6.0 has an additive effect in the transfection activity and therefore is a promising excipient in the pharmaceutical formulation of PAA-PEG polyplexes for intradermal tattoo vaccination. In addition, it may be that the murine model, for instance because of significant differences in skin structure, is

not suited as a read-out model for the tattoo-application. Also, the assumption that a high transfection efficiency automatically will translate in a high immune response likely is too simple and dependent on other factors.

In **chapter 3.3** we investigated if lyophilization can preserve our PEGylated poly(amido amine) s PAA-polyplexes. Therefore, polyplexes formulated with potential lyoprotectants (trehalose, sucrose or HPßCD) and with or without L-histidine-buffer were investigated for physico-chemical characteristics (appearance, size, PDI and charge) as well as transfection efficiency before lyophilization and after reconstitution of the freeze-dried products. After initial testing, sucrose was selected as lyoprotectant and the development of this formulation was further pursued. Characteristics of the PAA-PEG polyplexes before and after lyophilization were different with respect to size, PDI, and charge. Before drying, polyplexes formulated in 10 mM L-histidine buffer pH 6.0 with 10 % sucrose had the largest size and charge. After lyophilization, all formulations increased in size and PDI. Only the polyplexes lyophilized in 10% sucrose showed an increased charge after freeze-drying. TEM measurements indicate that the sucrose formulation reconstituted with 10 mM L-histidine buffer pH 6.0 displays a significantly higher level of organization with the presence of particle-like structures sized < 100 nm. It is also shown that lyophilization induces differences in physico-chemical characteristics (appearance, size, PDI and charge) of polyplex formulations before freeze-drying and after reconstitution of the lyophilized product. As the polyplex formulations, analyzed by TEM, all appear highly disorganized before and after freeze-drying these characteristics may be of less importance. PEGylated PAA-polyplexes formulated in sucrose and reconstituted with L-histidine buffer, however, show a more organized, particle-like formulation after reconstitution of the lyophilized product. Indeed, this formulation shows preserved transfection efficiency in an *ex vivo* human skin model, probably related to a charge increase, related to more protonated polymers.

In conclusion, this thesis shows that optimizing the polyplex formulation can increase the performance of dermal DNA vaccines administered via DNA tattooing. In addition, we have developed a GMP compliant plasmid DNA production process of pDNA vaccines for the treatment of Human papilloma viruses (HPV) 16 induced malignancies. In order to translate the results of this thesis towards a clinical application, future experiments should focus on the limitations of our read-out models. The observation that increased transfection efficiency *ex vivo* and *in vivo* does not automatically lead to increased immunogenicity should be investigated. Additionally, to optimize polyplex formulation, the development of PAA-PEG polyplex formulations should be further pursued in combination with L-histidine based buffer systems.



Appendices

Samenvatting
Dankwoord
Curriculum vitae
List of publications

SAMENVATTING

Het promotieonderzoek 'Productie en farmaceutische formulering van plasmide DNA vaccins' kan opgedeeld worden in twee delen. Het eerste deel beschrijft de ontwikkeling van een productiemethode voor plasmide DNA voor klinisch onderzoek onder de vigerende richtlijnen (GMP). Het tweede deel van het proefschrift richt zich op de ontwikkeling van 'DNA polyplexen' bestaand uit plasmide DNA en polymeer met als oogmerk het optimaliseren van de effectiviteit (transfectie efficiëntie) van het pDNA.

In **hoofdstuk 1** wordt de huidige stand van zaken beschreven op het gebied van de farmaceutische productie en analyse van antikanker plasmide DNA vaccins. Tevens wordt een overzicht van uitgevoerde klinische studies bij kanker wordt gegeven. Voor de uitvoering van klinische studies zijn aanzienlijke hoeveelheden (mg-g) pDNA benodigd. De meeste productieprocessen zijn echter ontwikkeld voor veel kleinschaliger laboratoriumexperimenten en het opschalen van een dergelijk proces is een uitdaging, temeer omdat voldaan moet worden aan 'Goede Manier van Produceren' (GMP) richtlijnen. Er wordt ingegaan op de constructie en design van pDNA en op de verschillende processtappen (upstream en downstream) in de productie van pDNA. Dit hoofdstuk geeft verder een overzicht van de verschillende analysemethodes voor de kwaliteitscontrole van plasmide DNA en beschrijft op welke manier(en) plasmide DNA langdurig bewaard kan worden zonder dat het aan kwaliteit verliest.

Hoofdstuk 2.1 van dit proefschrift beschrijft een productieproces voor het DNA vaccin pVAX TTFC-E7SH, dat voldoet aan de GMP richtlijnen en gebruikt kan worden klinische studies. De ontwikkeling en resultaten van een zogenaamde 'fed-batch' productiemethode worden uiteengezet. Een hogere bacteriedichtheid in de cultuur en daaruit volgend hogere concentratie van plasmide DNA per productierun zijn voordelen van deze methode. Het ontwikkelde proces is reproduceerbaar en resulteert in een constante hoge kwaliteit, stabiliteit en opbrengst van het plasmide DNA. Het geproduceerde DNA vaccin pVAX TTFC-E7SH (coderend voor een niet functionele versie van E7, gecombineerd met een tetanus toxine fragment c) zal klinisch worden toegepast in een fase I studie als HPV 16 vaccinatie.

Hoofdstuk 2.2 beschrijft een onverwacht productieprobleem dat geïdentificeerd is als de introductie van een gastheer cel transposon in de vector van het plasmide DNA. Dat wil zeggen dat er een gedeelte van het genomisch DNA van de *E.coli* bacterie terecht gekomen is in de DNA sequentie van het plasmide DNA. Dat kan gevaarlijk zijn in het geval van humane toediening van plasmide DNA vanwege de mogelijke vorming van aberrante eiwitten. Minder waarschijnlijk, maar niet uit te sluiten is dat het transposon het humaan genoom zou kunnen contamineren. Tijdens het produceren kwam deze verontreiniging niet naar voren maar

tijdens kwaliteitscontrole kwam deze verontreiniging wel aan het licht. Tijdens dit onderzoek is de identiteit, herkomst en mate van verontreiniging onderzocht. Om deze contaminatie in de toekomst te voorkomen wordt het gebruik van genetisch gemodificeerde gastheercellen geadviseerd. Door het transposon uit het genoom van de bacterie te kloneren is contaminatie op deze wijze te voorkomen.

In **hoofdstuk 2.3** wordt de houdbaarheid van plasmide DNA beschreven als gevriesdroogd poeder dat gereconstitueerd dient te worden vlak voor intradermale toediening. Dit plasmide DNA vaccin is gebuikt in een fase I studie in ons instituut. Deze stabiliteitsstudie toont aan dat vriesdrogen van plasmide DNA een aantrekkelijke methode is om gedurende langere tijd de kwaliteit van het plasmide DNA te behouden. Aanvullende onvoordelige en langdurige producties zijn niet noodzakelijk in het geval van extensie van de studie, iets dat niet ongewoon is tijdens de eerste fases van geneesmiddelontwikkeling. Deze studie toont aan dat plasmide DNA meer dan 5 jaar stabiliteit behoudt opslag bij -20°C.

Om te bepalen of de effectiviteit van het plasmide DNA verhoogd kan worden door het pDNA te binden aan een polymeer, wordt in **hoofdstuk 3.1** de screening van een groot aantal verschillende polymeren beschreven. Deze polymeren met verscheidene molecuulstructuren en diverse eigenschappen waardoor onderzocht kan worden of deze specifieke eigenschappen invloed hebben op de verbetering van de transfectie efficiëntie. De meest optimale polymeer-pDNA combinatie is onderzocht en eigenschappen van de verschillende polyplexen zijn gekarakteriseerd door ze te analyseren op grootte, lading en mate van homogeniteit. Dit onderzoek resulteerde in de identificatie van poly(amido amine)s polymeren als ideale polyplexvormer. Met dit polyplex zijn vervolgexperimenten uitgevoerd om vast te stellen aan welke eigenschappen een polymeer moet voldoen om de werkzaamheid van het plasmide DNA te verbeteren.

Hoofdstuk 3.2 beschrijft de toepassing van een histidine buffer in de farmaceutische formulering van de poly(amido amine)s (PAA-PEG) polyplexen. Gebruik van deze buffer resulteerde in een verbeterde transfectie-efficientie met een verhoging van *in vitro* en *ex vivo* tranfectie effiecientie van 2-voud ten opzichte van 'naakt' pDNA. De verbeterde werking van de polyplexen in histidine zou verklaard kunnen worden doordat celopname van de polyplexen verhoogd is. De nucleus wordt efficiënter bereikt door de lagere pH van het polymeer en de buffer. Hoewel de *in vivo* antigen expressie is toegenomen, is de daaropvolgende immunogeniciteit niet verhoogd. Verschillen in muriene en humane huid zou een oorzaak kunnen zijn van dit verschil maar ook het verband tussen antigen expressie en immunogeniciteit zou verder opgehelderd moeten worden.

Hoofdstuk 3.3 beschrijft een vriesdroogproces van een farmaceutische formulering van PAA-PEG polyplexen. Om de stabiliteit van de polyplexen te vergroten wordt een vriesdroogmethode voor polyplexen onderzocht waarin van verschillende cryoprotectants (trehalose, sucrose en HPßCD) de stabiliserende eigenschappen getest worden. Fysisch-chemische eigenschappen, degradatie, transfectie efficiëntie en uiterlijke kenmerken van de gevriesdroogde formuleringen worden gekarakteriseerd voor en na vriesdrogen. Het vriesdrogen van farmaceutische formuleringen van polyplexen was succesvol met behoud van transfectie efficiëntie na reconstitutie.

Concluderend heeft het promotieonderzoek geleid tot de ontwikkeling van een efficiënt en reproduceerbaar GMP productieproces. Daarnaast zijn belangrijke stappen gezet in de ontwikkeling van een polyplex richting klinisch onderzoek.

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Iris

CURRICULUM VITAE

Iris van der Heijden is geboren op 16 januari 1985 in Tilburg. Na het behalen van haar gymnasiumdiploma in 2003 aan het Theresialyceum in Tilburg is zij begonnen aan de studie Farmacie aan de Universiteit Utrecht. In 2007 behaalde zij haar bachelordiploma. Tijdens haar masteropleiding volgde zij een wetenschappelijke stage aan de Universiteit van Californië, San Diego (UCSD), Faculteit Psychiatrie in La Jolla, CA in de Verenigde Staten. Hier deed zij psychofarmacologisch onderzoek naar de rol van de serotoninereceptor in de behandeling van schizofrenie onder begeleiding van prof. dr. M.A. Geyer en prof. dr. B. Olivier. Ook volgde zij een wetenschappelijke stage aan de Universiteit van Newcastle, departement klinische toxicologie in het Calvary Mater ziekenhuis in Newcastle, NSW, Australië. Hier ontwikkelde zij populatie farmacokinetische en farmacodynamische modellen na overdosering van amulsipride onder begeleiding van dr. G. Isbister. In 2009 behaalde zij haar apothekerstitel en masterdiploma Farmacie. Direct na haar studie begon zij in de apotheek van het Slotervaartziekenhuis en het Nederlands Kanker Instituut aan het onderzoek dat beschreven staat in dit proefschrift. Het onderzoek werd uitgevoerd onder begeleiding van promotor prof. dr. J.H. Beijnen en co-promotor dr. B Nuijen.

Iris van der Heijden was born on January 16th, 1985 in Tilburg, the Netherlands. After graduating from high school in 2003 at Theresialyceum in Tilburg, she enrolled at Pharmacy School to obtain her Bachelor's degree in Pharmaceutical Sciences in 2007. During her Master's studies she was accepted into a psychopharmacology research internship in the Psychiatry department of the University of California, San Diego (UCSD), United States. Under the supervision of Professors M.A. Geyer PhD. and B. Olivier PhD., she investigated the role of the serotonin receptor in an animal model of schizophrenia. She expanded her research experience by accepting an internship in the department of clinical toxicology at Newcastle University, in the Calvary Mater Hospital, Newcastle NSW, Australia. Under the supervision of G. Isbister PhD. she developed population pharmacokinetic and pharmacodynamic models after amisulpride overdose. After obtaining her PharmD and Master's degree in 2009, she started her PhD project described in this thesis under the supervision of Professor J.H. Beijnen, PhD. and B. Nuijen PhD. at the pharmacy of the Slotervaart Hospital and the Netherlands Cancer Institute.

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